

University of Groningen

## The role of human CBX proteins in human benign and malignant hematopoiesis

Jung, Johannes

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2018

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Jung, J. (2018). *The role of human CBX proteins in human benign and malignant hematopoiesis*. University of Groningen.

### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

**THE ROLE  
OF HUMAN CBX PROTEINS  
IN HUMAN  
BENIGN AND MALIGNANT  
HEMATOPOIESIS**

**JOHANNES JUNG**

Research was funded by:  
Mildred-Scheel scholarship of the German Cancer Aid, Bonn  
Dutch Cancer Society, Amsterdam, The Netherlands

Printing: Eikon +  
Cover & layout:  Lovebird design.  
[www.lovebird-design.com](http://www.lovebird-design.com)

ISBN (print): 978-94-034-0905-4

Copyright 2018.



university of  
 groningen

# **The role of human CBX proteins in human benign and malignant hematopoiesis**

**PhD thesis**

to obtain the degree of PhD at the  
 University of Groningen  
 on the authority of the  
 Rector Magnificus Prof. E. Sterken  
 and in accordance with  
 the decision by the College of Deans.

This thesis will be defended in public on

Monday 17 September 2018 at 09.00 hours

by

**Johannes Benjamin Jung**

born on 13 December 1981  
 in Freiburg im Breisgau, Germany



**Supervisor**

Prof. G. de Haan

**Co-supervisor**

Dr. L. Bystrykh

**Assessment Committee**

Prof. G.A. Huls

Prof. J. Gil

Prof. C. Lengerke

## **TABLE OF CONTENTS**

### **CHAPTER 1**

Introduction and Outline of the thesis **9**

### **CHAPTER 2**

Do Hematopoietic Stem Cells get old? **35**

### **CHAPTER 3**

Hematopoiesis during development, aging and disease **47**

### **CHAPTER 4**

The role of CBX proteins in human benign and malignant hematopoiesis **65**

### **CHAPTER 5**

Summary and Future Perspectives **125**

### **APPENDICES**

Nederlandse samenvatting **143**

Acknowledgments **158**

CV **161**



*For my wife Sonja, my daughter Sarah  
and my parents*



# **1** INTRODUCTION AND OUTLINE OF THE THESIS





## HEMATOPOIESIS

In humans, postnatal steady-state hematopoiesis is mainly taking place in the bone marrow, which constitutes 4 % of the total body mass. Bone marrow is a soft highly vascular modified, semi-solid and gelatinous connective tissue that is located in the medullary cavity.

Bone marrow of human adults can macroscopically be further subdivided into red and yellow bone marrow. Whereas the first one mainly consists of hematopoietic tissue, the latter is characterized by the presence of fat cells which colors the bone marrow yellowish by the carotenoids in the intracellular fat droplets. Whereas in newborns the majority of the bones consist of red bone marrow, during childhood and aging the red bone marrow, especially in the facial bones and the diaphysis of the long bones, is gradually replaced by fat cells.

Although in adults nearly half of the bone marrow consists of fat cells, at times of physiological stress such as bleedings, the fat cells can be replaced by hematopoietic tissue, showing that the bone marrow and the hematopoietic system are highly flexible and respond to demands and external stimuli.

## HEMATOPOIETIC STEM CELLS

The term “stem cell” was introduced into the scientific literature by the German biologist Ernst Haeckel in the second half of the 19<sup>th</sup> century (Haeckel, 1868). He used the term to describe fertilized eggs but also a unicellular organism from which, in his eyes, all multicellular organisms originated. In 1877 Paul Ehrlich discovered that cells can be stained with aniline-derived dyes making it possible to distinguish between different kinds of leukocytes (Ehrlich, 1879) (Ramalho-Santos and Willenbring, 2007).

This knowledge divided the hematologic field into two parties. Followers of the dualistic model believed that there are two types of committed precursor cells: myeloid precursors which are located in the bone marrow and lymphatic precursor cells, which are hosted in the lymphoid organs, like spleen and lymph nodes. In contrast, followers of the unitarian model presumed that one single cell exist, that could differentiate into all three lineages. In the early 20<sup>th</sup> century the Russian-American embryologist Alexander Maximow and others were the first to use the term ‘hematopoietic stem cell’ and hypothesized that this primitive



hematopoietic stem cell could differentiate into all mature blood cells (Ramalho-Santos and Willenbring, 2007).

Nearly 50 years later experimental proof was provided for the existence of such a multipotent hematopoietic stem cell (HSC) by radiation experiments. Upon exposure of a lethal dose of radiation, mice could be rescued through intravenous injection of bone marrow cells of a donor mouse (Lorenz et al., 1951) by cellular reconstitution of a new hematopoietic system (Ford et al., 1956). Later, Till and McCulloch were able to show that numbers of colonies detected in host spleens were proportional to the number of transplanted bone marrow cells and that these colonies consisted of myeloid and erythroid cells, thus developing the first quantitative stem cell assay (Till and Mc, 1961).

All these early landmark studies show that primitive hematopoietic cells, located in the bone marrow, can engraft and generate multi-lineage progenies *in vivo*. In line with this concept it is now well known that hematopoiesis is hierarchical organized with hematopoietic stem cells on the top, which are able to differentiate into all mature blood cell types. Hematopoietic stem cells furthermore have the ability to self-renew and can undergo symmetric cell division thereby expanding the pool of hematopoietic stem cells. There are cell-intrinsic as well as extrinsic mechanisms that control this balance of self-renewal and differentiation.

## **EXTRINSIC REGULATION OF HEMATOPOIETIC STEM CELLS**

The bone marrow provides a so-called hematopoietic stem cell niche, which is believed to be the local microenvironment influencing maintenance, quiescence, and self-renewal of hematopoietic stem cells (Morrison and Scadden, 2014).

Arguably the first molecular evidence for a niche that would support hematopoietic stem cell maintenance and self-renewal was generated in a mouse model overexpressing parathyroid hormone or parathyroid hormone-related peptide, specifically in osteolineage cells. Overexpression of either protein resulted in an increased release of the Notch ligand Jagged 1 by osteoclasts, which consequently increased the number of hematopoietic stem cells *in vivo*. Furthermore, these HSC showed upregulation of the *Notch1* intracellular domain (Calvi et al., 2003; Hoffman, 2018).

The discovery that murine hematopoietic stem cells are enriched in the CD150<sup>+</sup>CD45<sup>-</sup>CD41<sup>-</sup> population made it possible to detect murine hematopoietic stem cells using immunohistochemical stainings of bone marrow sections by a two-color stain. This allowed mapping of murine hematopoietic stem cells to a perivascular niche in the bone marrow (Kiel et al., 2005).

However, these new techniques also put the initial findings from Calvi et al. into perspective, because only a small subset of murine hematopoietic stem cells was localized next to osteolineage cells, suggesting a more indirect effect of these cells on hematopoietic stem cells (Kiel et al., 2005). Nowadays, with the use of multi-photon microscopy, light sheet microscopy, and transgenic reporter animals it is possible to obtain images from the central cavity of the bone marrow and acquire spatial as well as temporal information to track hematopoietic stem cells in the bone marrow. These techniques also allowed to show that different stem- and progenitor cells occupy various and specialized niches created by distinct cell types: whereas -in line with previous publications- hematopoietic stem cells were mapped to a perivascular niche, lymphoid progenitors were mapped to an endosteal niche (Ding and Morrison, 2013). Cells which are located next to blood vessels are of course putative candidates for being an integral part of the stem cell niche by producing factors essential for hematopoietic stem cells. Perivascular mesenchymal stromal cells are very heterogeneous and produce multiple factors important for hematopoietic stem cell maintenance, including SCF and CXCL12 (Ding and Morrison, 2013) (Sugiyama et al., 2006) (Mendez-Ferrer et al., 2010). Multiple lines of evidence show that endothelial cells are not only located next to hematopoietic stem cells (Kiel et al., 2005) but are also functionally important for maintenance of hematopoietic stem cells *in vitro* and *in vivo*. Conditional deletion of membrane-bound SCF in perivascular stromal cells as well as endothelial cells resulted in a reduction of hematopoietic stem cells (Ding et al., 2012). Furthermore, endothelial cells exclusively express *E-selectin* which regulates quiescence (Winkler et al., 2012). Next to mesenchymal and endothelial cells there is also growing evidence that bone marrow macrophages and megakaryocytes are relevant regulators of stem cells, as well as adipocytes and neural cells (Hoffman, 2018).

The number of different cellular and molecular components that define the niche, as well as the fact that there are probably different niches for different hematopoietic stem cells and progenitors, testify to the

dynamics and complexity of the interaction between hematopoietic stem cells and their niche. The fact that hematopoietic stem cells, in comparison to other multipotent stem cells, cannot be sufficiently expanded *in vitro* without loss of function, suggests that unknown soluble or membrane-bound factors essential for hematopoietic stem cells are missing (Morrison and Scadden, 2014). Additionally, there are still many interesting niche-related questions which are of great scientific interest and of clinical importance. Does the niche contribute to the development of hematological malignancies? Do benign and leukemic stem cells use the same spatial niche? If not, is it possible to target niche cells which are only essential for leukemic stem cells? (Morrison and Scadden, 2014)

The growing field of niche-research will probably not only shed light on basic scientific questions but will potentially also discover new potential therapeutic targets for treating hematological malignancies.

## **INTRINSIC REGULATION OF SELF-RENEWAL AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS**

Beyond external niche-derived stimuli, hematopoietic stem cells are also regulated through cell-intrinsic mechanisms, such as transcription factors or epigenetic proteins. Transcription factors are proteins which are directly binding to promoter, enhancer or silencer regions in the genome, thereby regulating the transcriptional activity of genes. Whereas according to Gene Ontology the human genome contains 1.052 genes that belong to the class of transcription factors, Vaquerizas et. al. present a list of 1.391 manually curated transcription factors (Vaquerizas et al., 2009). How many of these are real transcription factors remains unclear.

Transcription factors have pleiotropic functions and thereby obtain various roles in different subsets of hematopoietic cells (Bodine, 2017). Furthermore, lineage-specific transcription factors are regulating each other antagonistically thereby providing intricate feedback loops (Orkin and Zon, 2008).

Of clinical interest is the fact that in many hematopoietic malignancies transcription factors are often dysregulated or mutated, as a single genetic event or as part of translocations (Bodine, 2017; Orkin and Zon, 2008). For example, MLL, RUNX1, TEL/ETV6, SCL/Tal1 and LMO2 are important transcription factors for regulation of hematopoietic stem

cells, that are quite often rearranged through chromosomal translocations in leukemic patients. These translocations can either result in dysregulation or oncogenic fusion proteins (Orkin and Zon, 2008).

Beyond transcription factors epigenetic regulators play a key role in regulating self-renewal and differentiation of hematopoietic stem cells.

## CHROMATIN AND EPIGENETICS

Although the genetic code of essentially all somatic cells is identical, the differentiation process of immature hematopoietic stem cells towards differentiated blood cells is associated with massive changes in the transcriptome. This implies that next to gene regulation through basic genetic elements like promoters and enhancers, alternative mechanisms exist which control gene expression.

In 1942 the term epigenetics was introduced by the embryologist Conrad Waddington although with a slightly different meaning (Deichmann, 2016; Slack, 2002). In modern biology, the term epigenetic refers to mechanisms which regulate gene transcription without changes in the DNA sequence. The fact that epigenetic mechanisms regulate transcription on top of “classical genetics” is literally described with the ancient Greek prefix “epi” meaning “on top of”. Already before the word epigenetic was introduced, the cytologist Walther Flemming in 1879 established the term “chromatin” for describing stainable structures in the nucleus of a cell during mitosis. Nowadays the word chromatin is used to describe the DNA with its associated proteins which mostly includes the basic histone proteins (Deichmann, 2016).

The total length of the DNA of a single diploid human cell is two meters. For ensuring that this amount of DNA can be stored in the nucleus with a diameter of only 6  $\mu\text{m}$ , DNA is organized in a non-random, highly organized structure which include many DNA associated proteins (Alberts B, 2002). The smallest unit of the chromatin, the nucleosomes, consists of 147 bps of double helix DNA, which is wrapped tightly around an octamer assembled of dimers of the core histone proteins (Luger et al., 1997). The core histone proteins (H2A, H2B, H3 and H4) are highly evolutionary conserved and consist of two functionally distinct domains—the globular domain and a protruding tail. The globular domains of all four core histones form the histone scaffold around which the DNA is

wrapped tightly around. The part of the globular domain which is in direct contact with the DNA, is referred to as the “lateral surface”. Beyond this globular domain, all four highly basic core histone proteins have protruding tails (Tropberger and Schneider, 2013). Multiple nucleosomes are connected with each other through linker DNA and the linker histone protein H1. Two different ground states of chromatin can be distinguished: hetero- and euchromatin. Heterochromatin contains mostly genes which are repressed. In contrast, euchromatin is more openly configured and thereby facilitates the binding of proteins of the transcriptional machinery (Allis and Jenuwein, 2016).

Epigenetic mechanisms include all chemical or structural modifications of the chromatin that influence transcriptional activity (Deichmann, 2016). Interestingly, some of these modifications are heritable so that cells can give a blueprint of their epigenome to their daughter cells (Heard and Martienssen, 2014). The majority of these modifications are reversible, rendering epigenetic proteins potentially clinically relevant target structures.

## **DNA METHYLATION**

DNA methylation was one of the first epigenetic mechanisms which was identified. Although chemical modifications of DNA had already been detected in 1948 (Hotchkiss, 1948), the proof that methylation of cytosine repressed transcription of genes was provided in 1980 (Allis and Jenuwein, 2016; Razin and Riggs, 1980).

Methylation of cytosine residues occurs mainly if the cytosine is followed directly by guanine (CpG: cytosine-phosphate-guanine) (Esteller, 2008). CpGs are distributed across the genome in a nonrandom manner and occur in GC-rich regions, so-called CpG islands. These CpG islands are very often localized in gene-regulatory regions like promoters in proximity to the transcriptional start sites. CpG islands in healthy cells localized in these regions are protected from DNA methylation, whereas CpG' sites localized elsewhere in the genome are frequently methylated (Bird, 2002).

The methylation of cytosine is catalyzed by three different DNA methyltransferases (DNMT1, DNMT3A and DNMT3B).

DNMT1 is a “maintenance” methyltransferase, establishing the methylation mark of the daughter strand after recognition of the methylated CpG site of the parent strand. In contrast to DNMT1, DNMT3A and DNMT3B

are predominantly *de novo* methyltransferases, which can methylate previous non-methylated cytosine residues (Bird, 2002; Rose and Klose, 2014).

Interestingly, 75% of the methylome (the collection of methylated loci) is consistent across all cell types, whereas 25% were cell-type specific either hyper- or hypomethylated (Liu et al., 2016). Comparison of DNA methylation patterns of tumor cells in comparison to their healthy counterparts have shown that DNA of cancer cells is globally less methylated (Feinberg and Vogelstein, 1983). Differences in methylation patterns can occur in promoter regions of oncogenes, which are either low or not expressed in healthy cells. Upon demethylation, these oncogenes are expressed and can contribute to tumorigenesis (Esteller, 2008). Interestingly, increased demethylation has also been observed in regions containing repetitive DNA sequences (Feinberg and Tycko, 2004), such as peri-centromeric regions. These might result in chromosomal instability and aneuploidy, which are both frequently observed in cancer cells (Esteller, 2008; Rodriguez et al., 2006). In line with this hypothesis, functional knockout of cytosine DNA methyltransferases results in chromosomal instability in human cancer cells (Karpf and Matsui, 2005).

Although cancer cells show global demethylation, hypermethylation of promoter-regions located next to or in CpG islands can specifically silence tumor suppressor genes involved in cell cycle control (e.g. *Rb*) (Ohtani-Fujita et al., 1993), DNA-repair (e.g. *BRCA1*) (Zhang and Long, 2015) or apoptosis (e.g. *Caspase 8*) (Wu et al., 2010). Hypermethylation of such CpG islands occurs in a cancer-type specific way (Costello et al., 2000).

The fact that malignant cells show massive aberrations of the methylome in comparison to their healthy counterparts, suggests that overexpression, abnormal recruitment or mutations in genes coding for DNA-methyltransferases might contribute to cancerogenesis. Overexpression of *DNMT1*, *DNMT3A* and *DNMT3B* methyltransferases was found in a variety of malignant diseases like AML (Mizuno et al., 2001). Furthermore, mutations in *DNMT1*, *DNMT3A* and *DNMT3B* can be detected in different cancer subtypes. Whereas mutations in *DNMT1* are most frequently found in colon cancer cells (Kanai et al., 2003), is *DNMT3A* often mutated in hematological diseases like acute myeloid leukemia (Ley et al., 2010), myeloid dysplastic syndrome (Haferlach et al., 2014) and acute lymphatic leukemia (Neumann et al., 2013).

Indeed, *DNMT3A* is the most frequently mutated epigenetic modifier in AML with a frequency of 6 to 36% (Cancer Genome Atlas Research

et al., 2013; Wouters and Delwel, 2015). Almost 50 % of *DNMT3A* mutations in AML patients occur in a DNA base triplet coding for arginine-882, leading to an amino acid exchange to histidine (R882H) or cysteine (R882C) within the catalytic domain, resulting in a missense mutation. AML cells overexpressing R882H protein show a ca. 80% reduced methyltransferase activity in comparison to the wild-type, suggesting a dominant negative phenotype (Russler-Germain et al., 2014). In line with these results conditional knockout studies in murine hematopoietic stem cells show that loss of *DNMT3A* results in increased self-renewal of hematopoietic stem cells and hampered differentiation (Challen et al., 2012).

The first hint that *DNMT3A* mutations are early events in the multi-step model of leukemogenesis arose after deep-sequencing analysis of different hematopoietic subsets in AML patients harboring mutations in *DNMT3A* and *NPM1*. Whereas both mutations could be detected in AML blasts, only *DNMT3A* mutations were detectable in the most primitive hematopoietic compartment, including hematopoietic stem cells and multipotent progenitors. Xenotransplantation of non-leukemic *DNMT3A*<sup>mut</sup> immunophenotypic hematopoietic stem cells in immunodeficient mice resulted in multilineage differentiation, indicating that these cells are fully functional hematopoietic stem cells. Furthermore, these cells showed a repopulation advantage over wild-type hematopoietic stem cells in xenotransplantation assays, suggesting that hematopoietic stem cells harboring *DNMT3A* mutations have more self-renewal and can be considered as pre-leukemic stem cells. Interestingly, CD33+ cells in the peripheral blood of patients in remission showed exclusively expression of mutated *DNMT3A*, but not expression of the mutated *NPM1* allele (Shlush et al., 2014). This shows that *DNMT3A*<sup>mut</sup> hematopoietic stem cells are capable of escaping the classical cytotoxic chemotherapy and thereby represent a pool of expanded stem cells which might acquire secondary mutations resulting in a relapse of the disease. In line with these results, *DNMT3A* R882H mediates resistance to anthracyclines (Guryanova et al., 2016), a drug class which is an essential part of the induction therapy of acute myeloid leukemia (Yates et al., 1973). In general, are AML patients harboring a *DNMT3A* mutation at diagnosis older, have higher white blood cell counts and a worse outcome (Ley et al., 2010).

Because hypermethylation of CpG islands in promoter regions can be the consequence of dysfunction of DNA methyltransferases, but may



also be due to reduced DNA demethylation, dysregulation or mutations in genes coding for these enzymes appear to be causally involved in AML pathogenesis. Demethylation of 5-methylcytosine can occur during mitosis, when DNMT1 fails to copy the methyl group from the parent strand. DNMT1 can sense methylated and non-methylated cytosine but not 5-hydroxymethylcytosine, which is an intermediate product of the passive DNA demethylation process. 5-hydroxymethylcytosine can then be replaced by cytosine through the base excision repair pathway (Chan and Majeti, 2013). The conversion of 5-methylcytosine towards 5-hydroxymethylcytosine is mediated through three enzymes TET1, TET2 and TET3 (Ko et al., 2010). Especially *TET2* mutations can be found in a variety of myeloid malignancies like myelodysplastic syndrome, acute myeloid leukemia, chronic myelomonocytic leukemia and systemic mastocytosis (Delhommeau et al., 2009; Tefferi et al., 2009). The vast majority of these mutations is heterozygous and results in a dysfunctional enzyme (Delhommeau et al., 2009). In line with this, conditional *TET2* knockout mice display hematopoietic stem cells with increased self-renewal activity and furthermore show common features of myeloproliferative syndromes like monocytosis, splenomegaly and extramedullary hematopoiesis (Moran-Crusio et al., 2011).

Additionally, whole-genome and exome-sequencing studies showed that mutations in *IDH1* and *IDH2* can be detected in 15% of all AML patients (Cancer Genome Atlas Research et al., 2013; Chan and Majeti, 2013). Similar to mutations in *DNMT3A*, mutations in *IDH* are heterozygous and occur at different hotspots in highly conserved arginine residues, namely *IDH1* R132, *IDH2* R140 and *IDH2* R172 (Chan and Majeti, 2013). The mutant protein catalyzes the conversion of alpha-ketoglutarate to D-2-hydroxyglutarate under the consumption of NADPH. This results in aberrantly high levels of 2-hydroxyglutarate and a competitive inhibition of multiple alpha-ketoglutarate dependent dioxygenases like *TET2* (Gross et al., 2010). The fact that *IDH* and *TET2* mutations are mutually exclusive and that both have highly similar hypermethylation signatures strongly suggests that *IDH2* mutations mainly function through *TET2* inhibition (Chan and Majeti, 2013).

The fact that so many genes that are directly or indirectly involved in DNA methylation are mutated in hematological malignancies, clearly indicate that aberrant DNA-methylation is a crucial step in malignant transformation of hematopoietic cells.



## POST-TRANSLATIONAL MODIFICATIONS OF HISTONE PROTEINS

Histone proteins can undergo a plethora of different post-translational covalent modifications including acetylation, methylation, SUMOylation, citrullination, phosphorylation and ribosylation. Such modifications are mainly covalently bound to lysine (K), but can also be added to other amino acids like arginine, serine or threonine (Rothbart and Strahl, 2014). Proteins involved in post-translational modifications can be functionally subdivided into writers (proteins which establish the mark), readers (proteins which can recognize the histone mark) and erasers (proteins which can remove the mark) (Zhang et al., 2015).

So far, the best studied modifications are located on the protruding tail of the histone proteins. These covalent modifications are easily accessible for writers, erasers and readers and thereby influence transcription, replication and DNA repair (Tropberger and Schneider, 2013). Post-translational modifications of histone tails are affecting the degree of compaction of chromatin regions, either directly by changing histone-DNA or histone-histone interactions, or indirectly by recruitment of effector proteins (Cosgrove et al., 2004; Rothbart and Strahl, 2014). Through integrative analysis of transcriptome and Chip-seq data it has become possible to associate different post-translational modifications of histone tails with different transcriptional states.

For example, H3K27ac and H3K4me1 mark active enhancers (Creyghton et al., 2010; Zhang et al., 2015). Accordingly, actively transcribed genes are marked with H3K4me3 and acetylation of H3 and H4 in promoter regions (Barrera et al., 2008; Deckert and Struhl, 2001; Liang et al., 2004). Furthermore, actively transcribed genes are marked with H3K79me3 (Ng et al., 2003), H2BK120ub (Batta et al., 2011), H3K36me3 (Pokholok et al., 2005) and acetylated H3 and H4 in the gene body (Myers et al., 2001) (Zhang et al., 2015).

Different mass spectrometry approaches targeted to histone proteins have shown that up to seven post-translational modifications can be added to the N-terminal region of histone 3 (H3<sub>1-50</sub>). Interestingly, specific combinations of different modifications were found more frequently than others (Young et al., 2009). This suggests that some modifications facilitate or prevent other modifications through crosstalk to other epigenetic pathways. Because nucleosomes contain homodimers of histone

proteins, it remains elusive if crosstalks between different pathways can only occur on one single histone polypeptide (symmetrically) or adjacent polypeptides (asymmetrically) (Rothbart and Strahl, 2014).

Voigt et al. showed via an MS approach with custom-made antibodies against H3K27me2, H3K27me3, H4K20me1, that H3 and H4 dimers are not always modified in the same pattern (Rothbart and Strahl, 2014; Voigt et al., 2012). These data, of course, leave a lot of room for speculations and suggest that different epigenetic marks may influence each other even if they are located on different histone tails.

Besides multiple publications focusing on post-translational modifications of histone tails, mass spectrometry experiments have shown that histone proteins can also undergo post-translational modifications of the globular domain. The most prominent of these post-translational modifications is located on the lateral surface and thereby affects residues of the protein which directly interact with DNA (Cosgrove et al., 2004; Tropberger and Schneider, 2013). These modifications can directly regulate accessibility without recruitment of further effector proteins by influencing mobility and stability of nucleosomes and DNA-histone interactions (Rothbart and Strahl, 2014; Tropberger and Schneider, 2013). So far, there are hardly any epigenetic reader proteins described as binding partners of post-translational modifications of the lateral surface (Tropberger and Schneider, 2013; Yu et al., 2012).

In contrast, many epigenetic reader, writer and eraser proteins have been described to post-translationally modify histone tails. One of the best-studied post-translational modifications of histones is acetylation. Enzymes transferring acetyl groups to lysine residues of histone proteins are called histone acetyltransferases (HAT), whereas enzymes removing acetyl groups are called histone deacetylases (HDAC) (Greenblatt and Nimer, 2014; Haberland et al., 2009). Because dense chromatin states are to some degree the resultant of electrostatic attraction of positively charged histone proteins and negatively charged phosphate groups of the DNA, neutralization of the positive charge of histones through covalent binding of a negatively charged acetyl group results in loss of dense chromatin states. The dynamic balance of acetylated and histone proteins is antagonistically controlled by these two prominent families of histone-modifying enzymes (Haberland et al., 2009).

Acetylated lysine residues, as described above, are in general associated with actively transcribed chromatin regions. Of note, mutations or

transcriptional dysregulation of histone acetyltransferases are linked to several malignant hematological diseases. For example 18% of all relapsed acute lymphatic leukemia patients present with a mutation in the acetyltransferase *CREBBP* (Greenblatt and Nimer, 2014). The overwhelming majority (89%) of mutations in *CREBBP* detected in highly hyperdiploid acute lymphoblastic leukemia patient samples is located in the functional histone acetyltransferase domain (Inthal et al., 2012). Functionally, these mutations result in impaired histone acetylation of H3K18 and transcriptional dysregulation of *CREBBP*-target genes (Mullighan et al., 2011).

Histone deacetylases have also shown to be involved in regulation of cell proliferation and cancer. Chip-seq experiments in murine embryonic stem cells showed that HDAC1 directly represses the tumor suppressor gene *CDKN1A* (Zupkovitz et al., 2010). Equivalent results were obtained through knockdown of *HDAC2* in HeLa cells which resulted in *CDKN1A* upregulation and morphological signs of differentiation (Huang et al., 2005), suggesting that different family members of HDACs have at least to some degree overlapping functions.

Histone deacetylases also play a role in the development of malignant hematological diseases. Expression of the fusion protein PML-RARalpha in acute promyelocytic leukemia cells results in aberrant recruitment of histone deacetylases to gene and promoter regions (Grignani et al., 1998; Tambaro et al., 2010). Similar results were obtained for the oncogenic fusion-protein RUNX1-ETO, which is able to bind HDAC1-3 (Amann et al., 2001).

In contrast to acetylation of histone proteins, the transfer of methyl groups to histone proteins is associated with distinct transcriptional states, dependent on the localization and the number of methyl groups. Whereas H3K9me3 and H3K27me3 are involved by transcriptional repression, H3K4me3 is associated with transcriptionally active genes. Because, in contrast to acetylation, histone methylation does not result in physical changes, effector proteins are necessary to translate methylation patterns in corresponding transcriptional and chromatin changes.

There is increasing evidence that enzymes that were once believed to only acetylate or methylate histone proteins, in fact are also able to modify non-histone proteins and thereby to alter their functional activity. This includes especially the tumor suppressor p53 (Tang et al., 2008), but also a key regulator of hematopoietic stem cells like RUNX1 (Yamaguchi et al., 2004).

## EPIGENETIC DRUGS AND THERAPIES IN MALIGNANT DISEASES

As described in the previous paragraphs, epigenetic changes due to mutations or dysregulation of genes coding for epigenetic readers, writers, or erasers, are frequently observed in cancer cells. Because epigenetic changes like DNA-methylation or post-translational covalent histone modifications in general are reversible, these are quite attractive putative pharmacological targets (Greenblatt and Nimer, 2014).

The first example of a successful epigenetic therapy was provided by the use of drugs inhibiting DNA methyltransferases, like 5-azacytosine (= 5-azacytosine, Vidaza®) and decitabine (= 5-aza-2'-deoxycytidine, Dakogen®) in acute myeloid leukemia and myelodysplastic syndrome (Jones et al., 2016). Whereas 5-azacytosine can be incorporated into DNA as well as RNA (Navada et al., 2014), its 2'-deoxy-derivate decitabine can only be incorporated into DNA. In the 1960's these drugs were initially tested as cytarabine analogues and antimetabolites in clinical trials using high doses, without resounding success due to their toxicity profiles (Crujisen et al., 2014; Jones et al., 2016). Later, the two drugs received further attention after it was shown that their use at lower doses induces cellular differentiation and demethylation of DNA in embryonic cells (Jones and Taylor, 1980).

The demethylating effect of 5-azacytosine is not due to direct demethylation of DNA, but through incorporation into the DNA during DNA replication and subsequent irreversible binding to and degradation of DNMT1 (Juttermann et al., 1994). In line with these data, cancer cells treated with demethylating agents show demethylation of some tumor suppressor genes like *p15/INK4B* (Daskalakis et al., 2002) (Jones et al., 2016). After demonstrating their efficiency in multiple clinical trials (Fenaux et al., 2009; Lubbert et al., 2016) both drugs were approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome. Furthermore, 5-azacytosine is approved by EMA for the treatment of chronic myelomonocytic leukemia (CMML) and AML in older patients, who are not eligible for standard induction chemotherapy and decitabine is approved for the treatment of secondary AML patients, who are not eligible for conventional therapy.

The toxicity profiles of both substances at lower doses are more acceptable so that especially older people, whose general and medical

conditions are more frequently not sufficient for classical induction cytotoxic chemotherapy regimens, are benefitting from epigenetic therapy approaches using 5-azacytidine and decitabine. This is especially important because more than 60% of all newly diagnosed AML patients are older than 60 years (National Cancer Institute, 2013). Interestingly, *DNMT3A*<sup>mut</sup> and *TET2*<sup>mut</sup> MDS patients have a statistically significant better response and progression-free survival to demethylating therapy approaches (Traina et al., 2014), suggesting that cancer cells with mutations in genes coding for proteins involved in DNA methylation are more sensitive to demethylating agents. As mentioned above, mutations in *IDH2* are observed in ca. 15% of all AML patients. In 2017 the first *IDH2* inhibitor, Enasidenib, was approved for the treatment of relapsed or refractory *IDH2*<sup>mut</sup> AML. The approval was the consequence of impressive data of a phase I/II single arm multicenter study showing that after a median follow-up of 6,6 months 19% of all AML *IDH2*<sup>mut</sup> patients experienced a complete remission which lasted at median 8,2 months (FDA, 2017).

The largest group of epigenetic drugs being tested and approved by FDA or EMA represent inhibitors of enzymes involved in post-translational modifications of histone tails.

Histone deacetylase-Inhibitors (HDACi) were initially discovered in drug screens aimed to search for differentiation inducers in leukemias (Jones et al., 2016; Richon et al., 1998). In line with the observation that HDACs are abnormally recruited through the fusion oncogenes AML1-ETO and PML-RARalpha, exposure of AML cells harboring these translocations to histone deacetylase inhibitors (HDACi) induces apoptosis and terminal differentiation (Insinga et al., 2004; West and Johnstone, 2014). Because the catalytic active domain of 11 of the 18 different HDACs in mammals is highly conserved and because the majority of the HDACs have functional redundancy, many HDACi show activity against multiple HDACs (Haberland et al., 2009; Halsall and Turner, 2016).

In 2006 the first HDACi, Vorinostat, which showed activity against class I, IIa, IIb and IV HDACs, was approved for the treatment of cutaneous and peripheral T-cell lymphoma (Halsall and Turner, 2016). In the meanwhile, also Belinostat and Romidepsin have been approved for the same indications (Jones et al., 2016). Recently, the histone-deacetylase inhibitor Panobinostat was approved for the treatment of multiple myeloma, in combination with the proteasome inhibitor Bortezomib (Jones

et al., 2016). Next to these already approved indications, HDACi are also tested in preclinical and early-clinical studies in other malignant diseases.

Activating mutations in the H3K27 methyltransferase EZH2, part of catalytic subunit of the Polycomb Repressive Complex 2, can be detected in diffuse large B-cell lymphoma (DLCL) and follicular lymphomas, and result in increased H3K27me3 due to altered substrate preferences. The EZH2 inhibitor GSK126 decreased H3K27me3 levels, which was associated with an inhibition of proliferation, especially in *EZH2<sup>mut</sup>* DLCL cell lines as well as in DLCL xenografts (McCabe et al., 2012). The EZH2 inhibitor Tazemostat was recently tested in heavily pretreated, relapsed and refractory follicular and diffuse large B-cell lymphoma patients. The overall response rate of patients suffering from *EZH2<sup>mut</sup>* follicular lymphoma was very high (92%), in contrast to *EZH2<sup>wt</sup>* follicular lymphoma patients. Similar to follicular lymphoma patients, also diffuse large B-cell lymphoma patients benefitted from the use of Tazemostat: 29% of the *EZH2<sup>mut</sup>* patients showed an overall response, whereas only 15% of *EZH2<sup>wt</sup>* patients did so. Fortunately, the drug was very well tolerated and showed a favorable side effect profile (Morschhauser F, 2017).

Beyond single epigenetic agents, more and more combinational therapeutic approaches are being tested. Because methylated DNA is frequently associated with other repressive histone marks, such as deacetylated histones (Eden et al., 1998; Jones et al., 2016), the combinatorial use of HDACi and demethylating agents might be beneficial. In line with this hypothesis, exposure of colon carcinoma cells to the histone deacetylase inhibitor TSA upon pretreatment of 5-azacytidine results in re-expression of *CDKN2A*, whereas their use as single agents cannot re-express *CDKN2A* robustly (Cameron et al., 1999; Jones et al., 2016). In 2014 data from a randomized study showed that the combination therapy of Entinostat and 5-azacytidine in AML and MDS patients resulted in a lower median overall survival. Curiously, this combination therapy resulted in less demethylation leading the authors of this study to propose eventually antagonistic effects (Prebet et al., 2014). Next to testing drug combinations of only selective epigenetic agents, more and more regimes are explored which combine the use of classic cytotoxic agents with these epigenetic drugs. The rationale behind this approach is that in some cancer cells pre-exposure to demethylating agents like decitabine or 5-azacytidine results in reduced resistance to classical cytotoxic chemotherapeutic drugs. For example, preclinical studies in platinum-resistant

ovarian cancer cells showed that exposure of decitabine to these cells restores their sensitivity against platinum-based cytotoxic therapeutic approaches (Li et al., 2009). Supporting this hypothesis, a phase two trial of heavily-pretreated carboplatin-resistant ovarian cancer patients of treatment with decitabine and carboplatin showed a very good response and progression-free survival (Matei et al., 2012).

The beneficial effect of the combinatorial use of epigenetic drugs and checkpoint inhibitors was accidentally discovered in a study which enrolled advanced treatment-refractory non-small lung cancer patients for testing the immune checkpoint inhibitor anti PD-1. Five out of 6 patients, who participated in a previous trial for testing the efficiency of the combinatorial use of azacytidine and Entinostat, showed no signs of any disease progression in six months (Juergens et al., 2011; Wrangle et al., 2013).

In conclusion, the previous paragraphs show that the increased knowledge in basic epigenetic science is more and more translated in clinical applications for treatment of cancer patients. In the next decades the epigenetic treatment options for patients with malignant diseases will increase.

## **OUTLINE OF THE THESIS**

The overall aim of the research described in this thesis project was to unravel the role of human CBX-2, 4, 6, 7 and 8 proteins, in regulating normal human hematopoietic stem and progenitor cells and to explore whether (one of) these proteins could potentially be a therapeutic target in leukemia.

Chapter 1 provides an introduction to hematopoiesis and hematopoietic stem cells. Furthermore, we describe briefly the concept of the hematopoietic stem cell niche, their molecular and cellular components, and how the niche contributes to hemostasis of hematopoietic stem cells as one example of extrinsic regulation of hematopoietic stem cells. Furthermore, we give a short overview of transcription factors as one class of intrinsic hematopoietic stem cell regulators. The predominant part of the introduction will provide an overview about epigenetics, their role in cancerogenesis and epigenetic therapeutical approaches.

Chapter 2 introduces the reader to characteristics of the aged hematopoietic system and hematopoietic stem cells. We discuss potential



mechanisms that may contribute to hematopoietic stem cell (HSC) aging, and elude to the reversibility of aging associated changes in old HSCs.

Chapter 3 gives a broad overview of Polycomb proteins and their role in the development of the hematopoietic system, cancerogenesis and discuss a potential role in aging.

In Chapter 4 we studied the function of different human CBX proteins in regulating hematopoietic stem and progenitor cells by enforced overexpression of human *CBX2*, 4, 6, 7 and 8 in human CD34+ cord blood cells. We show that *in vitro* overexpression of human *CBX7* has the most profound effect on self-renewal of primitive human CD34+ cord blood cells. Furthermore, we show that xenotransplantation of human CD34+ cord blood cells overexpressing *CBX7* results in higher engraftment, enhanced myelopoiesis and increased self-renewal of huCD34+CD38- cells. Furthermore, we performed RNA-seq and Chip-seq of *CBX7* overexpressing CD34+ cord blood cells to identify direct targets of huCBX7.

We assessed expression levels of *CBX7* in AML and performed knockdown experiments of *CBX7* in two AML cells. In both cell lines knockdown of *CBX7* was associated with an inhibition of proliferation. Additionally, knockdown of *CBX7* in the HL60 cell line induced upregulation of *CD11b* and morphological signs of differentiation. Besides that, knockdown of *CBX7* in OCI-AML3 cells results in upregulation of *CD14*.

Furthermore, we identified novel interaction partners of human CBX proteins via a mass spectrometry approach in *CBX7* overexpressing human and murine cells. We identified multiple H3K9-methyltransferase as binding partners of huCBX7. All of these three H3K9 methyltransferases have at least one tri-methylated lysine. Motif search of these trimethylated peptides revealed a sequence motif highly similar to H3K9me3 and H3K27me3. Chip-Seq experiments for H3K9me3 and *CBX7* showed that ca. 1/3 of all *CBX7* peaks are associated with H3K9me3 peaks. Furthermore, we show that knockdown of *SETDB1*, one of the H3K9 trimethylating enzymes, results in upregulation of *CD11b* and *CD14* in HL60 and OCI-AML3 cells as well as inhibition of proliferation.

In Chapter 5 we summarize the findings and discuss future perspectives.



## REFERENCES:

- Alberts B, J. A., Lewis J, et al (2002). *Molecular Biology of the Cell*. (<https://www.ncbi.nlm.nih.gov/books/NBK26834/>: Garland Science).
- Allis, C. D., and Jenuwein, T. (2016). The molecular hallmarks of epigenetic control. *Nat Rev Genet* 17, 487-500.
- Amann, J. M., Nip, J., Strom, D. K., Lutterbach, B., Harada, H., Lenny, N., Downing, J. R., Meyers, S., and Hiebert, S. W. (2001). ETO, a Target of t(8:21) in Acute Leukemia, Makes Distinct Contacts with Multiple Histone Deacetylases and Binds mSin3A through Its Oligomerization Domain. *Molecular and Cellular Biology* 21, 6470-6483.
- Barrera, L. O., Li, Z., Smith, A. D., Arden, K. C., Cavenee, W. K., Zhang, M. Q., Green, R. D., and Ren, B. (2008). Genome-wide mapping and analysis of active promoters in mouse embryonic stem cells and adult organs. *Genome Res* 18, 46-59.
- Batta, K., Zhang, Z., Yen, K., Goffman, D. B., and Pugh, B. F. (2011). Genome-wide function of H2B ubiquitylation in promoter and genic regions. *Genes Dev* 25, 2254-2265.
- Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes & Development* 16, 6-21.
- Bodine, D. M. (2017). Introduction to the review series on transcription factors in hematopoiesis and hematologic disease. *Blood* 129, 2039.
- Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., Martin, R. P., Schipani, E., Divieti, P., Bringhurst, F. R., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841-846.
- Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G., and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 21, 103-107.
- Cancer Genome Atlas Research, N., Ley, T. J., Miller, C., Ding, L., Raphael, B. J., Mungall, A. J., Robertson, A., Hoadley, K., Triche, T. J., Jr., Laird, P. W., et al. (2013). Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 368, 2059-2074.
- Challen, G. A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J. S., Bock, C., Vasanthakumar, A., Gu, H., Xi, Y., et al. (2012). Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* 44, 23-31.
- Chan, S. M., and Majeti, R. (2015). Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. *Int J Hematol* 98, 648-657.
- Cosgrove, M. S., Boeke, J. D., and Wolberger, C. (2004). Regulated nucleosome mobility and the histone code. *Nat Struct Mol Biol* 11, 1037-1043.
- Costello, J. F., Fruhwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., Wright, F. A., Feramisco, J. D., Peltomaki, P., Lang, J. C., et al. (2000). Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 24, 132-138.
- Creyghton, M. P., Cheng, A. W., Welstead, G. G., Kooistra, T., Carey, B. W., Steine, E. J., Hanna, J., Lodato, M. A., Frampton, G. M., Sharp, P. A., et al. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A* 107, 21931-21936.
- Crujisen, M., Lubbert, M., Wijermans, P., and Huls, G. (2014). Clinical Results of Hypomethylating Agents in AML Treatment. *J Clin Med* 4, 1-17.
- Daskalakis, M., Nguyen, T. T., Nguyen, C., Guldberg, P., Kohler, G., Wijermans, P., Jones, P. A., and Lubbert, M. (2002). Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. *Blood* 100, 2957-2964.
- Deckert, J., and Struhl, K. (2001). Histone acetylation at promoters is differentially affected by specific activators and repressors. *Mol Cell Biol* 21, 2726-2735.
- Deichmann, U. (2016). Epigenetics: The origins and evolution of a fashionable topic. *Dev Biol* 416, 249-254.
- Delhommeau, F., Dupont, S., Valle, V. D., James, C., Trannoy, S., Massé, A., Kosmider, O., Le Couedic, J.-P., Robert, F., Alberdi, A., et al. (2009). Mutation in TET2 in Myeloid Cancers. *New England Journal of Medicine* 360, 2289-2301.
- Ding, L., and Morrison, S. J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* 495, 231-235.
- Ding, L., Saunders, T. L., Enikolopov, G., and Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457-462.

- Eden, S., Hashimshony, T., Keshet, I., Cedar, H., and Thorne, A. W. (1998). DNA methylation modulates histone acetylation. *Nature* 394, 842.
- Ehrlich, P. (1879). *Arch Anat u Physiol* 3, 571-579.
- Esteller, M. (2008). Epigenetics in cancer. *N Engl J Med* 358, 1148-1159.
- FDA (2017). FDA granted regular approval to enasid- enib for the treatment of relapsed or refractory AML. In, U.S.F.D. Administration, ed.
- Feinberg, A. P., and Tycko, B. (2004). The history of cancer epigenetics. *Nat Rev Cancer* 4, 143-153.
- Feinberg, A. P., and Vogelstein, B. (1983). Hypomethylation Distinguishes Genes of Some Human Cancers from Their Normal Counterparts. *Nature* 301, 89-92.
- Fenaux, P., Mufti, G. J., Hellstrom-Lindberg, E., Santini, V., Finelli, C., Giagounidis, A., Schoch, R., Gattermann, N., Sanz, G., List, A., et al. (2009). Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncology* 10, 223-232.
- Ford, C. E., Hamerton, J. L., Barnes, D. W., and Loutit, J. F. (1956). Cytological identification of radiation-chimaeras. *Nature* 177, 452-454.
- Greenblatt, S. M., and Nimer, S. D. (2014). Chromatin modifiers and the promise of epigenetic therapy in acute leukemia. *Leukemia* 28, 1396-1406.
- Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioco, M., Fanelli, M., Ruthardt, M., Ferrara, F. E., Zamir, I., et al. (1998). Fusion proteins of the retinoic acid receptor- $\alpha$  recruit histone deacetylase in promyelocytic leukaemia. *Nature* 391, 815-818.
- Gross, S., Cairns, R. A., Minden, M. D., Driggers, E. M., Bittinger, M. A., Jang, H. G., Sasaki, M., Jin, S., Schenkein, D. P., Su, S. M., et al. (2010). Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. *J Exp Med* 207, 339-344.
- Guryanova, O. A., Shank, K., Spitzer, B., Luciani, L., Koche, R. P., Garrett-Bakelman, F. E., Ganzel, C., Durham, B. H., Mohanty, A., Hoermann, G., et al. (2016). DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling. *Nat Med* 22, 1488-1495.
- Haberland, M., Montgomery, R. L., and Olson, E. N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet* 10, 32-42.
- Haeckel (1868). *Natürliche Schöpfungsgeschichte* (Berlin Georg Reimer).
- Haferlach, T., Nagata, Y., Grossmann, V., Okuno, Y., Bacher, U., Nagae, G., Schnittger, S., Sanada, M., Kon, A., Alpermann, T., et al. (2014). Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia* 28, 241-247.
- Halsall, J. A., and Turner, B. M. (2016). Histone deacetylase inhibitors for cancer therapy: An evolutionarily ancient resistance response may explain their limited success. *Bioessays* 38, 1102-1110.
- Heard, E., and Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* 157, 95-109.
- Hoffman, R. (2018). *Hematology : basic principles and practice*.
- Hotchkiss, R. D. (1948). The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *J Biol Chem* 175, 315-332.
- Huang, B. H., Laban, M., Leung, C. H., Lee, L., Lee, C. K., Salto-Tellez, M., Raju, G. C., and Hooi, S. C. (2005). Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ* 12, 395-404.
- Insinga, A., Monestiroli, S., Ronzoni, S., Gelmetti, V., Marchesi, F., Viale, A., Altucci, L., Nervi, C., Minucci, S., and Pelicci, P. G. (2004). Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. *Nature Medicine* 11, 71.
- Inthal, A., Zeitlhofer, P., Zeginigg, M., Morak, M., Grausenburger, R., Fronkova, E., Fahrner, B., Mann, G., Haas, O. A., and Panzer-Grumayer, R. (2012). CREBBP HAT domain mutations prevail in relapse cases of high hyperdiploid childhood acute lymphoblastic leukemia. *Leukemia* 26, 1797-1803.
- Jones, P. A., Issa, J. P., and Baylin, S. (2016). Targeting the cancer epigenome for therapy. *Nat Rev Genet* 17, 630-641.
- Jones, P. A., and Taylor, S. M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. *Cell* 20, 85-93.
- Juergens, R. A., Wrangle, J., Vendetti, F. P., Murphy, S. C., Zhao, M., Coleman, B., Sebree, R., Rodgers,

- K., Hooker, C. M., Franco, N., et al. (2011). Combination Epigenetic Therapy Has Efficacy in Patients with Refractory Advanced Non-Small Cell Lung Cancer. *Cancer Discovery* 1, 598.
- Juttermann, R., Li, E., and Jaenisch, R. (1994). Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci U S A* 91, 11797-11801.
- Kanai, Y., Ushijima, S., Nakanishi, Y., Sakamoto, M., and Hirohashi, S. (2005). Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. *Cancer Letters* 192, 75-82.
- Karpf, A. R., and Matsui, S. (2005). Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. *Cancer Res* 65, 8635-8639.
- Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., and Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109-1121.
- Ko, M., Huang, Y., Jankowska, A. M., Pape, U. J., Tahiliani, M., Bandukwala, H. S., An, J., Lamperti, E. D., Koh, K. P., Ganetzky, R., et al. (2010). Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* 468, 839-845.
- Ley, T. J., Ding, L., Walter, M. J., McLellan, M. D., Lamprecht, T., Larson, D. E., Kandoth, C., Payton, J. E., Baty, J., Welch, J., et al. (2010). DNMT3A Mutations in Acute Myeloid Leukemia. *New England Journal of Medicine* 363, 2424-2433.
- Li, Y., Hu, W., Shen, D. Y., Kavanagh, J. J., and Fu, S. (2009). Azacitidine enhances sensitivity of platinum-resistant ovarian cancer cells to carboplatin through induction of apoptosis. *Am J Obstet Gynecol* 200, 177 e171-179.
- Liang, G., Lin, J. C., Wei, V., Yoo, C., Cheng, J. C., Nguyen, C. T., Weisenberger, D. J., Egger, G., Takai, D., Gonzales, F. A., and Jones, P. A. (2004). Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. *Proc Natl Acad Sci U S A* 101, 7357-7362.
- Liu, H., Liu, X., Zhang, S., Lv, J., Li, S., Shang, S., Jia, S., Wei, Y., Wang, F., Su, J., et al. (2016). Systematic identification and annotation of human methylation marks based on bisulfite sequencing methylomes reveals distinct roles of cell type-specific hypomethylation in the regulation of cell identity genes. *Nucleic Acids Research* 44, 75-94.
- Lorenz, E., Uphoff, D., Reid, T. R., and Shelton, E. (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst* 12, 197-201.
- Lubbert, M., Suci, S., Hagemeijer, A., Ruter, B., Platzbecker, U., Giagounidis, A., Selleslag, D., Labar, B., Germing, U., Salih, H. R., et al. (2016). Decitabine improves progression-free survival in older high-risk MDS patients with multiple autosomal monosomies: results of a subgroup analysis of the randomized phase III study 06011 of the EORTC Leukemia Cooperative Group and German MDS Study Group. *Ann Hematol* 95, 191-199.
- Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. E., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 389, 251.
- Matei, D., Fang, F., Shen, C., Schilder, J., Arnold, A., Zeng, Y., Berry, W. A., Huang, T., and Nephew, K. P. (2012). Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res* 72, 2197-2205.
- McCabe, M. T., Ott, H. M., Ganji, G., Korenchuk, S., Thompson, C., Van Aller, G. S., Liu, Y., Graves, A. P., Iii, A. D. P., Diaz, E., et al. (2012). EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature* 492, 108-112.
- Mendez-Ferrer, S., Michurina, T. V., Ferraro, F., Mazloom, A. R., Macarthur, B. D., Lira, S. A., Scadden, D. T., Ma'ayan, A., Enikolopov, G. N., and Frenette, P. S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829-834.
- Mizuno, S., Chijiwa, T., Okamura, T., Akashi, K., Fukumaki, Y., Niho, Y., and Sasaki, H. (2001). Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* 97, 1172-1179.
- Moran-Crusio, K., Reavie, L., Shih, A., Abdel-Wahab, O., Ndiaye-Lobry, D., Lobry, C., Figueroa, M. E., Vasanthakumar, A., Patel, J., Zhao, X., et al. (2011). Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell* 20, 11-24.
- Morrison, S. J., and Scadden, D. T. (2014). The bone

- marrow niche for haematopoietic stem cells. *Nature* 505, 327-334.
- Morschhauser F, S. G., McKay P, et al. (2017). Interim report from a phase 2 multicenter study of taze-metostat, an EZH2 inhibitor, in patients with relapsed or refractory B-cell non-Hodgkin lymphomas. In 14th International Conference on Malignant Lymphoma, (Lugano, Switzerland).
- Mullighan, C. G., Zhang, J., Kasper, L. H., Lerach, S., Payne-Turner, D., Phillips, L. A., Heatley, S. L., Holmfeldt, L., Collins-Underwood, J. R., Ma, J., et al. (2011). CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature* 471, 235-239.
- Myers, F. A., Evans, D. R., Clayton, A. L., Thorne, A. W., and Crane-Robinson, C. (2001). Targeted and Extended Acetylation of Histones H4 and H3 at Active and Inactive Genes in Chicken Embryo Erythrocytes. *Journal of Biological Chemistry* 276, 20197-20205.
- National Cancer Institute (2013). SEER Stat Fact Sheets. In.
- Navada, S. C., Steinmann, J., Lubbert, M., and Silverman, L. R. (2014). Clinical development of demethylating agents in hematology. *J Clin Invest* 124, 40-46.
- Neumann, M., Heesch, S., Schlee, C., Schwartz, S., Gokbuget, N., Hoelzer, D., Konstandin, N. P., Ksienzyk, B., Vosberg, S., Graf, A., et al. (2015). Whole-exome sequencing in adult ETP-ALL reveals a high rate of DNMT3A mutations. *Blood* 121, 4749-4752.
- Ng, H. H., Ciccone, D. N., Morshhead, K. B., Oettinger, M. A., and Struhl, K. (2005). Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: A potential mechanism for position-effect variegation. *Proceedings of the National Academy of Sciences* 100, 1820-1825.
- Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N. E., Robbins, P. D., and Sakai, T. (1993). CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* 8, 1063-1067.
- Orkin, S. H., and Zon, L. I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 152, 631-644.
- Pokholok, D. K., Harbison, C. T., Levine, S., Cole, M., Hannett, N. M., Lee, T. I., Bell, G. W., Walker, K., Rolfe, P. A., Herbolzheimer, E., et al. (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* 122, 517-527.
- Prebet, T., Sun, Z., Figueroa, M. E., Ketterling, R., Melnick, A., Greenberg, P. L., Herman, J., Juckett, M., Smith, M. R., Malick, L., et al. (2014). Prolonged administration of azacitidine with or without entinostat for myelodysplastic syndrome and acute myeloid leukemia with myelodysplasia-related changes: results of the US Leukemia Intergroup trial E1905. *J Clin Oncol* 32, 1242-1248.
- Ramalho-Santos, M., and Willenbring, H. (2007). On the origin of the term "stem cell". *Cell Stem Cell* 1, 35-38.
- Razin, A., and Riggs, A. D. (1980). DNA methylation and gene function. *Science* 210, 604-610.
- Richon, V. M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R. A., and Marks, P. A. (1998). A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc Natl Acad Sci U S A* 95, 3003-3007.
- Rodriguez, J., Frigola, J., Vendrell, E., Risques, R.-A., Fraga, M. F., Morales, C., Moreno, V., Esteller, M., Capellà, G., Ribas, M., and Peinado, M. A. (2006). Chromosomal Instability Correlates with Genome-wide DNA Demethylation in Human Primary Colorectal Cancers. *Cancer Research* 66, 8462-9468.
- Rose, N. R., and Klose, R. J. (2014). Understanding the relationship between DNA methylation and histone lysine methylation. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1839, 1362-1372.
- Rothbart, S. B., and Strahl, B. D. (2014). Interpreting the language of histone and DNA modifications. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1839, 627-643.
- Russler-Germain, D. A., Spencer, D. H., Young, M. A., Lamprecht, T. L., Miller, C. A., Fulton, R., Meyer, M. R., Erdmann-Gilmore, P., Townsend, R. R., Wilson, R. K., and Ley, T. J. (2014). The R882H DNMT3A mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers. *Cancer Cell* 25, 442-454.
- Shlush, L. I., Zandi, S., Mitchell, A., Chen, W. C., Brandwein, J. M., Gupta, V., Kennedy, J. A., Schimmer, A. D., Schuh, A. C., Yee, K. W., et al. (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* 506, 328-333.
- Slack, J. M. W. (2002). Conrad Hal Waddington: the last Renaissance biologist? *Nat Rev Genet* 3, 889-895.

- Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25, 977-988.
- Tambaro, F. P., Dell'aversana, C., Carafa, V., Nebbio, A., Radic, B., Ferrara, F., and Altucci, L. (2010). Histone deacetylase inhibitors: clinical implications for hematological malignancies. *Clin Epigenetics* 1, 25-44.
- Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. *Cell* 133, 612-626.
- Tefferi, A., Levine, R. L., Lim, K. H., Abdel-Wahab, O., Lasho, T. L., Patel, J., Finke, C. M., Mullally, A., Li, C. Y., Pardanani, A., and Gilliland, D. G. (2009). Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. *Leukemia* 23, 900-904.
- Till, J. E., and Mc, C. E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 14, 213-222.
- Traina, F., Visconte, V., Elson, P., Tabarrok, A., Janowska, A. M., Hasrouni, E., Sugimoto, Y., Szpurka, H., Makishima, H., O'Keefe, C. L., et al. (2014). Impact of molecular mutations on treatment response to DNMT inhibitors in myelodysplasia and related neoplasms. *Leukemia* 28, 78-87.
- Tropberger, P., and Schneider, R. (2013). Scratching the (lateral) surface of chromatin regulation by histone modifications. *Nat Struct Mol Biol* 20, 657-661.
- Vaquerez, J. M., Kummerfeld, S. K., Teichmann, S. A., and Luscombe, N. M. (2009). A census of human transcription factors: function, expression and evolution. *Nat Rev Genet* 10, 252-263.
- Voigt, P., LeRoy, G., Drury, W. J., 3rd, Zee, B. M., Son, J., Beck, D. B., Young, N. L., Garcia, B. A., and Reinberg, D. (2012). Asymmetrically modified nucleosomes. *Cell* 151, 181-195.
- West, A. C., and Johnstone, R. W. (2014). New and emerging HDAC inhibitors for cancer treatment. *J Clin Invest* 124, 30-39.
- Winkler, I. G., Barbier, V., Nowlan, B., Jacobsen, R. N., Forristal, C. E., Patton, J. T., Magnani, J. L., and Levesque, J. P. (2012). Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med* 18, 1651-1657.
- Wouters, B. J., and Delwel, R. (2015). Epigenetics and approaches to targeted epigenetic therapy in acute myeloid leukemia. *Blood* 127, 42-52.
- Wrangle, J., Wang, W., Koch, A., Easwaran, H., Mohammad, H. P., Vendetti, F., Vancrinkinge, W., Demeyer, T., Du, Z., Parsana, P., et al. (2013). Alterations of immune response of Non-Small Cell Lung Cancer with Azacytidine. *Oncotarget* 4, 2067-2079.
- Wu, Y., Alvarez, M., Slamon, D. J., Koeffler, P., and Vadgama, J. V. (2010). Caspase 8 and maspin are downregulated in breast cancer cells due to CpG site promoter methylation. *BMC Cancer* 10, 32.
- Yamaguchi, Y., Kurokawa, M., Imai, Y., Izutsu, K., Asai, T., Ichikawa, M., Yamamoto, G., Nitta, E., Yamagata, T., Sasaki, K., et al. (2004). AML1 is functionally regulated through p300-mediated acetylation on specific lysine residues. *Journal of Biological Chemistry* 279, 15630-15638.
- Yates, J. W., Wallace, H. J., Jr., Ellison, R. R., and Holland, J. F. (1973). Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. *Cancer chemotherapy reports* 57, 485-488.
- Young, N. L., DiMaggio, P. A., Plazas-Mayorca, M. D., Baliban, R. C., Floudas, C. A., and Garcia, B. A. (2009). High throughput characterization of combinatorial histone codes. *Mol Cell Proteomics* 8, 2266-2284.
- Yu, Y., Song, C., Zhang, Q., DiMaggio, P. A., Garcia, B. A., York, A., Carey, M. F., and Grunstein, M. (2012). Histone H5 lysine 56 methylation regulates DNA replication through its interaction with PCNA. *Mol Cell* 46, 7-17.
- Zhang, L., and Long, X. (2015). Association of BRCA1 promoter methylation with sporadic breast cancers: Evidence from 40 studies. *Sci Rep* 5, 17869.
- Zhang, T., Cooper, S., and Brockdorff, N. (2015). The interplay of histone modifications – writers that read. *EMBO reports*.
- Zupkovitz, G., Grausenburger, R., Brunmeir, R., Senese, S., Tischler, J., Jurkin, J., Rembold, M., Meunier, D., Egger, G., Lagger, S., et al. (2010). The Cyclin-Dependent Kinase Inhibitor p21 Is a Crucial Target for Histone Deacetylase 1 as a Regulator of Cellular Proliferation. *Molecular and Cellular Biology* 30, 1171-1181.





# **2** DO HEMATOPOIETIC STEM CELLS GET OLD?

---

Johannes Jung, Sonja Buisman and Gerald de Haan

European Research Institute for the Biology of Ageing, University Medical  
Center Groningen, University of Groningen, the Netherlands

Published in *Leukemia* 31, 529-531 (2017)





In many countries of the world the proportion of elderly people will rise very substantially in the upcoming decades. As a result, the number of patients that present with age-related diseases will also increase. This relates to neurodegenerative conditions such as Alzheimer's disease that many people will instantly link to an aging society, but it also includes multiple hematological syndromes that display clear increases in incidence with advanced age (Figure 1). Whereas in the United States for a long time the leading cause of death has been heart disease, this was recently replaced by cancer (Heron M, 2016). More old people will result in more patients with leukemia and in increasing health care costs for their treatment (Figure 2).

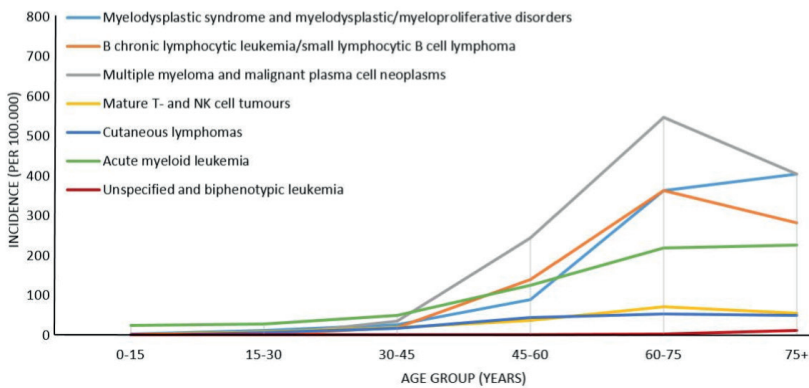


Figure 1:

Observed incidence of several hematological diseases by age in the Netherlands in 2015.

Source: Netherlands Cancer Registry, managed by IKNL © June 2016.

In addition to these clear-cut hematological diseases, there are multiple other (pre)-clinical manifestations that may be affected by malfunctioning of the hematopoietic system. These include for example an increased susceptibility to infections (due to reduced numbers and functioning of lymphocytes) (Frasca et al., 2008), reduced vaccination efficiency (Goodwin et al., 2006) and an increased risk of arteriosclerosis (due to altered macrophage activity), anemia (Tettamanti et al., 2010), and maybe even some neurological conditions (as a result of loss of microglia functioning) (Mosher and Wyss-Coray, 2014).

To explore why many hematological diseases occur much more frequently in older people, we first need to assess what changes with age in blood (precursor) cells. Functionally, hematopoietic stem cells produce

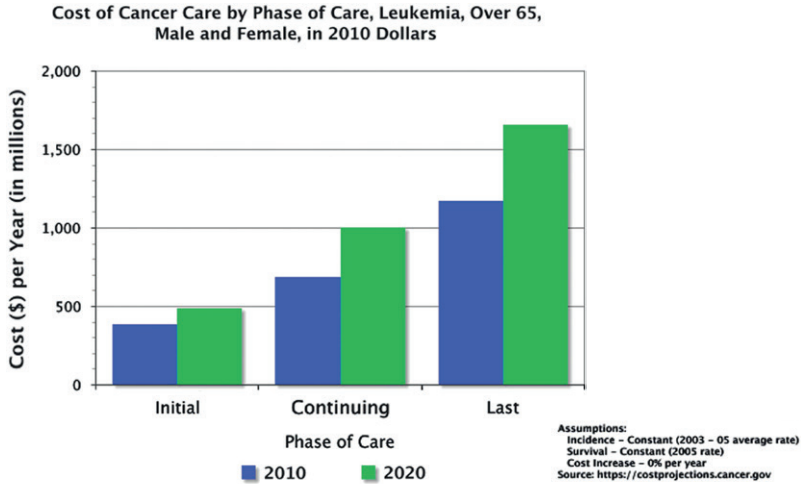


Figure 2:  
 Cost of leukemia care by phase of care in male and female patients older than 65, in 2010 US dollars.

fewer progeny as they age. This has been best studied in mice. While it is clear that old mice do not run out of stem cells, and indeed classical serial transplantation studies have documented that hematopoietic stem cells can outlive their original donor mouse (Harrison, 1979), many age-dependent detrimental stem cell phenotypes have been reported. Most notably, the levels of engraftment upon transplantation of a single, or a low number, of purified hematopoietic stem cells are much lower when the donor cells originate from an old mouse, compared to young cells (Dykstra et al., 2011). These data strongly suggest that the number of mature cells produced per stem cell declines with age. However, it is not only the absolute number of mature produced cells that is declining, aging is also associated with lineage-skewing, which refers to the observation that the relative proportion myeloid and lymphoid cells changes in favor of myeloid cell production (Beerman et al., 2010).

It has not been very well studied to what extent the functional activity of mature, fully differentiated, blood cells such as erythrocytes, platelets, granulocytes and macrophages is reduced upon normal aging.

Most of the above observations have been made in mouse models, and although there is little reason to believe that human hematopoietic stem cells age differently compared to those in mice, it is important to note

that we have not fully assessed to what extent aging of hematopoietic stem cells is evolutionary conserved in these two species.

What is also largely unknown is the extent to which aging of stem cells is conserved across multiple regenerating tissues. Although intuitively one would expect that mechanisms that contribute to stem cell aging may be operating in all adult stem cell populations, it is also possible that major differences exist. For example, where it is generally believed that hematopoietic stem cells are normally largely quiescent, and in fact stem cell activation is believed to be detrimental (Walter et al., 2015), in the intestinal system this appears to be quite the opposite. Intestinal stem cells have been reported to cycle very actively, yet these cells seem to be exempt from the aging process (Clevers, 2013). In muscle stem cells, in contrast, several aging characteristics that are observed in the hematopoietic system appear to be also present (Brack et al., 2007). If the mechanisms that contribute to aging are conserved in multiple tissues, it is conceivable that interventions to prevent stem cell aging in one tissue may in fact also affect those in others.

We hypothesize that the age-dependent loss of hematopoietic homeostasis finds its origin in detrimental molecular events that first occur in primitive hematopoietic stem cells. The impaired ability of aged hematopoietic stem cells to properly balance the choice between self-renewal and differentiation may predispose to hematological and -possibly- other disorders. Therefore, efforts to prevent such age-dependent hematopoietic stem cell deterioration are expected to be beneficial at multiple levels.

Our understanding of the molecular causes that underly stem cell aging is still limited. A great unknown is whether impaired functioning of hematopoietic stem cells results from cell-intrinsic or rather cell-extrinsic causes. This is not only of academic interest but is also highly relevant if approaches are developed to delay, prevent, or indeed reverse, stem cell aging. What would be the cell type to target, the stem cell itself, or the microenvironmental niche cell next to which it lives? Experimental transplantation studies have shown that transplanting old stem cells in a young recipient does not erase functional decline, strongly suggesting that at least a major component that contributes to stem cell aging must be a cell-intrinsic feature (Rossi et al., 2005). However, it is also very clear that the constitution of aged bone marrow is very different compared to young. In aged human bone marrow adipogenesis is much more prevalent than in young, and bones become very brittle upon aging (Rozman

et al., 1989). The molecular and cellular composition of the bone marrow microenvironment, which contains the elusive hematopoietic stem cell niche, has only recently been studied in significant detail (Birbrair and Frenette, 2016) and at current it is far from clear how this microenvironment changes during aging, and how this might contribute to decreased stem cell functioning.

An interesting observation in elderly humans is that the hematopoietic system appears to become more clonal, i.e. leukocytes in the peripheral blood are derived from fewer and fewer stem cells. Initial observations on increased clonal hematopoiesis were based on skewed X-inactivation patterns in elderly females (Busque et al., 1996), but more recently the same phenomenon has been observed using whole genome sequencing approaches (Genovese et al., 2014; Xie et al., 2014). At current it remains unclear whether oligoclonal hematopoiesis is of any clinical relevance. Several reports document very significant clonal dominance in normal elderly people, without any signs of hematological disease (Busque et al., 2012; Jaiswal et al., 2014; van den Akker et al., 2016). In experimental settings it has never been documented that mice that were transplanted with a single, or very few stem cells were more prone to develop hematological disorders.

It is of great interest to assess to what extent stem cell-intrinsic aging parameters may be reversible. An interesting experimental approach showed that hematopoietic stem cells derived from iPS cells generated from aged HSCs, were functionally equivalent to cells derived from iPS cells generated from young HSCs (Wahlestedt et al., 2015). This strongly suggests that at least a major part of the stem cell intrinsic age-dependent decline can be reversed. This also suggest that although aged HSCs appear to display increased levels of DNA damage (Beerman et al., 2014), and indeed DNA repair deficient mice and human show bone marrow pathology (Salob et al., 1992; Zhang et al., 2011), dysfunctioning of normal stem cells during aging is unlikely to result from an accumulation of random genetic mutations (which obviously would not be corrected during reprogramming).

If reprogramming indeed is able to reverse hematopoietic stem cell aging, it seems plausible that epigenetic mechanisms contribute to stem cell aging. Many studies in the last decade have demonstrated that, like any adult cell type, hematopoietic stem cells show quite a distinct gene expression profile. This stem cell transcriptome must be carefully controlled by the collective consequences of a multitude of epigenetic

modifications that compact or relax locally the stem cell genome. Many epigenetic writers and erasers have been shown to play important roles in hematopoietic stem cell activity. This includes for example Ezh2 (Kamminga et al., 2006), Bmi1 (Rizo et al., 2008; Rizo et al., 2009), Cbx7 (Klauke et al., 2013) and Dnmt3a (Challen et al., 2012). It appears very likely that upon a single stem cell division, some of the activating or repressing marks that are deposited or read by these proteins are not properly copied to the daughter cells, which would result in an aberrant i.e., stem cell incompatible, gene expression pattern, and thus to loss of stem cell functionality. We envision that such erosion from a stem cell gene expression profile is not a digital -all or nothing- event, but rather occurs very gradually every time a cell divides.

Due to the unavoidable epigenetic differences that accumulate with each of these cell divisions, it seems very well possible that old hematopoietic stem cells require quantitatively or qualitatively different mitogenic signals from their environment compared to young stem cells. It has been well documented in mice and man that hematopoietic stem cells from fetal liver, cord blood, or newly borns are fundamentally different from adult stem cells (Bowie et al., 2007; Rebel et al., 1996). Some of the molecular circuitry that is associated with these stage/age specific signals has been elucidated (Copley et al., 2013; Kim et al., 2007; Rossi et al., 2005), but it is likely that much remains to be explored. Clinically, this may be of interest when cord blood-derived stem cells in the future will be used to transplant aged recipients. Will these heterochronic transplants cause problems?

In contrast to genetic lesions, aberrant epigenetic modifications can in principle be erased and corrected. This would offer ways to reverse aspects of the aging process. The most profound example of such epigenetic resetting is obviously exemplified by the process of reprogramming adult cells to pluripotency by overexpression of several transcription factors. However, it is also possible to interfere in specific epigenetic pathways by exposing cells to (small) molecules such as histone deacetylase inhibitors or DNA demethylating agents that inhibit specific epigenetic enzymes.

It is interesting to note that the clonal hematopoiesis that is observed in a sizeable fraction of elderly people often is associated with mutations in DNMT3A (Xie et al., 2014). Also, mutations in several other epigenetic genes, including EZH2 (Morin et al., 2010) and TET2 (Kandath et al., 2013; Langemeijer et al., 2011), are frequently found in hematological

disorders. This suggests that mutations in genes coding for epigenetic enzymes may confer a proliferative advantage to a cell (Jung et al., 2016). Such a mutant cell may be considered preleukemic, either due to the fact that the first mutation increases its proliferative activity and thus the odds that this preleukemic cell is hit by a second oncogenic event, or alternatively, the first epigenetic mutation may change the epigenetic landscape of a preleukemic cell in such a way that a second mutation is more oncogenic compared to if it had first occurred in an epigenetically unperturbed cell.

This latter scenario should be testable and would predict that a similar oncogenic insult in an old stem cell would have different pathological consequences compared to if it had first occurred in a young cell. There is actually evidence for this; enforced overexpression of Bcr-abl in old stem cells causes different disease kinetics compared to overexpression in young stem cells (Signer et al., 2007). Thus, it is conceivable that leukemia that originates in the elderly is molecularly and functionally distinct from leukemia in young adults.

We expect that many of the questions raised above will be answered in the not too distant future, as more and more laboratories have become interested in the fundamental question as to how a self-renewing stem cell ages.

## REFERENCES:

- Beerman, I., Bhattacharya, D., Zandi, S., Sigvardsson, M., Weissman, I. L., Bryder, D., and Rossi, D. J. (2010). Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proceedings of the National Academy of Sciences* 107, 5465-5470.
- Beerman, I., Seita, J., Inlay, M. A., Weissman, I. L., and Rossi, D. J. (2014). Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell* 15, 37-50.
- Birbrair, A., and Frenette, P. S. (2016). Niche heterogeneity in the bone marrow. *Annals of the New York Academy of Sciences* 1370, 82-96.
- Bowie, M. B., Kent, D. G., Dykstra, B., McKnight, K. D., McCaffrey, L., Hoodless, P. A., and Eaves, C. J. (2007). Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. *Proceedings of the National Academy of Sciences* 104, 5878-5882.
- Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., and Rando, T. A. (2007). Increased Wnt Signaling During Aging Alters Muscle Stem Cell Fate and Increases Fibrosis. *Science* 317, 807.
- Busque, L., Mio, R., Mattioli, J., Brais, E., Blais, N., Lalonde, Y., Maragh, M., and Gilliland, D. G. (1996). Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. *Blood* 88, 59-65.
- Busque, L., Patel, J. P., Figueroa, M. E., Vasanthakumar, A., Provost, S., Hamilou, Z., Mollica, L.,

- Li, J., Viale, A., Heguy, A., *et al.* (2012). Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet* 44, 1179-1181.
- Challen, G. A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J. S., Bock, C., Vasanthakumar, A., Gu, H., Xi, Y., *et al.* (2012). Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* 44, 25-31.
- Clevers, H. (2013). The intestinal crypt, a prototype stem cell compartment. *Cell* 154, 274-284.
- Copley, M. R., Babovic, S., Benz, C., Knapp, D. J. H. F., Beer, P. A., Kent, D. G., Wohrer, S., Treloar, D. Q., Day, C., Rowe, K., *et al.* (2013). The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. *Nat Cell Biol* 15, 916-925.
- Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M., and de Haan, G. (2011). Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med* 208, 2691-2705.
- Frasca, D., Landin, A. M., Lechner, S. C., Ryan, J. G., Schwartz, R., Riley, R. L., and Blomberg, B. B. (2008). Aging Down-Regulates the Transcription Factor E2A, Activation-Induced Cytidine Deaminase, and Ig Class Switch in Human B Cells. *The Journal of Immunology* 180, 5283-5290.
- Genovese, G., Kahler, A. K., Handsaker, R. E., Lindberg, J., Rose, S. A., Bakhoum, S. F., Chambert, K., Mick, E., Neale, B. M., Fromer, M., *et al.* (2014). Clonal Hematopoiesis and Blood-Cancer Risk Inferred from Blood DNA Sequence. *New England Journal of Medicine* 371, 2477-2487.
- Goodwin, K., Viboud, C., and Simonsen, L. (2006). Antibody response to influenza vaccination in the elderly: A quantitative review. *Vaccine* 24, 1159-1169.
- Harrison, D. E. (1979). Mouse erythropoietic stem cell lines function normally 100 months: loss related to number of transplantations. *Mechanisms of ageing and development* 9, 427-435.
- Heron M, A. R. (2016). Changes in the leading cause of death: Recent patterns in heart disease and cancer mortality. NCHS data brief No. 254.
- Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., Lindsley, R. C., Mermel, C. H., Burt, N., Chavez, A., *et al.* (2014). Age-Related Clonal Hematopoiesis Associated with Adverse Outcomes. *New England Journal of Medicine* 371, 2488-2498.
- Jung, J., Buisman, S., and de Haan, G. (2016). Hematopoiesis during development, aging, and disease. *Exp Hematol* 44, 689-695.
- Kamminga, L. M., Bystrykh, L. V., de Boer, A., Hower, S., Douma, J., Weersing, E., Dontje, B., and de Haan, G. (2006). The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood* 107, 2170-2179.
- Kandath, C., McLellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J. F., Wyczalkowski, M. A., *et al.* (2015). Mutational landscape and significance across 12 major cancer types. *Nature* 502, 333-339.
- Kim, I., Saunders, T. L., and Morrison, S. J. (2007). Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* 130, 470-483.
- Klauke, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., *et al.* (2015). Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. *Nat Cell Biol* 15, 353-362.
- Langemeijer, S. M. C., Jansen, J. H., Hooijer, J., van Hoogen, P., Stevens-Linders, E., Massop, M., Waanders, E., van Reijmersdal, S. V., Stevens-Kroef, M. J. P. L., Zwaan, C. M., *et al.* (2011). TET2 mutations in childhood leukemia. *Leukemia* 25, 189-192.
- Morin, R. D., Johnson, N. A., Severson, T. M., Mungall, A. J., An, J., Goya, R., Paul, J. E., Boyle, M., Woolcock, B. W., Kuchenbauer, F., *et al.* (2010). Somatic mutation of EZH2 (Y641) in Follicular and Diffuse Large B-cell Lymphomas of Germinal Center Origin. *Nature genetics* 42, 181-185.
- Mosher, K. I., and Wyss-Coray, T. (2014). Microglial dysfunction in brain aging and Alzheimer's disease. *Biochemical Pharmacology* 88, 594-604.
- Rebel, V., Miller, C., Eaves, C., and Lansdorp, P. (1996). The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. *Blood* 87, 3500-3507.
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* 111, 2621-2630.



- Rizo, A., Olthof, S., Han, L., Vellenga, E., de Haan, G., and Schuringa, J. J. (2009). Repression of BMI1 in normal and leukemic human CD34+ cells impairs self-renewal and induces apoptosis. *Blood* 114, 1498-1505.
- Rossi, D. J., Bryder, D., Zahn, J. M., Ahlenius, H., Sonu, R., Wagers, A. J., and Weissman, I. L. (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Sciences of the United States of America* 102, 9194-9199.
- Rozman, C., Feliu, E., Berga, L., Reverter, J. C., Climent, C., and Ferran, M. J. (1989). Age-related variations of fat tissue fraction in normal human bone marrow depend both on size and number of adipocytes: a stereological study. *Exp Hematol* 17, 34-37.
- Salob, S. P., Webb, D. K. H., and Atherton, D. J. (1992). A child with xeroderma pigmentosum and bone marrow failure. *British Journal of Dermatology* 126, 372-374.
- Signer, R. A. J., Montecino-Rodriguez, E., Witte, O. N., McLaughlin, J., and Dorshkind, K. (2007). Age-related defects in B lymphopoiesis underlie the myeloid dominance of adult leukemia. *Blood* 110, 1851-1859.
- Tettamanti, M., Lucca, U., Gandini, F., Recchia, A., Mosconi, P., Apolone, G., Nobili, A., Tallone, M. V., Detoma, P., Giacomini, A., et al. (2010). Prevalence, incidence and types of mild anemia in the elderly: the "Health and Anemia" population-based study. *Haematologica* 95, 1849-1856.
- van den Akker, E. B., Pitts, S. J., Deelen, J., Moed, M. H., Potluri, S., van Rooij, J., Suchiman, H. E., Lakenberg, N., de Dijkster, W. J., Uitterlinden, A. G., et al. (2016). Uncompromised 10-year survival of oldest old carrying somatic mutations in DNMT3A and TET2. *Blood* 127, 1512-1515.
- Wahlestedt, M., Norddahl, G. L., Sten, G., Ugale, A., Frisk, M.-A. M., Mattsson, R., Deierborg, T., Sigvardsson, M., and Bryder, D. (2015). An epigenetic component of hematopoietic stem cell aging amenable to reprogramming into a young state. *Blood* 121, 4257-4264.
- Walter, D., Lier, A., Geiselhart, A., Thalheimer, F. B., Huntscha, S., Sobotta, M. C., Moehrer, B., Brocks, D., Bayindir, I., Kaschutnig, P., et al. (2015). Exit from dormancy provokes DNA-damage-induced attrition in hematopoietic stem cells. *Nature* 520, 549-552.
- Xie, M., Lu, C., Wang, J., McLellan, M. D., Johnson, K. J., Wendl, M. C., McMichael, J. F., Schmidt, H. K., Yellapantula, V., Miller, C. A., et al. (2014). Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 20, 1472-1478.
- Zhang, S., Yajima, H., Huynh, H., Zheng, J., Callen, E., Chen, H.-T., Wong, N., Bunting, S., Lin, Y.-F., Li, M., et al. (2011). Congenital bone marrow failure in DNA-PKcs mutant mice associated with deficiencies in DNA repair. *The Journal of Cell Biology* 193, 295-305.





# **3** HEMATOPOIESIS DURING DEVELOPMENT, AGING AND DISEASE

---

Johannes Jung, Sonja Buisman and Gerald de Haan

European Research Institute for the Biology of Ageing, University Medical  
Center Groningen, University of Groningen, the Netherlands

published in *Experimental Hematology* 2016;44:689-695

## **ABSTRACT:**

Hematopoietic stem cells were once considered to be all alike. However, in the mid-90's it became apparent that stem cells from early developmental phases were superior to those from adults, and aged stem cells were defective compared to young (Van Zant et al., 1997). It has since become clear that Polycomb group (PcG-) proteins are important regulators of stem cell functioning. PcG proteins are chromatin-associated proteins involved in writing or reading epigenetic histone modifications. PcG proteins are not only involved in normal blood cell formation, but have also been shown to be involved in cancer, and possibly aging. In this review we describe how the different phases that comprise birth, maintenance, functional decline, derailment, and death of hematopoietic stem cell, are continuous processes that may all be controlled by Polycomb group proteins.

## INTRODUCTION:

When hematopoietic stem cells were first 'discovered' and methods to purify these cells had been developed, it was generally assumed that all hematopoietic stem cells were functionally identical, and contributed to blood cell formation equally during the lifetime of an organism (Harrison, 1983). In a series of elegant experiments, partly published by Gary Van Zant and co-workers in *Experimental Hematology*, it was shown that in fact hematopoietic stem cells do age (Van Zant et al., 1990), that the rate of aging is related to their (strain-dependent) proliferative activity (De Haan and Van Zant, 1999) and that stem cell quiescence is reversible (Van Zant et al., 1992). Collectively, these and other studies led to a model in which it was proposed that any time a hematopoietic stem cell divides, its two daughter cells inherit a somewhat lower stem cell potential, thus directly linking hematopoietic stem cell turnover with loss of stem cell quality (Van Zant et al., 1997). Enhanced cell turnover, due to (serial) transplantation, repeated rounds of chemotherapy, or normal aging, all would lead to loss of stem cell functioning. At the time it was postulated that telomere shortening could provide the molecular clock that would restrict stem activity.

In recent years it has become evident that the classical divide between development, normal aging, and indeed malignant degeneration of the hematopoietic system, may not be very distinct and in fact may rather constitute a continuum. Although molecular mechanisms that specify the birth of the first hematopoietic stem cells during development are likely to be different compared to those required for the maintenance of blood cell formation, key genes have shown to be important for both. Similarly, the gradual demise of blood cell production during aging can often not clearly be distinguished from preclinical conditions that may culminate in hematological malignancies.

The fact that (pre-)hematological malignancies mutations have been found in genes encoding for proteins involved in epigenetic regulation, underscores the relevance of this process for normal blood cell development, blood cell aging, and their causal role in hematological malignancies. We will here postulate that the mitotic clock that restricts hematopoietic stem cell functioning may be governed by the correct deposition of epigenetic modifications at hundreds of loci in stem cell daughter cells. A key class of epigenetic regulators consists of the Polycomb Group (PcG)

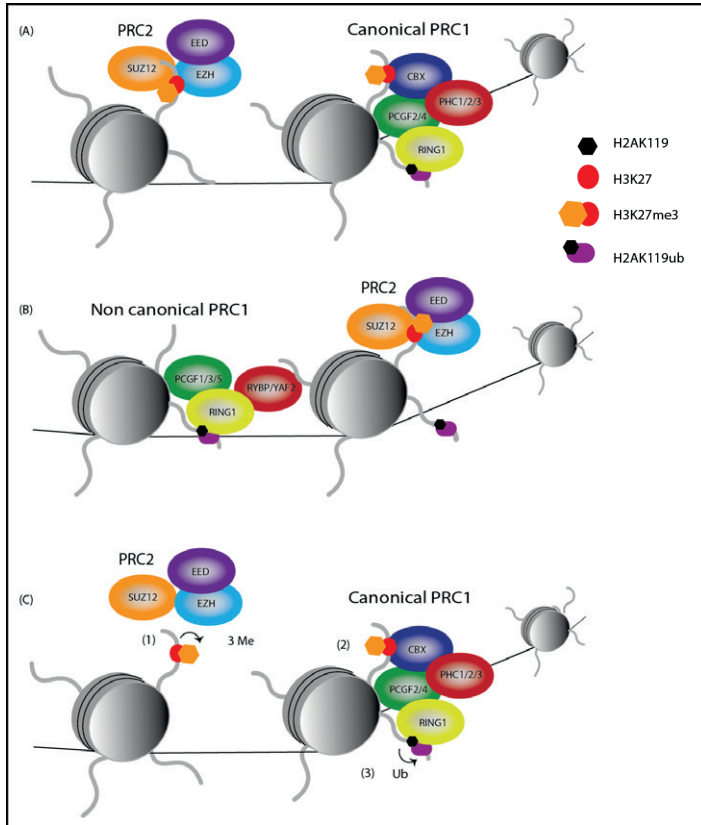
proteins which play essential roles in embryogenesis, adult life, possibly aging, and finally in the development of malignancies. In this review we will provide a brief overview of PcG proteins, the composition of the complexes in which they occur and their functioning in stem cells, with a special focus on benign, aging and malignant hematopoiesis.

## **POLYCOMB GROUP PROTEINS: COMPOSITION AND FUNCTION**

PcG proteins are chromatin-associated proteins, which were first discovered in *Drosophila melanogaster*, as repressors of HOX-genes to control body segmentation along the anterior-posterior axis during development (Lewis, 1978). The function of PcG proteins in mammals as repressors of developmental genes is highly conserved (Morey and Helin, 2010).

PcG proteins are involved in essential cellular processes like senescence (Bracken et al., 2007; Dietrich et al., 2007), cancer (Sparmann and van Lohuizen, 2006; Tan et al., 2011), cell cycle control (Martinez and Cavalli, 2006; Sparmann and van Lohuizen, 2006) and stem cell self-renewal (Rajasekhar and Begemann, 2007). PcG proteins assemble in *Drosophila*, as well as in humans, in multi-protein complexes that are involved in altering chromatin compaction, thereby regulating transcription of genes. During evolution the number of genes coding for the various Polycomb group proteins increased from 15 in *Drosophila* to 37 in mammals (Di Croce and Helin, 2013), establishing substantial functional diversity.

The best characterized complexes are the canonical Polycomb Repressive Complex (PRC) -1 and -2 (**Figure 1**) (Levine et al., 2002). The PRC2 complex is highly conserved from flies to mammals, and consists of four different components, in mammals referred to as SUZ12, EED, EZH1/2 and RbAp 46/48 (Margueron and Reinberg, 2011; Simon and Kingston, 2009). In mammals there are two orthologs of the Enhancer of Zeste subunit (EZH1 and EZH2), which are mutual exclusively present in the complex and are both able to methylate H3K27. Although both proteins share substantial similarities (ca. 65%) and can assemble with the same PRC2 components, they seem to harbor different methyltransferase activities via their SET-domains and are often differentially expressed (Simon and Kingston, 2009). Whereas EZH2 is more abundant in dividing cells and its knockdown leads to a global loss of H3K27me2 and



**Figure 1:**

Composition of the canonical (A) and non-canonical (B) Polycomb repressive complex (PRC) 1. (B) Non-canonical PRC1 variants can be recruited to chromatin by, for instance, KDM2B that results in ubiquitination of lysine 119 on histone 2A, which results in formation of PRC2 with subsequent trimethylation of lysine 27 of histone 3A. (C) According to the hierarchical model the enzymatic subunit of PRC2, EZH1 or EZH2, mediate the methylation of lysine 27 on histone 3A (1), H3K27me<sub>3</sub>. This mark is recognized by the chromodomain of the CBX proteins of PRC1 (2), and will lead to monoubiquitination of lysine 119 on histone 2A (3), H2AK119.

H3K27me<sub>3</sub>, EZH1 is expressed in both dividing and non-dividing cells and its knockdown leads to only marginal changes in the methylation pattern of H3K27 (Margueron et al., 2008).

These findings suggest a model in which EZH2-containing PRC2 is more important for de novo methylation of H3K27 and EZH1-containing PRC2 plays a role in maintaining and restoration of H3K27 methylation



(Margueron and Reinberg, 2011). Interestingly, EZH2 is overexpressed in many cancer cell lines and mutations of EZH2 can be found in myeloid neoplasms (Ernst et al., 2010; Schuettengruber and Cavalli, 2009).

For proper functioning of PRC2 the assembly of all subunits is important (Aloia et al., 2013; Cao and Zhang, 2004; Ketel et al., 2005; Pasini et al., 2004).

Next to the four core components of PRC2, there are additional proteins, which can integrate into PRC2, eventually either facilitating the recruitment of PRC2 to its target genes or increasing the enzymatic activity of EZH1 or -2. For further details on the PRC2 complex, we recommend the review from Margueron et al. (Margueron and Reinberg, 2011).

Whereas the composition of PRC2 is rather constant, the PcG genes that encode for proteins that assemble into PRC1 experienced much more diversification, so that in humans every PRC1 subunit can be assembled by many homologs, leading to more than 180 theoretical permutations of canonical PRC1 (Gao et al., 2012). In fact, it is probably more appropriate to refer to the PRC1 complex as a family of different PRC1 complexes (Schuettengruber and Cavalli, 2009). Nevertheless, in all different types of PRC1 the enzymatic active subunit, RING1A or RING1B, is present and this protein promotes the ubiquitination of the histone tail of H2A on lysine 119 (Gao et al., 2012).

It has become common to distinguish between canonical and non-canonical PRC1 complexes (Comet and Helin, 2014). In mammals, canonical complexes are characterized by the presence of one of the five chromobox-domain proteins (Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8), which are able to recognize the H3K27me3 mark set by PRC2. In addition, canonical PRC1 exists of one of the six members of the PCGF-family (PCGF1-6), one of the three members of the HPH-family (HPH1-3) and the E3-ligase RING1A or RING1B (Cao et al., 2005; Di Croce and Helin, 2013; Wang et al., 2004).

Non-canonical PRC1 complexes contain either RYBP, or the demethylase Kdm2b or E2F6/ L3MBTL (Gao et al., 2012; Morey et al., 2013; Tavares et al., 2012). The exact biological function of all the different PRC1 complexes remains so far elusive. (Figure 1)

Because Cbx proteins can recognize H3K27me3 with their chromobox domain (Fischle et al., 2005) and because PRC1 and PRC2 have largely overlapping target sites, it was originally postulated that transcriptional repression is achieved by the initial trimethylation of H3K27 by PRC2 with subsequent ubiquitination of lysine 119 of H2A through PRC1 (Comet and Helin, 2014; Simon and Kingston, 2013; Wang et al., 2004).

However, after the discovery of non-canonical PRC1 complexes which contain proteins like RYBP, Kdm2b or E2F6/ L3MBTL that bear no chromodomain at all, this classical model had to be revised (Comet and Helin, 2014). Therefore, at current it is unclear how collectively PRC1 and PRC2 are achieving gene repression. For further reading on this topic we recommend the review from Blackledge et al. (Blackledge et al., 2015).

## **POLYCOMB PROTEINS IN DEVELOPMENT AND THEIR ROLE IN STEM CELLS**

As already mentioned above, Polycomb proteins were initially discovered as repressors of Hox genes during early embryonic development in *Drosophila melanogaster* (Lewis, 1978). Mice lacking one of the three PRC2 components Ezh2 (O'Carroll et al., 2001), Eed (Faust et al., 1998) or Suz12 (Pasini et al., 2004) are not viable and show severe defects during gastrulation, emphasizing their role in embryonic development.

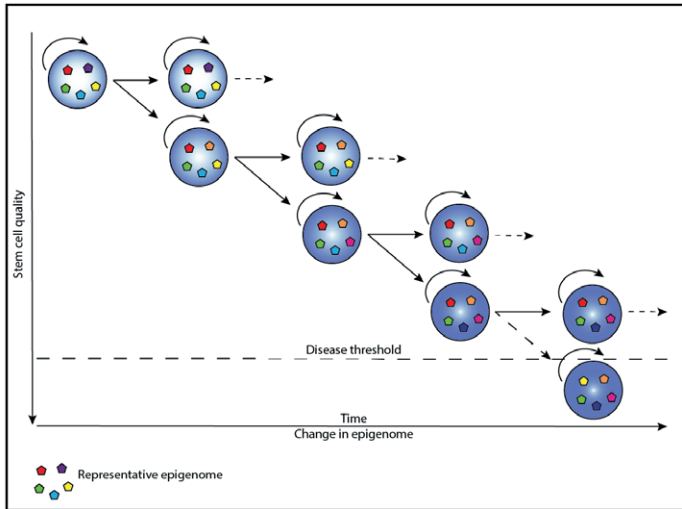
The majority of the PRC1 proteins seem also to have important roles in mouse development, but in later stages. The exception is Ring1B; deletion of Ring1B in murine embryonic stem cells leads to a lethal phenotype due to impaired gastrulation. Although Ring1B and Ring1A proteins are quite homologous, Ring1A is not able to compensate for loss of Ring1B in embryonic stem cells (Voncken et al., 2003).

Bmi1 deficient mice are viable but have a shorten lifespan due to various defects in the nervous and hematopoietic system. For instance, bone marrow, spleen and the thymic cortex of Bmi1<sup>-/-</sup> mice show signs of severe hypoplasia, which is associated with reduced absolute cell count, especially of B-lymphoid and myeloid cells (van der Lugt et al., 1994). Deletion of chromobox proteins in murine embryonic stem cells seems not to impair embryogenesis, but rather affects later stages of development. For instance, knockout of Cbx2 results in retarded growth, homeotic transformations, malformations, and reduced expansion of lymphocytes and fibroblasts *in vitro* (Core et al., 1997). In contrast to Cbx2, deletion of Cbx7 results in an increased body length and a shorten lifespan due to the development of liver and lung carcinomas (Forzati et al., 2012). Expression studies suggest that Cbx7 and Cbx6 are the most abundant Cbx proteins in self-renewing murine embryonic stem cells. Chip-seq experiments showed that the promoters of Cbx4 and Cbx8 were

decorated with the repressive epigenetic mark H3K27me<sub>3</sub>. During differentiation to embryoid bodies the Cbx7 locus was repressed by H3K27me<sub>3</sub>, which coincided with a release of the repression of Cbx4 and Cbx8. These data indicate that Cbx proteins are balancing self-renewal and differentiation in embryonic stem cells and form an intricate feedback regulatory loop (Camahort and Cowan, 2012).

## EPIGENETIC PROTEINS IN AGING

One hallmark of aging is the declining function and physiological integrity of tissue (Lopez-Otin et al., 2013). Because normal tissue function is characterized by proper balancing self-renewal and differentiation of adult stem cells, in aged tissues stem cell functioning is often impaired. In contrast to what was reported in the early days, when hematopoietic stem cell assays did not allow single cell analyses, the aged murine and human hematopoietic system is characterized by an increase of the number of stem cells, but these are impaired in their differentiation towards the lymphoid lineage, resulting in a shift towards the myeloid lineage (de Haan and Van Zant, 1999) (Morrison et al., 1996) (Rossi et al., 2005). As described earlier, normal hematopoietic stem cell function is strongly controlled by epigenetic mechanisms, and therefore dysregulation of epigenetic writers or erasers might contribute to the aged phenotype of stem cells. It seems plausible that the deposition of key epigenetic stem cell modifications in the two stem cell daughter cells is compromised upon (repeated) cell division, in such a way that daughter cells epigenetically and transcriptionally drift away from the pristine ground state which specifies optimal stem cell functioning (**Figure 2**). Indeed, epigenomic profiling of aged murine hematopoietic stem cells showed broader H3K4me<sub>3</sub> peaks and hypomethylation of transcription factor binding sites of genes important for hematopoietic stem cell self-renewal or maintenance. Simultaneously, transcription factor binding sites of genes important for differentiation were hypermethylated. Also, the PRC2 mediated H3K27me<sub>3</sub> mark showed an increase length of coverage and an increased intensity at many promoters of genes (Sun et al., 2014). The expression levels of epigenetic proteins was changed in old hematopoietic stem cells compared to their young counterparts. While expression of EZH1 was increased, expression of EZH2 and Cbx2 was decreased (Sun et al., 2014).



**Figure 2:**

Young “high quality” stem cells give rise to high quality stem cells and differentiate into high quality progeny. This process is at least partly regulated by the proper establishment of key epigenetic modifications. Changes in epigenome occur with each cell division, which can lead to a decline of stem- and progenitor cell quality. Such, potentially stochastic, changes in the epigenome accompany the normal aging process. However, when epigenomic aberrations accumulate and proper stem cell potential is below a certain level (the disease threshold), hematological disease ensues. It is important to note not all cells cross the disease threshold with time and, conversely, young stem cells can also give rise to disease.

During aging also the DNA methylome in human cells is changing. One of the key enzymes promoting de novo methylation of DNA is the methyltransferase DNMT3A. This gene is one of the most mutated genes in cancer patients, notably in hematological malignancies like AML, myelodysplastic syndrome and T-cell acute lymphoblastic leukemia (Kandoth et al., 2013; Ley et al., 2010; Roller et al., 2013; Thol et al., 2011). Hematopoietic stem cells bearing *DNMT3A* mutations have a proliferative advantage over “healthy” hematopoietic stem cells in xenotransplantation studies, indicating that mutant DNMT3A might transform healthy hematopoietic stem cells into pre-leukemic ones, which are still contributing to hematopoiesis but lead to clonal hematopoiesis. Sequencing studies of patient samples from diagnosis, remission and relapse, indicate that DNMT3A mutated pre-leukemic stem cells are relatively chemoresistant and contribute to hematopoiesis after remission,

but also represent a pool of pre-leukemic stem cells for relapse (Shlush et al., 2014). These findings reinforce the notion that normal aging and overt disease are tightly linked and controlled by epigenetic mechanisms.

## POLYCOMB PROTEINS IN CANCER

The first link between Polycomb proteins and the development of cancer was the discovery that Bmi1 collaborates with c-myc in murine lymphomagenesis (van Lohuizen et al., 1991). Although enforced overexpression of BMI1 in murine and human hematopoietic stem cells lead to an increased self-renewal activity, the development of hematological malignancies was not observed, indicating that increased *BMI1* expression in hematopoietic stem and progenitor cells as a single event is not sufficient for malignant transformation (Iwama et al., 2004; Rizo et al., 2008). Yet, Bmi1 is crucial for the maintenance of malignant hematopoietic stem cells *in vivo*, as only leukemic cells derived from Bmi1 wild-type mice are able to generate leukemia in secondary recipients (Lessard and Sauvageau, 2003). In human myeloid leukemia, down-regulation of *BMI1* resulted in reduced self-renewal activity of leukemic stem cells *in vitro* and *in vivo*. (Rizo et al., 2009)

Such a proliferative advantage of *BMI1* overexpressing hematopoietic stem cells may lead to a clonal expansion of premalignant stem cells, which can acquire additional genetic or epigenetic changes that may result in an overt malignancy (Valent et al., 2012).

Indeed, *BMI1* expression in CD34+ cells of CML patients in accelerated phase is higher in comparison to patients in chronic phase (Mohty et al., 2007). Also, in MDS patients higher BMI1 levels correlated positively with disease progression (Mihara et al., 2005). In a subset of chronic lymphocytic leukemia and mantle cell lymphoma, higher BMI1 levels can be detected (Beà et al., 2001; Teshima et al., 2014). Interestingly, high expression of BMI1 can also be observed in non-hematological cancers like non-small cell lung cancer (Vonlanthen et al., 2001) and breast cancer (Paranjape et al., 2014), indicating that BMI1 may play a universal role in different cancers. The mechanism of high BMI1 expression levels is not clear, and BMI1 is not frequently mutated in cancer patients.

Unlike BMI, mutations of EZH2 are found in patients suffering from various lymphoid (diffuse large B-cell lymphoma, follicular lymphoma) (Morin et al., 2010) and myeloid malignancies (myelodysplastic/

myeloproliferative overlap syndrome, myelofibrosis) (Ernst et al., 2010). *EZH2* is also frequently overexpressed in breast, bladder and prostate cancer and its expression levels correlate with higher proliferation rates and affect prognosis (Bachmann et al., 2006). Interestingly, *EZH2* mutations in hematological malignancies can lead to both a loss or gain of function of the methyltransferase activity, which indicates that *EZH2* can act either as a tumor suppressor or an oncogene depending on the cellular context and type of alteration (Ernst et al., 2010).

Such bimodal behavior is also observed for the PRC1 member *Cbx7*. *Cbx7* can act as an oncogene in the hematopoietic compartment and as a tumor suppressor in epithelial cancers. Mice transplanted with bone marrow cells overexpressing *Cbx7* developed different types of leukemia (Klauke et al., 2013). Similarly, in human follicular lymphoma overexpression of *CBX7* can be detected (Scott et al., 2007). On the other hand, loss of *CBX7* expression in some epithelial cancers was associated with a more aggressive phenotype (Pallante et al., 2008). In general, Polycomb proteins are involved in crucial pathways involved in both stem cell regulation and cancerogenesis.

In recent years it has become evident that there is ample crosstalk between the Polycomb protein system and DNA methylation. In embryonic stem cells loci enriched for the *EZH2* mark H3K27me<sub>3</sub> show nearly no overlap with those enriched for DNA methylation. In contrast, in somatic cells and cancer cells there is substantial overlap between these two epigenetic marks (Brinkman et al., 2012; Rose and Klose, 2014; Statham et al., 2012). Furthermore, in embryonic stem cells promoters, which are enriched for H3K27me<sub>3</sub>, show increased gain of DNA methylation during differentiation and carcinogenesis (Mohn et al., 2008; Schlesinger et al., 2007). There are some indications that DNA methylating enzymes can be recruited by PRC2, as it was shown that *EZH2* is able to interact with all three DNMTs and thereby recruiting them to H3K27me<sub>3</sub> loci (Vire et al., 2006).

## **POLYCOMB GROUP PROTEINS AS THERAPEUTICAL TARGETS IN CANCER**

Because epigenetic changes, unlike genetic lesions, are in principle reversible, pharmaceutical perturbation of the activity or composition of Polycomb complexes might be a promising therapeutical approach. Such

an approach may liberate cancer stem cells from excessive self-renewal and in fact introduce differentiation or apoptosis of cancer stem cells. Recent data show that pharmacological inhibition of Polycomb proteins may be a viable therapeutic strategy. However, these potentially drugable chromatin-modifying enzymes also occur in healthy cells. In addition, these enzymes may also display functions beyond their histone-modifying role, and thus intervening in their functioning may result in off-target and side effects (Wouters and Delwel, 2015).

In the near future, increased molecular understanding of epigenetic mechanisms and their role in oncogenesis is likely to result in better and more targeted therapies in cancer patients. Eventually, this knowledge may also let us to reconsider the aging process. If aging of the hematopoietic system can at least partly be explained by potentially reversible epigenetic changes, the aging process might be amenable to interventions aimed to slow down some of its deleterious aspects. This could offer an option to prevent or treat some of the initial steps of a process that may ultimately lead to hematological malignancy.

## ACKNOWLEDGMENTS:

This work was supported by the Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung and Dutch Cancer Society KWF (grant RUG 2014-7178)

## REFERENCES:

- Aloia, L., Di Stefano, B., and Di Croce, L. (2013). Polycomb complexes in stem cells and embryonic development. *Development* 140, 2525-2534.
- Bachmann, I. M., Halvorsen, O. J., Collett, K., Stefansson, I. M., Straume, O., Haukaas, S. A., Salvesen, H. B., Otte, A. P., and Akslen, L. A. (2006). EZH2 Expression Is Associated With High Proliferation Rate and Aggressive Tumor Subgroups in Cutaneous Melanoma and Cancers of the Endometrium, Prostate, and Breast. *Journal of Clinical Oncology* 24, 268-273.
- Beà, S., Tort, F., Pinyol, M., Puig, X., Hernández, L., Hernández, S., Fernández, P. L., van Lohuizen, M., Colomer, D., and Campo, E. (2001). BMI-1 Gene Amplification and Overexpression in Hematological Malignancies Occur Mainly in Mantle Cell Lymphomas. *Cancer Research* 61, 2409-2412.
- Blackledge, N. P., Rose, N. R., and Klose, R. J. (2015). Targeting Polycomb systems to regulate gene expression: modifications to a complex story. *Nat Rev Mol Cell Biol* 16, 643-649.
- Bracken, A. P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theilgaard-Mönch, K., Minucci, S., Porse, B. T., Marine, J.-C., et al. (2007). The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes & Development* 21, 525-530.

- Brinkman, A. B., Gu, H., Bartels, S. J. J., Zhang, Y., Matarese, F., Simmer, F., Marks, H., Bock, C., Gnirke, A., Meissner, A., and Stunnenberg, H. G. (2012). Sequential ChIP-bisulfite sequencing enables direct genome-scale investigation of chromatin and DNA methylation cross-talk. *Genome Research* 22, 1128-1138.
- Camahort, R., and Cowan, C. A. (2012). Cbx proteins help ESCs walk the line between self-renewal and differentiation. *Cell Stem Cell* 10, 4-6.
- Cao, R., Tsukada, Y., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol Cell* 20, 845-854.
- Cao, R., and Zhang, Y. (2004). SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell* 15, 57-67.
- Comet, I., and Helin, K. (2014). Revolution in the Polycomb hierarchy. *Nat Struct Mol Biol* 21, 573-575.
- Core, N., Bel, S., Gaunt, S. J., Aurrand-Lions, M., Pearce, J., Fisher, A., and Djabali, M. (1997). Altered cellular proliferation and mesoderm patterning in Polycomb-M35-deficient mice. *Development* 124, 721-729.
- de Haan, G., and Van Zant, G. (1999). Dynamic Changes in Mouse Hematopoietic Stem Cell Numbers During Aging. *Blood* 93, 3294-3301.
- De Haan, G., and Van Zant, G. (1999). Genetic analysis of hemopoietic cell cycling in mice suggests its involvement in organismal life span. *The FASEB Journal* 13, 707-713.
- Di Croce, L., and Helin, K. (2013). Transcriptional regulation by Polycomb group proteins. *Nat Struct Mol Biol* 20, 1147-1155.
- Dietrich, N., Bracken, A. P., Trinh, E., Schjerling, C. K., Koseki, H., Rappsilber, J., Helin, K., and Hansen, K. H. (2007). Bypass of senescence by the polycomb group protein CBX8 through direct binding to the INK4A-ARF locus. *The EMBO Journal* 26, 1637-1648.
- Ernst, T., Chase, A. J., Score, J., Hidalgo-Curtis, C. E., Bryant, C., Jones, A. V., Waghorn, K., Zoi, K., Ross, F. M., Reiter, A., et al. (2010). Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nature genetics* 42, 722-726.
- Faust, C., Lawson, K. A., Schork, N. J., Thiel, B., and Magnuson, T. (1998). The Polycomb-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development* 125, 4495-4506.
- Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes & Development* 17, 1870-1881.
- Forzati, F., Federico, A., Pallante, P., Abbate, A., Esposito, F., Malapelle, U., Sepe, R., Palma, G., Troncone, G., Scarfo, M., et al. (2012). CBX7 is a tumor suppressor in mice and humans. *J Clin Invest* 122, 612-623.
- Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF Homologs, CBX Proteins, and RYBP Define Functionally Distinct PRC1 Family Complexes. *Molecular Cell* 45, 344-356.
- Harrison, D. E. (1983). Long-term erythropoietic repopulating ability of old, young, and fetal stem cells. *The Journal of Experimental Medicine* 157, 1496-1504.
- Iwama, A., Oguro, H., Negishi, M., Kato, Y., Morita, Y., Tsukui, H., Ema, H., Kamijo, T., Kato-Fukui, Y., Koseki, H., et al. (2004). Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity* 21, 843-851.
- Kandath, C., McLellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J. F., Wyczalkowski, M. A., et al. (2013). Mutational landscape and significance across 12 major cancer types. *Nature* 502, 333-339.
- Ketel, C. S., Andersen, E. F., Vargas, M. L., Suh, J., Strome, S., and Simon, J. A. (2005). Subunit Contributions to Histone Methyltransferase Activities of Fly and Worm Polycomb Group Complexes. *Molecular and Cellular Biology* 25, 6857-6868.
- Klauke, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., et al. (2015). Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. *Nat Cell Biol* 15, 353-362.
- Lessard, J., and Sauvageau, G. (2005). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* 423, 255-260.
- Levine, S. S., Weiss, A., Erdjument-Bromage, H., Shao, Z., Tempst, P., and Kingston, R. E. (2002). The Core of the Polycomb Repressive Complex Is Compositionally and



- Functionally Conserved in Flies and Humans. *Molecular and Cellular Biology* 22, 6070-6078.
- Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* 276, 565-570.
- Ley, T. J., Ding, L., Walter, M. J., McLellan, M. D., Lamprecht, T., Larson, D. E., Kandath, C., Payton, J. E., Baty, J., Welch, J., *et al.* (2010). DNMT3A Mutations in Acute Myeloid Leukemia. *New England Journal of Medicine* 363, 2424-2433.
- Lopez-Otin, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194-1217.
- Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C. L., Dynlacht, B. D., and Reinberg, D. (2008). Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Mol Cell* 32, 503-518.
- Margueron, R., and Reinberg, D. (2011). The Polycomb complex PRC2 and its mark in life. *Nature* 469, 343-349.
- Martinez, A.-M., and Cavalli, G. (2006). The role of Polycomb Group Proteins in Cell Cycle Regulation During Development. *Cell Cycle* 5, 1189-1197.
- Mihara, K., Chowdhury, M., Nakaju, N., Hidani, S., Ihara, A., Hyodo, H., Yasunaga, S. i., Takihara, Y., and Kimura, A. (2005). Bmi-1 is useful as a novel molecular marker for predicting progression of myelodysplastic syndrome and patient prognosis. *Blood* 107, 305-308.
- Mohn, F., Weber, M., Rebhan, M., Roloff, T. C., Richter, J., Stadler, M. B., Bibel, M., and Schubeler, D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. *Mol Cell* 30, 755-766.
- Mohty, M., Yong, A. S. M., Szydlo, R. M., Apperley, J. F., and Melo, J. V. (2007). The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. *Blood* 110, 380-383.
- Morey, L., Aloia, L., Cozzuto, L., Benitah, S. A., and Di Croce, L. (2013). RYBP and Cbx7 define specific biological functions of polycomb complexes in mouse embryonic stem cells. *Cell Rep* 3, 60-69.
- Morey, L., and Helin, K. (2010). Polycomb group protein-mediated repression of transcription. *Trends Biochem Sci* 35, 323-332.
- Morin, R. D., Johnson, N. A., Severson, T. M., Mungall, A. J., An, J., Goya, R., Paul, J. E., Boyle, M., Woolcock, B. W., Kuchenbauer, F., *et al.* (2010). Somatic mutation of EZH2 (Y641) in Follicular and Diffuse Large B-cell Lymphomas of Germinal Center Origin. *Nature genetics* 42, 181-185.
- Morrison, S. J., Wandycz, A. M., Akashi, K., Glogerson, A., and Weissman, I. L. (1996). The aging of hematopoietic stem cells. *Nat Med* 2, 1011-1016.
- O'Carroll, D., Erhardt, S., Pagani, M., Barton, S. C., Surani, M. A., and Jenuwein, T. (2001). The Polycomb-Group Gene Ezh2 Is Required for Early Mouse Development. *Molecular and Cellular Biology* 21, 4350-4356.
- Pallante, P., Federico, A., Berlingieri, M. T., Bianco, M., Ferraro, A., Forzati, F., Iaccarino, A., Russo, M., Pierantoni, G. M., Leone, V., *et al.* (2008). Loss of the CBX7 Gene Expression Correlates with a Highly Malignant Phenotype in Thyroid Cancer. *Cancer Research* 68, 6770-6778.
- Paranjape, A. N., Balaji, S. A., Mandal, T., Krushik, E. V., Nagaraj, P., Mukherjee, G., and Rangarajan, A. (2014). Bmi1 regulates self-renewal and epithelial to mesenchymal transition in breast cancer cells through Nanog. *BMC Cancer* 14, 1-14.
- Pasini, D., Bracken, A. P., Jensen, M. R., Denchi, E. L., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *The EMBO Journal* 23, 4061-4071.
- Rajasekhar, V. K., and Begemann, M. (2007). Concise Review: Roles of Polycomb Group Proteins in Development and Disease: A Stem Cell Perspective. *STEM CELLS* 25, 2498-2510.
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* 111, 2621-2650.
- Rizo, A., Olthof, S., Han, L., Vellenga, E., de Haan, G., and Schuringa, J. J. (2009). Repression of BMI1 in normal and leukemic human CD34+ cells impairs self-renewal and induces apoptosis. *Blood* 114, 1498-1505.
- Roller, A., Grossmann, V., Bacher, U., Poetzinger, F., Weissmann, S., Nadarajah, N., Boeck, L., Kern, W., Haferlach, C., Schnittger, S., *et al.* (2013). Landmark analysis of DNMT3A mutations in hematological malignancies. *Leukemia* 27, 1573-1578.

- Rose, N. R., and Klose, R. J. (2014). Understanding the relationship between DNA methylation and histone lysine methylation. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1839, 1362-1372.
- Rossi, D. J., Bryder, D., Zahn, J. M., Ahlenius, H., Sonu, R., Wagers, A. J., and Weissman, I. L. (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proceedings of the National Academy of Sciences of the United States of America* 102, 9194-9199.
- Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B. E., *et al.* (2007). Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nature genetics* 39, 232-236.
- Schuettengruber, B., and Cavalli, G. (2009). Recruitment of Polycomb group complexes and their role in the dynamic regulation of cell fate choice. *Development* 136, 3531-3542.
- Scott, C. L., Gil, J., Hernando, E., Teruya-Feldstein, J., Narita, M., Martinez, D., Visakorpi, T., Mu, D., Cordon-Cardo, C., Peters, G., *et al.* (2007). Role of the chromobox protein CBX7 in lymphomagenesis. *Proc Natl Acad Sci U S A* 104, 5389-5394.
- Shlush, L. I., Zandi, S., Mitchell, A., Chen, W. C., Brandwein, J. M., Gupta, V., Kennedy, J. A., Schimmer, A. D., Schuh, A. C., Yee, K. W., *et al.* (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* 506, 328-333.
- Simon, J. A., and Kingston, R. E. (2009). Mechanisms of Polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol* 10, 697-708.
- Simon, Jeffrey A., and Kingston, Robert E. (2013). Occupying Chromatin: Polycomb Mechanisms for Getting to Genomic Targets, Stopping Transcriptional Traffic, and Staying Put. *Molecular Cell* 49, 808-824.
- Sparmann, A., and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* 6, 846-856.
- Statham, A. L., Robinson, M. D., Song, J. Z., Coolen, M. W., Stirzaker, C., and Clark, S. J. (2012). Bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq) directly informs methylation status of histone-modified DNA. *Genome Research* 22, 1120-1127.
- Sun, D., Luo, M., Jeong, M., Rodriguez, B., Xia, Z., Hannah, R., Wang, H., Le, T., Faull, Kym F., Chen, R., *et al.* (2014). Epigenomic Profiling of Young and Aged HSCs Reveals Concerted Changes during Aging that Reinforce Self-Renewal. *Cell Stem Cell* 14, 673-688.
- Tan, J., Jones, M., Koseki, H., Nakayama, M., Muntean, A. G., Maillard, I., and Hess, Jay L. (2011). CBX8, a Polycomb Group Protein, Is Essential for MLL-AF9-Induced Leukemogenesis. *Cancer Cell* 20, 563-575.
- Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., *et al.* (2012). RYBP-PRC1 Complexes Mediate H2A Ubiquitylation at Polycomb Target Sites Independently of PRC2 and H3K27me3. *Cell* 148, 664-678.
- Teshima, K., Nara, M., Watanabe, A., Ito, M., Ikeda, S., Hatano, Y., Oshima, K., Seto, M., Sawada, K., and Tagawa, H. (2014). Dysregulation of BMI1 and microRNA-16 collaborate to enhance an anti-apoptotic potential in the side population of refractory mantle cell lymphoma. *Oncogene* 33, 2191-2203.
- Thol, F., Winschel, C., Lüdeking, A., Yun, H., Friesen, I., Damm, F., Wagner, K., Krauter, J., Heuser, M., and Ganser, A. (2011). Rare occurrence of DNMT3A mutations in myelodysplastic syndromes. *Haematologica* 96, 1870-1873.
- Valent, P., Bonnet, D., De Maria, R., Lapidot, T., Copland, M., Melo, J. V., Chomienne, C., Ishikawa, F., Schuringa, J. J., Stassi, G., *et al.* (2012). Cancer stem cell definitions and terminology: the devil is in the details. *Nat Rev Cancer* 12, 767-775.
- van der Lugt, N. M., Domen, J., Linders, K., van Roon, M., Robanus-Maandag, E., te Riele, H., van der Valk, M., Deschamps, J., Sofroniew, M., and van Lohuizen, M. (1994). Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. *Genes & Development* 8, 757-769.
- van Lohuizen, M., Verbeek, S., Scheljen, B., Wientjens, E., van der Guidon, H., and Berns, A. (1991). Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. *Cell* 65, 737-752.
- Van Zant, G., de Haan, G., and Rich, I. N. (1997). Alternatives to stem cell renewal from a developmental viewpoint. *Exp Hematol* 25, 187-192.

- Van Zant, G., Holland, B. P., Eldridge, P. W., and Chen, J. J. (1990). Genotype-restricted growth and aging patterns in hematopoietic stem cell populations of allophenic mice. *The Journal of Experimental Medicine* 171, 1547-1565.
- Van Zant, G., Scott-Micus, K., Thompson, B. P., Fleischman, R. A., and Perkins, S. (1992). Stem cell quiescence/activation is reversible by serial transplantation and is independent of stromal cell genotype in mouse aggregation chimeras. *Exp Hematol* 20, 470-475.
- Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J.-M., *et al.* (2006). The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 439, 871-874.
- Voncken, J. W., Roelen, B. A. J., Roefs, M., de Vries, S., Verhoeven, E., Marino, S., Deschamps, J., and van Lohuizen, M. (2003). Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition. *Proceedings of the National Academy of Sciences* 100, 2468-2473.
- Vonlanthen, S., Heighway, J., Altermatt, H. J., Gugger, M., Kappeler, A., Borner, M. M., Lohuizen, M. v., and Betticher, D. C. (2001). The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer* 84, 1572-1576.
- Wang, L., Brown, J. L., Cao, R., Zhang, Y., Kassisi, J. A., and Jones, R. S. (2004). Hierarchical recruitment of polycomb group silencing complexes. *Mol Cell* 14, 637-646.
- Wouters, B. J., and Delwel, R. (2015). Epigenetics and approaches to targeted epigenetic therapy in acute myeloid leukemia. *Blood* 127, 42-52.





# 4 THE ROLE OF CBX PROTEINS IN HUMAN BENIGN AND MALIGNANT HEMATOPOIESIS

---

Jung J<sup>1</sup>, Buisman SC<sup>1</sup>, Weersing E<sup>1</sup>,  
Dethmers-Ausema B<sup>1</sup>, Zwart E<sup>1</sup>, Schepers H<sup>2</sup>,  
Dekker MR<sup>3</sup>, Lazare SS<sup>1</sup>, Hammerl F<sup>1</sup>, Paeschke K<sup>1</sup>,  
Juraneck S<sup>1</sup>, Skokova J<sup>4</sup>, Kooistra SM<sup>5</sup>,  
Klauke K<sup>1</sup>, Poot RA<sup>3</sup>, Bystrykh LV<sup>1,\*</sup> and de Haan G<sup>1,\*</sup>

## Affiliations:

1. European Research Institute for the Biology of Ageing, University Medical Center Groningen, University of Groningen, the Netherlands
2. Department of Hematology, University Medical Center Groningen, University of Groningen, the Netherlands
3. Department of Cell Biology, ErasmusMC, Rotterdam, the Netherlands
4. University Hospital of Tübingen, Germany
5. Department of Neuroscience, Section Medical Physiology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

\*Corresponding authors: (l.bystrykh@umcg.nl and g.de.haan@umcg.nl).

*In preparation*

## **SUMMARY:**

In this study we demonstrate that among all five CBX Polycomb proteins, only CBX7 possesses the ability to control self-renewal of human hematopoietic stem- and progenitor cells (HSPCs). Xenotransplantation of *CBX7*-overexpressing HSPCs resulted in increased multi-lineage long-term engraftment and myelopoiesis. Gene expression and chromatin analyses revealed perturbations in genes involved in differentiation, DNA and chromatin maintenance, and cell cycle control. *CBX7* is up-regulated in AML and its genetic or pharmacological repression in AML cells inhibited proliferation and induced differentiation. Mass spectrometry analysis revealed novel non-histone protein interactions between CBX7 and the H3K9 methyltransferases SETDB1 and EHMT1 and -2. These CBX7-binding proteins possess a trimethylated lysine peptide motif highly similar to the canonical CBX7 target H3K27me<sub>3</sub>. Depletion of SETDB1 in AML cells phenocopied repression of CBX7. We identify CBX7 as an important regulator of self-renewal and uncover novel, non-canonical crosstalk between epigenetic pathways revealing new therapeutic opportunities for leukemia.

## **SIGNIFICANCE:**

Epigenetic modifiers are important regulators of hematopoietic stem cells and are frequently mutated or aberrantly expressed in hematological malignancies. Because epigenetic changes are in general reversible, they represent putative druggable targets. Here we show that CBX7, known to direct the Polycomb Repressive Complex-1 to loci marked by H3K27me<sub>3</sub>, regulates self-renewal of benign hematopoietic stem as well as leukemic cells. We demonstrate that CBX7 does not only bind to H3K27me<sub>3</sub>, but also interacts with multiple non-histone proteins, including enzymes involved in H3K9 methylation, which themselves harbor trimethylated lysine residues. This non-canonical role for CBX7 mediates crosstalk between Polycomb activity and other pathways involved in epigenetic repression. Disruption of canonical and non-canonical interactions leads to differentiation of leukemic cells, and thus suggests novel therapeutic opportunities.

## INTRODUCTION

Hematopoietic stem cells (HSCs) are able to self-renew and differentiate into all mature blood cells to ensure peripheral blood cell homeostasis during adult lifespan. In these primitive cells, the choice between self-renewal and differentiation must be well balanced to avoid either cytopenia or hyperproliferative conditions like leukemia. Self-renewal and differentiation are accompanied and controlled by a multitude of epigenetic changes of DNA and of histone proteins (Kamminga et al., 2006; Klauke et al., 2013; Rizo et al., 2008; Tadokoro et al., 2007). One important family of epigenetic regulators that is critical for stem cells is represented by the Polycomb group (PcG) genes.

PcG genes encode for chromatin-associated proteins, which assemble in various multimeric protein complexes and contribute to the regulation of gene expression patterns by posttranslational modifications of histone tails (Bracken et al., 2006; Cao et al., 2005).

The two best-characterized PcG protein complexes are the canonical Polycomb Repressive Complex 1 (PRC1) and -2 (PRC2). The canonical PRC1 is characterized by the presence of at least one of the five Polycomb chromobox proteins (CBX2, 4, 6, 7 and 8). Many functional and molecular studies have shown similar and overlapping binding patterns of PRC1- and PRC2-protein containing complexes (Comet and Helin, 2014; Morey et al., 2012). Although the enzymatic activity of many individual epigenetic writers and erasers has been elucidated, our understanding of the biological role and the molecular dynamics of epigenetic protein complexes is still limited.

CBX proteins are characterized as chromodomain-containing proteins, recognizing trimethylated lysine 27 on histone H3 (H3K27me<sub>3</sub>), which is deposited by EZH1/2 (Fischle et al., 2003; Min et al., 2003). After recognition of H3K27me<sub>3</sub> by the CBX proteins, the catalytic subunit of PRC1, RING1A/B, ubiquitinates H2AK119 (Cao et al., 2005) leading to repression of transcription through chromatin compaction and inhibition of RNA Polymerase II (Stock et al., 2007). Beyond this classical PRC2/PRC1 recruitment model, evidence is emerging for a far more diverse and complicated composition and recruitment process. Most notably, it has become apparent that a plethora of distinct PRC1 complexes exist, some of which contain RYBP instead of CBX (Tavares et al., 2012). Furthermore, PRC1 can be present at genomic loci in the absence of any PRC2 activity (Kahn et al., 2016).



Notwithstanding our limited understanding of the complex protein-protein and protein-DNA interactions in which the PcG proteins are involved, it has become evident that PcG proteins are important regulators of self-renewal and differentiation of many types of pluripotent and adult stem cells (Morey et al., 2013). Indeed, deregulation of their expression or mutations in genes coding for PcG proteins can result in cancer development. We have previously shown that overexpression of the H3K27 methyltransferase *Ezh2* in murine HSCs prevents their exhaustion in serial transplantation experiments (Kamminga et al., 2006). Furthermore, both EZH2 and BMI1 are important regulators of self-renewal of normal murine and human hematopoietic stem cells (Rizo et al., 2008). Interestingly, mutations in the *EZH2* gene were later found in patients with myelodysplastic syndromes and acute myeloid leukemia (Cancer Genome Atlas Research et al., 2013; Nikoloski et al., 2010)

More recently, we showed that *Cbx7*, but not *Cbx2*, -4, or -8, is a potent regulator of self-renewal of murine hematopoietic stem cells and its enforced overexpression resulted in increased self-renewal and in phenotypically diverse leukemias (Klauke et al., 2013). In human cells, systematic short hairpin-mediated repression of all CBX proteins in CD34<sup>+</sup> cord blood cells resulted in decreased proliferation and colony-forming unit ability. In this experiment knockdown of *CBX2* was shown to be most detrimental (van den Boom et al., 2013).

Collectively these studies highlight the relevance of PcG proteins, and particularly CBX proteins, in maintaining blood cell homeostasis. As epigenetic changes are in principle reversible, elucidating the function of epigenetic writers, readers, and erasers in the context of healthy and malignant hematopoiesis is indispensable for identifying novel therapeutic targets.

Therefore, in the current study, we asked to what extent different CBX proteins are able to affect the balance between self-renewal and production of mature blood cells of normal human cord blood-derived primitive CD34<sup>+</sup> cells. We identify *CBX7* as a potent inducer of self-renewal. Reversely, repression of *CBX7* in AML cells results in their terminal differentiation. In addition, we identify novel evolutionary conserved non-histone interaction partners of *CBX7*. These novel interaction partners include multiple epigenetic enzymes, most notably SETDB1, EHMT1, and EHMT2, which are all H3K9 methyltransferases that carry a potential lysine site for trimethylation. These sites are in a conserved peptide context, which is similar to H3K9me3 and H3K27me3. Importantly, depletion of SETDB1, similar to

CBX7, also induced differentiation of AML cells, suggesting that at least part of the self-renewal potential of CBX7 is dependent on its interaction with an H3K9-methyltransferase. H3K27me3 and H3K9me3 ChIP-seq and RNA-seq experiments revealed direct and indirect CBX7 targets which comprise a complex network of both classical histone modifications and novel epigenetic interactions that collectively control the balance between self-renewal and differentiation in primitive human hematopoietic cells.

## RESULTS

### **CBX7 enhances self-renewal of human CD34<sup>+</sup> cord blood cells *in vitro***

To assess the role of the five different PRC1-CBX proteins on hematopoietic progenitor function, we overexpressed *CBX2*, *4*, *6*, *7* and *8* in CD34<sup>+</sup> cord blood cells and performed colony-forming unit (CFU) assays. Whereas overexpression of *CBX7* and *CBX8* resulted in increased CFU-frequencies, overexpression of *CBX2* and *-4* resulted in lower CFU-frequencies in comparison to empty vector control (EV). Overexpression of *CBX6* had no discernable effect (Figure 1A). Although *CBX8* overexpression resulted in a slightly higher CFU frequency in comparison to *CBX7*, replating of *CBX7* overexpressing cells from a primary plate resulted in higher CFU-frequency (Figure 1B). In line with these data, *CBX8* overexpressing CD34<sup>+</sup> HSPCs showed no proliferative advantage in comparison to control cells in a cytokine-driven suspension culture, whereas *CBX7* overexpressing CD34<sup>+</sup> HSPCs showed a strong proliferative advantage and could be kept in culture up to ten weeks (Figure 1C and Supplementary Figure 1A). To determine the role of the five different CBX proteins in regulating the most primitive cell compartment, we performed cobblestone area-forming cell (CAFC) assays, that were evaluated 35 days after seeding of transduced and sorted CD34<sup>+</sup> cells. *CBX7* overexpression increased the CAFC frequency ~10 fold, whereas *CBX8* overexpression resulted in a smaller increase in CAFC frequency (Figure 1D). In contrast, overexpression of *CBX4* decreased the cobblestone area-forming cell frequency dramatically (~50-fold), while overexpression of *CBX2* and *CBX6* had no effect. We next tested whether *CBX7* is essential for self-renewal of human primitive CD34<sup>+</sup> cells by performing short hairpin

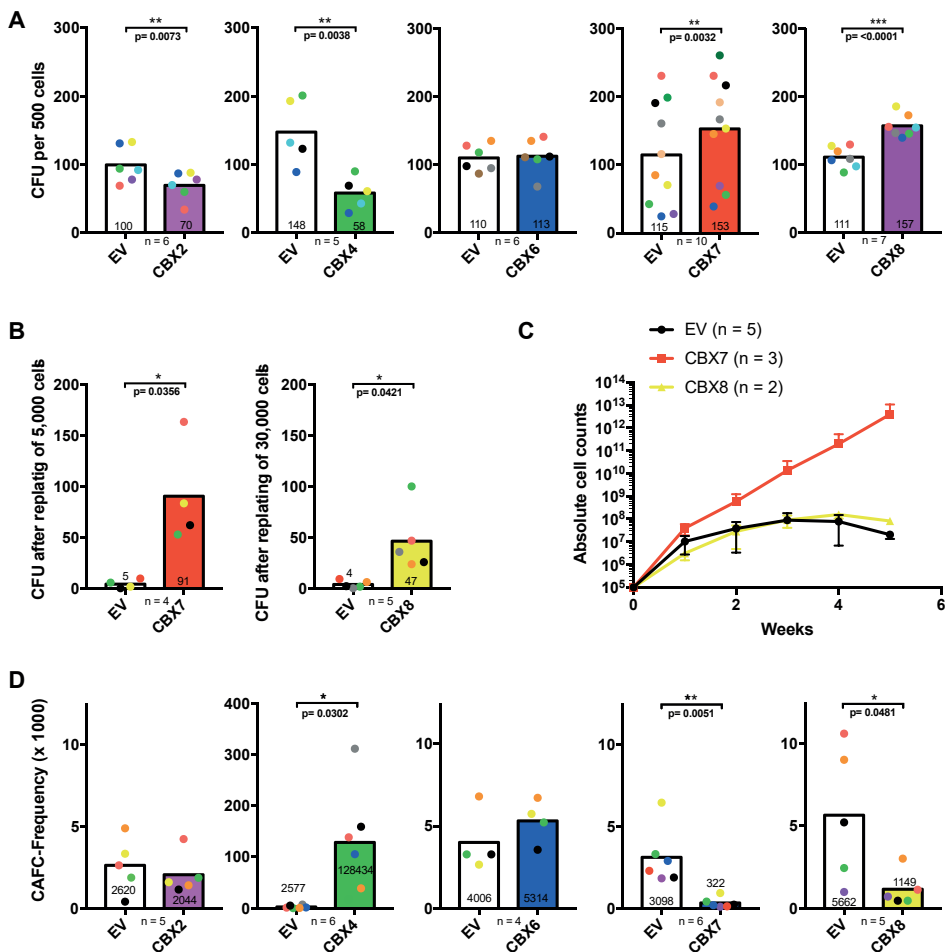
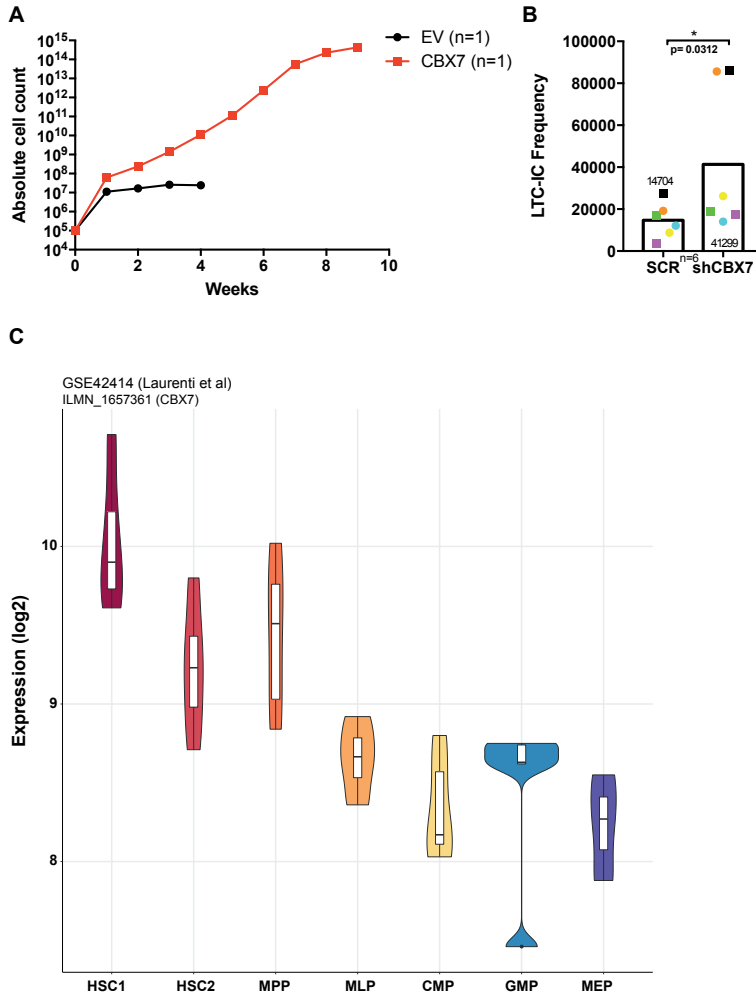


Figure 1:

Enforced retroviral overexpression of *CBX2*, *4*, *6*, *7* or *8* reveals distinct effects on human  $CD34^+$  cord blood-derived progenitors and primitive cells in vitro.

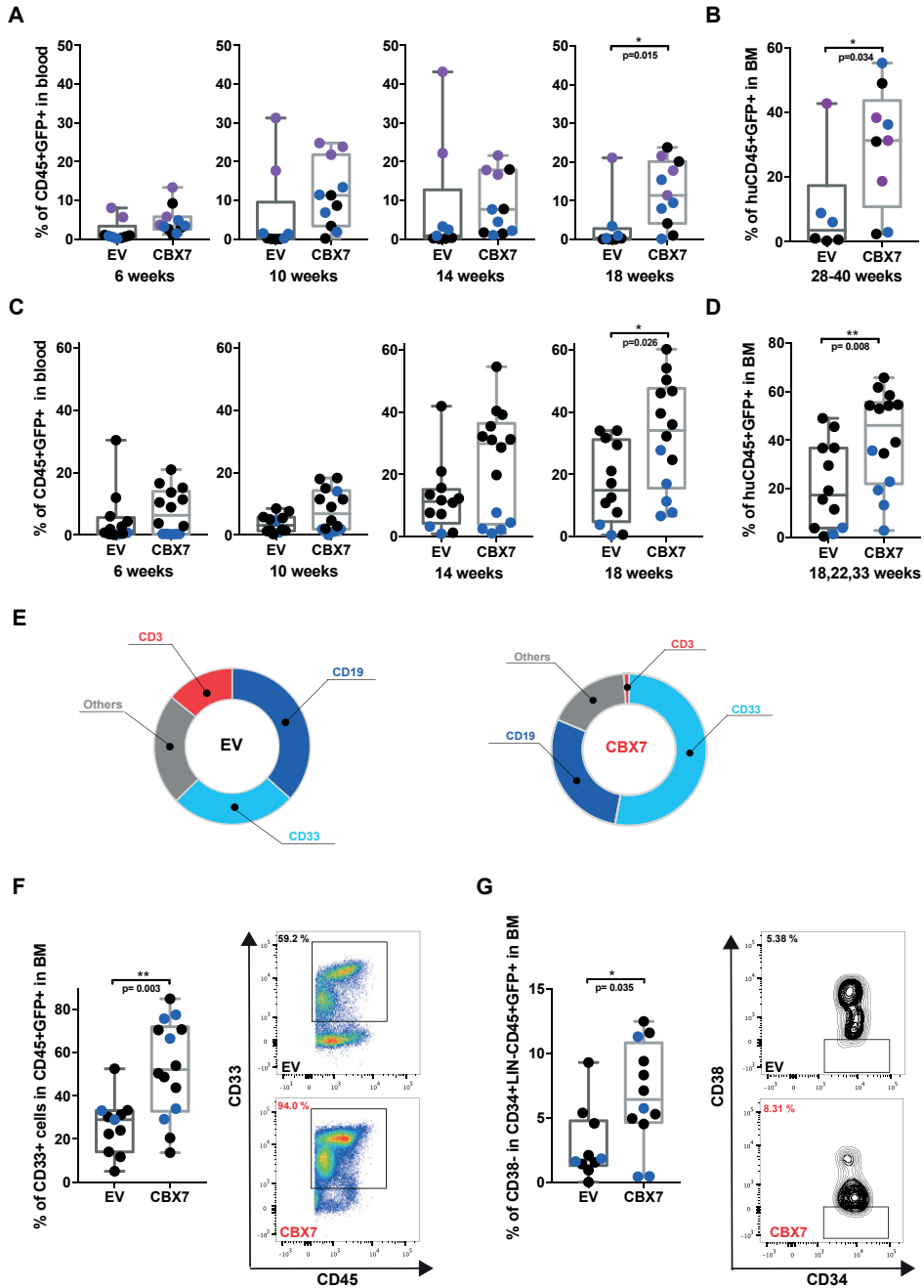
(A) CFU-frequencies of cord blood-derived  $CD34^+$  cells overexpressing *CBX2*, *4*, *6*, *7*, or *8*. (B) CFU-frequencies after replating of 5,000 *CBX7* overexpressing cells or 30,000 *CBX8* overexpressing cells. (C) Absolute cell counts in cytokine-driven liquid culture of cord blood-derived  $CD34^+$  cells transduced with an empty vector (EV), or upon *CBX7* or *CBX8* overexpression. (D) Day 35 cobblestone area-forming cell frequency (CAFC) of cord blood-derived  $CD34^+$  cells overexpressing *CBX2*, *4*, *6*, *7*, or *8*. (The Y-axis indicates the number of cells that need to be plated for a CAFC to develop.)

[Each graph represents multiple independent experiments with different cords. Identically colored circles indicate paired experimental and control samples that originate from the same cord. Statistical analysis was performed using a two-tailed paired t-test.]



Supplementary Figure 1, related to Figure 1

(A) Absolute cell counts in cytokine-driven liquid culture of cord blood-derived CD34<sup>+</sup> cells transduced with an empty vector (EV) or CBX7 overexpressing vector. (B) Frequency of long-term culture initiating cells of cord blood-derived CD34<sup>+</sup> cells upon knockdown with two different short-hairpins against CBX7. The number of cells that need to be plated for one LTC-IC to develop is indicated. Each graph represents multiple independent experiments with different cords. Identically colored circles indicate paired experimental and control samples that originate from the same cord. Rectangles represent knockdown with shCBX7#1, circles represent knockdown with shCBX7#2 (n = 6). Statistical significance was determined using Wilcoxon matched-pairs signed rank test. (C) Log<sub>2</sub> mRNA expression levels of CBX7 (ILMN\_1657361) in different subsets of sorted CD34<sup>+</sup> cord blood derived hematopoietic stem and progenitor cells (data were derived from GSE42414, Laurenti et al, Nature Immunology, 2013).



mediated knockdown of *CBX7* in  $CD34^+$  HSPCs with two distinct short hairpins. Indeed, knockdown of *CBX7* resulted in a 3-fold reduced LTC-IC frequency (Supplementary Figure 1B).

These *in vitro* phenotypes prompted us to analyze endogenous *CBX7* expression levels in different primitive hematopoietic cell subsets using previously published microarray experiments (Laurenti et al., 2013). *CBX7* expression decreased during differentiation from HSCs (HSC1 =  $Lin^- CD34^+ CD38^- CD45RA^- CD90^+ CD49f^+$ , HSC2 =  $Lin^- CD34^+ CD38^- CD45RA^- CD90^- CD49f^+$ ) to more mature MPPs, CMPs and GMPs subsets (Supplementary Figure 1C).

### ***CBX7* overexpression enhances engraftment of human $CD34^+$ cord blood *in vivo***

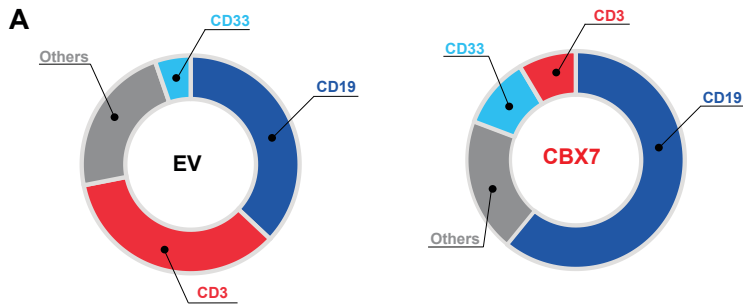
As *CBX7* proved to be the most potent inducer of  $CD34^+$  HSPC proliferation *in vitro*, we assessed whether its overexpression improved engraftment of these cells *in vivo*. To this end, we transduced  $CD34^+$  cord blood cells with a *CBX7* expressing vector and transplanted the equivalent of  $2 \times 10^5$   $CD34^+ GFP^+$  cells in sub-lethally irradiated female NOD-SCID

4

Figure 2:

***CBX7* overexpression induces enhanced long-term engraftment, myelopoiesis and self-renewal of primitive  $CD34^+ CD38^-$  HSPCs *in vivo*.**

(A) Human chimerism levels in the peripheral blood of NSG mice upon transplantation of 200,000  $CD34^+ GFP^+$  *CBX7* overexpressing or *EV* control cord blood cells. (B) Primary recipients were sacrificed after 28-40 weeks, and human engraftment in bone marrow was evaluated. (C) Human chimerism levels in the peripheral blood of NSG mice upon transplantation of 7 days *ex vivo* cultured *CBX7* overexpressing or empty vector control  $CD34^+$  cord blood cells. (D) Human engraftment in bone marrow of mice shown in panel C, combined analysis of mice sacrificed after 18, 22 and 33 weeks post-transplant. (E) Relative engraftment of human  $CD19^+$ ,  $CD3^+$  and  $CD33^+$  cells within human  $CD45^+ GFP^+$  bone marrow cells of mice shown in panel C, combined analysis of mice sacrificed after 18, 22 and 33 weeks post transplantation. (F) Human myeloid engraftment in bone marrow of mice shown in panel C, combined analysis of mice sacrificed after 18, 22 and 33 weeks post transplantation. (G) Frequency of  $CD34^+ CD38^-$  cells in  $huCD45^+ GFP^+ lin^- CD34^+$  cells in bone marrow of mice shown in panel C, combined analysis of mice sacrificed after 18, 22 and 33 weeks post transplantation. (Identically colored circles indicate paired experimental and control samples that originate from the same cord. Statistical analysis was performed using a two-tailed Mann-Whitney test).



**Supplementary Figure 2, related to Figure 2**

(A) Relative engraftment of human CD19<sup>+</sup>, CD3<sup>+</sup> and CD33<sup>+</sup> cells within the human CD45<sup>+</sup>GFP<sup>+</sup> peripheral blood cell fraction, measured 18 weeks post transplantation of CBX7 or EV overexpressing cells into NSG mice (EV n=10, CBX7 n=14, two cords).

IL2r $\gamma$ null (NSG) mice. After transduction, cells were kept in culture for 24 hours before transplantation. We used three freshly isolated cords and from 6 weeks post-transplantation onwards, we measured chimerism every 4 weeks in the peripheral blood (Figure 2A). Mice transplanted with CBX7 overexpressing CD34<sup>+</sup> cord blood cells showed significantly higher engraftment of CD45<sup>+</sup>GFP<sup>+</sup> cells in peripheral blood 18 weeks after transplantation. After 28-40 weeks mice were sacrificed and engraftment of GFP<sup>+</sup> cells in the bone marrow was analyzed. Mice transplanted with CBX7 overexpressing cord blood cells showed ~3 fold (EV 10%, CBX7 29.5%) higher bone marrow engraftment compared to mice transplanted with the EV-control (Figure 2B).

To explore whether CBX7 overexpression would be able to maintain human CD34<sup>+</sup> HSPCs in a more primitive state for a longer period *ex vivo*, we prolonged total *in vitro* culture time from 3 to 7 days and transplanted the equivalent of  $1.5 \times 10^6$  GFP<sup>+</sup>CD34<sup>+</sup> cord blood cells in irradiated NSG mice. Mice transplanted with CBX7 overexpressing cord blood cells displayed significantly higher engraftment in peripheral blood after 18 weeks (Figure 2C). Mice were sacrificed after 18, 22, or 33 weeks and bone marrow cells were analyzed for the presence of human donor-derived cells. Overall, mice transplanted with CBX7 overexpressing cells showed significantly higher levels of GFP<sup>+</sup> cells in the bone marrow (Figure 2D). Furthermore, mice transplanted with CBX7 overexpressing CD34<sup>+</sup> cells showed a reduced percentage of CD3<sup>+</sup> T-cells and a significantly increased percentage of CD33<sup>+</sup> cells in the human CD45<sup>+</sup>GFP<sup>+</sup>

compartment in the bone marrow suggesting that overexpression of *CBX7* enhances myelopoiesis roughly twofold (Figure 2E and F). Similar results were obtained in the peripheral blood after 18 weeks (Supplementary Figure 2A). Furthermore, these mice showed a significantly higher percentage of primitive  $CD38^-$  cells in the  $GFP^+ Lin^-CD34^+$  compartment (Figure 2G), indicating that *CBX7* controls *in vivo* proliferation or maintenance of human primitive hematopoietic stem- and progenitor cells.

## Genome-wide transcriptional consequences of *CBX7* and *-8* overexpression

We next assessed the effect of *CBX7* and *CBX8* overexpression on the transcriptional program of human  $CD34^+$  HSPCs. We used  $CD34^+$  cord blood cells from 5 female newborns and transduced these with *CBX7*, *CBX8* or an empty vector control, sorted 100,000  $CD34^+GFP^+$  cells 96 hours post-transduction and performed transcriptome analysis using high-throughput RNA-sequencing.

Differential expression analysis showed a total of 1463 genes significantly up- and 1183 genes significantly down-regulated when these five independent replicate cords with *CBX7* overexpression were compared with controls.

To annotate *CBX7*-induced up- and down-regulated genes we first used traditional GO enrichment analysis, which reports significantly enriched categories of genes grouped by molecular function or biological process. Screening downregulated genes after *CBX7* overexpression for enriched biological processes revealed that more than 100 genes were associated with “cell differentiation”, including “leucocyte differentiation”, “lymphocyte differentiation”, “epithelial cell differentiation”, “T and B cells differentiation”, “myeloid cell differentiation”, “neuron differentiation”, and “macrophage differentiation” (Figure 3A). Furthermore, we found repression of genes associated with cell cycle arrest and negative regulation of cell cycle. In contrast, *CBX7*-induced upregulated genes revealed transcripts related to cell cycle (“cell cycle”, “cell cycle process”, “G1/S transition of mitotic cell cycle”) (Supplementary Figure 3A) and DNA replication (“DNA replication initiation”, “DNA replication”, “DNA conformation change”, “G1/S transition of mitotic cell cycle”, “chromatin assembly or disassembly”) (Supplementary Figure 3B). The list of upregulated genes did not contain any GO-group associated with differentiation



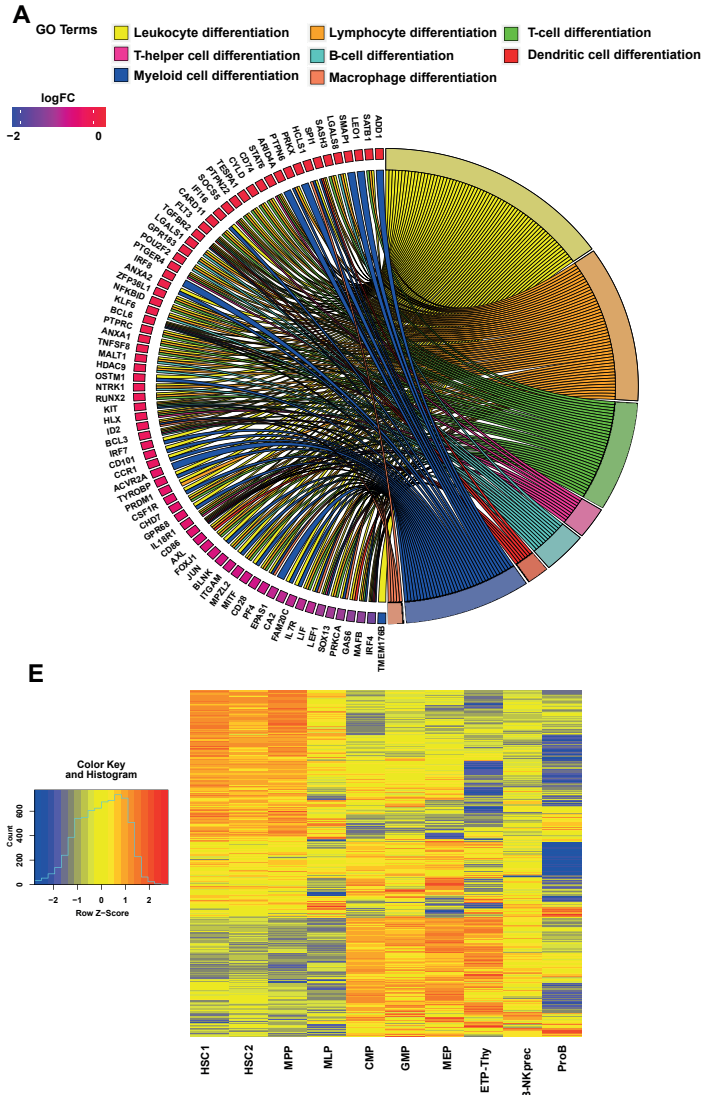
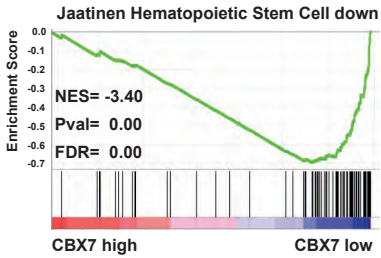
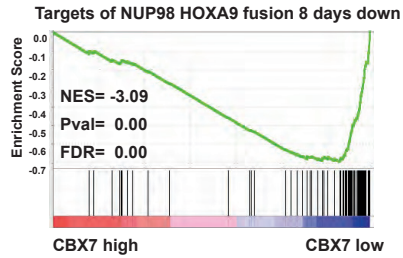
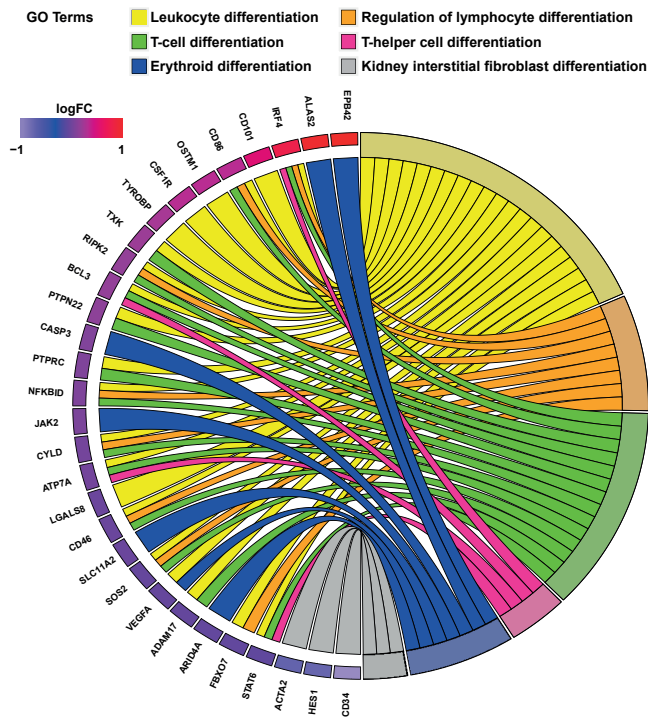


Figure 3:

RNA-Seq analysis of *CBX7*, *CBX8* and *EV* overexpressing *CD34+* HSPC.

Sets of differentially expressed genes were screened for Gene Ontology (GO) enrichment. GO categories were enriched for “differentiation”, “cell cycle”, “chromatin” and “DNA”, shown using GO Chord plots. Preranked gene set enrichment analysis was performed for differentially expressed genes ( $FDR < 0.1$ ) upon overexpression of *CBX7* in comparison to empty vector control cells. (A) GO Chord plot of genes repressed upon overexpression of *CBX7* in comparison to control cells, associated with

**B****C****D**

the GO terms “differentiation” of various hematopoietic cells. (B and C) Gene Set Enrichment plots for 2 out of the top 3 gene sets ( $p < 0.001$ ) with the highest enrichment in genes downregulated upon overexpression of *CBX7* compared to control values. (D) GO Chord plot of genes differentially expressed upon overexpression of *CBX7* in comparison to *CBX8* overexpressing *CD34+* HSPCs associated with GO terms “differentiation” of various cell types. (E) Heatmap containing genes upregulated upon overexpression of *CBX7* and their expression in multiple normal hematopoietic subsets according to previously published data from (Laurenti et al. Nature Immunology, 2013).

**4**





of any kind of hematopoietic cells. These GO annotations are in good agreement with the *in vitro* and *in vivo* observation that overexpression of *CBX7* leads to elevated self-renewal.

The transcriptional consequences of *CBX7* overexpression were—as anticipated—complex, but revealed perturbations of multiple genes known to be crucial for HSC behavior. In total, there were 146 genes downregulated upon *CBX7* overexpression which were related to “transcription”. From those, 36 were transcription factors, and 22 genes were related to histone modifications, 2 of which were Polycomb associated genes (*BMI1* and *SCML1*).

Reversely, genes upregulated upon *CBX7* overexpression revealed 160 genes related to “transcription”. From those, 19 genes contained the term “transcription factor” (including *GATA1*, *CITED2*, *RUNX1*, *MYC*, *HOXA7*). No Polycomb genes were upregulated (except for *CBX7*). Another 19 genes were related to the term “histone modifications” (including *DNMT3A* and *KDM1A*). Further, we observed upregulation of genes important for myelopoiesis, including *CEBPA*, *MPO* and the *G-CSF-receptor*.

Complementary, we performed Gene Set Enrichment Analysis (GSEA) on a pre-ranked list containing all genes differentially expressed (FDR<0.1) upon *CBX7* overexpression in comparison to the empty vector control. We sorted gene-sets with a FDR<0.25 and  $p<0.01$  according to their normalized enrichment-score (NES). Interestingly, GSEA revealed a strong negative correlation (high NES) with a gene set containing genes with low abundance in  $CD133^+$  HSCs, indicating that increased levels of *CBX7* results in maintained repression of genes which are usually barely expressed in HSCs (Figure 3B). Furthermore, we identified two other sets with a high negative correlation, both containing genes downregulated upon overexpression of *HOXA9* either with *NUP98* or *Meis1*, suggesting that *CBX7* targets overlap with targets of these fusion oncogenes (Figure 3C and Supplementary Figure 3C). Furthermore, we found a strong negative correlation with a gene set containing genes lower expressed in leukemic stem cells ( $CD34^+CD38^-$ ) in comparison to leukemic blasts ( $CD34^+CD38^+$ ) suggesting that genes downregulated by *CBX7* overexpression are indeed lower expressed in immature leukemic stem cells than in more differentiated leukemic blasts (Supplementary Figure 3D).

Transcriptome analysis of *CBX8* overexpressing  $CD34^+$  cells resulted in 1444 significantly upregulated and 815 downregulated genes in comparison to empty vector. As the cell biological consequences of *CBX7* and

*CBX8* overexpression were quite distinct, we compared differential gene expression patterns of *CBX7* with *CBX8* and identified a fraction of genes specifically up- (334) or down-regulated (346) by either *CBX7* or *CBX8*. Interestingly, GO-analysis of differentially expressed genes upon *CBX8* overexpression in comparison to EV revealed similar suppression of differentiation pathways. However, lymphoid pathways were suppressed to a lesser extent, and many of the replication/cell cycle genes were missing from the list of upregulated genes (Supplementary Figure 3E). GO-Analysis for genes differentially expressed between *CBX7* and *CBX8* overexpression conditions revealed that genes specifically upregulated upon overexpression of *CBX8* in comparison to *CBX7* were associated with differentiation of hematopoietic cells (Figure 3D, Supplementary Figure 3B). These genome-wide transcriptome analyses are in accordance with *CBX7* overexpressing  $CD34^+$  cells having a higher CFU-replating efficiency and a higher CAFC-frequency compared to *CBX8*.

To further characterize differentially expressed genes upon *CBX7* overexpression, we compared these with steady state transcriptomes of multiple subsets of hematopoietic cell types, using a previously published expression data set as a cross reference (Laurenti et al., 2013). This analysis revealed that 378 transcripts that were higher expressed upon *CBX7* overexpression were preferentially abundant in the more primitive cell compartments (HSC1, HSC2, MPP versus MLP, CMP, GMP, MEP, ETP-Thy, B-NKprec, ProB) (Figure 3E). This suggests their involvement in maintaining elevated levels of self-renewal upon overexpression of *CBX7* in HSCs.

This group of primitive-signature genes includes *CCND2*, *ERG*, *FLI1*, *HMGA2*, *IGF1R*, *LMO2*, *MEIS1*, *MYCN*, *PER2*, *PTK2*, *RUNX1*, *SPINT1*, *ZBTB16*, and *ZEB1*, and all belong to the KEGG pathway GO group “Transcriptional misregulation in cancer”. Also, overexpression of *CBX7* resulted in downregulation of *CD38* and upregulation of *CD34*, two markers which are used for identifying primitive hematopoietic cells in FACS stainings.

In summary, our transcriptome analysis clearly reveals that *CBX7* mediates its activity through repression of genes important for differentiation of hematopoietic cells and upregulation of genes important for cell cycle. These include multiple well-known upregulated oncogenes and downregulated tumor suppressor genes.



## **CBX7 expression is elevated in AML and its repression result in differentiation of AML cells.**

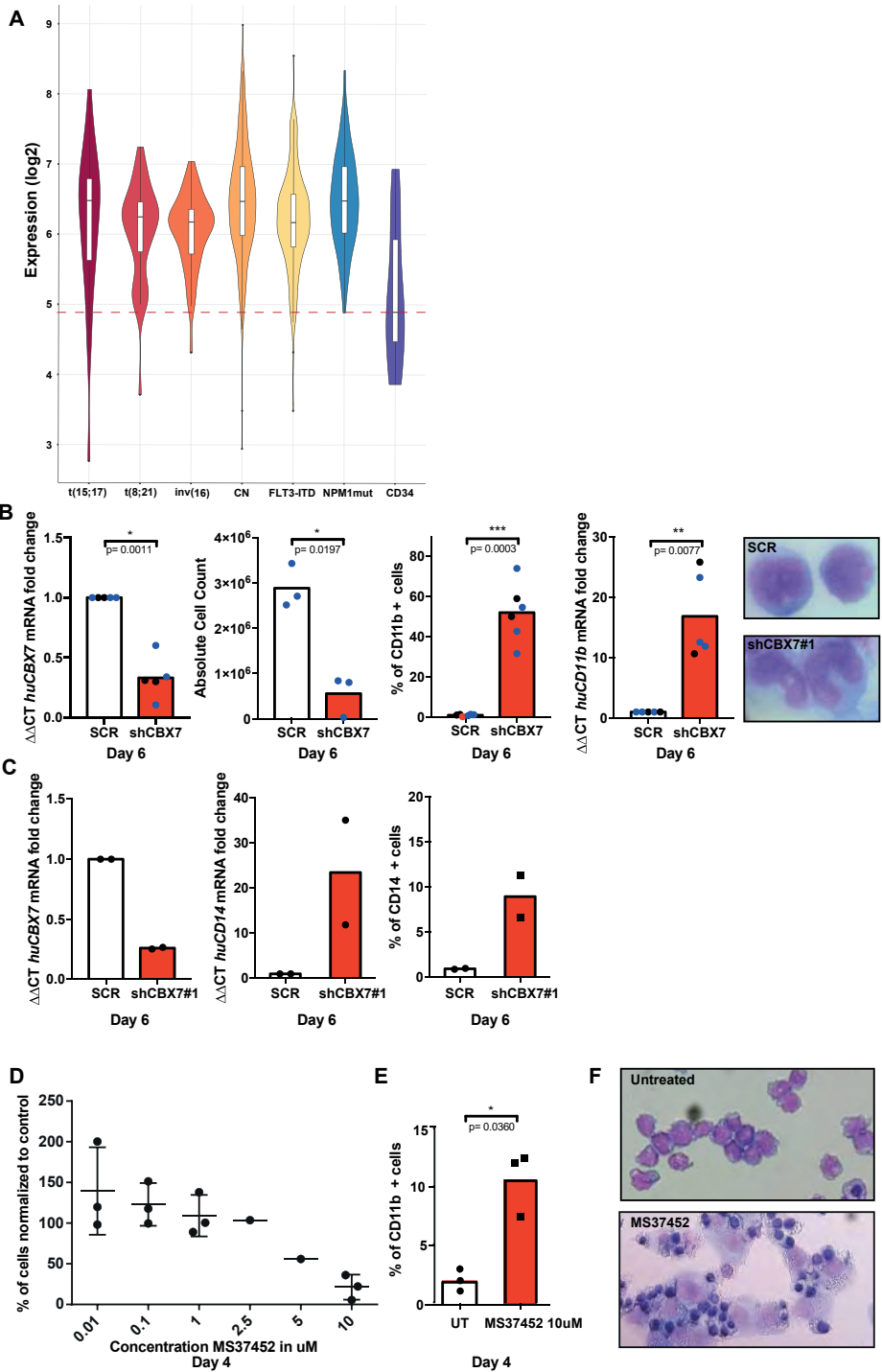
Our data show that CBX7 is able to increase self-renewal of normal human hematopoietic stem and progenitor cells. To explore a putative role for CBX7 in the maintenance of AML cells, we first analyzed *CBX7* mRNA expression levels in AML patient samples in two previously published data sets. In the first data set, containing 529 AML patient samples from patients treated at the Erasmus MC (Rotterdam, The Netherlands), *CBX7* expression was significantly upregulated in comparison to peripheral blood mobilized CD34<sup>+</sup> cells (Verhaak et al., 2009). The highest expression was observed in acute promyelocytic leukemia (t(15;17); APL), leukemias with a normal karyotype and *NPM1* mutated leukemia (Figure 4A). We additionally analyzed data from the Cancer Genome Atlas via Bloodspot (Bagger et al., 2016). Also in this patient cohort, *CBX7* level was significantly higher in multiple AML subtypes (Supplementary Figure 4A).

To explore a functional role for high *CBX7* expression in human leukemia, we assessed to what extent depletion of *CBX7* would affect leukemic cell growth. As *CBX7* is more abundantly expressed in APL (Figure 4A),

**Figure 4:**

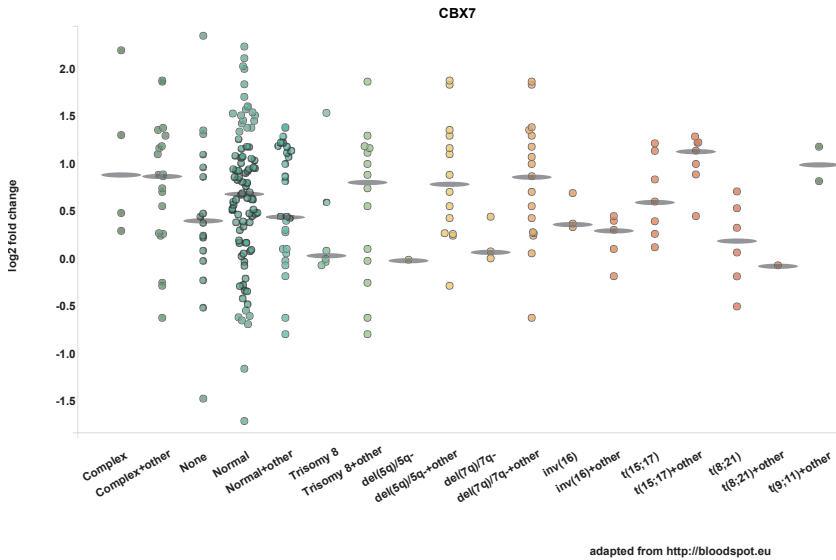
**CBX7 is significantly higher expressed in AML patient samples, and its knockdown induces differentiation in AML cells.**

(A) Analysis of *CBX7* expression in 529 AML patient samples by microarray. Figure 4A shows a violin plot displaying expression of various AML subtypes and CD34<sup>+</sup> peripheral blood mobilized stem cells. (B) Short-hairpin mediated knockdown of *CBX7* mRNA (3B, 1st panel) in HL60 cells results in upregulation of CD11b on mRNA and protein levels (3B, 3rd + 4th panel) after six days. Multiple cells showed signs of differentiation upon knockdown of *CBX7*. (3B, 5th panel) (black= shCBX7#1, blue = shCBX7#2). (C) Short-hairpin mediated knockdown of *CBX7* mRNA (3C, 1st panel) in OCI-AML3 cells results in upregulation of CD14 on mRNA (3C, 2nd panel) and protein levels (3C, 3rd panel) after six days. (D) Growth of OCI-AML3 cells treated with the *CBX7* chromodomain inhibitor MS37452 in different concentrations after four days in culture. (E) Treatment of OCI-AML3 cells with MS37452 at a concentration of 10 $\mu$ M results in increased expression of CD11b. (F) MS37452 induces monocyte/macrophage differentiation in OCI-AML3 cells. After treatment for 4 days with MS37452 at concentration of 10 $\mu$ M, cytospin preparations were stained with May-Grünwald Giemsa stain. Magnification 40x



4



**A****Supplementary Figure 4, related to Figure 4**

(A) Log<sub>2</sub> fold change of *CBX7* mRNA expression in the AML TCGA dataset analyzed with the nearest normal counterpart method (Rapin et al, Blood 2014). (Source <http://bloodspot.eu>)

we downregulated *CBX7* mRNA using a short-hairpin approach in HL60 cells, which harbor a t(15;17) translocation. Knockdown of *CBX7* was associated with a reduced abundance of *CBX7* mRNA to ~40% of normal levels (Figure 4B, first panel) and lower absolute cell numbers after 6 days in culture (Figure 4B, second panel). Strikingly, downregulation of *CBX7* resulted in a significant increase of CD11b expression, which is usually not expressed on primitive APL-blasts but rather on mature monocytes, macrophages and granulocytes (Figure 4B, third and fourth panel). The changes of CD11b protein levels were associated with an increased expression of *CD11b* on mRNA level (Figure 4B, fourth panel), and morphological signs of cellular maturation upon May-Grünwald Giemsa staining (Figure 4 B, fifth panel).

It has been reported that *CBX7* can interact with mutated DNMT3A(R882), but not with wild type DNMT3A in AML patient samples (Koya et al., 2016). Therefore, we decided to downregulate *CBX7* in OCI-AML3 cells, a cell line carrying DNMT3A R882 and mutant NPM1.

Similar as in HL60 cells, upon knockdown of CBX7, OCI-AML3 cells started to differentiate and upregulated the differentiation marker CD14 on protein and mRNA level (Figure 4C). In summary, these experiments indicate that CBX7 is necessary for maintaining leukemic cells in an undifferentiated state, independent of DNMT3A.

We tested whether pharmacological inhibition of CBX7 would result in similar effects as short hairpin mediated repression. To this end we cultured OCI-AML3 cells in the presence of increasing concentrations of the small molecule MS37452, which has been shown to bind to residues in the chromodomain of CBX7 so that protein-protein interactions are disturbed. This loss of normal chromodomain function resulted in derepression of PRC target genes in prostate cancer cells (Ren et al., 2015). In OCI-AML3 cells MS37452 resulted in loss of cell growth in a time- and dose-dependent manner. Furthermore, MS37452 treatment induced differentiation in leukemic cells, as evidenced by upregulation of the differentiation marker (and CBX7 target) CD14 and by the strong increase of cells with a highly differentiated morphology (Figure 4E and F).

## **CBX7 interacts with trimethylated non-Polycomb proteins**

To further unravel the molecular mechanism by which CBX7 exerts its potent activity and taking into account that PcG proteins are known to operate in large protein complexes, we decided to identify proteins directly interacting with CBX.

We performed label-free mass-spectrometry analysis of benzoase treated proteins that co-precipitated with FLAG-tagged CBX7, FLAG-tagged CBX8 and FLAG-tagged CBX4, using murine and human cells. A protein fraction that co-precipitated with FLAG-tagged GFP was used as a negative control. To prioritize candidates, we first removed proteins with low spectral counts (<10% of the cumulative spectral count) and then ranked proteins in relation to their spectral counts. We compared all MS sets and screened for consistent binding partners of both murine and human CBX proteins. As expected, multiple members of PRC1 and -2 complexes were identified, including PCGF1, PCGF2, PCGF6, SCML2, PHC1, PHC2, PHC3, BMI1, RING2, RING1, EED, PCGF5, and SUZ12. The finding of those known CBX interaction partners confirmed that other proteins in the pull downs could be potential members of CBX-containing protein complexes. Interestingly, a considerable number of histone modifiers (63 proteins), transcription

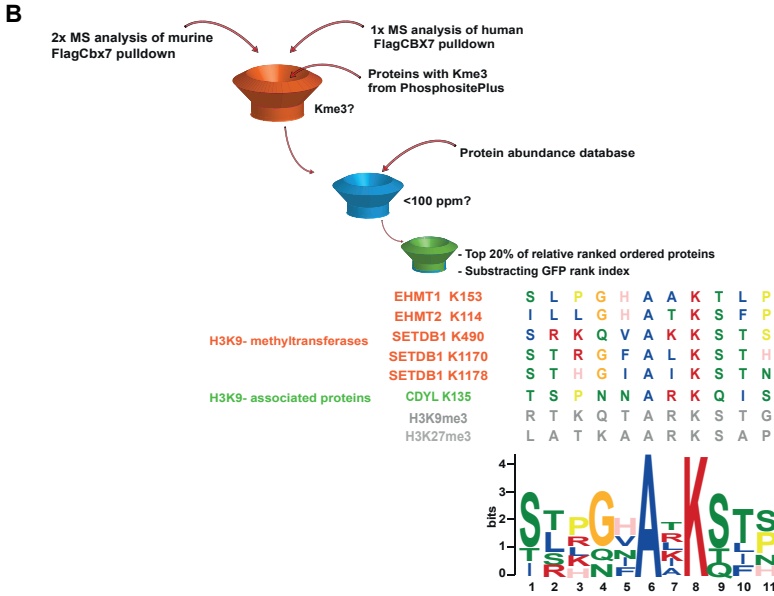
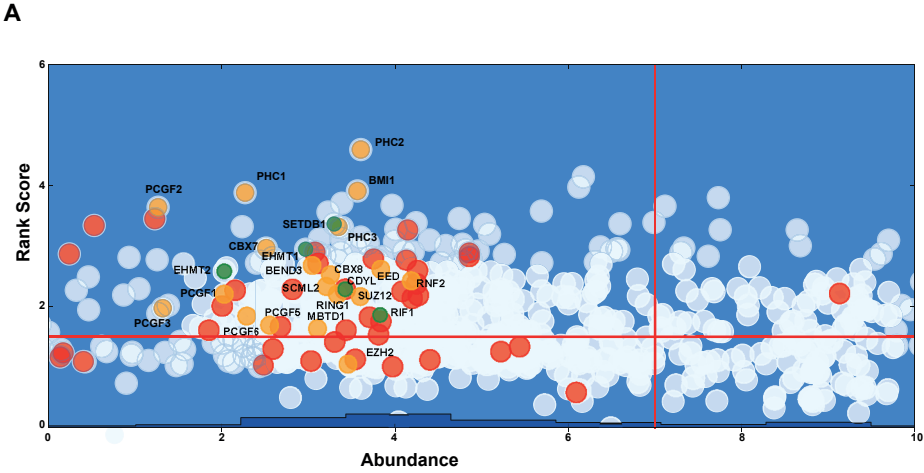
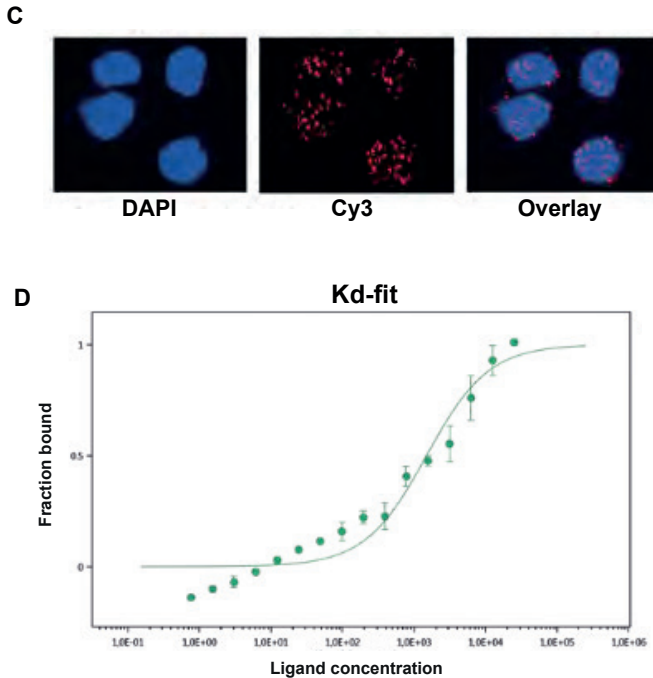


Figure 5:

Mass spectrometry analysis of FLAG-pulldowns reveals multiple H3K9 methyltransferases as CBX7-binding partners harboring a trimethylated-lysine embedded in a motif highly similar to H3K9me3 and H3K27me3

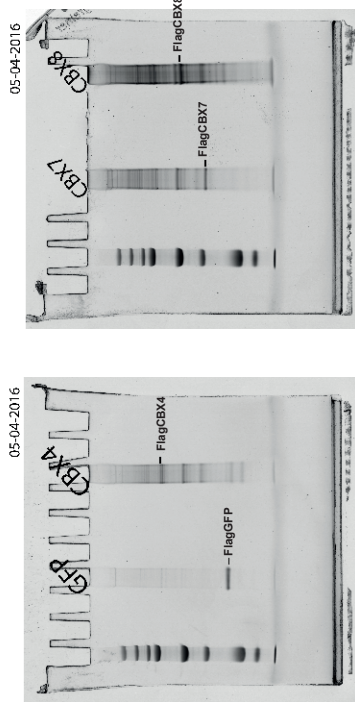
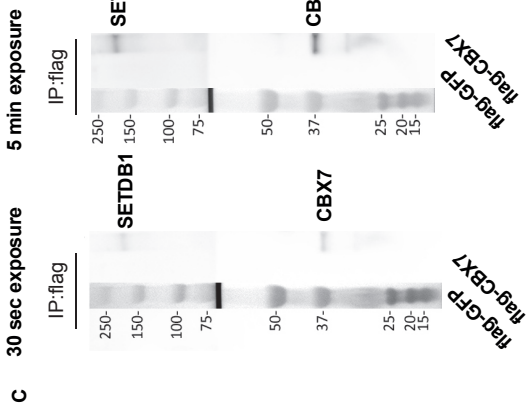
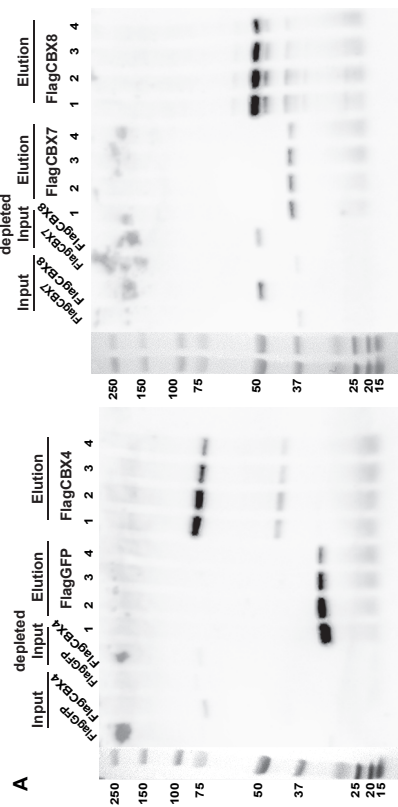
(A) Search for putative interaction partners of CBX proteins by label-free mass spectrometry. The 2D plot depicts CBX7 interaction partners ranked on the basis of their cumulative rank score (derived from the frequency of spectral counts, corrected for GFP control samples) and the average abundance of these proteins in the human PaxDB database. The top-left corner represents priority candidates.



Red symbols indicate known transcription factors, orange symbols refer to Polycomb proteins, and green symbols are proteins with known lysine trimethylation sites. (B) Schematic overview of the analysis resulting in the identification of tri-methylated CBX7-binding proteins, the corresponding peptides and their CBX7 tri-methylated binding motif. (C) Duolink proximity ligation assay (PLA) of endogenous SETDB1 and CBX7 performed in HL60 cells. Each PLA signal (Cy3, red) is indicative of one detected interaction event. Nuclei (blue) are stained with DAPI. (D) Dissociation constant (Kd)-fit of labelled SETDB1 used at a constant concentration of 25nM and (unlabelled) CBX7 titrated until a final concentration of 25 $\mu$ M (1:1) determined by Microscale Thermophoresis (MST).

factors (20 proteins: GTF3C1, YY1, ZFPM1, MGA, GATA1, BPTF, GTF3C5, AHCTF1, LDB1, ADNP, ELF2, GTF3C3, PELP1, E4F1, CBFA2T3, GTF3C4, PRDM2, NCOR1, CDYL, HLTf) and DNA repair associated proteins (DDB1, RPS3, RFC1, MSH6, LIG3, TRIP12, BRCA2, RNF169, MRE11A, INO80, BRCA1, POLB, EPC2) were also detected (Figure 5 A).

Since canonically CBX7 binds to the trimethylated lysine of H3K27 through its chromodomain, we hypothesized that the chromodomain could potentially associate with other trimethylated lysines in non-histone proteins when they contain a peptide context similar to H3K27.



Therefore, we screened the list of CBX7 human and murine binding partners for proteins harboring a putative trimethylated lysine using the PhosphoSitePlus database (Hornbeck et al., 2015). This screen revealed a list of 218 human and murine trimethylated proteins, corresponding to 335 known trimethylated human and murine peptides. We only considered proteins with high spectral counts (top 15% of the relative rank ordered proteins) and low protein abundance (<100 ppm, pax-db.org, average of all samples) and corrected for the binding of each candidate to GFP. This strict filtering narrowed our list down to four proteins (CDYL, SETDB1, EHMT1 and EHMT2) (Figure 5B). We then applied the same filtering for murine Cbx7-binding proteins and identified SETDB1, EHMT1 and EHMT2 as evolutionary conserved binding partners. When using less strict filtering rules (top 70%) we also identified CDYL as a binding partner of murine Cbx7. Interestingly, this list of trimethylated CBX7 interaction partners contains three H3K9 methyltransferases (EHMT1, EHMT2, SETDB1) and one H3K9me3 associated proteins (CDYL). Next to our approach to use spectral counts for identifying putative interaction partners, we also calculated the relative enrichment in the exponentially modified protein abundance index (emPAI) in the CBX7-sample over the control-sample (FlagGFP). All our candidate proteins (SETDB1, EHMT1, EHMT2, CDYL) showed a relative enrichment of at least 12.

We next assessed whether these binding partners to human CBX7 contain a common signature. Indeed, the consensus-binding motif for CDYL, EHMT1, EHMT2, SETDB1 was found to be S[LT]PGHA.Kme3ST[PS], which is highly similar to the peptide sequences to which human CBX7 is known to bind: A[RILFYV]Kme3[ST], (Kaustov et al., 2011) (Figure 5B). The similarity of these motifs suggests that CBX7 interacts with these non-histone proteins via the chromodomain. To validate the interaction of SETDB1 and CBX7 in FLAG-tagged CBX7 overexpressing K562 cells

#### Supplementary Figure 5, related to Figure 5

(A) Western blot of nuclear lysates of K562 cells after FLAG-immunoaffinity purification of FLAG-CBX7, FLAG-CBX8, FLAG-CBX4 and FLAG-GFP. (B) Coomassie-stained SDS-polyacrylamide gels of FLAG-CBX7, FLAG-CBX8, FLAG-CBX4 and FLAG-GFP purification Eluates 1-3 were combined. (C) Western blot analysis of combined eluates (1-3) upon FLAG-immunoaffinity purification of FLAG-tagged CBX7 and FLAG-tagged GFP. The upper part of the membrane was probed with an antibody against SETDB1, the lower part of the membrane was stained against CBX7.

we stained the immunoblots for CBX7 and SETDB1 (Supplementary Figure 5C).

Finally, we used two independent approaches to confirm that CBX7 and SETDB1 are indeed bona fide binding partners. First, we performed Proximity Ligation Assays (PLA) of endogenous SETDB1 and CBX7 in HL60 cells and found intense co-staining throughout the nucleus (Figure 5C). To assess whether CBX7 and SETDB1 are direct interaction partners we used Microscale Thermophoresis. In a cell-free environment these two proteins showed strong binding to each other, with a dissociation constant ( $K_d$ )-fit of labelled SETDB1 and (unlabelled) CBX7 of 1.5  $\mu$ M, which in fact is much lower than the previously reported  $K_d$ -value of CBX7 to H3K27me3 (Figure 5D) (Kaustov et al., 2011).

In summary, our data show that human Polycomb CBX7 is able to interact with H3K9 methylation-associated proteins that harbor a trimethylated lysine. These interactions expand the role of CBX far beyond reading H3K27me3 and identify a non-canonical role for CBX7 in providing crosstalk to epigenetic pathways governing H3K9 methylation.

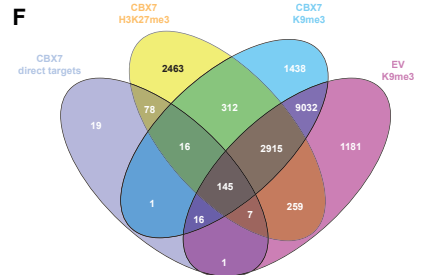
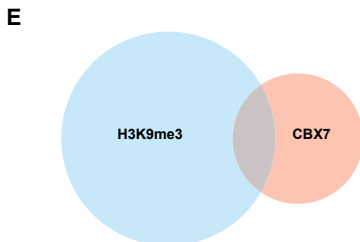
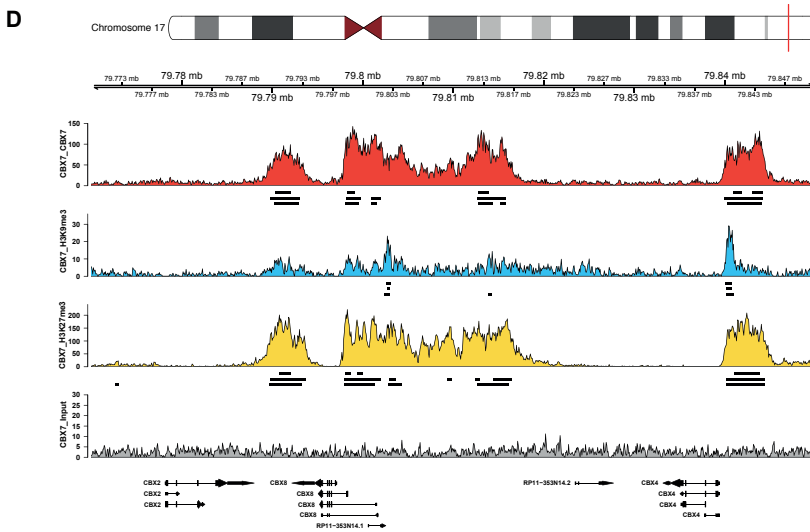
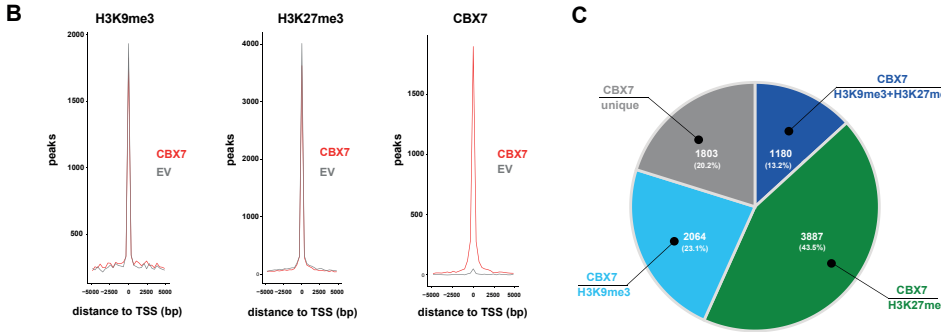
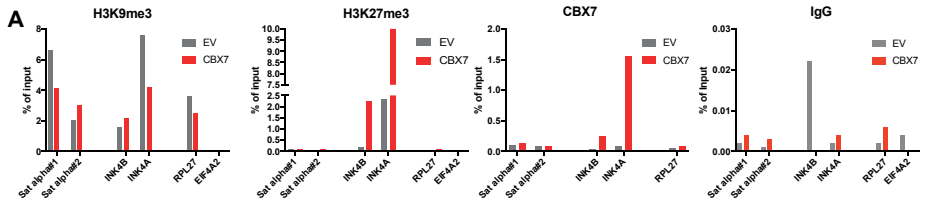
## Identification of CBX7 target loci and their association with H3K9me3 and H3K27me3

To identify genes directly controlled by CBX7 and to unravel their association with repressive histone marks, we performed multiple ChIP-Seq

Figure 6:

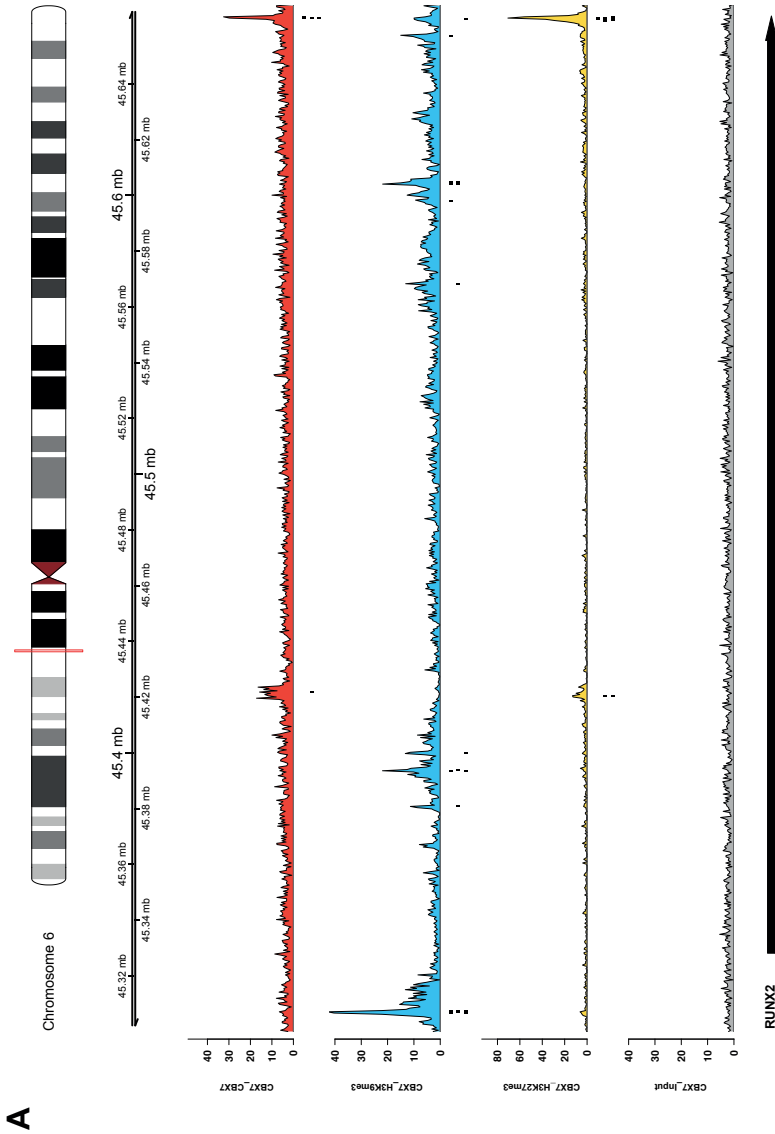
Identification of CBX7 genome-wide binding sites in primary human CD34+ cells and their association with H3K9me3 and H3K27me3.

(A) Chip-qPCR validation of selected positive and negative H3K9me3, H3K27me3, and CBX7 target loci, and IgG (control). (Data from one representative experiment are shown). (B) Genome-wide distribution of H3K9me3-, H3K27me3- and CBX7- peaks to nearest TSS in bps. (C) Pie-chart showing absolute and relative numbers of genome-wide CBX7 peaks and their overlap with H3K9me3 and/or H3K27me3 peaks. (D) Merged ChIP-Seq tracks of CBX7, H3K9me3, H3K27me3-experiments compared to input controls highlighting the genomic CBX2, CBX4 and CBX8 locus. Peak calling tracks are shown for each biological replicate individually. (E) Euler diagram showing overlap of TSS marked with CBX7 and H3K9me3, using a +/- 5000 bp threshold. (F) Venn diagram showing overlap of genes marked with H3K27me3 (CBX7 H3K27me3) and H3K9me3 (CBX7 H3K9me3) in CBX7 overexpressing CD34+ HSPCs, H3K9me3 in control CD34+ HSPCs (EV H3K9me3) and direct targets of CBX.





experiments, all using primary transduced and expanded CD34<sup>+</sup> cord blood cells. As we found that CBX7 interacted with multiple H3K9 methyltransferases, we did not only analyze canonical H3K27me3 peaks, but also searched for loci covered with H3K9me3 upon overexpression of *CBX7* in primary cells. We pooled CD34<sup>+</sup> cord blood cells from different donors, transduced these with *CBX7* overexpressing or empty





control vectors, expanded these cells and sorted CD34<sup>+</sup>GFP<sup>+</sup> cells before cross-linking.

Three independent replicate experiments were performed, each with different cord blood batches. Before deep sequencing we tested each sample for enrichment at known target loci by qPCR (Figure 6A). Following sequencing, we only considered peaks that were present in at least two independent ChIP-Seq samples, and when the adjusted p-value was below 0.05. We detected only few CBX7 peaks in control samples and these were widespread across the genome. A substantial number of these peaks were not associated with promoter regions (classified by distances within 5kb of the transcriptional start site (TSS)). In contrast, peaks were strongly enriched at the TSS upon overexpression of *CBX7*, indicating that CBX7 acted specifically at core promoter regions. *CBX7* overexpression did not significantly alter the genome-wide occupancy of H3K27me3 and H3K9me3 in relation to TSSs (Figure 6B).

We next searched genome-wide for loci that were targeted by CBX7 and in addition covered by H3K9me3 or H3K27me3. We observed that 23 % of all CBX7 peaks were overlapping with H3K9me3, while 44 % were overlapping with H3K27me3. Furthermore, 13 % of all CBX7 peaks were associated with both H3K9me3 and H3K27me3 (Figure 6C), accumulating in ~1/3 of all CBX7 peaks being associated with H3K9me3. In agreement with our data, analysis of published ENCODE datasets of H3K9me3 and H3K27me3 ChIP-seq experiments in hematopoietic cell lines (K562 and GM12878) also showed partial overlap of genes decorated with H3K27me3 and H3K9me3 peaks (data not shown).

We then asked whether CBX7 and H3K9me3 were co-localized around TSSs, which would provide further evidence of a joint gene regulatory function. Indeed, in *CBX7* overexpressing CD34<sup>+</sup> cells ~20% of all TSSs marked with CBX7 were also marked with H3K9me3 (Figure 6E).

These molecular patterns are compatible with a model in which H3K9 methyltransferases act as binding partners of CBX7, at least for a subset of the genomic sites bound by CBX7. Interestingly, the genomic regions around TSSs of *CBX4* and *CBX8* loci were decorated with CBX7, as well as with H3K27me3 and H3K9me3, suggesting that *CBX7* antagonizes the two other *CBX* genes, whose expression resulted in a reduction in the CAFC-frequency upon overexpression (Figure 6D and Figure 1D).

To refine the list of direct targets of CBX7, we performed an integrative analysis of RNA-Seq and ChIP-Seq data and searched for genes that

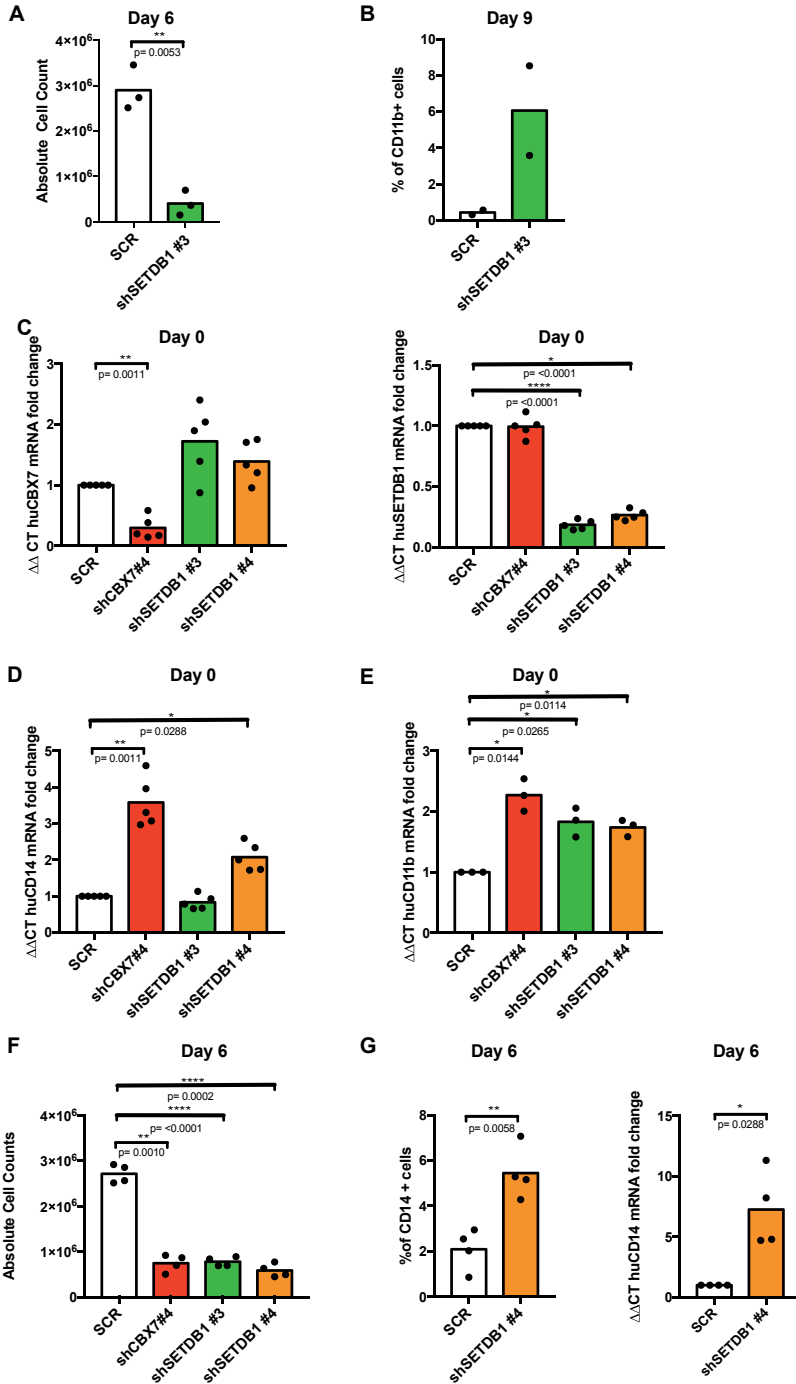
were downregulated upon overexpression of *CBX7* while at the same time covered with *CBX7* ChIP-seq peaks. Out of 1183 repressed genes, 220 showed *CBX7* peaks within 5kb around their transcriptional start site. Interestingly, an additional 63 genes were not marked within a region of 5kb around the TSS but instead around or within the gene body (Supplementary Figure 6A), indicating that *CBX7* may also influence gene expression from regulatory regions outside the immediate vicinity of the TSS. All these 283 genes are likely to be primary targets of *CBX7* in human HSPCs (Supplementary Table 1).

The large majority of these primary targets (246 out of the 283) were also marked by H3K27me<sub>3</sub> within 5kb around the gene body, confirming the well know-interaction of CBX with the Polycomb repressive mark set by EZH2. Interestingly, and in agreement with our finding that *CBX7* directly interacts with various H3K9 methyltransferases, 178 (i.e. 62%) of these direct *CBX7* targets were also marked with H3K9me<sub>3</sub> (Figure 6F). Most of those genes were marked by both H3K9me<sub>3</sub> and H3K27me<sub>3</sub>, yet some repressed genes were exclusively marked with H3K9me<sub>3</sub> (Figure 6F). Furthermore, we found 17 direct targets of *CBX7* which showed increased H3K9me<sub>3</sub> signal intensities upon overexpression of *CBX7*.

Collectively, these molecular signatures reveal functional non-canonical cross talk between Polycomb CBX proteins and H3K9 methylation, as first suggested by the physical interaction of *CBX7* and *SETDB1*, *EHMT1*, and *EHMT2*.

### **SETDB1 and CBX7 share functional activity**

As we identified the H3K9 methyltransferase *SETDB1* as a novel *CBX7* interacting protein, and as we found that approximately one third of the *CBX7* genomic target loci were also covered by H3K9me<sub>3</sub>, we evaluated the function of *SETDB1* in leukemic cells. Mutations in *SETDB1* are associated with the development of clonal hematopoiesis and *SETDB1* shows higher expression in leukemic stem cells compared to leukemic blasts in AML patient samples (Eppert et al., 2011; Steensma et al., 2015). Here we set out to investigate whether shRNA mediated knockdown of *SETDB1* in myeloid leukemic cells would phenocopy the effects observed upon *CBX7*-repression. Indeed, *SETDB1* knockdown strongly impaired proliferation of HL60 cells (Figure 7A). Strikingly, *SETDB1* knockdown resulted in increased expression of CD11b, similar as *CBX7* does in HL60



cells (Figure 7B). Furthermore, *SETDB1* knockdown increased expression of CD11b in OCI-AML3 cells.

Also, similar to *CBX7*, knockdown of *SETDB1* in OCI-AML3 cells reduced proliferation and induced increased *CD14* mRNA expression immediately after transduction as well as six days later on protein level (Figure 7C right panel, 7D, 7G). We next confirmed that the *CD14* locus is a direct *CBX7* target, as in primary CD34<sup>+</sup> cells *CD14* is in the top three downregulated genes upon overexpression of *CBX7* and is marked with *CBX7*, H3K27me3 and H3K9me3 (Supplementary Figure 6B). Surprisingly, knockdown of *SETDB1* resulted in increased expression of *CBX7* suggesting the presence of feedback-loops (Figure 7C). These experimental data indicate that *CBX7* and *SETDB1* jointly repress genes that are important for differentiation of leukemic cells towards mature myeloid cells.

## DISCUSSION

In this study, we identify *CBX7* as a regulator of self-renewal in normal and leukemic hematopoietic cells. We describe the complex molecular architecture of *CBX7*-induced self-renewal and discover a novel, biologically relevant, non-canonical role for *CBX7* as a binding partner of multiple H3K9 methyltransferases, including *SETDB1*.

Polycomb *CBX* proteins are key components of the PRC1 complex, where their function is believed to be essential for recruitment of PRC1

**Figure 7:**

### Repression of *SETDB1* phenocopies repression of *CBX7* in leukemic cells

(A) Absolute cell numbers after 6 days of culturing 150,000 HL60 cells upon short hairpin-mediated knockdown of *SETDB1*. (B) Percentage of CD11b<sup>+</sup> HL60 cells after 9 days in culture upon knockdown of *SETDB1*. (C) Fold change of *CBX7* mRNA (left panel) and *huSETDB1* mRNA (right panel) expression in OCI-AML3 cells 24 hours after transduction with multiple short hairpins targeting *CBX7* or *SETDB1*. (D+E) Fold change of *CD11b* (D) and *CD14* (E) mRNA expression in OCI-AML3 cells 24 hours after transduction with multiple short hairpins targeting *CBX7* or *SETDB1*. (F) Absolute cell numbers after 6 days of culturing 150,000 OCI-AML3 cells upon short hairpin-mediated knockdown of *SETDB1* or *CBX7*. (G) Percentage of CD14<sup>+</sup> cells (left panel) and fold change of *CD14* mRNA expression (right panel) upon knockdown of *SETDB1* in OCI-AML3 cells six days after sort. (All statistical analyses were performed using paired t-test, two-tailed)

to H3K27me3-modified genomic loci. Thus, the chromobox domain contained in all CBX proteins is able to recognize H3K27me3 modifications deposited by EZH1/2 as part of the PRC2 complex, which contributes to repression of target genes. Whereas the *Drosophila* genome contains a single *cbx* gene, during evolution amplification of CBX homologs has occurred in mammals. CBX2, -4, -6, -7, and -8 have all been described to be part of the PRC1 complex, and it is likely that various assemblies of PRC1 have distinct biological targets. In this project, we investigated the role of all five PRC1-CBX proteins in regulating human CD34<sup>+</sup> HSPCs. We show that CBX7 is uniquely able to enhance cell growth of primitive hematopoietic cell subsets. Additionally, transplantation of CBX7 overexpressing CD34<sup>+</sup> cells resulted in enhanced long-term engraftment, multi-lineage differentiation potential, and an increased frequency of myeloid CD33<sup>+</sup> cells and primitive CD34<sup>+</sup>CD38<sup>-</sup> cells in the bone marrow. These results are reminiscent of data of mouse *Cbx7* which we reported earlier (Klaue et al., 2013), and establish CBX7 as an important evolutionary conserved regulator of self-renewal of human CD34<sup>+</sup> HSPCs.

Overexpression of CBX7 resulted in repression of genes associated with differentiation and led to an upregulation of genes involved in cell cycle and DNA replication. ChIP-seq analysis showed that ~1/3 of the repressed differentiation-associated genes were direct CBX7 targets. Furthermore, many genes which were upregulated upon overexpression of CBX7 are preferentially expressed by primitive hematopoietic cell subsets, and thus are likely to contribute to maintenance of the primitive phenotype. Overexpression of CBX8 resulted in transcriptional consequences distinct from those observed after CBX7 perturbation, illustrating that different CBX proteins regulate the expression of different genes, which likely explains the cell biological differences that we observed.

Our *in vitro*, as well as *in vivo*, data indicate that CBX7 regulates self-renewal activity of primitive cells. As we show that CBX7 represses genes important for differentiation, we hypothesized that CBX7 may also play a role in AML, where self-renewal is enhanced and conversely, differentiation is repressed. Here we show that knockdown of CBX7 in leukemic cell lines affects their proliferation and results in derepression of genes that are normally expressed on differentiated cells.

The molecular mechanism by which CBX7 represses differentiation-inducing genes remains to be elucidated, but our studies strongly suggest that the interplay between the canonical, H3K27me3-mediated, and a newly

discovered, non-canonical, H3K9 mediated pathway plays an important role. Whereas the *Drosophila* Polycomb Cbx protein can only recognize H3K27me3 *in vitro* but not H3K9me3, biochemical studies have revealed that multiple mammalian CBX homologs can also bind to H3K9me3 in cell free systems, each with different binding affinities (Bernstein et al., 2006; Kaustov et al., 2011). So far, no H3K9 methyltransferases were described to interact with CBX proteins *in vivo*. As CBX proteins interact with trimethylated lysine residues on histone proteins via their chromodomain, we hypothesized that CBX proteins might also interact with non-histone proteins harboring a trimethylated lysine embedded in a motif highly similar to histone proteins. Indeed, our mass spectrometry analysis revealed multiple of such candidates. Interestingly, all four evolutionary conserved CBX interacting proteins (EHMT1 -a.k.a GLP-, EHMT2 a.k.a. G9A, SETDB1, and CDYL) have been shown to physically interact and are strongly associated with H3K9 methylation (Fritsch et al., 2010).

We focused our further studies on the interaction between CBX7 and SETDB1. SETDB1 is an H3K9 methyltransferase that is best known for its role in repressing the expression of endogenous retroviral elements in the genome (Collins et al., 2015). Interestingly, both SETDB1 and CBX7 have been identified as regulators of embryonic stem cell states (Bilodeau et al., 2009), but the role of SETDB1 in hematopoiesis has only recently emerged. Interestingly, mutations in *SETDB1* have been associated with clonal hematopoiesis in elderly individuals (Steensma et al., 2015). Recently, it has been shown that deletion of *Setdb1* in murine hematopoietic stem cells results in bone marrow failure (Koide et al., 2016).

Biochemical studies have revealed that the chromodomain of CBX7 has high affinity for a trimethylated 24 amino-acid peptide, representing exactly the consensus amino-acid sequence of SETDB1 (amino acids 1157 to 1181). In fact, the affinity of CBX7 for this sequence is higher for peptides representing the amino-acid sequence of H3K27me3 or H3K9me3 (Kaustov et al., 2011). Three lysines residues of SETDB1 have been shown to be trimethylated (K490, K1170 and K1178), all could serve as putative binding sites for CBX7 (Hornbeck et al., 2015).

In accordance with the direct *in vivo* interaction between CBX7 and SETDB1, nearly one third of all CBX7 target loci were simultaneously covered with H3K9me3. In addition, the fact that 62 out of the 95 differentially expressed direct CBX7 target genes associated with differentiation were marked by both CBX7 and H3K9me3, strongly suggest that



self-renewal of human HSPCs is dependent on CBX7-mediated joint repression of target loci by methylation of both H3K27 and H3K9.

Reversely, we demonstrate that proliferation is decreased in leukemic cells when either *CBX7* or *SETDB1* is downregulated, or when CBX7 is pharmacologically inhibited. Similarly, it has been shown that exposure of murine AML blasts to the EHMT2 inhibitor UNC0638 leads to inhibition of growth and induction of myeloid differentiation (Lehnertz et al., 2014).

As for the exact molecular mechanism by which CBX7, SETDB1 and H3K9me3 interact, we hypothesize that such interactions are locus-specific and dependent on the exact composition of the protein complex involved. We propose that regulation follows a step-wise program, where trimethylated SETDB1 initially converts H3K9me or H3K9me2 into H3K9me3, resulting in attraction of PRC1 by binding of CBX7 to SETDB1. An alternative, not mutually exclusive possibility is that CBX7 first recognizes trimethylated SETDB1, by which it is then recruited to H3K9me2 loci to ensure further chromatin compaction. These recruitment models would be independent of H3K27me3/PRC2. At loci where both H3K27me3 and H3K9me3 histone marks are present, CBX7 could be recruited to both. It is interesting to note that one of the CBX7 binding proteins we identified, CDYL, can bind to EZH2 as well as to SETDB1 (Escamilla-Del-Arenal et al., 2013; Fritsch et al., 2010; Zhang et al., 2011) allowing for multiple alternative Polycomb and H3K9 methyltransferase interactions. Further elucidation of the daunting complexities by which seemingly independent epigenetic pathways converge will allow the understanding of the molecular machinery by which self-renewal is ensured. Disruption of these self-renewal pathways is likely to offer novel therapeutic opportunities in leukemia.

## ACKNOWLEDGEMENTS

The authors wish to thank Geert Mesander, Johan Teunis and Theo Bijma of the Central Flowcytometry Unit of the University Medical Center Groningen, and Dick H Deckers and Jeroen Demmers of the Mass Spectrometry Facility of Erasmus MC for their excellent support. Furthermore, we would like to thank Kazemier I. for performing the MST measurements. This study was supported by a grant from the Dutch Cancer Society (RUG 2014-7178), the Netherlands Organization for Scientific Research (Mouse Clinic for Cancer and Aging) and a personal fellowship of the Deutsche Krebshilfe to JJ.

## REFERENCES

- Bagger, F. O., Sasivarevic, D., Sohi, S. H., Laursen, L. G., Pundhir, S., Sonderby, C. K., Winther, O., Rapin, N., and Porse, B. T. (2016). BloodSpot: a database of gene expression profiles and transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Res* 44, D917-924.
- Bernstein, E., Duncan, E. M., Masui, O., Gil, J., Heard, E., and Allis, C. D. (2006). Mouse Polycomb Proteins Bind Differentially to Methylated Histone H3 and RNA and Are Enriched in Facultative Heterochromatin. *Molecular and Cellular Biology* 26, 2560-2569.
- Bilodeau, S., Kagey, M. H., Frampton, G. M., Rahl, P. B., and Young, R. A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes & Development* 23, 2484-2489.
- Bracken, A. P., Dietrich, N., Pasini, D., Hansen, K. H., and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev* 20, 1123-1136.
- Cancer Genome Atlas Research, N., Ley, T. J., Miller, C., Ding, L., Raphael, B. J., Mungall, A. J., Robertson, A., Hoadley, K., Triche, T. J., Jr., Laird, P. W., et al. (2013). Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 368, 2059-2074.
- Cao, R., Tsukada, Y., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol Cell* 20, 845-854.
- Collins, P. L., Kyle, K. E., Egawa, T., Shinkai, Y., and Oltz, E. M. (2015). The histone methyltransferase SETDB1 represses endogenous and exogenous retroviruses in B lymphocytes. *Proc Natl Acad Sci U S A* 112, 8367-8372.
- Comet, I., and Helin, K. (2014). Revolution in the Polycomb hierarchy. *Nat Struct Mol Biol* 21, 573-575.
- Eppert, K., Takenaka, K., Lechman, E. R., Waldron, L., Nilsson, B., van Galen, P., Metzeler, K. H., Poepl, A., Ling, V., Beyene, J., et al. (2011). Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med* 17, 1086-1093.
- Escamilla-Del-Arenal, M., da Rocha, S. T., Spruijt, C. G., Masui, O., Renaud, O., Smits, A. H., Margueron, R., Vermeulen, M., and Heard, E. (2015). Cdy1, a new partner of the inactive X chromosome and potential reader of H3K27me3 and H3K9me2. *Mol Cell Biol* 33, 5005-5020.
- Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes & Development* 17, 1870-1881.
- Fritsch, L., Robin, P., Mathieu, J. R. R., Souidi, M., Hinaux, H., Rougeulle, C., Harel-Bellan, A., Ameyar-Zazoua, M., and Ait-Si-Ali, S. (2010). A Subset of the Histone H3 Lysine 9 Methyltransferases Suv39h1, G9a, GLP, and SETDB1 Participate in a Multimeric Complex. *Molecular Cell* 37, 46-56.
- Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015). PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Research* 43, D512-D520.
- Kahn, T. G., Dorafshan, E., Schultheis, D., Zare, A., Stenberg, P., Reim, I., Pirrotta, V., and Schwartz, Y. B. (2016). Interdependence of PRC1 and PRC2 for recruitment to Polycomb Response Elements. *Nucleic Acids Res* 44, 10132-10149.
- Kamminga, L. M., Bystrykh, L. V., de Boer, A., Houwer, S., Douma, J., Weersing, E., Dontje, B., and de Haan, G. (2006). The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood* 107, 2170-2179.
- Kaustov, L., Ouyang, H., Amaya, M., Lemak, A., Nady, N., Duan, S., Wasney, G. A., Li, Z., Vedadi, M., Schapira, M., et al. (2011). Recognition and specificity determinants of the human cbx chromodomains. *J Biol Chem* 286, 521-529.
- Klaue, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., et al. (2015). Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. *Nat Cell Biol* 15, 353-362.
- Koide, S., Oshima, M., Takubo, K., Yamazaki, S., Nitta, E., Saraya, A., Aoyama, K., Kato, Y., Miyagi, S., Nakajima-Takagi, Y., et al. (2016). Setdb1 maintains hematopoietic stem and progenitor cells by restricting the ectopic

- activation of nonhematopoietic genes. *Blood* 128, 638-649.
- Koya, J., Kataoka, K., Sato, T., Bando, M., Kato, Y., Tsuruta-Kishino, T., Kobayashi, H., Narukawa, K., Miyoshi, H., Shirahige, K., and Kurokawa, M. (2016). DNMT3A R882 mutants interact with polycomb proteins to block hematopoietic stem and leukaemic cell differentiation. *Nat Commun* 7.
- Laurenti, E., Doulatov, S., Zandi, S., Plumb, I., Chen, J., April, C., Fan, J. B., and Dick, J. E. (2013). The transcriptional architecture of early human hematopoiesis identifies multilevel control of lymphoid commitment. *Nat Immunol* 14, 756-765.
- Lehnertz, B., Pabst, C., Su, L., Miller, M., Liu, F., Yi, L., Zhang, R., Kros, J., Yung, E., Kirschner, J., et al. (2014). The methyltransferase G9a regulates HoxA9-dependent transcription in AML. *Genes Dev* 28, 317-327.
- Min, J., Zhang, Y., and Xu, R. M. (2005). Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. *Genes Dev* 17, 1823-1828.
- Morey, L., Aloia, L., Cozzuto, L., Benitah, S. A., and Di Croce, L. (2013). RYBP and Cbx7 define specific biological functions of polycomb complexes in mouse embryonic stem cells. *Cell Rep* 3, 60-69.
- Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S. A., and Di Croce, L. (2012). Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. *Cell Stem Cell* 10, 47-62.
- Nikoloski, G., Langemeijer, S. M. C., Kuiper, R. P., Knops, R., Massop, M., Tonnissen, E. R. L. T. M., van der Heijden, A., Scheele, T. N., Vandenbergh, P., de Witte, T., et al. (2010). Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 42, 665-667.
- Ren, C., Morohashi, K., Plotnikov, Alexander N., Jakoncic, J., Smith, Steven G., Li, J., Zeng, L., Rodriguez, Y., Stojanoff, V., Walsh, M., and Zhou, M.-M. (2015). Small-Molecule Modulators of Methyl-Lysine Binding for the CBX7 Chromodomain. *Chemistry & Biology* 22, 161-168.
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* 111, 2621-2650.
- Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hasserjian, R. P., and Ebert, B. L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 126, 9-16.
- Stock, J. K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A. G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* 9, 1428-1435.
- Tadokoro, Y., Ema, H., Okano, M., Li, E., and Nakachi, H. (2007). De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *The Journal of Experimental Medicine* 204, 715.
- Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., et al. (2012). RYBP-PRC1 Complexes Mediate H2A Ubiquitylation at Polycomb Target Sites Independently of PRC2 and H3K27me3. *Cell* 148, 664-678.
- van den Boom, V., Rozenveld-Geugien, M., Bonardi, F., Malanga, D., van Gosliga, D., Heijink, A. M., Viglietto, G., Morrone, G., Fusetti, F., Vellenga, E., and Schuringa, J. J. (2013). Nonredundant and locus-specific gene repression functions of PRC1 paralog family members in human hematopoietic stem/progenitor cells. *Blood* 121, 2452-2461.
- Verhaak, R. G., Wouters, B. J., Erpelinck, C. A., Abbas, S., Beverloo, H. B., Lugthart, S., Lowenberg, B., Delwel, R., and Valk, P. J. (2009). Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica* 94, 151-154.
- Zhang, Y., Yang, X., Gui, B., Xie, G., Zhang, D., Shang, Y., and Liang, J. (2011). Corepressor Protein CDYL Functions as a Molecular Bridge between Polycomb Repressor Complex 2 and Repressive Chromatin Mark Trimethylated Histone Lysine 27. *Journal of Biological Chemistry* 286, 42414-42425.

## SUPPLEMENTARY TABLE 1

### Direct targets of CBX7 +/- 5000 bps around TSS

KDM7A	WNT5B	GPAT3	B3GALNT1	GOLGA2P10
MMP25	UST	PDE5A	CD14	OTUD7B
GIPR	HES1	PRDM5	TRH	DPYSL2
VCAN	FRMD4B	LEF1	CHD7	NDFIP2
DSG2	PODXL2	FGD4	KRT19	CA2
ARAP2	EPB41L5	GAS2L3	ZNF518	ID2
HDAC9	IGFBP2	PIF1	COL24A1	BAHD1
PTGER3	EPAS1	IRF8	LPAR3	SLC18A2
BCAR1	NID1	TOM1L1	SMPDL3A	ARHGAP42
NCKAP1	MYCL	RP5-862P8.2	NBEA	BMI1
LTK	CAMSAP2	CDC42BPA	RAPH1	NR1D2
SNX24	LGALS1	SOX15	NRIP3	FAM20C
NTN1	CNRIP1	RHOB	BNIP3	
ME1	MOB3B	CAMK2D	JUN	
PFKP	PLEKHG1	PLK2	MAF	
SPTB	DUSP4	PLA2G7	KCTD12	
MGAT4A	STX16	FBXO25	THBD	
ARHGAP10	CHST8	PARD3	FJX1	
VASH1	CDKN1A	HTR7	ZADH2	
TRIB2	PSD4	PTPRJ	FZD2	
PTPN18	PODXL	ST14	SGSH	
PDE8A	IL13RA1	CRIM1	EXT1	
FERMT2	LGALS5	DLG5	AP1S2	
ZNF532	PTPRE	SLC25A4	GAS6	
ARHGEF10L	RAP1GAP2	DST	KCNQ5	
RASAL2	WASF3	PTPN14	ZDHHC23	
CTTNBP2	CCNA1	TRIM36	MYBL1	
CRYBG3	MORC4	BCL2L11	MITF	
MAG3	ARNTL	RBMS1	SHTN1	
OSTM1	SORT1	PID1	SHISA7	
SYDE2	PTGFRN	MCOLN2	RP11-43F13.1	
NRP1	FST	ANKH	ARL4C	
H2AFY2	DTNA	PRKCA	SPATS2L	
TPTEP1	SLC37A2	ZFYVE9	NTNG2	
KCNK10	GOLM1	FZD1	SULF2	
COCH	PSAT1	KIT	MYO6	
PPP1R16B	KCNMB4	FMNL2	IGF2R	
RGCC	NPL	AGAP1	MYO5A	
DGKH	USP44	CXCL16	DPP4	
CTSH	SLC41A2	ADCY9	S100A10	
CD276	ADAMTS7	PROK2	DNM3	
FZD5	TM6SF1	HSPA4L	TUSC1	
PTPRS	KLF4	ANXA5	UNC13B	
CPVL	TMOD1	NFIL3	PPP1R14C	
UNC5B	CTSV	OTUD1	COLGALT2	
TBC1D12	IRF4	UBTD1	MAFB	
RASD1	SLC22A23	FBN1	ZDBF2	
TBC1D9	THBS1	TRIM44	LGR4	
GLRB	PAQR5	PBK	AC104655.3	
DTX4	UACA	RAB31	ANKRD18B	
SLC15A3	TTLL7	STXBP6	FOXD2-AS1	
PRDM4	RASGEF1B	PCSK9	TMEM150C	

**63 genes direct targets with peak in genebody +/- 5000**

POU2F2	FBN2
COL23A1	PHLDA1
PDE4A	TACC1
RPS6KA2	EPS8
CHFR	CAST
COBLL1	DDAH1
C20orf194	UCHL1
CACNA1I	TIAM1
JPH1	MYO1E
BNIP3L	AUTS2
TMEM176B	KALRN
PMP22	NFIA
UNC93B1	FCMR
PHACTR1	SFMBT1
IL1R1	ARHGEF3
SGK1	SEPT8
RARRES1	DLC1
PLBD1	GPRC5C
EGR2	CLCN5
RUNX2	GATM
HSPA2	MYO1D
TNFRSF19	CACNB4
FOXJ1	CNR2
SH5BP5	FAM169A
KCNC3	MFAP3L
DSC2	TBC1D8
CEP162	SLFN12L
PKIB	RP13-104F24.2
CPM	PLXNA4
TRPC6	MIR54A
PLCB2	FRG1HP
CYP1B1	

## MATERIAL AND METHODS

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Blocking agents for excluding unspecific binding:</b>		
Human BD Fc block™	BD Bioscience	564220
CD16/ CD32 Mouse BD Fc block™	BD Bioscience	555142
<b>Antibodies used for staining of differentiated hematopoietic cells</b>		
Mouse anti human CD3 APC-Cy7 (SK7)	BD Bioscience	557832
Mouse anti human CD4 PE-Cy7 (SK3)	BD Bioscience	557852
Mouse anti human CD33 BV421(WM53)	BD Bioscience	562854
Mouse anti human CD45 APC (HI30)	BD Bioscience	560973
Mouse anti human CD19 PE (HIB19)	BD Bioscience	555415
BD Horizon Brilliant Stain buffer	BD Bioscience	563794
<b>Antibodies used for staining of immature CD34<sup>+</sup> hematopoietic cells and LIN<sup>+</sup> cells</b>		
Mouse anti human CD38 PE (HIT2)	BD Bioscience	555460
Mouse anti human CD90 AF700 (5E10)	BioLegend	328120
Mouse anti human CD34 APC (581)	BD Bioscience	555824
Mouse anti human CD45 RA PE-Cy7 (L48)	BD Bioscience	337186
Mouse anti human CD45 BV421 (HI30)	BioLegend	304032
Mouse anti human CD2 PE-Cy5 (RPA-2.10)	BioLegend	300210
Mouse anti human CD3 PE-Cy5 (UCHT1)	BioLegend	300410
Mouse anti human CD4 PE-Cy5 (RPA-T4)	BioLegend	300510
Mouse anti human CD7 PE-Cy5 (6B7)	BioLegend	345110
Mouse anti human CD8 PE-Cy5 (RPA-T8)	BioLegend	301010
Mouse anti human CD19 PE-Cy5 (HIB19)	BioLegend	302210
Mouse anti human CD20 PE-Cy5 (2H7)	BioLegend	302308
Mouse anti human CD235a PE-Cy5 (HIR2)	BioLegend	306606
Mouse anti human CD11b PE-Cy5 (ICRF44)	BioLegend	301308
Mouse anti human CD14 PE-Cy5 (TuK4), TRI-COLOR	ThermoFisher Scientific	MHCD1406
Mouse anti human CD56 PE-Cy5 (MEM-188)	BioLegend	304608
<b>Antibodies used for staining of OCI-AML3/HL60 cells for <i>in vitro</i> experiments</b>		
Mouse Anti-Human Alexa Fluor® 700 CD14 Clone M5E2 (RUO)	BD Bioscience	557923
Mouse Anti-Human BV421 CD11b/MAC-1 (RUO)	BD Bioscience	562632
<b>Antibodies used for staining of CD34<sup>+</sup> cells for <i>in vitro</i> experiments</b>		
Human anti CD34 PE-Cy7 (8G12)	BD Bioscience	348811
<b>Materials for Isolation of CD34<sup>+</sup> cells from Cord blood</b>		
CD34 <sup>+</sup> MicrobeadKit	Miltenyi Biotec	150-056-702
Lymphoprep	Stem cell technologies	7861
LS Columns	Miltenyi Biotec	150-042-401
<b>Antibodies used for Chip-Seq</b>		
H3K9me3 polyclonal antibody-Premium	Diagenode	C15410193
Anti H3K27me3	Merck	07-449
Anti CBX7	Merck	07-981
Pierce Protein A/G Magnetic beads	Thermo Scientific	#88803
<b>Antibodies used for Western-Blot and Proximity Ligation Assay</b>		
Monoclonal ANTI-FLAG® M2 antibody	Sigma	F3165-2 mg
Polyclonal rabbit anti human/mouse CBX7 p15	Santa Cruz Biotechnology	SC 70-232
SETDB1 Antibody (5H6A12)	Pierce Protein	MA5-15722
<b>Medium</b>		
IMDM 2% FCS	StemCell Technologies	# 07700
MethoCult™ H4435 Enriched	StemCell Technologies	#04435 and 04445
Myelocult™ H5100	StemCell Technologies	#05100 and 05150

REAGENT or RESOURCE	SOURCE	IDENTIFIER
StemSpan™ SFEM	StemCell Technologies	# 09650
Source of consensus cDNA		
cDNA of huCBX2 (NM_005189), transcript variant 1	Origene	SC303599
cDNA of huCBX4 (NM_003655)	Origene	SC117841
cDNA of huCBX6	ThermoFisher	MHS6278-202759205 (now available via Dharmacon)
cDNA of huCBX7	ThermoFisher	MHS6278-202760094 (now available via Dharmacon) (site-directed mutagenesis to consensus cDNA, base exchange G230A and C715G)
cDNA of huCBX8	Kind gift from K. Hansen, Copenhagen	(Dietrich et al., 2007)
cDNA of huSETDB1	Kind gift from Frank Rauscher, The Wistar Institute, Philadelphia, USA and Lingwen Ding, Cancer Science Institute of Singapore Republic of Singapore	Rauscher FJ 3 <sup>rd</sup> et al, Genes & Development 16(8): 919-32, April 15 2002.
Sequences of short hairpins		
pLKO.1_GFP_SCR	CAACAAGATGAAGAGCACCAA	A kind gift from Vincent van den Boom
pLKO.1_GFP_shCBX7 (#1)	CGGAAGGGTAAAGTCGAGTAT	A kind gift from Vincent van den Boom
pLKO.1_mCherry_SCR	CAACAAGATGAAGAGCACCAA	
pLKO.1_mCherry_shCBX7	GCTTCTGTGAGGTGTTTAGC	
pLKO.1_mCherry_shSETDB1#3	GCTCAGATGATAACTTCTGTA	Was subloned from Sigma shSETDB1 (TRCN 276169)
pLKO.1_mCherry_shSETDB1#4	AGTTAGAGACATGGGTAATA	Was subloned from Sigma shSETDB1 (TRCN 276105)
Primer sequences for cloning		
GFP_MluI_fw	CGCAATTGATGGTGAGCAAGGG-CGAGGA	
FLAG_MluI_rv	CGCAATTGCTTGTTCATCGTC-GTCCTTGTAGT	
huCBX7_Not1_fw	CGG CGG CCG CATGGAGCTGT-CAGCCATCGGC	
FLAG_huCBX7_Not1_fw	CGG CGG CCG C ATG GAC TAC AAG GAC GAC GAT GAC AAG ATG GAG CTG TCA GCC ATC GGC	
huCBX7_Sal1_rv	CGGTCGACTCAGAACTTCCCACT-GCGGTCT	
huCBX2_Not1_fw	CGG CGG CCG C ATGGAGGAGCT-GAGCAGCGTG	
huCBX2_Sal1_rv	CGGTCGAC TCAGTAATGCCT-CAGGTTGAAG	
huCBX4_Not1_fw	CGG CGG CCG C ATGGAGCTGC-CAGCTGTTGG	
huCBX4_Sal1_rv	CGGTCGAC CTACACCGTACG-TACTCCTTG	
FLAG_huCBX4_Not1_fw	CGG CGG CCG C ATG GAC TAC AAG GAC GAC GAT GAC AAG ATGGAGCTGCCAGCTGTTGG	
huCBX6_Not1_fw	CGG CGG CCG C ATG GAC TAC AAG GAC GAC GAT GAC AAG ATGGAGCTGCCAGCTGTTGG	

REAGENT or RESOURCE	SOURCE	IDENTIFIER
huCBX6_Sal1_rv	CGGTCGAC TCACTTGCTCGC- CCCAATGC	
FLAG_huCBX8_Not1_fw	CGG CGG CCG C ATG GAC TAC AAG GAC GAC GAT GAC AAG ATGGAGCTTTCAGCGGTG	
FLAG_huSETDB1_Mlu1_fw	CGA CGC GTA TGG ACT ACA AGG ACG ACG ATG ACA AGA TGT CTT CCC TTC CTG GGT G	
huSETDB1_Xba_rv	CGTCTAGACTAAAGAAGAC- GTCCTCTGCATTCA	
<b>Primer sequences for measuring gene expression analysis by qPCR</b>		
hubetaAktin_fw	TCCCTGGAGAAGAGCTACGA	
Hubeta_Aktin_rv	AGCACTGTGTGGCGTACAG	
huHPRT_fw	GAACGTCTTGGCTCGAGATGTG	
huHPRT_rv	TCCAGCAGGTCAGCAAAGAAT	
huCBX7_fw	GCGGAAGGGTAAAGTTCGAGT	
huCBX7_rv	ACCTCTCTTCCATACCCCGA	
huCBX8_fw	TGGTCGCAGAAGTACAGCAC	
huCBX8_rv	CACGCTTTTGGGGCCATAG	
huSETDB1_fw	CCAAATATGGGTGCTGTG AGGA	
huSETDB1_rv	TTC CAC TGG CTT GAA CTG GG	
huCD11b_fw	ACT TGC AGT GAG AAC ACG TAT G	
huCD11b_rv	AGA GCC ATC AAT CAA GAA GGC	
huCD14_fw	ACTTGCACTTTCAGCTTGC	(Haghparsat et al., 2011)
huCD14_rv	GCCCACTCCAGGATTGTCAG	
<b>Chip-qPCR primers</b>		
Alpha Sat_fw_Primer#1	CTGCACTACCTGAAGAGGAC	(Wang et al., 2013)
Alpha_Sat_rv_Primer#1	GATGGTTCAACACTGTTTACA	
Alpha Sat_fw_Primer#2	AAGGTCAATGGCAGAAAAGAA	(Moralli et al., 2015)
Alpha_Sat_rv_Primer#2	CAACGAAGGCCACAAGATGTC	
INK4B_fw	ATCACGGAGCAATAAACCCCAAC	(Kheradmand Kia et al., 2009)
INK4B_rv	CAAGAGAAACAGCGACCTAACC	
INK4A_fw	ACCAAGACTTCGCTGACC	(Kheradmand Kia et al., 2009)
INK4A_rv	CAAGGAGGACCATAATTCTACC	
RPL27_fw	TCCGGACGCAAAGCTGTGTCATCG	(Kheradmand Kia et al., 2009)
RPL27_rv	TCCGGACGCAAAGCTGTGTCATCG	
EIF4A2_fw	TTTTTGTAGCTGACCGAAGCA	(Takayama et al., 2014)
EIF4A2_rv	GCGCCCTATGACCTTCACTA	
<b>Chemicals, Peptides and recombinant protein</b>		
Hydrocortisone	StemCell Technologies	#07904
Human recombinant TPO	RnD	288-TP-200
Human recombinant FLT3L	RnD	308-FK-025
Human recombinant SCF	RnD	255-SC-050
Human recombinant IL3	Sigma	I1646
Mouse recombinant IL3	RnDSystems	403-ml-50
RetroNectin® 2,5 mg	Westburg/ Takara	T100/B
Hexadimethrine bromide	Sigma	h-9268
FuGene HD transfection reagent	Promega	E2312
Formaldehyde, 37% formaldehyde solution	Santa Cruz Biotechnology	Sc-203049
SDS Solution, 20%	Fisher Scientific	BP 1311-1
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Merck	#000000011873580001
QIAquick PCR Purification Kit	Quiagen	#28104



REAGENT or RESOURCE	SOURCE	IDENTIFIER
Novagen	Merck Millipore	70664
Benzoase		
Nuclease, Purity 99%		
SnakeSkin	Thermo Scientific	68700
Dialysis Tubing, 7K MWCO, 22mm		
3xFlag® peptide	Sigma	F5290-4MG
CryoStor® CS10	StemCell Technologies	#07950
Protease Inhibitor Cocktail	Sigma	P8340
Anti-Flag M2 Magnetic beads	Sigma	M8823
NT Protein Labelling kit Red-NHS	Nanotemper	MO-L001
MS37452	Sigma	SML1405
<b>Experimental Models: Cell Lines</b>		
MS5 cells	DSMZ	ACC 441
HL60 cells	ATCC	CCL-240
	DSMZ	ACC 3
OCI-AML3 cells	DSMZ	ACC 582
32D cells		Kind gift from Ivo Touw
PG13 cells	ATCC	CRL-10686
Phoenix-ECO cells	ATCC	CRL-3214
K562 cells	ATCC	CCL-243
293FT	ThermoFisher Scientific	R70007
<b>Experimental Models: Organisms/ Strains</b>		
NOD.Cg-Prkdcscid Il2rgtm1Wjl/Szj		Mice were purchased from Charles River Laboratory (L'Arbesle Cedex, France) and bred in house.
<b>Software and Algorithms</b>		
Graphpad Prism (v5-7)	Graphpad Prism	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
ELDA	Hu, Y, and Smyth, GK (2009). ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. <i>Journal of Immunological Methods</i> 347, 70-78.	<a href="http://bioinf.wehi.edu.au/software/elda/">http://bioinf.wehi.edu.au/software/elda/</a>
FlowJo	Version X.0.7	

## Method details:

### Cell Culture:

Phoenix-ECO cells were culture in DMEM + 1% P/S + 10% heat-inactivated FCS. PG13 cells were culture in DMEM + 1% P/S + 10% heat-inactivated FCS. HEK293FT cells were culture in DMEM +1% P/S + 10% heat-inactivated FCS. HL60 cells were cultured in RPMI+ 1% P/S+ 20% heat-inactivated FCS. OCI-AML3 cells were cultured in RPMI + 1%P/S + 10% heat-inactivated FCS. K562 cells were cultured in RPMI + 1% P/S + 10% heat-inactivated FCS.

### **Cloning of retroviral vector constructs:**

The consensus cDNA of CBX2,4,6,7 and 8 and FLAG-tagged versions of the cDNA were inserted in the retroviral vector backbone of SF91-IRES-GFP (Klauke et al., 2013) upstream of IRES by PCR based cloning using Not1 and Sal1 restriction sites. Primers used for PCR based cloning are listed above. FLAG-tagged GFP vector was cloned by vector-PCR of SF91-FLAG tagged muCbx7 (Klauke et al., 2013) with MluI restriction site containing primers and subsequent ligation.

### **Cloning of FLAG-tagged huSETDB1 cDNA in pRRLA:**

A FLAG-tagged versions of SETDB1 cDNA was inserted in the lentiviral vector backbone of pRRLA IRES-GFP upstream of IRES by PCR based cloning using Mlu1 and Xba1 restriction sites. Primers used for PCR based cloning are listed above.

### **Cloning of short-hairpins in lentiviral expression vectors:**

Corresponding oligos for SCR, shCBX7, shSETDB1#3 and shSETDB1#4 were annealed and cloned into the empty pLKO.1\_mCherry vector upon digestion with Age1 and EcoR1.

### **CD34<sup>+</sup> cord blood isolation:**

Cord blood was obtained from healthy full-term pregnancies after informed consent in accordance with the Declaration of Helsinki from the obstetrics department at the Isala Hospital in Zwolle, the Netherlands. Initially, cord blood volume and cell counts were measured and then diluted 1:1 with PBS+ 2 mM EDTA+0.5% BSA. Maximum 30 ml of diluted cord blood was carefully layered on 15 ml of Lymphoprep™ in a 50 ml falcon tube and centrifuged for 20 minutes, 800g, without brakes. Middle layer containing mononuclear cells was harvested and diluted 1:1 with PBS 2 mM EDTA 0.5% BSA and then centrifuged for 5 minutes at 800g. Cell pellets were collected and washed with PBS 2mM EDTA 0.5% BSA and centrifuged for 10 minutes at 200g. Immunomagnetic labeling and separation were performed according to the manufacturer's manual of the CD34 MicroBead Kit, human (Miltenyi Biotec). Cells were either used immediately for experiments or frozen in Cryostor CS10.

### **Transduction of 32D cells:**

Initially, 300,000 Phoenix-ECO cells/well (of a six-well plate) were seeded in DMEM + 1%P/S + 10% FCS. On day 2 cells were transfected with 1 µg of plasmid

with the help of FuGene® in a 1:3 ratio. 24 hours after transfection medium was changed to RPMI+10%FCS+1%P/S. On day 4 non-treated six-well plates were coated with RetroNectin® according to the manufacturer's manual. Viral supernatant was harvested and filtered through a sterile syringe filter with a 0.45 µm pore size hydrophilic PVDF membrane. Then 2 ml of viral supernatant, 300,000 32D cells and hexadimethrine bromide (2 µg/ml) and muIL3 (10 ng/ml) were added/well. Six-well plates were centrifuged for 45 minutes at room temperature for 45 minutes at 400 g. 24 hours after transduction virus-supernatant was replaced by RPMI+10%FCS+1%P/S+ muIL3 (10 ng/ml).

### **Production of a stable retroviral producer cell line (PG13):**

Initially, 300,000 Phoenix-ECO cells/well (of a six-well plate) were seeded in DMEM + 1%P/S + 10% FCS. On day 2 cells were transfected with 1 µg of plasmid with the help of FuGene® in a 1:3 ratio. 24 hours after transfection medium was refreshed and 10,000 PG13 cells were plated out/well in a tissue culture treated six-well plate. 48 hours after transfection viral supernatant was harvested and used to transduce the retroviral packaging cell line PG13 with the help of Hexadimethrine bromide (2 µg/ml). One day after transduction medium of transduced PG13 was changed to DMEM+1%P/S+10%FCS and cultured at 37°C/5% CO<sub>2</sub>.

### **Production of lentiviral supernatant:**

$2.75 \times 10^6$  293FT cells were plated in gelatin coated cell-culture treated 10 cm dishes in DMEM+10%FCS+1%P/S and incubated overnight at 37°C/5%CO<sub>2</sub>. On the next day cells were transfected with 3 µg of the pLKO.1 or pRRLA vector, 3 µg of the packaging plasmid pCMV8.91 and 0.7 µg of envelope plasmid VSV-G and 21 µl of FuGene®. On the next day medium was changed to either StemSpan™ SFEM or RPMI. Two days after transfection the virus was collected, filtered through a sterile syringe filter with a 0.45 µm pore size hydrophilic PVDF membrane and used either immediately for transduction or was frozen.

### **Retroviral virus production and transduction of CD34<sup>+</sup> cells:**

24 hours before the first transduction round CD34<sup>+</sup> cells were prestimulated in StemSpan™ SFEM with SCF 100 ng/ml, FLT3L 100 ng/ml and TPO 100 ng/ml at 37°C and 5% CO<sub>2</sub>. Medium of transduced PG13 cells was changed to StemSpan™ SFEM. On the day of transduction not tissue-cultured six-well plates were coated with RetroNectin® according to the manufacturer's manual.

Then viral supernatant of virus-producing PG13 cells was harvested and filtered through a sterile syringe filter with a 0.45 µm pore size hydrophilic PVDF membrane. Between 500,000 and 1,000,000 CD34<sup>+</sup> cells were transduced with 2 ml of viral supernatant in the presence of SCF 100 ng/ml, FLT3L 100 ng/ml, TPO 100 ng/ml and Hexadimethrine bromide to a final concentration of 2 µg/ml. Six-well plates were centrifuged at 400g for 1 hour at room temperature. Transduction was repeated two times in 8-12 hour time intervals. After last transduction round medium was changed to StemSpan™ SFEM with SCF 100 ng/ml, FLT3L 100 ng/ml and TPO 100 ng/ml.

#### **Lentiviral transduction of CD34<sup>+</sup> cells:**

CD34<sup>+</sup> cells were cultured in StemSpan™ SFEM with SCF 100 ng/ml, FLT3L 100 ng/ml and TPO 100 ng/ml 24 hours before first transduction round at 37°C and 5% CO<sub>2</sub>. On the day of transduction not tissue-cultured six-well plates were coated with RetroNectin according to the manufacturer's manual. Lentiviral supernatant was thawed on ice. Between 500,000 and 1,000,000 CD34<sup>+</sup> cells were transduced with 2 ml of viral supernatant in the presence of SCF 100 ng/ml, FLT3L 100 ng/ml, TPO 100 ng/ml and Hexadimethrine bromide 2 µg/ml. Six-well plates were centrifuged at 400g for 1 hour at room temperature. Transduction was repeated once in 8-12 hour time intervals. After last transduction round medium was changed back to StemSpan™ SFEM containing SCF 100 ng/ml, FLT3L 100 ng/ml and TPO 100 ng/ml.

#### **Lentiviral transduction of HL60 and OCI-AML3 cells:**

On the day of transduction not tissue-cultured six-well plates were coated with RetroNectin according to the manufacturer's manual. Between 300,000 and 500,000 cells were transduced in 2 ml of viral supernatant containing Hexadimethrine bromide 2 µg/ml. Six-well plates were centrifuged at 400G for 1 hour at room temperature. Transduction was repeated once in 8–12 hour time intervals. After last transduction round medium was changed back to RPMI+1%P/S+10% (OCI-AML3) or 20% of FCS (HL60). At several time points cells were counted manually with a hemacytometer.

#### **MS37452 treatment of OCI-AML3 cells:**

Initially, 500,000 OCI-AML3 cells/well (of a six-well plate) were seeded in RPMI+1%P/S+10% heat-inactivated FCS supplemented with MS37452 (dissolved in DMSO at a concentration of 50µM) at different concentrations. After four days cells were counted manually using a hemacytometer.

### **FACS analysis of HL60 and OCI-AML3 cells:**

Samples were incubated with Human BD Fc block™ to prevent unspecific binding at 4°C in the dark. After blocking, 5 µl mouse anti huCD11b BV421 and/or 5 µl mouse anti huCD14 Alexa Fluor 700 antibodies were added and samples were incubated for 20-25 minutes at 4°C in the dark. Afterwards, cells were washed and resuspended with PBS+BSA 0.2% containing a viability dye (PI). Samples were analyzed on a BD FACSCanto II.

### **Sort of GFP<sup>+</sup>CD34<sup>+</sup> cells (MoFlo Astrios and MoFloXDP):**

24 hours after the last transduction round cells were harvested, washed and resuspended in PBS+BSA 0.2%. Cells were incubated with Human BD Fc block™ for 15 minutes according to the manufacturer's manual to prevent unspecific binding. After blocking, 5 µl of mouse anti huCD34 PE-Cy7 was added and incubated at 4°C for 20 minutes. Cells were washed with PBS + BSA 0.2% and resuspended in PBS +BSA 0.2% with the viability dye (PI).

### **Colony-forming unit assay:**

All experiments with CD34<sup>+</sup> cells were performed with single (not pooled) cords (except Chip-Seq experiments). 5,000 CD34<sup>+</sup>GFP<sup>+</sup> cells were sorted in a FACS tube containing 1 ml of IMDM 2% FBS™. 0.3 ml of the sorted cells in IMDM 2%FCS™ were added to a pre-aliquoted 3 ml MethoCult™ tube. Afterwards the tube was vortexed for at least 4 seconds and then let stand for a minimum of 5 minutes. For dispensing the MethoCult™ mixture into 35 mm culture dishes a 3 ml syringe with a 16 gauge blunt-end needle was used to add 1.1 ml per dish. Dishes were cultured at 37°C / 5% CO<sub>2</sub> conditions. Colonies were counted and typed after 14 days. For replating cells from primary dishes were harvested, centrifuged and counted with a hemocytometer. For CBX7 5,000 cells and for CBX8 30,000 cells were plated out as described above. Control cells were plated out at same cell numbers as experimental groups. Figure 1A+B show single data points of each experiment. Each single experiment was performed in duplicates and the average of the technical replicates was plotted.

### **Cobblestone area-forming cell assay:**

96-well flat-bottom plates were pre-coated with 0.1% gelatin. Two days before sort 10,000 MS5 cells were plated in 200 µl of Myelocult™ H5100 supplemented with 10<sup>-6</sup> M hydrocortisone + 1% P/S. On the next day cells were radiated with 30 Gy. One day post radiation CD34<sup>+</sup>GFP<sup>+</sup> cells were sorted directly into 96-well plates at limiting dilution and cultured for 5 weeks with weekly performed

half-medium changes. Cobblestones were analyzed with a phase-contrast microscope. Frequency of each experiment was calculated with ELDA software. (Hu and Smyth, 2009) Figure 1D shows single data points of each experiment. (The Y-axis indicates the number of cells that need to be plated for a CAFC to develop.)

#### **Long-term culture initiating cell assay:**

96-well flat-bottom plates were pre-coated with 0.1% gelatin. Two days before sort 10,000 MS5 cells were plated out in 200  $\mu$ l of Myelocult™ H5100 supplemented with  $10^{-6}$  M hydrocortisone.

Cells were sorted directly into 96-well plates at limiting dilution and cultured for 5 weeks with weekly performed half-medium changes. After 5 weeks medium was replaced by Methocult H4335 and incubated for two further weeks at 37°C/5% CO<sub>2</sub>. Colony-formation was accessed by phase-contrast microscopy. Frequency of each experiment was calculated with ELDA software (Hu and Smyth, 2009).

(The Y-axis indicates the number of cells that need to be plated for a LTC-IC to develop)

#### **Suspension culture experiment with CBX7, CBX8 and EV overexpressing cells:**

100,000 CD34<sup>+</sup>GFP<sup>+</sup> cells were sorted in a six-well plate containing 2 ml of Myelocult™ H5100, 1% P/S,  $10^{-6}$  M hydrocortisone, TPO 100 ng/ml, IL3 50 ng/ml, SCF 100 ng/ml, FLT3L 100 ng/ml. Cells were cultured at 37°C/5% CO<sub>2</sub>. Cells were counted manually with a hemacytometer every week and 100,000 cells were re-seeded under the same conditions.

#### **Xenotransplantation of transduced CD34<sup>+</sup> cells 24 hours after last transduction round:**

Mouse experiments were performed in line with international and national guidelines. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RUG).

For all xenotransplantation studies, we performed single cord transplantations of freshly isolated CD34<sup>+</sup> cord blood cells. Female 11-22 weeks old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were radiated three hours before transplantation with 1.8 Gy. In each experiment age of mice was balanced with maximum 2 weeks of difference between the experimental and control group. No antibiotic prophylaxes after radiation was given.

Isolation of CD34<sup>+</sup> cells and transduction was performed as described above. 24 hours after transduction the percentage of CD34<sup>+</sup>GFP<sup>+</sup> cells was determined after Fc blocking and staining with CD34<sup>+</sup>PE-Cy7 as described above. Cells were harvested and counted manually with a hemocytometer and trypan blue and resuspended in PBS. In total equivalents of 200,000 CD34<sup>+</sup>GFP<sup>+</sup> cells were transplanted per mouse via retro-orbital injection. A small aliquot was kept in culture for determining the exact number of transplanted CD34<sup>+</sup>GFP<sup>+</sup> cells 24 hours later. Because the transduction efficiency of experimental group was always lower than in the control group only absolute percentages of GFP engraftment are illustrated.

### **Xenotransplantation of transduced CD34<sup>+</sup> cells after one week in vitro culture:**

Mouse experiments were performed in line with international and national guidelines and all experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RUG).

For all xenotransplantation studies, we performed single cord transplantation of freshly isolated transduced CD34<sup>+</sup> cord blood cells. Female 10–20 weeks old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were irradiated three hours before transplant with 1.8 Gy. In the first experiment two control mice and one experimental mouse were 9 weeks older than 4 experimental mice. In the second experiment mice were between 15.5 and 19.5 weeks old upon transplant, age between both groups was balanced.

Isolation of CD34<sup>+</sup> cells was performed as described above. After isolation, cells were cultured in StemSpan™ SFEM exposed to FLT3L, TPO and SCF each with 100 ng/ml. Then three transduction rounds in a 24 hours time intervals were performed. 24 hours after the last transduction round cells were cultured for further 96 hours, so that cells were after isolation for one week *in vitro* cultured before transplantation.

On the day of transplantation percentage of CD34<sup>+</sup>GFP<sup>+</sup> cells was determined after Fc blocking and staining with CD34<sup>+</sup>PE-Cy7 as described above. Cells were harvested and counted manually with a hemocytometer and trypan blue and resuspended in PBS. In total equivalents of 1.5 million of CD34<sup>+</sup>GFP<sup>+</sup> cells were transplanted per mouse via retro-orbital injection under general anesthesia. Because transduction efficiency of experimental group was always lower than in the control group only absolute percentages of GFP engraftment are illustrated.

**Bleeding of xenotransplanted NSG mice:**

Beginning 6-weeks after transplantation chimerism in peripheral blood was determined in 4-week intervals. Blood samples were taken under general anesthesia via retro-orbital bleeding. Blood was lysed with ammonium chloride, washed two times with PBS+BSA 0.2% and resuspended in 50  $\mu$ l of PBS+BSA 0.2%. Samples were incubated with Human BD Fc block™ and CD16/CD32 Mouse BD Fc block™ to prevent unspecific binding. After incubation for 10 minutes at room temperature antibody master mix and BV stain buffer was added and samples were incubated for 20-25 minutes at 4°C in the dark. Afterwards, cells were washed and resuspended with PBS+BSA 0.2% containing a viability dye (PI). Samples were analyzed on a BD FACSCanto II.

**Bone marrow analysis of NSG mice:**

Mice were sacrificed and dissected under general anesthesia after the end of the experiment or reaching the human endpoint of the experiment. Bones (Femur, tibia, fibula and pelvis) were collected and cleaned. Bones were crushed in the presence of PBS+0.2% PBS with a mortar and pestle. The obtained cell suspension was filtered through a 40  $\mu$ m filter. Remaining erythrocytes were lysed with ammonium chloride. Cells were then pelleted by centrifugation and resuspended in PBS+BSA 0.2%. For preventing unspecific binding samples were incubated with Human BD Fc block™ and CD16/CD32 Mouse BD Fc block™.

After incubation for 10 minutes at room temperature, antibody master mix and BV stain (only for lineage-specific staining) buffer was added and samples were incubated for 20–25 minutes at 4°C in the dark. Afterwards, cells were washed and resuspended with PBS+BSA 0.2% containing a viability dye (PI). Samples were analyzed on a BD FACSCanto II (Lineage staining) and LSR II (progenitor staining).

**RNA-Seq of CD34<sup>+</sup> cells:**

100,000 GFP<sup>+</sup> CD34<sup>+</sup> cells were sorted into lysis buffer 4 days post-transduction. RNA was extracted using the Nucleospin RNA XS Kit, with the addition of a second elution step to increase yield. RNA quality and quantity was assessed using the Bioanalyzer RNA total Pico Assay. RNA samples with an RNA-integrity >8 were processed for RNA-seq library preparation using the SMARTer Stranded Total RNA-seq Kit. Briefly, 10 ng of total RNA was reverse transcribed using random primers and amplified via PCR during which barcoded Illumina adapters were added. After amplification, ribosomal RNA and mitochondrial cDNA were removed by annealing specific R-probes,



resulting in cleavage of ribosomal and mitochondrial cDNA in the presence of ZapR. After cleavage of ribosomal and mitochondrial cDNA, the remaining cDNA was amplified again during another round of PCR. The nM concentration of RNA-seq libraries were quantified based on library size (Bioanalyzer) and cDNA concentration (Qubit) and normalized to 2 nM prior to pooling. RNA-sequencing was performed on an Illumina HiSeq 2500 machine, three single-end runs with a read length of 63-64nt, resulting in fastq samples consisting of 26 to 64 million reads. Sample mapping was done with STAR (version 2.5.1b-2.5.2b), a custom genome index was build using Genecodegenes.org release 24 (GRCh38.p5) Ensembl 83, December 2015. STAR outputs read counts per gene, these were filtered by removing ribosomal and transfer RNA. Differential expression (DE) analyses by EdgeR (version 3.16.2), with upper quartile normalization. DE genes per condition ranked by p-value and adjusted for multiple testing using a Benjamini- Hochberg method. The final DE gene lists were filtered by FDR<0.05.

The RNA-seq data are deposited at ENA (PRJEB22831).

Gene Annotation and GO search was done using String database (<https://string-db.org>). Corresponding annotated and GO files were downloaded from the site and further analysed with custom scripts. GOChord plots were done in R (GOplot packages).

RNA expression data from Laurenti et al. (Laurenti et al., 2013), were obtained from GEO (GSE42141). Expression heatmap was done using R using heatmap.2 function in gplot library. Data were clustered by correlation and z-transformed. Gene Set-Enrichment Analysis (Subramanian, Tamayo, et al. 2005, PNAS 102, 15545-15550 and Mootha, Lindgren, et al. (2003, Nat Genet 34, 267-273)) was performed on a preranked list of all differentially expressed genes (FDR<0.1). The number of permutations was set to 1000, with exclusion of filtersets <10. (Furthermore, we applied the following filters scoring\_scheme weighted, norm meandiv, make\_sets true, gui false, set\_max 500, set\_min 15, npmerm 1000)

### **Chip-Seq of transduced CD34<sup>+</sup> cells:**

Frozen human CD34<sup>+</sup> enriched cord blood cells were thawed, pooled (Batch 1= 21 cords, Batch 2= 21 cords, Batch 3= 31 cords) and cultured in StemSpan™ SFEM with TPO, SCF and FLT3L (each 100 ng/ml) for prestimulation. After 24–48 hours three transduction rounds with retroviral supernatant for either EV or CBX7 at 24-hour time intervals were performed. Transduction was performed as described above. After the final transduction round, cells were

further expanded in StemSpan™ SFEM with TPO, SCF and FLT3L (each 100 ng/ml). One week after thawing, cells were stained and CD34<sup>+</sup>GFP<sup>+</sup> cells sorted as described above. Sorted cells were washed in ice-cold PBS+BSA 0,2%, centrifuged (450G, 5 min, 4°C) and resuspended in 1% cold formaldehyde for fixation. Tubes were incubated on a rotator at 4°C for 10 minutes. Fixation was stopped by adding glycine to a final concentration of 0.125 M. After addition of glycine cells were incubated on a rotator at 4°C for 5 minutes, washed two times with cold PBS, transferred to a low-adherent tube and resuspended in SDS buffer (NaCl 100mM, Tris-Cl pH8.1 50 mM, NaN<sub>3</sub> 0.2%, 0.5 % SDS) + cComplete™ protease inhibitors (1 tablet/ 50 ml SDS buffer). Samples were snapfrozen on dry ice and then transferred to -80°C for storage. The following table describes the number of transduced CD34<sup>+</sup> cells/ experiment and per antibody in million of cells.

	H3K9me3	H3K27me3	CBX7	IgG
1 <sup>st</sup> Experiment	1.4	1.4	2	0.2
2 <sup>nd</sup> Experiment	0.65	0.8	0.8	0.125
3 <sup>rd</sup> Experiment	1	1	2	0.2

For chromatin-immunoprecipitation, samples were thawed, centrifuged for 5 minutes at 900 g at room temperature and the supernatant was discarded. Pellets were resuspended in 500 µl of IP buffer (30 ml SDS buffer + 15 ml Triton dilution buffer (100 mM Tris-Cl pH8.6 + 100 mM NaCl + 5 mM EDTA pH 8.0 + 0.2% NaN<sub>3</sub> + 5% Triton X-100 + cComplete™ protease inhibitors) and sonicated to an average length of 400-500 bps (Bioruptor 30s on/30s off/cycle, high, in total 3 cycles). 5% of each sample was reversed crosslinked in TE with 1% SDS and 200 mM NaCl overnight at 65°C. On the next day decrosslinked DNA was isolated with the QIAquick PCR purification kit (Qiagen) and appropriate fragment length was confirmed via agarose gel electrophoresis. Protein A/G magnetic beads were washed three times with cold PBS and once with cold IP buffer. Crosslinked and fragmented samples were thawed and centrifuged for 30 min at 17,000g at 4°C. Samples were precleared by rotating at 4°C with 7.5 µl of washed beads for 1 hour. Samples were then distributed into several low adherence tubes for incubation with 5 µg of antibody and incubated overnight at 4°C on a rotating platform. The next day, samples were incubated with 20 µl of washed beads for 4 hours on a rotating platform at 4°C. Using a magnetic stand, the supernatant was removed and beads were washed 4x in a low salt buffer (150 mM) and once with TE. After the last washing step samples were

incubated in 1% SDS, 200 nM NaCl overnight at 65°C with 1100 RPM to reverse crosslinks. DNA was isolated with QIAquick PCR purification kit and enrichment of positive and negative loci was confirmed via qPCR.

ChIP libraries for sequencing were prepared with the Microplex Library Preparation Kit V2 (Diagenode, C05010012) according to the manufacturers protocol. The concentration of individual Chip-seq samples was determined based on library size (Agilent, Bioanalyzer 2100) and DNA concentration (Thermo Fisher, Qubit) and diluted to 2nM prior to pooling.

ChIP-Seq was performed on an Illumina NextSeq 500 machine, paired-end 79–80 bp,, a custom genome index was build using Genecodegenes.org release 25 (GRCh38.p7) Ensembl 85, July 2016. BAM mem (version 0.7.15) produced BAM files were generated and processed with Samtools (version 1.3.1). MACS2 (version 2.1.0) with the settings -f BAMPE --nomodel --broad --broad-cutoff -g 2.7e9 --keep-dup 1. The output BED files where analysed using bedtools (version 2.26.0) functions intersect, closest and Deeptools (version 2.4.2).

The Chip-seq data are deposited at ENA (PRJEB22344).

### **Mass spectrometry of pull-downs of FLAG-tagged-huCBX7, -huCBX8, -huCBX4 and -GFP in K562 cells and FLAG-tagged-muCbx7 and -GFP in 32D-cells:**

32D cells and K562 cells were transduced as described above, sorted and expanded. Cells were harvested and washed. For each experiment 2-4 ml of cell pellets were used. Cell pellets were resuspended in 4-5 pellet volumes of ice-cold buffer A (10mM HEPES ph 7.6, 1.5mM MgCl<sub>2</sub> and 10mM KCl, 0.5 mM DTT+ complete protease inhibitor), lysed for 10 minutes on ice and centrifuged for 10 min at 3000 rpm. Pellets were resuspended in 2 pellet volumes of buffer A. The cell suspension was homogenized with a Dounce Homogenizer with 10 strokes (pestle A). The homogenized suspension was centrifuged for 10 minutes with 3000 rpm. Supernatant was removed and pellets were centrifuged for 1 minute with 3000 rpm. Supernatant was removed and pellets were resuspended in 1.5 pellet volumes of buffer C (20 mM HEPES ph 7.6, 20% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, complete protease inhibitor). Nuclei suspension was homogenized with Dounce homogenizer with 10 strokes (pestle B). The suspension was then rotated on a rotor suspension at 4°C for 30 minutes and afterwards centrifuged for 15 min with maximal speed. Nuclear extracts were dialyzed with a SnakeSkin Dialysis tube to buffer D (20 mM HEPES [pH 7.6], 0.2 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 100 mM KCl, 20% glycerol, 0.5 mM DTT, 0.2 mM PMSF, 9.5 mg/l sodium Metabisulfite).

60  $\mu$ l of anti-FLAG M2 agarose beads (Sigma) equilibrated and washed in buffer C-100 (20 mM Hepes pH 7.6, 20% glycerol, 1.5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.2 mM EDTA, 0.02% NP40, 0.5 mM DTT, complete proteaseinhibitor) were added to 1.5 ml of nuclear extract in low-adherence microcentrifuge tubes and incubated for 3 hr at 4°C in the presence of 225 units of Benzonase (Novagen) on a rotator. Afterwards beads were washed 5-times with buffer C-100. Bound proteins were eluted four times with 60  $\mu$ l of 3xFLAG-peptide solution (buffer c-100 + 0.2 mg/ml 3xFLAG peptide) for 15 minutes at 4°C. Efficiency of co-immunoprecipitation and elution was checked via Western blot with staining against FLAG. Most efficient elutions were pooled, TCA precipitated, and proteins separated by polyacrylamide gel electrophoresis stained with (Invitrogen). MS was done in a label free format. For each identified peptide and protein spectral counts were calculated using commercial PEAK studio software, using standard filtering settings. Further, data were merged for all 10 slices into a single table, the sum of all spectral counts (per slice) was taken as a measure of the protein amount in the sample. Data were grouped into three experiments (Human, mouse 1 and mouse 2), sorted by spectral count abundance. The last 10% of the least abundant proteins were removed from each pull down list. The rest of the proteins were ranked in relative scale (0 for the most abundant to 0.9 as the least abundant). The cumulative abundance rank index was calculated for every candidate protein by subtracting each abundance rank index from the control rank index (GFP).

The list of proteins with Kme3 modifications was downloaded from Phosphosite database. Information for proteins abundance was downloaded from PaxDB site average abundance across all human samples was used for this analysis.

Cross comparison of the gene/protein lists from different databases was done using custom scripts. All illustrations were prepared in R and Python using standard graphic packages.

For identification of trimethylated interaction partners of CBX7 we only considered proteins which were in the top 20% of the relative ranked ordered CBX7-binding proteins and which have an abundance score below 100 (ppm) over all cell types and whose relative ranked score in the experimental sample is higher than in the control sample.

To confirm interaction of CBX7 and SETDB1 in K562 cells we performed Co-IP in FLAG-tagged CBX7 overexpressing K562 cells as described above and performed SDS Page. After protein transfer membrane was stained against CBX7 and SETDB1.

### **Detection of CBX7 and SETDB1 interaction in HL60 cells by DUOLINK in situ proximity ligation assay (PLA):**

$5 \times 10^4$  HL60 cells were fixed in ice-cold methanol for 5 minutes on cytospin slides. Interaction of the endogenous CBX7 and SETDB1 proteins in HL60 cells was assessed using the Duolink in situ Proximity Ligation Assay (PLA) (Olink Bioscience, Uppsala, Sweden), as described by the manufacturer. Used antibodies:

- Polyclonal rabbit anti human/mouse CBX7 p15 Santa Cruz Biotechnology, SC 70-232
- anti- SETDB1 Antibody (5H6A12) Pierce Protein, MA5-15722

### **Purification of FLAG-tagged protein:**

Retroviral supernatant for overexpressing FLAG-tagged huCBX7 and lentiviral supernatant for overexpressing FLAG-tagged huSETDB1 was produced as described above. 293FT cells were transduced with two transduction rounds and expanded.

FLAG-tagged SETDB1 and CBX7 was purified using anti-Flag M2 magnetic beads (Sigma). Frozen pellets were resuspended in 10 vol (v/v) lysis buffer (50 mM Tris-HCl pH 7.5, 600 mM NaCl, 2 mM EDTA, 1.0% NP40 (v/v), 1:1000  $\mu$ l Protease Inhibitor Mix (Sigma-Aldrich). Lysates were incubated for 30 min on ice while being inverted (3 times) every 5 min. After incubation the lysate was forced 10 times through a 26 g needle. Debris was pelleted by centrifugation (20000 g, 30 min, 4°C) and the supernatant transferred to a new tube. 1 original pellet vol. (v/v) anti-Flag M2 magnetic beads (Sigma-Aldrich) was washed 4 times with 1 ml TBS (50 mM Tris-Cl, pH 7.6; 150 mM NaCl) and 1 time with lysis buffer. The supernatant and the washed beads were combined and antigen capture was performed for 4 hr at 4°C using a head-over-tail rotator (HOT). After incubation, samples were placed on a magnetic stand for 1 min. The supernatant was removed and the bead-bound protein washed 3 times with 1ml lysis buffer (5 min HOT, 1 min magnetic stand) and 3 times with 1 ml wash buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA, 0.1% NP40 (v/v)). The FLAG-tagged protein was eluted by adding 150  $\mu$ l elution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 3xFlag-Peptide 1mg/ml) and incubation for 5 hr at 4°C. 20  $\mu$ l 85% glycerol were added to the eluted protein. Proteins were stored at -20°C.

### **Microscale thermophoresis (MST):**

SETDB1 was labeled with Monolith NT Protein Labelling kit Red-NHS (Nanotemper). For binding reactions, a constant of 25 nM NT-647 labeled

SETDB1 was used and a 1:1 serial dilution of CBX7, with 25 nM as the highest concentration

Binding reactions were prepared in 1x MST buffer supplemented with 0,05% Tween-20 to a total volume of 10 µl. Microscale thermophoresis (MST) analysis was performed on a Monolith NT.115 (Nanotemper) with 40% LED and 20% MST power using standard treated capillaries (Nanotemper). Kd was calculated using the MO affinity analysis software (Nanotemper).

## REFERENCES

- Bagger, F. O., Sasivarevic, D., Sohi, S. H., Laursen, L. G., Pundhir, S., Sonderby, C. K., Winther, O., Rapin, N., and Porse, B. T. (2016). BloodSpot: a database of gene expression profiles and transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Res* 44, D917-924.
- Bernstein, E., Duncan, E. M., Masui, O., Gil, J., Heard, E., and Allis, C. D. (2006). Mouse Polycomb Proteins Bind Differentially to Methylated Histone H3 and RNA and Are Enriched in Facultative Heterochromatin. *Molecular and Cellular Biology* 26, 2560-2569.
- Bilodeau, S., Kagey, M. H., Frampton, G. M., Rahl, P. B., and Young, R. A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes & Development* 23, 2484-2489.
- Bracken, A. P., Dietrich, N., Pasini, D., Hansen, K. H., and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev* 20, 1123-1136.
- Cancer Genome Atlas Research, N., Ley, T. J., Miller, C., Ding, L., Raphael, B. J., Mungall, A. J., Robertson, A., Hoadley, K., Triche, T. J., Jr., Laird, P. W., et al. (2013). Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 368, 2059-2074.
- Cao, R., Tsukada, Y., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol Cell* 20, 845-854.
- Collins, P. L., Kyle, K. E., Egawa, T., Shinkai, Y., and Oltz, E. M. (2015). The histone methyltransferase SETDB1 represses endogenous and exogenous retroviruses in B lymphocytes. *Proc Natl Acad Sci U S A* 112, 8367-8372.
- Comet, I., and Helin, K. (2014). Revolution in the Polycomb hierarchy. *Nat Struct Mol Biol* 21, 573-575.
- Eppert, K., Takenaka, K., Lechman, E. R., Waldron, L., Nilsson, B., van Galen, P., Metzeler, K. H., Poepl, A., Ling, V., Beyene, J., et al. (2011). Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med* 17, 1086-1093.
- Escamilla-Del-Arenal, M., da Rocha, S. T., Spruijt, C. G., Masui, O., Renaud, O., Smits, A. H., Margueron, R., Vermeulen, M., and Heard, E. (2013). Cdy1, a new partner of the inactive X chromosome and potential reader of H3K27me3 and H3K9me2. *Mol Cell Biol* 33, 5005-5020.
- Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., and Khorasanizadeh, S. (2005). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes & Development* 17, 1870-1881.
- Fritsch, L., Robin, P., Mathieu, J. R. R., Souidi, M., Hinaux, H., Rougeulle, C., Harel-Bellan, A., Ameyar-Zazoua, M., and Ait-Si-Ali, S. (2010). A Subset of the Histone H3 Lysine 9 Methyltransferases Suv39h1, G9a, GLP, and SETDB1 Participate in a Multimeric Complex. *Molecular Cell* 37, 46-56.
- Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015). PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Research* 43, D512-D520.
- Kahn, T. G., Dorafshan, E., Schultheis, D., Zare, A., Stenberg, P., Reim, I., Pirrotta, V., and Schwartz, Y. B. (2016). Interdependence of

PRC1 and PRC2 for recruitment to Polycomb Response Elements. *Nucleic Acids Res* 44, 10132-10149.

- Kamminga, L. M., Bystrykh, L. V., de Boer, A., Houwer, S., Douma, J., Weersing, E., Dontje, B., and de Haan, G. (2006). The Polycomb group gene *Ezh2* prevents hematopoietic stem cell exhaustion. *Blood* 107, 2170-2179.
- Kaustov, L., Ouyang, H., Amaya, M., Lemak, A., Nady, N., Duan, S., Wasney, G. A., Li, Z., Vedadi, M., Schapira, M., *et al.* (2011). Recognition and specificity determinants of the human *cbx* chromodomains. *J Biol Chem* 286, 521-529.
- Klaue, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., *et al.* (2013). Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. *Nat Cell Biol* 15, 353-362.
- Koide, S., Oshima, M., Takubo, K., Yamazaki, S., Nitta, E., Saraya, A., Aoyama, K., Kato, Y., Miyagi, S., Nakajima-Takagi, Y., *et al.* (2016). *Setdb1* maintains hematopoietic stem and progenitor cells by restricting the ectopic activation of nonhematopoietic genes. *Blood* 128, 638-649.
- Koya, J., Kataoka, K., Sato, T., Bando, M., Kato, Y., Tsuruta-Kishino, T., Kobayashi, H., Narukawa, K., Miyoshi, H., Shirahige, K., and Kurokawa, M. (2016). DNMT3A R882 mutants interact with polycomb proteins to block haematopoietic stem and leukaemic cell differentiation. *Nat Commun* 7.
- Laurenti, E., Doulatov, S., Zandi, S., Plumb, I., Chen, J., April, C., Fan, J. B., and Dick, J. E. (2013). The transcriptional architecture of early human hematopoiesis identifies multilevel control of lymphoid commitment. *Nat Immunol* 14, 756-763.
- Lehnertz, B., Pabst, C., Su, L., Miller, M., Liu, F., Yi, L., Zhang, R., Kros, J., Yung, E., Kirschner, J., *et al.* (2014). The methyltransferase G9a regulates HoxA9-dependent transcription in AML. *Genes Dev* 28, 317-327.
- Min, J., Zhang, Y., and Xu, R. M. (2003). Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. *Genes Dev* 17, 1825-1828.
- Morey, L., Aloia, L., Cozzuto, L., Benitah, S. A., and Di Croce, L. (2013). RYBP and Cbx7 define specific biological functions of polycomb complexes in mouse embryonic stem cells. *Cell Rep* 3, 60-69.
- Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S. A., and Di Croce, L. (2012). Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. *Cell Stem Cell* 10, 47-62.
- Nikoloski, G., Langemeijer, S. M. C., Kuiper, R. P., Knops, R., Massop, M., Tonnisson, E. R. L. T. M., van der Heijden, A., Scheele, T. N., Vandenbergh, P., de Witte, T., *et al.* (2010). Somatic mutations of the histone methyltransferase gene *EZH2* in myelodysplastic syndromes. *Nat Genet* 42, 665-667.
- Ren, C., Morohashi, K., Plotnikov, Alexander N., Jankovic, J., Smith, Steven G., Li, J., Zeng, L., Rodriguez, Y., Stojanoff, V., Walsh, M., and Zhou, M.-M. (2015). Small-Molecule Modulators of Methyl-Lysine Binding for the CBX7 Chromodomain. *Chemistry & Biology* 22, 161-168.
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of *BMI1*. *Blood* 111, 2621-2630.
- Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hassertjan, R. P., and Ebert, B. L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 126, 9-16.
- Stock, J. K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A. G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* 9, 1428-1435.
- Tadokoro, Y., Ema, H., Okano, M., Li, E., and Nakachi, H. (2007). De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *The Journal of Experimental Medicine* 204, 715.
- Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., *et al.* (2012). RYBP-PRC1 Complexes Mediate H2A Ubiquitylation at Polycomb Target Sites Independently of PRC2 and H3K27me3. *Cell* 148, 664-678.
- van den Boom, V., Rozenveld-Geugien, M., Bonardi, F., Malanga, D., van Gosliga, D., Heijink, A.

- M., Viglietto, G., Morrone, G., Fusetti, F., Vel-  
lenga, E., and Schuringa, J. J. (2013). Nonre-  
dundant and locus-specific gene repression  
functions of PRC1 paralog family members in  
human hematopoietic stem/progenitor cells.  
*Blood* 121, 2452-2461.
- Verhaak, R. G., Wouters, B. J., Erpelinck, C. A., Ab-  
bas, S., Beverloo, H. B., Lugthart, S., Lowen-  
berg, B., Delwel, R., and Valk, P. J. (2009).  
Prediction of molecular subtypes in acute my-  
eloid leukemia based on gene expression pro-  
filing. *Haematologica* 94, 131-134.
- Zhang, Y., Yang, X., Gui, B., Xie, G., Zhang, D.,  
Shang, Y., and Liang, J. (2011). Corepres-  
sor Protein CDYL Functions as a Molecular  
Bridge between Polycomb Repressor Complex  
2 and Repressive Chromatin Mark Trimethyl-  
ated Histone Lysine 27. *Journal of Biological  
Chemistry* 286, 42414-42425.





# 5 SUMMARY AND FUTURE PERSPECTIVES





## SUMMARY

The hematopoietic system is hierarchically organized, with hematopoietic stem cells, able to differentiate into all mature blood cells at the apex. Besides the capacity to differentiate, hematopoietic stem cells are also characterized by the ability to self-renew and thus control the size of the hematopoietic stem cell pool. Epigenetic proteins are important regulators of this equilibrium of self-renewal and differentiation and thereby maintain homeostasis of the hematopoietic tissue. Dysregulation of this balance can result in stem cell exhaustion, or in proliferative syndromes like leukemia. Identifying proteins or pathways involved in controlling this balance will provide insight into potential disease-relevant target structures for therapeutical approaches.

One particularly important class of epigenetic proteins is represented by the group of Polycomb proteins, which are involved in regulation of totipotent (O'Loghlen et al., 2012) as well as multipotent stem cells (Klauke et al., 2013; Rizo et al., 2008), X-chromosome inactivation (O'Loghlen et al., 2012), DNA-damage response (Vissers et al., 2012) and cancerogenesis (Mohty et al., 2007; Nikoloski et al., 2010).

The highly evolutionary conserved Polycomb group proteins are chromatin-associated proteins, which assemble in multimeric protein complexes and which repress target genes through post-translational modifications of histone tails (Cao et al., 2002; Stock et al., 2007), inhibition of RNA-polymerase II (Stock et al., 2007) and chromatin compaction (Endoh et al., 2012). Some Polycomb proteins possess catalytic activity for writing epigenetic marks like EZH1/2, which catalyzes the trimethylation of H3K27me<sub>3</sub>. Polycomb CBX proteins harbor a chromodomain for reading trimethylated lysine residues on histone proteins.

Although Polycomb Cbx proteins are evolutionary conserved, their number increased during evolution. Whereas invertebrates like *Drosophila* have only one Cbx protein, human have five Polycomb CBX proteins, namely CBX 2, 4, 6, 7 and 8, which increases the diversity of the composition of the PRC1 and thus probably also reflects different biological functions.

Overexpression of murine *Cbx7* in 5-fluoruracil treated bone marrow cells resulted upon transplantation in increased HSC self-renewal activity and in the development of immunophenotypically different subtypes of leukemia (Klauke et al., 2013). Short-hairpin mediated knockdown experiments of different human CBX proteins in CD34<sup>+</sup> cord blood cells

showed that knockdown of *CBX2* had the most detrimental effect and is associated with a strong reduction in progenitor and hematopoietic stem cell function (van den Boom et al., 2013).

In this PhD project, we investigated the role of human CBX proteins in the regulation of human hematopoietic stem and progenitor-derived cord blood cells. Using an overexpression approach, we ensured that one specific CBX protein is incorporated in the majority of PRC1 complexes, enabling to study the function of single CBX proteins. Specifically, we wanted to explore the role of *CBX7* in normal hematopoiesis and leukemia and to discover new, functionally relevant, interaction partners of murine and human CBX proteins.

In **Chapter 1** we provide an overview of epigenetics and hematopoiesis. We define the concept of a hematopoietic stem cell from a historical perspective, and introduce the reader to the first landmark studies that proved the existence of hematopoietic stem cells. We then briefly describe molecular and cellular components and function of the hematopoietic stem cell niche as one class of extrinsic regulators of hematopoietic stem cells and present transcription factors as an example of intrinsic regulators of hematopoietic stem cells. We concentrate on a second group of intrinsic regulators of hematopoietic stem cells: epigenetic proteins. We focus on DNA-methylation and post-translation modifications of histones as two key epigenetic mechanisms. In the last part of this chapter we show how the increasing knowledge of dysregulation of and mutations in genes coding for epigenetic proteins and their putative druggability is transferred from bench to bedside. Because epigenetic proteins do not only play a role in oncogenesis but also in other disease relevant pathways such as inflammation, epigenetic therapeutic approaches very likely will find their way in daily clinical practice also for treatment of non-malignant diseases like autoimmune disorders.

In Chapter 2 we discuss how hematopoietic stem cells are ageing and what the consequences this may have. We describe how the incidence of hematological malignancies is age-dependent and how aging is associated with a functional decline of the hematopoietic system, such as reduced vaccination efficiency (Goodwin et al., 2006) and increased susceptibility to infections (Frasca et al., 2008).

We briefly outline the current understanding of age-dependent changes of murine hematopoietic stem cells and how, to some extent, we lack knowledge about such changes of human stem cells. Furthermore, we

hypothesize that most features of an aged hematopoietic system are functional consequences of molecular events happening within primitive hematopoietic stem cells. However, beyond these cell-intrinsic mechanisms probably also extrinsic factors, like changes in the composition, or functionality of the niche, may result in functional impairment of hematopoietic stem cells. In the last part of this chapter, we speculate whether aged-dependent changes in hematopoietic stem cells may be reversible. Interestingly, hematopoietic stem cells that were derived from induced pluripotent stem (iPS) cells, generated from aged hematopoietic stem cells, have been shown to be functionally equivalent to young hematopoietic stem cells derived from iPS cells generated from young hematopoietic stem cells (Wahlestedt et al., 2013). This suggests reversibility of the aging process, and a functional role of epigenetic proteins in the ageing hematopoietic system. This is especially interesting as several studies have shown that mutations in epigenetic proteins, including DNMT3A, TET2, ASXL1 and SETDB1, are associated with the development of clonal hematopoiesis (Jaiswal et al., 2014; Steensma et al., 2015; Xie et al., 2014).

In **Chapter 3** we review the role of Polycomb proteins in hematopoiesis, during development, ageing and disease. We introduce the reader to the composition of different Polycomb group complexes (PRC), with a particular focus on PRC2 and canonical PRC1 and their function. The fact that mice deficient for one of the three core components of PRC2 are not viable (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004) shows how crucial these proteins are for proper embryonic development. Mice deficient for PRC1 members show multiple defects in later stages of the development (Core et al., 1997; Forzati et al., 2012; van der Lugt et al., 1994). All these studies highlight the crucial function of these proteins for regulation of omnipotent and multipotent stem cells.

The significant importance of these proteins for regulation of cell cycle and stem cell self-renewal is also evident from the fact that dysregulation of and mutations in genes coding for Polycomb proteins can be detected in hematological diseases (e.g. *BMI1* overexpression in chronic lymphatic leukemia (Beà et al., 2001), *EZH2* mutations in diffuse large B-cell lymphoma (Morin et al., 2010)) as well as in carcinomas ((*BMI1* in non-small lung cancer (Vonlanthen et al., 2001) and breast cancer (Paranjape et al., 2014)). Targeting Polycomb proteins using inhibitors like tazemostat for *EZH2* have now increased the therapeutic repertoire in follicular lymphoma (Morschhauser F, 2017).

In general, this Chapter serves as an introduction to the main topic and experimental results presented in Chapter 4.

In **Chapter 4** we studied the role of different human CBX proteins in the regulation of CD34+ cord blood-derived hematopoietic stem and progenitor cells by their enforced retroviral overexpression. As already previously mentioned, the number of CBX proteins increased during evolution from one to five. All CBX Polycomb proteins harbor a chromodomain with which they are able to recognize trimethylated lysine residues, like H3K27me3 (Kaustov et al., 2011). Because Polycomb CBX proteins are the only members of the PRC1 family harboring a reading domain for post-translational modifications of histone tails, different CBX proteins probably guide the PRC1 to different genomic loci thereby controlling diverse but also partly overlapping subsets of genes and thus obtain different functions.

We show through retroviral overexpression that CBX7 and, to a lesser extent CBX8, enhanced the function of hematopoietic CD34+ progenitor cells *in vitro*, whereas overexpression of CBX 2, 4 and 6 did not show comparable, but in fact partly opponent phenotypes. Similar results were obtained when we assessed the consequences for the most primitive hematopoietic compartment *in vitro*. CBX7 enhanced the self-renewal activity of human hematopoietic stem and progenitor cells. Analysis of a previously published microarray of different human CD34+ subsets showed decreasing expression of *CBX7* mRNA in CD34+ compartment during differentiation from primitive hematopoietic stem cells towards lineage-restricted progenitor cells.

In line with our *in vitro* observations, immunodeficient mice transplanted with *CBX7* overexpressing CD34+ cord blood cells, showed higher multi-lineage engraftment levels, even after one week of *in vitro* culture, enhanced myelopoiesis and an increased percentage of CD34+CD38- cells in the bone marrow. Transcriptome analysis of *CBX7* overexpressing cells revealed that genes important for differentiation were repressed, whereas genes involved in regulating cell cycle were upregulated. Because higher self-renewal activity of hematopoietic stem and progenitor cells, accompanied by repression of genes crucial for differentiation, is a hallmark of leukemia, we studied *CBX7* expression in AML. We analyzed two cohorts of AML patients and found, in both datasets, in multiple subtypes higher *CBX7* expression in comparison to their healthy counterparts.

To explore the functional role of *CBX7* expression in AML we down-regulated *CBX7* mRNA via short hairpins and observed an inhibition in

proliferation as well as an upregulation of myeloid differentiation markers like *CD11b* and *CD14*.

To identify novel interaction partners of CBX proteins we performed mass spectrometry analysis of FLAG-pull downs of FLAG-tagged CBX7 and FLAG-tagged GFP overexpressing cells. We hypothesized that CBX proteins may also bind to non-histone proteins if these harbor a trimethylated lysine in a similar peptide context as H3K27me3. Indeed, after performing multiple stringent filtering steps we identified multiple novel CBX7 partner proteins that contained such a peptide context. Interestingly, several of these were H3K9 methyltransferases and one was an H3K9 associated protein. Downregulation of *SETDB1*, one the newly identified CBX7-binding H3K9 methyltransferases, in AML cell lines was associated with upregulation of *CD11b* and *CD14*, and loss of cell proliferation, indicating that CBX7 and *SETDB1* co-repress genes important for differentiation.

## DISCUSSION AND FUTURE PERSPECTIVES

In the following paragraphs, I discuss and speculate on the results and future perspectives of this project with a particular focus on the functionality of CBX7 and possible translational aspects of our findings.

### Interaction partners and recruitment of CBX7

Our overexpression studies show that CBX7, in comparison to other CBX proteins, has a unique and evolutionary conserved function in regulating hematopoietic stem and progenitor cells. Although chromodomains of all Polycomb CBX proteins are evolutionary conserved and highly similar, overexpression of distinct CBX proteins results in expression of distinct and only partly overlapping subsets of genes. This suggests that other domains of the CBX proteins, which have been less evolutionary conserved, may be responsible for the different phenotypes, possibly by binding to different genetic target loci or by binding to distinct interaction partners, resulting in a variety of recruitment mechanisms.

During evolution, the number of Polycomb CBX homologs increased from one to five. Whereas the single *Drosophila* Polycomb Cbx protein only recognizes H3K27me3, human Polycomb CBX proteins can bind H3K27me3 as well as H3K9me3 with different binding affinities *in vitro*.



Interestingly, at least *in vitro*, human CBX7 has the highest binding affinity towards H3K9me<sub>3</sub>, whereas CBX2 *in vitro* only recognizes H3K27me<sub>3</sub> (Kaustov et al., 2011). So far, no genome-wide binding studies have been performed to assess whether CBX7 and H3K9me<sub>3</sub> have common targets. Our results show that CBX7 is, at least under certain conditions, localized close to sites of H3K9me<sub>3</sub>. In line with these findings we identified multiple H3K9 methyltransferases, including SETDB1, as putative binding partners for CBX7. We confirmed the interaction between CBX7 and SETDB1 on endogenous expression levels in HL60 cells via Proximity ligation assay. Furthermore, we showed direct interaction of both proteins in a cell-free environment via micro-scale thermophoresis. Although we did not perform a quantitative mass spectrometry analysis, our data suggest that CBX7 binds with higher affinity to SETDB1 compared to CBX8. In agreement with our *in vivo* functional data, it has been shown that the chromodomain of CBX7 binds with higher affinity to a 20-aminoacid long peptide harboring three trimethylated lysine residues representing parts of the protein of human SETDB1 in comparison to CBX8 (Kaustov et al., 2011).

In general, these different binding affinities could contribute to the variety of phenotypes that we observed upon overexpression of different CBX proteins. Interestingly, CBX7, as well as SETDB1, were identified in a knockout-screen for genes which prevent differentiation of embryonic stem cells (Bilodeau et al., 2009). In the same publication, it was shown that in embryonic stem cells especially genes classified as developmental regulators were bound by H3K9me<sub>3</sub> and H3K27me<sub>3</sub>. Furthermore, 20% of all euchromatic genes bound by H3K9me<sub>3</sub> were also bound by SETDB1, indicating that at least in some parts of the chromatin the Polycomb system and SETDB1 jointly regulate expression of target genes (Bilodeau et al., 2009). Furthermore, SETDB1 peaks in Chip-seq experiments can either occur coinciding with H3K9me<sub>3</sub> or as single peaks without evidence of H3K9me<sub>3</sub> presence. Interestingly, peaks of SETDB1 were associated with binding of EZH2 and RING1B, further suggesting crosstalk between these two pathways (Fei et al., 2015).

The fact that all three H3K9 methyltransferases have been observed in previous mass spectrometry experiments to harbor trimethylated lysine residues embedded in a motif highly similar to H3K9me<sub>3</sub> and H3K27me<sub>3</sub> (Hornbeck et al., 2015) suggests that CBX7 binds to these via the chromodomain. According to the classical hierarchical recruitment model, the PRC2 complex is guided to non-methylated CpG-islands, resulting

in the trimethylation of H3K27 through EZH2. The canonical PRC1 complex can subsequently recognize H3K27me<sub>3</sub> through binding of the chromodomain of one out of the five Polycomb CBX proteins to H3K27me<sub>3</sub> (Comet and Helin, 2014).

The identification of three H3K9 methyltransferases, as well as CDYL, as CBX7 binding partners, which were found to be trimethylated in previous mass spectrometry experiments, suggests an alternative, PRC2-independent, recruitment model.

The methyltransferase SETDB1 contains multiple functional important domains allowing interaction to other epigenetic pathways: the MBD domain for sensing methylated DNA, and two tudor domains allowing binding of mSin3A/B and HDAC1/2 (Karanth et al., 2017). In a hypothetical PRC2 independent recruitment model, trimethylated SETDB1 could initially be recruited to H3K9me<sub>2</sub> or methylated DNA and attract a CBX7 containing PRC1 resulting in chromatin compaction and repression of target genes.

It remains unclear whether H3K9me<sub>3</sub> and H3K27me<sub>3</sub> modifications, as well as CBX7 and SETDB1 binding, are occurring at the same histone protein (symmetrical) or at the other H3 protein with which it forms the dimer (asymmetrical). Performing mass spectrometry analysis of single histone proteins could potentially answer this question (Rothbart and Strahl, 2014). As described in the Introduction of this thesis, post-translation modifications of histones can occur on the protruding tail as well as on the globular domain. Profiling of single histone proteins from CBX7 overexpressing cells would allow to screen for other marks co-occurring with CBX7 either on the tail as well as on the globular domain.

In colon cancer cells, it has been shown that CBX7 interacts with all three DNA methyltransferases, and that its overexpression resulted in hypermethylation of CpG containing promoters (Mohammad et al., 2009). Whether and to what extent CBX7 mediated repression of genes is associated with hypermethylation of promoter regions, could be easily assessed by performing DNA methylation arrays or by bisulfite sequencing.

CBX7 can bind to H3K27me<sub>3</sub> and H3K9me<sub>3</sub> but also directly to long non-coding RNA (Yap et al., 2010), which offers an alternative recruitment mechanism. So far, non-coding RNA binding of a CBX protein has been described for CBX7 as well as CBX4, two proteins whose chromodomains are extremely similar except for one amino acid (Gil and O’Loughlin, 2014). Recently, an unexpected role was shown for CBX7 as a mRNA binding protein resulting in upregulation of the target gene (Rosenberg et al.,

2017). CLIP-Seq (cross-linking immunoprecipitation-high-throughput sequencing) in CBX7 overexpressing CD34+ HSPCs could reveal direct mRNA and long non-coding RNA targets for CBX7, and an integrated analysis with our transcriptome could further answer whether CBX7 and mRNA interaction leads to differential expression of genes.

## **Global epigenetic and chromatin changes upon overexpression of CBX7**

In the previous paragraph, we discussed and speculated on direct CBX7 interaction partners resulting in local epigenetic changes. Beyond these local changes, overexpression of CBX7 may result in more global changes of the epigenetic landscape, far away from initial CBX7 binding sites through differential expression of additional epigenetic modifiers. Indeed, in the list of differential expressed genes we observed multiple epigenetic modifier and reader proteins, which may result in global epigenetic changes. This included genes involved in DNA methylation, such as *DNMT3A* and *IDH2*, as well as genes involved in methylation and demethylation of H3K4me3, such as the H3K4 methyltransferase *PRDM16*, and the demethylase *KDM1A*. Furthermore, we observed repression of genes involved in demethylation of H3K9 and H3K27, including *KDM7A*, and genes involved in methylation of arginine residues on histone proteins, *PRMT2*.

## **CBX7- a putative oncogene in hematopoietic neoplasms and potential clinical implications**

Overexpression of murine *Cbx7* in 5-Fluoruracil treated bone marrow cells resulted in 90% of all transplanted animals in a leukemic phenotype with rapid onset. 60% of these mice developed a T-cell lymphocytosis, with enlarged spleens and lymph nodes. Twenty percent of all mice developed a leukocytosis without expression of lineage markers, and 10% of all mice developed a leukopenia and anemia with reticulocytosis (Klauke et al., 2013). Furthermore, elevated CBX7 levels were detected in patient samples in follicular lymphoma (Scott et al., 2007) and SNPs in the promoter and enhancer region of CBX7 have been associated with a higher risk to develop multiple myeloma (Chubb et al., 2013).

In line with its potential role in the development or progression of malignant hematopoietic neoplasms, upon overexpression of *CBX7* we

observed higher self-renewal activity of human primitive hematopoietic stem and progenitor cells, higher rates of proliferation in a cytokine-driven suspension culture and higher engraftment in immunodeficient mice upon transplantation. Interestingly, we did not observe increased lymphopoiesis which could have been due to the use of cytokine combinations that mostly promote the growth of primitive hematopoietic stem as well as myeloid progenitor cells, but not of lymphoid progenitor cells.

In contrast, we observed a higher percentage of CD33+ cells in the compartment of CD45+GFP+ cells, which led us to evaluate the expression of CBX7 in AML patient samples in a previously published microarray. This analysis revealed a significantly higher expression of CBX7 in CD34+ cells of AML-patients in comparison to CD34+ peripheral mobilized stem cells of healthy individuals. CBX7 was also higher expressed in The Cancer Genome Atlas dataset. Interestingly, it was recently shown that a CBX7 containing PRC1 interaction with DNMT3AR882 blocks differentiation of murine hematopoietic stem cells (Koya et al., 2016). This finding complements our transcriptome data, in which we find especially repression of genes important for differentiation of diverse hematopoietic cell types. Knockdown of CBX7 *mRNA* via short-hairpins resulted in the expression of markers of myeloid differentiation, like *CD11b* and *CD14*. Furthermore, knockdown of CBX7 in OCI-AML3, as well as HL60, cells resulted in inhibition of proliferation, what nicely corresponds to the observation that overexpression of CBX7 results in upregulation cell cycle genes. Our transcriptome data also identified a subset of genes expressed predominantly in primitive CD34+ cells which belong to the KEGG pathway GO group “Transcriptional misregulation in cancer” like *HMGA2*, *CCND2*, *ERG*, *IGF1R*, *LMO2*, *MEIS1* and *MYCN*.

These facts make it worthwhile to aim for targeting CBX7 in hematological diseases. So far, three chemical compounds have been described to inhibit the chromodomain of CBX7 (Ren et al., 2015; Simhadri et al., 2014; Stuckey et al., 2016). Because chromodomains of all human Polycomb CBX proteins are evolutionary conserved, and also similar to other proteins harboring a chromodomain, like heterochromatin-associated proteins or CDYL, development of inhibitors which uniquely target the chromodomain of human CBX7 without having any kind of off-target effects is quite challenging. Furthermore, these compounds will have to be able to cross the cell membrane to reach a sufficiently high intracellular concentration to prevent binding of CBX7 to trimethylated lysine residues. So far, two of the three chemical probes showed intracellular

activity. Both compounds were tested in a prostate carcinoma cell line. Whereas one inhibitor resulted in increased expression of *p16/CDKN2A* (Ren et al., 2015), a classical CBX7 target, the other inhibitor was additionally able to inhibit proliferation (Stuckey et al., 2016).

Furthermore, targeting of CBX7 with chromodomain-inhibitors could also impair self-renewal of benign hematopoietic cells. Our LTC-IC assay data suggest that self-renewal of human benign hematopoietic stem cells is at least *in vitro* impaired upon knockdown of CBX7 in CD34+ cord blood cells. In contrast, *Cbx7*<sup>-/-</sup> mice show no hematological abnormalities, suggesting that CBX7 would be not essential for steady state hematopoiesis (Forzati et al., 2012). However, functional *in vitro* as well as *in vivo* tests of hematopoietic stem cells were not performed in these mice, leaving room for speculation that CBX7 may be essential for hematopoiesis under stress conditions like infections, bleeding or stem cell transplantation.

Besides that, CBX7 is expressed in a variety of non-hematopoietic cells, suggesting that the use of such an inhibitor may provoke side-effects in non-hematopoietic tissues. For targeting CBX7 specifically in AML cells, approaches which use vehicle strategies like GO (Mylotarg®) could be useful. Using the same approach CBX7 could be linked to antibodies specifically recognizing CD33, an antigen expressed mainly on myeloid progenitors, monocytes, neutrophil granulocytes and to some extent on multipotent hematopoietic stem cells (Linenberger, 2005; Taussig et al., 2005). Furthermore, CD33 is expressed in 85-90 % of adult and pediatric AML cells and the expression of CD33 is on average 3-times higher on leukemic blasts in comparison to CD33+ healthy bone marrow cells (Linenberger, 2005). Due to expression differences of CD33 between benign and AML cells one could expect to observe mainly CBX7 inhibition in the myeloid and especially in the malignant compartment.

As our data suggest that CBX7 is able to bind by its chromodomain to H3K27m<sub>3</sub>, to H3K9me<sub>3</sub>, as well as to trimethylated non-histone proteins, its inhibition will probably also result in interruption of the interaction of these proteins, including SETDB1. Interestingly, like CBX7, SETDB1 is also overexpressed in prostate carcinoma cells (Sun et al., 2014).

Because CBX proteins interact in multimeric protein complexes and in concert with functional repressing systems like PRC2, histone deacetylases, as well as DNA methyltransferases, it might be worthwhile to consider combinational therapy approaches with demethylating agents, histone deacetylases as well as EZH2-Inhibitors.

In the early beginnings of cancer therapy most therapeutic regimes contained only classical cytotoxic reagents, using a ‘one size fits all’ approach for each histological cancer subtype. In the last decade multiple drugs have been approved targeting specifically proteins mutated or over-expressed in cancer cells, allowing to design individual treatment protocols based on expression and genomic data of the individual cancer at diagnosis resulting in personalized medicine. The increasing knowledge about epigenetic mechanisms in benign and malignant cells will probably result in the development of new compounds specifically targeting the hugely complex epigenetic machinery. Incorporation of epigenetic profiles of cancer cells at diagnosis might allow to use compounds targeting epigenetic “Achilles heels”, which promote growth advantage or resistance to other drugs in cancer cells.

## REFERENCES

- Beà, S., Tort, F., Pinyol, M., Puig, X., Hernández, L., Hernández, S., Fernández, P. L., van Lohuizen, M., Colomer, D., and Campo, E. (2001). BMI-1 Gene Amplification and Overexpression in Hematological Malignancies Occur Mainly in Mantle Cell Lymphomas. *Cancer Research* 61, 2409-2412.
- Bilodeau, S., Kagey, M. H., Frampton, G. M., Rahl, P. B., and Young, R. A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes & Development* 23, 2484-2489.
- Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002). Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing. *Science* 298, 1039-1043.
- Chubb, D., Weinhold, N., Broderick, P., Chen, B., Johnson, D. C., Forsti, A., Vijaykrishnan, J., Migliorini, G., Dobbins, S. E., Holroyd, A., et al. (2013). Common variation at 3q26.2, 6p21.33, 17p11.2 and 22q13.1 influences multiple myeloma risk. *Nat Genet* 45, 1221-1225.
- Comet, I., and Helin, K. (2014). Revolution in the Polycomb hierarchy. *Nat Struct Mol Biol* 21, 573-575.
- Core, N., Bel, S., Gaunt, S. J., Aurrand-Lions, M., Pearce, J., Fisher, A., and Djabali, M. (1997). Altered cellular proliferation and mesoderm patterning in Polycomb-M33-deficient mice. *Development* 124, 721-729.
- Endoh, M., Endo, T. A., Endoh, T., Isono, K., Sharif, J., Ohara, O., Toyoda, T., Ito, T., Eskeland, R., Bickmore, W. A., et al. (2012). Histone H2A mono-ubiquitination is a crucial step to mediate PRC1-dependent repression of developmental genes to maintain ES cell identity. *PLoS Genet* 8, e1002774.
- Faust, C., Lawson, K. A., Schork, N. J., Thiel, B., and Magnuson, T. (1998). The Polycomb-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development* 125, 4495-4506.
- Fei, Q., Yang, X., Jiang, H., Wang, Q., Yu, Y., Yu, Y., Yi, W., Zhou, S., Chen, T., Lu, C., et al. (2015). SetDB1 modulates PRC2 activity at developmental genes independent of H3K9 trimethylation in mouse ES cells. *Genome Research*.
- Forzati, F., Federico, A., Pallante, P., Abbate, A., Esposito, F., Malapelle, U., Sepe, R., Palma, G., Troncone, G., Scarfo, M., et al. (2012). CBX7 is a tumor suppressor in mice and humans. *J Clin Invest* 122, 612-623.
- Gil, J., and O’Loughlin, A. (2014). PRC1 complex diversity: where is it taking us? *Trends Cell Biol* 24, 632-641.

- Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015). PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Research* 43, D512-D520.
- Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., Lindsley, R. C., Mermel, C. H., Burt, N., Chavez, A., et al. (2014). Age-Related Clonal Hematopoiesis Associated with Adverse Outcomes. *New England Journal of Medicine* 371, 2488-2498.
- Karanth, A. V., Maniswami, R. R., Prashanth, S., Govindaraj, H., Padmavathy, R., Jegatheesan, S. K., Mullangi, R., and Rajagopal, S. (2017). Emerging role of SETDB1 as a therapeutic target. *Expert Opin Ther Targets* 21, 319-331.
- Kaustov, L., Ouyang, H., Amaya, M., Lemak, A., Nady, N., Duan, S., Wasney, G. A., Li, Z., Vedadi, M., Schapira, M., et al. (2011). Recognition and specificity determinants of the human cbx chromodomains. *J Biol Chem* 286, 521-529.
- Klaue, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., et al. (2013). Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. *Nat Cell Biol* 15, 353-362.
- Koya, J., Kataoka, K., Sato, T., Bando, M., Kato, Y., Tsuruta-Kishino, T., Kobayashi, H., Narukawa, K., Miyoshi, H., Shirahige, K., and Kurokawa, M. (2016). DNMT3A R882 mutants interact with polycomb proteins to block haematopoietic stem and leukaemic cell differentiation. *Nat Commun* 7.
- Linenger, M. L. (2005). CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. *Leukemia* 19, 176-182.
- Mohammad, H. P., Cai, Y., McGarvey, K. M., Easwaran, H., Van Neste, L., Ohm, J. E., O'Hagan, H. M., and Baylin, S. B. (2009). Polycomb CBX7 Promotes Initiation of Heritable Repression of Genes Frequently Silenced with Cancer-Specific DNA Hypermethylation. *Cancer Research* 69, 6322-6330.
- Mohty, M., Yong, A. S. M., Szydlo, R. M., Apperley, J. F., and Melo, J. V. (2007). The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. *Blood* 110, 380-383.
- Morin, R. D., Johnson, N. A., Severson, T. M., Mungall, A. J., An, J., Goya, R., Paul, J. E., Boyle, M., Woolcock, B. W., Kuchenbauer, F., et al. (2010). Somatic mutation of EZH2 (Y641) in Follicular and Diffuse Large B-cell Lymphomas of Germinal Center Origin. *Nature genetics* 42, 181-185.
- Morschhauser F, S. G., McKay P, et al. (2017). Interim report from a phase 2 multicenter study of tazemetostat, an EZH2 inhibitor, in patients with relapsed or refractory B-cell non-Hodgkin lymphomas. In 14th International Conference on Malignant Lymphoma, (Lugano, Switzerland).
- Nikoloski, G., Langemeijer, S. M. C., Kuiper, R. P., Knops, R., Massop, M., Tonissen, E. R. L. T. M., van der Heijden, A., Scheele, T. N., Vandenbergh, P., de Witte, T., et al. (2010). Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 42, 665-667.
- O'Carroll, D., Erhardt, S., Pagani, M., Barton, S. C., Surani, M. A., and Jenuwein, T. (2001). The Polycomb-Group Gene *Ezh2* Is Required for Early Mouse Development. *Molecular and Cellular Biology* 21, 4330-4336.
- O'Loughlen, A., Munoz-Cabello, A. M., Gaspar-Maia, A., Wu, H. A., Banito, A., Kunowska, N., Racek, T., Pemberton, H. N., Beolchi, P., Laval, F., et al. (2012). MicroRNA regulation of *Cbx7* mediates a switch of Polycomb orthologs during ESC differentiation. *Cell Stem Cell* 10, 35-46.
- Paranjape, A. N., Balaji, S. A., Mandal, T., Krushik, E. V., Nagaraj, P., Mukherjee, G., and Rangarajan, A. (2014). *Bmi1* regulates self-renewal and epithelial to mesenchymal transition in breast cancer cells through Nanog. *BMC Cancer* 14, 1-14.
- Pasini, D., Bracken, A. P., Jensen, M. R., Denchi, E. L., and Helin, K. (2004). *Suz12* is essential for mouse development and for EZH2 histone methyltransferase activity. *The EMBO Journal* 23, 4061-4071.
- Ren, C., Morohashi, K., Plotnikov, Alexander N., Jankovic, J., Smith, Steven G., Li, J., Zeng, L., Rodriguez, Y., Stojanoff, V., Walsh, M., and Zhou, M.-M. (2015). Small-Molecule Modulators of Methyl-Lysine Binding for the CBX7 Chromodomain. *Chemistry & Biology* 22, 161-168.
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* 111, 2621-2630.



- Rosenberg, M., Blum, R., Kesner, B., Maier, V. K., Szanto, A., and Lee, J. T. (2017). Denaturing CLIP, dCLIP, Pipeline Identifies Discrete RNA Footprints on Chromatin-Associated Proteins and Reveals that CBX7 Targets 5' UTRs to Regulate mRNA Expression. *Cell Syst* 5, 368-385 e315.
- Rothbart, S. B., and Strahl, B. D. (2014). Interpreting the language of histone and DNA modifications. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1839, 627-643.
- Scott, C. L., Gil, J., Hernando, E., Teruya-Feldstein, J., Narita, M., Martinez, D., Visakorpi, T., Mu, D., Cordon-Cardo, C., Peters, G., et al. (2007). Role of the chromobox protein CBX7 in lymphomagenesis. *Proc Natl Acad Sci U S A* 104, 5389-5394.
- Simhadri, C., Daze, K. D., Douglas, S. F., Quon, T. T. H., Dev, A., Gignac, M. C., Peng, F., Heller, M., Boulanger, M. J., Wulff, J. E., and Hof, F. (2014). Chromodomain Antagonists That Target the Polycomb-Group Methyllysine Reader Protein Chromobox Homolog 7 (CBX7). *Journal of Medicinal Chemistry* 57, 2874-2883.
- Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hasseryan, R. P., and Ebert, B. L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 126, 9-16.
- Stock, J. K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A. G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* 9, 1428-1435.
- Stuckey, J. I., Dickson, B. M., Cheng, N., Liu, Y., Norris, J. L., Cholensky, S. H., Tempel, W., Qin, S., Huber, K. G., Sagum, C., et al. (2016). A cellular chemical probe targeting the chromodomains of Polycomb repressive complex 1. *Nat Chem Biol* 12, 180-187.
- Sun, Y., Wei, M., Ren, S. C., Chen, R., Xu, W. D., Wang, F. B., Lu, J., Shen, J., Yu, Y. W., Hou, J. G., et al. (2014). Histone methyltransferase SETDB1 is required for prostate cancer cell proliferation, migration and invasion. *Asian J Androl* 16, 319-324.
- Taussig, D. C., Pearce, D. J., Simpson, C., Rohatiner, A. Z., Lister, T. A., Kelly, G., Luongo, J. L., Danet-Desnoyers, G. A., and Bonnet, D. (2005). Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood* 106, 4086-4092.
- van den Boom, V., Rozenveld-Geugien, M., Bonardi, F., Malanga, D., van Gosliga, D., Heijink, A. M., Viglietto, G., Morrone, G., Fusetti, F., Vellenga, E., and Schuringa, J. J. (2013). Nonredundant and locus-specific gene repression functions of PRC1 paralog family members in human hematopoietic stem/progenitor cells. *Blood* 121, 2452-2461.
- van der Lugt, N. M., Domen, J., Linders, K., van Roon, M., Robanus-Maandag, E., te Riele, H., van der Valk, M., Deschamps, J., Sofroniew, M., and van Lohuizen, M. (1994). Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. *Genes & Development* 8, 757-769.
- Visser, J. H., van Lohuizen, M., and Citterio, E. (2012). The emerging role of Polycomb repressors in the response to DNA damage. *J Cell Sci* 125, 3939-3948.
- Vonlanthen, S., Heighway, J., Altermatt, H. J., Guger, M., Kappeler, A., Borner, M. M., Lohuizen, M. v., and Betticher, D. C. (2001). The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer* 84, 1372-1376.
- Wahlestedt, M., Norddahl, G. L., Sten, G., Ugale, A., Frisk, M.-A. M., Mattsson, R., Deierborg, T., Sigvardsson, M., and Bryder, D. (2013). An epigenetic component of hematopoietic stem cell aging amenable to reprogramming into a young state. *Blood* 121, 4257-4264.
- Xie, M., Lu, C., Wang, J., McLellan, M. D., Johnson, K. J., Wendl, M. C., McMichael, J. F., Schmidt, H. K., Yellapantula, V., Miller, C. A., et al. (2014). Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 20, 1472-1478.
- Yap, K. L., Li, S., Munoz-Cabello, A. M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M. J., and Zhou, M. M. (2010). Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. *Mol Cell* 38, 662-674.





# **A**PPENDICES

---

**NEDERLANDSE SAMENVATTING**

**ACKNOWLEDGMENTS**

**CV**



## NEDERLANDSE SAMENVATTING

Het hematopoïetische systeem is hiërarchisch georganiseerd met aan de basis hematopoïetische stamcellen die kunnen differentiëren tot alle volwassen bloedcellen. Naast de capaciteit om te differentiëren, worden hematopoïetische stamcellen gekarakteriseerd door de mogelijkheid om zichzelf te hernieuwen en zo bepalen zij de grootte van de hematopoïetische stamcelcompartiment. Epigenetische eiwitten zijn belangrijke regulatoren van dit evenwicht tussen zelfhernieuwing en differentiatie en behouden zo de homeostase van het hematopoïetische weefsel. Verstoring van deze balans kan leiden tot stamcel uitputting of tot proliferatieve syndromen zoals leukemie. Het identificeren van eiwitten en de routes betrokken bij het controleren van deze balans geeft inzicht in mogelijke ziekte-relevante targets voor therapeutische aanpak.

Eén met name belangrijke groep van epigenetische eiwitten wordt gerepresenteerd door de Polycomb eiwitten, die betrokken zijn bij de regulatie van zowel totipotente, (O’Loughlen et al., 2012), als multipotente stamcellen (Klauke et al., 2013; Rizo et al., 2008), X-chromosoom inactivatie (O’Loughlen et al., 2012), DNA-schade respons (Vissers et al., 2012) en carcinogenese (Mohty et al., 2007; Nikoloski et al., 2010). De in grote mate evolutionair geconserveerde Polycomb groep eiwitten zijn chromatine-geassocieerde eiwitten, die deel uitmaken van multimere eiwitcomplexen en die target genen kunnen onderdrukken door post-translationele modificaties van histonen (Cao et al., 2002; Stock et al., 2007), inhibitie van RNA- polymerase II (Stock et al., 2007) en verdichting van de chromatine structuur (Endoh et al., 2012). Sommige Polycomb eiwitten bezitten katalytische activiteit voor het schrijven van epigenetische markeringen, zoals EZH1/2, dat de trimethylering van H3K27 katalyseert. Polycomb CBX eiwitten bezitten een chromodomein dat getrimethyleerde lysine residuen op histoneiwitten kan lezen.

Hoewel de Polycomb Cbx eiwitten evolutionair geconserveerd zijn, neemt het aantal toe tijdens evolutie. Waar ongewervelden zoals *Drosophila* enkel één Cbx eiwit kennen, hebben mensen vijf Polycomb CBX eiwitten, namelijk CBX 2, 4, 6, 7 en 8. Hierdoor neemt de diversiteit in de samenstelling van het PRC1 complex toe, wat waarschijnlijk ook verschillende biologische functies reflecteert.

Overexpressie van Cbx7 in 5-fluoruracil behandelde muizen beenmergcellen resulteerde na transplantatie in verhoogde hematopoïetische

stamcel (HSC) zelfhernieuwingsactiviteit en in de ontwikkeling van immunofenotypisch verschillende subtypen van leukemie (Klauke et al., 2013). Short-hairpin gemedieerde knockdown experimenten van de verschillende humane CBX eiwitten in CD34+ navelstreng bloedcellen lieten zien dat knockdown van CBX2 het meest nadelige effect had en geassocieerd is met een sterke vermindering van de functie van hematopoietische stam- en voorlopercellen (van den Boom et al., 2013).

In dit PhD project, wordt de rol van de humane CBX eiwitten in de regulatie van humane hematopoietische stam- en voorlopercellen afkomstig uit navelstrengbloed onderzocht. De incorporatie van een specifiek CBX eiwit in de meerderheid van de PRC1 complexen werd bereikt door middel van een overexpressie aanpak, die het mogelijk maakte om de functie van enkele CBX eiwitten te bestuderen. We wilden met name de rol van CBX7 in normale hematopoëse en leukemie verder onderzoeken, en nieuwe, functioneel relevante, interactiepartners van muis en humane CBX eiwitten ontdekken.

In **hoofdstuk één** laten we een overzicht zien van epigenetica en hematopoiese. Het concept van de hematopoietische stamcel wordt vanuit historisch perspectief gedefinieerd en de studies die belangrijke mijlpalen zijn in het aantonen van het bestaan van de hematopoietische stamcel worden geïntroduceerd. Vervolgens worden kort de moleculaire en cellulaire componenten en functie van de hematopoietische stamcelniche als een van de groepen van extrinsieke regulatoren van hematopoietische stamcellen besproken en worden transcriptie factoren gepresenteerd als een voorbeeld van intrinsieke regulatoren van hematopoietische stamcellen. We concentreren ons op een tweede groep van intrinsieke regulatoren van hematopoietische stamcellen: epigenetische eiwitten. We focussen op DNA- methylering en post-translationele modificaties van histonen als twee belangrijke epigenetische mechanismen. In het laatste deel van dit hoofdstuk laten we zien hoe toenemende kennis over verstoring van en mutaties in genen die coderen voor epigenetische eiwitten en hun farmacologische toepasbaarheid wordt vertaald van onderzoek naar kliniek. Aangezien epigenetische eiwitten niet alleen een rol spelen in oncogenese, maar ook in andere ziekte-relevante routes, zoals ontsteking, is het erg waarschijnlijk dat epigenetische benaderingen hun weg vinden naar de dagelijkse klinische praktijk, ook voor behandeling van niet maligne ziekte zoals auto-immuunziekten.

In **hoofdstuk twee** bespreken we hoe hematopoietische stamcellen verouderen en welke consequenties dit kan hebben. We beschrijven hoe de

incidentie van hematologische ziekten leeftijdsafhankelijk is en hoe veroudering geassocieerd is met een functionele verslechtering van het hematopoietische systeem, zoals verminderde vaccinatie efficiëntie (Goodwin et al., 2006) en verhoogde infectiegevoeligheid (Frasca et al., 2008).

We beschrijven kort de huidige inzichten omtrent leeftijdsafhankelijke veranderingen in hematopoietische stamcellen in de muis en hoe, in zekere mate, kennis ontbreekt over zulke veranderingen in humane stamcellen. Bovendien veronderstellen we dat de meeste kenmerken van een verouderd hematopoietische systeem functionele gevolgen zijn van moleculaire gebeurtenissen in de primitieve hematopoietische stamcellen. Echter zijn er, naast deze celintrinsieke mechanismen waarschijnlijk ook extrinsieke factoren, zoals veranderingen in de samenstelling of functionaliteit van de niche, die kunnen resulteren in functionele beperkingen van hematopoietische stamcellen. In het laatste deel van dit hoofdstuk speculeren we of leeftijdsafhankelijke veranderingen in hematopoietische stamcellen omkeerbaar zijn. Interessant is dat hematopoietische stamcellen die afkomstig zijn van geïnduceerde pluripotente stamcellen (iPS), gegenereerd uit verouderde hematopoietische stamcellen, functioneel vergelijkbaar zijn met jonge hematopoietische stamcellen afkomstig van iPS cellen gegenereerd vanuit jongehematopoietische stamcellen (Wahlestedt et al., 2013). Dit suggereert omkeerbaarheid van het verouderingsproces en een functionele rol van epigenetische eiwitten in het verouderende hematopoietische systeem. Dit is met name interessant aangezien verschillende studies laten zien dat mutaties in epigenetische eiwitten, waaronder DNMT3A, TET2, ASXL1 en SETDB1, zijn geassocieerd met de ontwikkeling van klonale hematopoïese (Jaiswal et al., 2014; Steensma et al., 2015; Xie et al., 2014).

In **hoofdstuk drie** bekijken we de rol van de Polycomb eiwitten in hematopoïese, tijdens ontwikkeling, veroudering en ziekte. We introduceren de lezer met de samenstelling van de verschillende Polycomb groep complexen (PRC), met name gericht op PRC2 en het gebruikelijke PRC1 en hun functie. Het feit dat muizen die deficiënt zijn voor een van de drie kerncomponenten van het PRC2 niet levensvatbaar zijn (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004) laat zien hoe cruciaal deze eiwitten voor normale embryonale ontwikkeling zijn. Muizen die deficiënt zijn voor bestanddelen van PRC1 laten verschillende defecten zien in de latere stadia van hun ontwikkeling. (Core et al., 1997; Forzati et al., 2012; van der Lugt et al., 1994). Al deze studies benadrukken de cruciale

functie van deze eiwitten voor de regulatie van omni- en multipotente stamcellen.

De grote betekenis van deze eiwitten voor de regulatie van de celcyclus en stamcelzelfhernieuwing wordt ook duidelijk uit het feit dat dysregulatie van en mutaties in genen die coderen voor de Polycomb eiwitten kunnen worden gedetecteerd in hematologische ziekten (b.v. *BMI1* overexpressie in chronische lymfatische leukemie (Beà et al., 2001), *EZH2* mutaties in zowel diffuus groot B-cel lymfoom (Morin et al., 2010)) als in carcinomen ((*BMI1* in niet klein-cellig longkanker (Vonlanthen et al., 2001) en borstkanker (Paranjape et al., 2014)). Behandeling gericht op de Polycomb eiwitten door middel van inhibitoren zoals tazemostat voor *EZH2* hebben het huidige therapeutische repertoire in folliculair lymfoom vergroot (Morschhauser F, 2017).

In het algemeen dient dit hoofdstuk als een introductie op het hoofdonderwerp en de experimentele resultaten gepresenteerd in hoofdstuk vier.

In **hoofdstuk vier** bestudeerden we de rol van de verschillende humane CBX eiwitten via retrovirale overexpressie in de regulatie van uit navelstrengbloed afkomstige CD34+ hematopoietische stam- en voorlopercellen. Zoals eerder genoemd neemt het aantal CBX eiwitten toe tijdens evolutie van één naar vijf CBX eiwitten. Alle CBX Polycomb eiwitten bezitten een chromodomein waarmee ze getrimethyleerde lysine residuen kunnen herkennen, zoals H3K27me3 (Kaustov et al., 2011). Omdat de Polycomb CBX eiwitten de enige zijn binnen de PRC1 familie die een herkenningsdomein bezitten voor post-translationele modificaties van histonstaarten, kunnen de verschillende CBX eiwitten waarschijnlijk het PRC1 complex leiden naar verschillende loci in het genoom waarbij ze verschillende, maar ook deels overlappende subgroepen van genen controleren en zo verschillende functies krijgen.

We laten via retrovirale overexpressie zien dat CBX7 en, in mindere mate CBX8, de functie van hematopoietische CD34+ voorlopercellen *in vitro* versterkt. Waar overexpressie van CBX2, -4 en -6 geen vergelijkbare, maar in feite deels tegengestelde fenotypes laten zien. Vergelijkbare resultaten werden verkregen toen we de consequenties voor het meest primitieve hematopoietische compartiment *in vitro* beoordeelden. CBX7 versterkt de zelfhernieuwingsactiviteit van de humane hematopoietische stam- en voorlopercellen. Analyse van een eerder gepubliceerde microarray van verschillende humane CD34+ subgroepen liet een afname in expressie van CBX7 mRNA in het CD34+ compartiment tijdens

differentiatie van primitieve hematopoietische stamcellen naar meer uitgerijpte voorloper cellen.

In lijn met onze *in vitro* observaties, lieten immunodeficiënte muizen getransplanteerd met CD34+ uit navelstrengbloed afkomstige cellen na overexpressie van CBX7 hogere multi-lineage engraftment levels zien (zelfs na een week *in vitro* gekweekt te zijn), versterkte myelopoiese en een toegenomen percentage van CD34+CD38- cellen in het beenmerg. Transcriptoomanalyse van cellen met overexpressie van CBX7 liet zien dat genen die van belang zijn voor differentiatie werden onderdrukt, terwijl genen betrokken bij celcyclusregulatie meer tot expressie komen. Gezien hogere zelfhernieuwingsactiviteit van hematopoietische stam- en voorlopercellen samengaan met repressie van genen cruciaal voor differentiatie, een kenmerk is van leukemie, hebben we CBX7 expressie in AML bestudeerd. We hebben twee cohorten van AML patiënten bestudeerd en hebben, in beide datasets in meerdere subgroepen hogere CBX7 expressie gevonden in vergelijking met gezonde tegenhangers.

Om de functionele rol van CBX7 expressie in AML verder te onderzoeken hebben we CBX7 mRNA omlaag gereguleerd via short-hairpins en is zowel inhibitie in proliferatie als een up-regulatie van myeloïde differentiatie markeringsgenen, zoals CD11b en CD14, waargenomen.

Om nieuwe interactiepartners van de CBX eiwitten te identificeren hebben we massaspectrometrie analyses toegepast op flag-pulldowns van cellen met overexpressie van flag-gelabeld CBX7 en flag-gelabeld GFP (green fluorescent protein).

We veronderstelden dat CBX eiwitten ook kunnen binden aan niet-histon eiwitten als deze een getrimethyleerd lysine in een vergelijkbare peptide context als H3K27me3 bevatten. Inderdaad bleek na het uitvoeren van meerdere strikte filterstappen dat we meerdere nieuwe CBX7 partner eiwitten identificeerden die een dergelijke peptide context bevatten. Interessant genoeg waren enkele van deze eiwitten H3K9 methyltransferases en was één van hen een H3K9 geassocieerd eiwit. Down-regulatie van SETDB1, één van de nieuw geïdentificeerde CBX7- bindende H3K9 methyltransferases, in AML cellijnen was geassocieerd met up-regulatie van CD11b en CD14, en verlies van celproliferatie, wat indiceert dat CBX7 en SETDB1 samen genen onderdrukken die van belang zijn voor differentiatie.

**A**



## DISCUSSIE EN TOEKOMSTPERSPECTIEF

In de volgende paragrafen zullen de resultaten besproken worden en zal er gespeculeerd worden over de toekomstperspectieven van dit project met specifieke focus op de functionaliteit van CBX7 en de mogelijke translationele aspecten van onze bevindingen.

### Interactie partners en rekrutering van CBX7

Onze overexpressie studies laten zien dat CBX7, in vergelijking met de andere CBX eiwitten, een unieke en evolutionair behouden functie heeft in het reguleren van hematopoietische stam- en voorlopercellen. Alhoewel de chromodomeinen van alle Polycomb CBX eiwitten evolutionair behouden zijn en in grote mate overeenkomen, resulteert overexpressie van de verschillende CBX eiwitten in overexpressie van verschillende en slechts gedeeltelijk overlappende subgroepen van genen. Dit suggereert dat de andere domeinen van de CBX eiwitten, welke minder evolutionair behouden zijn, verantwoordelijk kunnen zijn voor de verschillende fenotypes. Dit komt mogelijk door het binden van verschillende genetische target loci of door het binden aan verschillende interactiepartners hetgeen resulteert in een variëteit aan rekruteringsmechanismen.

Gedurende de loop der evolutie is het aantal Polycomb CBX homologen toegenomen van één naar vijf. Waar het enkele *Drosophila* Polycomb Cbx eiwit enkel H3K27me<sub>3</sub> herkent, kunnen de humane Polycomb CBX eiwitten zowel H3K27me<sub>3</sub> binden als H3K9me<sub>3</sub> met verschillende bindingaffiniteit *in vitro*. Interessant is dat, ten minste *in vitro*, humaan CBX7 de hoogste bindingsaffiniteit heeft ten opzichte van H3K9me<sub>3</sub>, terwijl CBX2 *in vitro* enkel H3K27me<sub>3</sub> herkent (Kaustov et al., 2011). Tot nu toe waren er nog geen genoom-brede binding studies uitgevoerd om te beoordelen of CBX7 en H3K9me<sub>3</sub> overeenkomstige targets hebben. Onze resultaten laten zien dat CBX7, in ieder geval onder bepaalde condities, nabij H3K9me<sub>3</sub> is gelokaliseerd. In lijn met deze bevindingen hebben we meerdere H3K9 methyltransferases geïdentificeerd als vermeende bindingpartners voor CBX7, waaronder SETDB1.

We bevestigen de interactie tussen CBX7 en SETDB1 in K562 cellen met CBX7-overexpressie via immunoblots. Hoewel we geen kwantitatieve massa spectrometrie analyse hebben uitgevoerd, suggereert onze data dat CBX7, in vergelijking met CBX8, met hogere affiniteit SETDB1

bindt. In overeenstemming met onze functionele *in vivo* data, is er aangetoond dat het chromodomein van CBX7, in vergelijking met CBX8, met hogere affiniteit bindt aan een 20-aminozuur lang eiwit met de drie getrimethyleerde lysine residuen die de delen van het humane SETDB1 eiwit representeren (Kaustov et al., 2011).

In het algemeen kunnen deze verschillende bindingsaffiniteiten bijdragen aan een verscheidenheid van fenotypes die we observeren bij overexpressie van de verschillende CBX eiwitten.

Interessant is dat zowel CBX7 als SETDB1 werden geïdentificeerd in een knockout- screening voor genen welke differentiatie van embryonale stamcellen tegengaan. (Bilodeau et al., 2009). In dezelfde publicatie werd aangetoond dat in embryonale stamcellen met name genen geclassificeerd als ontwikkelingsregulators waren gebonden door H3K9me3 en H3K27me3.

Bovendien wordt 20% van alle euchromatische genen gebonden door H3K9me3 ook gebonden door SETDB1, wat indiceert dat ten minste in sommige delen van het chromatine het Polycomb systeem en SETDB1 gezamenlijk expressie van target genen reguleren (Bilodeau et al., 2009). Verder kunnen SETDB1 pieken in Chip-seq experimenten zowel samen vallen met H3K9me3 of als enkele piek zonder bewijs voor H3K9me3 aanwezigheid voorkomen. Opmerkelijk is dat SETDB1 pieken geassocieerd zijn met binding van EZH2 en RING1B, wat verder de suggestie wekt van crosstalk tussen deze twee pathways (Fei et al., 2015).

Het feit dat men in eerdere massaspectrometrie experimenten heeft waargenomen dat alle drie H3K9 methyltransferases een getrimethyleerd lysine residu hebben dat in een motief ligt dat in grote mate lijkt op H3K9me3 en H3K27me3 (Hornbeck et al., 2015), suggereert dat CBX7 hieraan bindt via het chromodomein. Volgens het klassieke hiërarchische rekruteringsmodel wordt het PRC2 complex geleid naar niet gemethyleerde CpG-eilanden, resulterend in trimethylering van H3K27 via EZH2. Het gebruikelijke PRC1 complex kan vervolgens H3K27me3 herkennen via binding van het chromodomein van één van de vijf Polycomb CBX eiwitten aan H3K27me3 (Comet and Helin, 2014).

De identificatie van zowel drie H3K9 methyltransferases, als CDYL, waarvan men in eerdere massaspectrometrie experimenten vond dat ze getrimethyleerd zijn, als CBX7 bindingspartners suggereert een alternatief, PRC2-onafhankelijk, rekruteringsmodel. Omdat we interactie van CBX7 met SETDB1 hebben aangetoond in cellen met CBX7 overexpressie

en knockdown van beide genen vergelijkbare effecten liet zijn, is het erg waarschijnlijk dat beide eiwitten ook op endogene expressie niveaus interacteren.

De methyltransferase SETDB1 bevat meerdere functioneel belangrijke domeinen welke interactie met andere epigenetische pathways toestaan: het MBD domein voor het waarnemen van gemethyleerd DNA, en twee tudor domeinen die binding met mSin3A/B en HDAC1/2 mogelijk maken (Karanth et al., 2017). In een hypothetisch PRC2 onafhankelijk recruitment model, zou getrimethyleerd SETDB1 initieel kunnen worden gerekruteerd door H3K9me2 of gemethyleerd DNA en een CBX7 bevattend PRC1 kunnen aantrekken resulterend in chromatinecompactie en repressie van target genen.

Het blijft onduidelijk of zowel H3K9me3 en H3K27me3 modificaties, als CBX7- en SETDB1-binding, voorkomen op het zelfde histoneiwit (symmetrisch) of op het andere H3-eiwit waarmee het de dimeer vormt (asymmetrisch). Een massaspectrometrie analyse van enkele histoneiwitten zou mogelijk deze vraag kunnen beantwoorden (Rothbart and Strahl, 2014). Zoals beschreven in de introductie van deze thesis kunnen post-translationele modificaties van histonen voorkomen op zowel de uitstekende staart als het globulaire domein. Profileren van enkele histoneiwitten van cellen met CBX7 expressie biedt de mogelijkheid te screenen voor andere markeringen die samengaan met CBX7 op zowel de staart als het globulaire domein.

In colonkankercellen is het aangetoond dat CBX7 interacteert met alle drie DNA methyltransferases en dat CBX7 overexpressie resulteerde in hypermethylering van CpG bevattende promotors (Mohammad et al., 2009). Of en in welke mate CBX7 gemedieerde repressie van genen geassocieerd is met hypermethylering van promotorregio's kan gemakkelijk worden nagegaan via DNA-methylering arrays of bisulfietsequencing.

CBX7 kan binden aan H3K27me3 en H3K9me3, maar ook direct aan lange, niet-coderend, RNAs (Yap et al., 2010), hetgeen alternatieve rekruteringsmechanismes biedt. Tot nu toe is het binden van CBX eiwitten aan niet-coderend RNA beschreven voor CBX7 en CBX4, twee eiwitten waarvan de chromodomeinen, op één aminozuur na, in zeer grote mate vergelijkbaar zijn (Gil and O'Loughlin, 2014). Recentelijk is een onverwachte rol van CBX7 aangetoond, als een mRNA bindend eiwit hetgeen resulteert in up-regulatie van het target gen (Rosenberg et al., 2017). CLIP-Seq (cross-linking immunoprecipitation-high-throughput sequencing) in CD34+ HSPCs met

CBX7 overexpressie zou directe mRNA en lange niet-coderend RNA targets voor CBX7 kunnen laten zien, en een geïntegreerde analyse met onze transcriptoom data zou verder kunnen beantwoorden of CBX7 en mRNA interactie leidt tot differentiële expressie van genen.

## **Globale epigenetische- en chromatineveranderingen na CBX7 overexpressie**

In de vorige paragraaf hebben we bediscussieerd en gespeculeerd over de directe CBX7 interactiepartners die leiden tot lokale epigenetische veranderingen. Afgezien van deze lokale veranderingen kan overexpressie van CBX7 resulteren in meer globale veranderingen van het epigenetische landschap, ver weg van de initiële CBX7 bindingsplaats via differentiële expressie van additionele epigenetische modificatoren. Het is inderdaad zo dat we in de lijst differentieel tot expressie gekomen genen meerdere epigenetische modificatie- en lezer-eiwitten observeren, welke kunnen resulteren in globale epigenetische veranderingen. Dit omvat zowel genen betrokken bij DNA-methylering, zoals *DNMT3A* en *IDH2*, als genen betrokken bij methylering en demethylering van H3K4me3, zoals de H3K4 methyltransferase *PRDM16*, en de demethylase *KDM1A*. Verder zagen we repressie van genen betrokken bij demethylering van H3K9 en H3K27, waaronder *KDM7A*, en genen betrokken bij methylering van arginine residuen op histoneiwitten, *PRMT2*.

## **CBX7- een verondersteld oncogen in hematopoïetische neoplasmen en mogelijke klinische implicaties**

Overexpressie van *Cbx7* in 5-Fluoruraciel behandelde muizen beenmergcellen resulteerde in 90% van de getransplanteerde dieren in een leukemisch fenotype met snelle aanvang. Zestig procent van deze muizen ontwikkelde een T-cel lymfocytose, met vergrote milt en lymfeklieren. Twintig procent van alle muizen ontwikkelde een leukocytose zonder expressie van lineagemarkers, en tien procent van alle muizen ontwikkelde een leukopenie en anemie met reticulocytose (Klauke et al., 2013). Verder werden verhoogde CBX7 levels gedetecteerd in samples van patiënten met folliculair lymfoom (Scott et al., 2007) en zijn SNPs in de promotor en enhancer regio van CBX7 geassocieerd met een verhoogd risico op multiple myeloom (Chubb et al., 2013).

**A**

In overeenstemming met de mogelijke rol van CBX7 in de ontwikkeling of progressie van maligne hematopoietische neoplasmies, observeerden we na overexpressie van CBX7 verhoogde zelfhernieuwingsactiviteit van humane primitieve hematopoietische stam- en voorlopercellen, hogere proliferatie snelheid in een cytokine gedreven suspensie cultuur en hogere engrafment in immunodeficiënte muizen na transplantatie. Het is interessant dat we geen verhoogde lymfopoiese hebben geobserveerd, wat mogelijk voortvloeit uit het gebruik van cytokine combinaties die met name de groei van zowel primitieve hematopoietische stamcellen, als myeloïde voorlopercellen, maar niet van lymfoïde voorlopercellen promoten.

Daarentegen observeerden we een hoger percentage CD33+ cellen in het compartiment van CD45+GFP+ cellen, hetgeen er toe heeft geleid dat we CBX7 expressie in AML patiënten samples zijn gaan evalueren in een eerder gepubliceerde microarray. Deze analyse liet een significant hogere expressie van CBX7 in CD34+ cellen van AML patiënten zien in vergelijking met CD34+ perifere gemobiliseerde stamcellen van gezonde individuen. Hogere CBX7 expressie was ook te vinden in The Cancer Genome Atlas dataset. Het is interessant dat recentelijk is aangetoond dat een CBX7 bevattende PRC1 interactie met DNMT3AR882 differentiatie van muizen hematopoietische stamcellen blokkeert (Koya et al., 2016). Deze bevinding complementeert onze transcriptoom data, waarin we met name repressie van genen belangrijk vinden voor de differentiatie van verschillende hematopoietische celtypen.

Knockdown van CBX7 mRNA via short-hairpins resulteerde in de expressie van markers van myeloïde differentiatie, zoals *CD11b* en *CD14*. Verder resulteerde knockdown van CBX7 in zowel OCI-AML3, als HL60 cellen in inhibitie van proliferatie, hetgeen goed correspondeert met de observatie dat overexpressie van CBX7 resulteert in up-regulatie van celcyclus genen. Onze transcriptoomdata identificeerde ook een subgroep van genen die met name tot expressie komen in primitieve CD34+ cellen die behoren tot de KEGG pathway GO groep “Transcriptional misregulation in cancer” zoals *HMG2*, *CCND2*, *ERG*, *IGF1R*, *LMO2*, *MEIS1* en *MYCN*.

Deze feiten maken het de moeite waard om te streven naar het targeten van CBX7 in hematologische ziekten. Tot nu toe zijn er drie chemische verbindingen beschreven die het chromodomein van CBX7 inhiberen (Ren et al., 2015; Simhadri et al., 2014; Stuckey et al., 2016). Omdat de

chromodomeinen van alle humane Polycomb CBX eiwitten evolutionair behouden zijn, en ook gelijkend aan andere eiwitten die een chromodomein bevatten, zoals heterochromatine-geassocieerde eiwitten of CDYL, is het ontwikkelen van inhibitoren die zich uniek richten op het chromodomein van CBX7 zonder off-target effecten zeer uitdagend. Daarnaast moeten deze verbindingen in staat zijn het celmembraan te doordringen om een voldoende hoge intracellulaire concentratie te bereiken om CBX7 binding aan getrimethyleerde lysine residuen tegen te gaan. Tot nu toe hebben twee van de drie chemische probes intracellulaire activiteit laten zien. Beide verbindingen zijn getest in een prostaatcarcinoom cellijn. Waar één inhibitor resulteerde in verhoogde expressie van *p16/CDKN2A* (Ren et al., 2015), een klassiek CBX7 target, was de andere inhibitor daarnaast ook in staat proliferatie te inhiberen (Stuckey et al., 2016).

Verder kan het beïnvloeden van CBX7 met chromodomein-inhibitoren ook zelfhernieuwing van benigne hematopoïetische cellen tegengaan. Onze LTC-IC data suggereert dat zelfhernieuwing van humane benigne hematopoïetische ten minste *in vitro* wordt beperkt na knockdown van CBX7 in CD34+ uit navelstrengbloed afkomstige cellen.

Daarentegen, laten *Cbx7*<sup>-/-</sup> muizen geen hematologische afwijkingen zien, suggererend dat CBX7 niet essentieel is voor steady state hematopoïese (Forzati et al., 2012). Echter zijn er in deze muizen zowel *in vitro* als *in vivo* geen functionele testen van hematopoïetische stamcellen uitgevoerd, dat suggereert dat CBX7 essentieel kan zijn voor hematopoïese onder stresscondities, zoals infecties, bloedingen en stamceltransplantaties.

Daarnaast komt CBX7 tot expressie in een variëteit van non-hematopoïetische cellen, dat suggereert dat gebruik van een dergelijke inhibitor bijwerkingen kan uitlokken in niet-hematopoïetische weefsels. Om specifiek in AML cellen CBX7 te targeten, kunnen methoden worden gebruikt waarbij vehikel strategieën zoals GO (Mylotarg®) nuttig zijn. Gebruikmakend van eenzelfde aanpak zouden CBX7 inhibitoren gelinkt kunnen worden aan antilichamen die specifiek CD33 herkennen, een antigeen dat tot expressie komt op met name myeloïde voorlopercellen, monocyten, neutrofiële granulocyten en in enige mate op multipotente hematopoïetische stamcellen (Linenberger, 2005; Taussig et al., 2005). Verder komt CD33 tot expressie in 85-90 % van de volwassen en pediatrische AML cellen en is de expressie van CD33 gemiddeld drie keer hoger op leukemische blasten in vergelijking met CD33+ cellen in

gezonde beenmerg cellen (Linenberger, 2005). Door verschillen in CD33 expressie tussen benigne en AML cellen kan men verwachten met name CBX7 inhibitie waar te nemen in het myeloïde en dan vooral het maligne compartiment.

Zoals onze data suggereert is CBX7 in staat via het chromodomein te binden aan zowel H3K27m3, H3K9me3, als getrimethyleerde niet-histon eiwitten, en zal CBX7 inhibitie waarschijnlijk ook resulteren in verstoring van de interactie van deze eiwitten, zoals SETDB1. Het is interessant dat, net als CBX7, SETDB1 ook meer tot expressie komt in prostaatkanker cellen (Sun et al., 2014).

Omdat de CBX eiwitten interacteren in multimere eiwitcomplexen en in samenspel met functionele repressieve systemen zoals PRC2, histon deacetylases en DNA methyltransferases, kan het waardevol zijn combinatie therapie te overwegen met demethylerende agentia, histon deacetylases of EZH2-inhibitoren.

In het vroege begin van kankertherapie bevatten de meeste therapeutische regimes enkel klassieke cytotoxische reagentia, die een 'one size fits all' aanpak voor elk histologisch kanker subtype gebruiken. In het laatste decennium zijn er meerdere medicijnen goedgekeurd die specifiek gericht zijn op bepaalde eiwitten, die gemuteerd zijn of tot overexpressie komen in kankercellen, hetgeen het ontwerpen van individuele behandelprotocollen gebaseerd op expressie en genomdata van de individuele kanker bij diagnose resulterend in personalized medicine mogelijk maakt. De toenemende kennis over epigenetische mechanismen in benigne en maligne cellen resulteert waarschijnlijk in de ontwikkeling van nieuwe verbindingen die zich specifiek richten op de uiterst complexe epigenetische machinerie.

Incorporatie van epigenetische profielen bij diagnose kan leiden tot het gebruik van verbindingen gericht op de epigenetische "Achilles hiel", die groeivoordeel of resistentie tegen andere medicatie in kankercellen induceren.

## REFERENCES

- Beà, S., Tort, F., Pinyol, M., Puig, X., Hernández, L., Hernández, S., Fernández, P. L., van Lohuizen, M., Colomer, D., and Campo, E. (2001). BMI-1 Gene Amplification and Overexpression in Hematological Malignancies Occur Mainly in Mantle Cell Lymphomas. *Cancer Research* 61, 2409-2412.
- Bilodeau, S., Kagey, M. H., Frampton, G. M., Rahl, P. B., and Young, R. A. (2009). SetDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. *Genes & Development* 23, 2484-2489.
- Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002). Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing. *Science* 298, 1039-1043.
- Chubb, D., Weinhold, N., Broderick, P., Chen, B., Johnson, D. C., Forsti, A., Vijaykrishnan, J., Migliorini, G., Dobbins, S. E., Holroyd, A., et al. (2013). Common variation at 5q26.2, 6p21.33, 17p11.2 and 22q13.1 influences multiple myeloma risk. *Nat Genet* 45, 1221-1225.
- Comet, I., and Helin, K. (2014). Revolution in the Polycomb hierarchy. *Nat Struct Mol Biol* 21, 573-575.
- Core, N., Bel, S., Gaunt, S. J., Aurrand-Lions, M., Pearce, J., Fisher, A., and Djabali, M. (1997). Altered cellular proliferation and mesoderm patterning in Polycomb-M33- deficient mice. *Development* 124, 721-729.
- Endoh, M., Endo, T. A., Endoh, T., Isono, K., Sharif, J., Ohara, O., Toyoda, T., Ito, T., Eskeland, R., Bickmore, W. A., et al. (2012). Histone H2A mono-ubiquitination is a crucial step to mediate PRC1-dependent repression of developmental genes to maintain ES cell identity. *PLoS Genet* 8, e1002774.
- Faust, C., Lawson, K. A., Schork, N. J., Thiel, B., and Magnuson, T. (1998). The Polycomb- group gene *ee* is required for normal morphogenetic movements during gastrulation in the mouse embryo. *Development* 125, 4495-4506.
- Fei, Q., Yang, X., Jiang, H., Wang, Q., Yu, Y., Yu, Y., Yi, W., Zhou, S., Chen, T., Lu, C., et al. (2015). SetDB1 modulates PRC2 activity at developmental genes independent of H3K9 trimethylation in mouse ES cells. *Genome Research*.
- Forzati, F., Federico, A., Pallante, P., Abbate, A., Esposito, F., Malapelle, U., Sepe, R., Palma, G., Troncone, G., Scarfo, M., et al. (2012). CBX7 is a tumor suppressor in mice and humans. *J Clin Invest* 122, 612-623.
- Gil, J., and O'Loughlen, A. (2014). PRC1 complex diversity: where is it taking us? *Trends Cell Biol* 24, 652-641.
- Hornbeck, P. V., Zhang, B., Murray, B., Kornhauser, J. M., Latham, V., and Skrzypek, E. (2015). PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Research* 43, D512-D520.
- Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., Lindsley, R. C., Mermel, C. H., Burt, N., Chavez, A., et al. (2014). Age-Related Clonal Hematopoiesis Associated with Adverse Outcomes. *New England Journal of Medicine* 371, 2488-2498.
- Karant, A. V., Maniswami, R. R., Prashanth, S., Govindaraj, H., Padmavathy, R., Jegatheesan, S. K., Mullangi, R., and Rajagopal, S. (2017). Emerging role of SETDB1 as a therapeutic target. *Expert Opin Ther Targets* 21, 319-331.
- Kaustov, L., Ouyang, H., Amaya, M., Lemak, A., Nady, N., Duan, S., Wasney, G. A., Li, Z., Vedadi, M., Schapira, M., et al. (2011). Recognition and specificity determinants of the human cbx chromodomains. *J Biol Chem* 286, 521-529.
- Klaue, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., et al. (2013). Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. *Nat Cell Biol* 15, 353-362.
- Koya, J., Kataoka, K., Sato, T., Bando, M., Kato, Y., Tsuruta-Kishino, T., Kobayashi, H., Narukawa, K., Miyoshi, H., Shirahige, K., and Kurokawa, M. (2016). DNMT3A R882 mutants interact with polycomb proteins to block haematopoietic stem and leukaemic cell differentiation. *Nat Commun* 7.
- Linberger, M. L. (2005). CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. *Leukemia* 19, 176-182.
- Mohammad, H. P., Cai, Y., McGarvey, K. M., Easwaran, H., Van Neste, L., Ohm, J. E., O'Hagan,



- H. M., and Baylin, S. B. (2009). Polycomb CBX7 Promotes Initiation of Heritable Repression of Genes Frequently Silenced with Cancer-Specific DNA Hypermethylation. *Cancer Research* 69, 6522-6530.
- Mohty, M., Yong, A. S. M., Szydlo, R. M., Apperley, J. F., and Melo, J. V. (2007). The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. *Blood* 110, 380-383.
- Morin, R. D., Johnson, N. A., Severson, T. M., Mungall, A. J., An, J., Goya, R., Paul, J. E., Boyle, M., Woolcock, B. W., Kuchenbauer, F., *et al.* (2010). Somatic mutation of EZH2 (Y641) in Follicular and Diffuse Large B-cell Lymphomas of Germinal Center Origin. *Nature genetics* 42, 181-185.
- Morschhauser F, S. G., McKay P, *et al.* (2017). Interim report from a phase 2 multicenter study of tazemetostat, an EZH2 inhibitor, in patients with relapsed or refractory B-cell non-Hodgkin lymphomas. In 14th International Conference on Malignant Lymphoma, (Lugano, Switzerland).
- Nikoloski, G., Langemeijer, S. M. C., Kuiper, R. P., Knops, R., Massouf, M., Tonnissen, E. R. L. T. M., van der Heijden, A., Scheele, T. N., Vandenberghe, P., de Witte, T., *et al.* (2010). Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 42, 665-667.
- O'Carroll, D., Erhardt, S., Pagni, M., Barton, S. C., Surani, M. A., and Jenuwein, T. (2001). The Polycomb-Group Gene Ezh2 Is Required for Early Mouse Development. *Molecular and Cellular Biology* 21, 4330-4336.
- O'Loughlin, A., Munoz-Cabello, A. M., Gaspar-Maia, A., Wu, H. A., Banito, A., Kunowska, N., Racek, T., Pemberton, H. N., Beolchi, P., Laval, F., *et al.* (2012). MicroRNA regulation of Cbx7 mediates a switch of Polycomb orthologs during ESC differentiation. *Cell Stem Cell* 10, 33-46.
- Paranjape, A. N., Balaji, S. A., Mandal, T., Krushik, E. V., Nagaraj, P., Mukherjee, G., and Rangarajan, A. (2014). Bmi1 regulates self-renewal and epithelial to mesenchymal transition in breast cancer cells through Nanog. *BMC Cancer* 14, 1-14.
- Pasini, D., Bracken, A. P., Jensen, M. R., Denchi, E. L., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *The EMBO Journal* 23, 4061-4071.
- Ren, C., Morohashi, K., Plotnikov, Alexander N., Jakoncic, J., Smith, Steven G., Li, J., Zeng, L., Rodriguez, Y., Stojanoff, V., Walsh, M., and Zhou, M.-M. (2015). Small-Molecule Modulators of Methyl-Lysine Binding for the CBX7 Chromodomain. *Chemistry & Biology* 22, 161-168.
- Rizo, A., Dontje, B., Vellenga, E., de Haan, G., and Schuringa, J. J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. *Blood* 111, 2621-2630.
- Rosenberg, M., Blum, R., Kesner, B., Maier, V. K., Szanto, A., and Lee, J. T. (2017). Denaturing CLIP, dCLIP, Pipeline Identifies Discrete RNA Footprints on Chromatin-Associated Proteins and Reveals that CBX7 Targets 3' UTRs to Regulate mRNA Expression. *Cell Syst* 5, 368-385 e515.
- Rothbart, S. B., and Strahl, B. D. (2014). Interpreting the language of histone and DNA modifications. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1839, 627-643.
- Scott, C. L., Gil, J., Hernando, E., Teruya-Feldstein, J., Narita, M., Martinez, D., Visakorpi, T., Mu, D., Cordon-Cardo, C., Peters, G., *et al.* (2007). Role of the chromobox protein CBX7 in lymphomagenesis. *Proc Natl Acad Sci U S A* 104, 5389-5394.
- Simhadri, C., Daze, K. D., Douglas, S. F., Quon, T. T. H., Dev, A., Gignac, M. C., Peng, F., Heller, M., Boulanger, M. J., Wulff, J. E., and Hof, F. (2014). Chromodomain Antagonists That Target the Polycomb-Group Methyl-lysine Reader Protein Chromobox Homolog 7 (CBX7). *Journal of Medicinal Chemistry* 57, 2874-2883.
- Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hasserjian, R. P., and Ebert, B. L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 126, 9-16.
- Stock, J. K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A. G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* 9, 1428-1435.

- Stuckey, J. I., Dickson, B. M., Cheng, N., Liu, Y., Norris, J. L., Cholensky, S. H., Tempel, W., Qin, S., Huber, K. G., Sagum, C., *et al.* (2016). A cellular chemical probe targeting the chromodomains of Polycomb repressive complex 1. *Nat Chem Biol* 12, 180-187.
- Sun, Y., Wei, M., Ren, S. C., Chen, R., Xu, W. D., Wang, F. B., Lu, J., Shen, J., Yu, Y. W., Hou, J. G., *et al.* (2014). Histone methyltransferase SETDB1 is required for prostate cancer cell proliferation, migration and invasion. *Asian J Androl* 16, 319-324.
- Taussig, D. C., Pearce, D. J., Simpson, C., Rohatiner, A. Z., Lister, T. A., Kelly, G., Luongo, J. L., Danet-Desnoyers, G. A., and Bonnet, D. (2005). Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood* 106, 4086-4092.
- van den Boom, V., Rozenveld-Geugien, M., Bonardi, F., Malanga, D., van Gosliga, D., Heijink, A. M., Viglietto, G., Morrone, G., Fusetti, F., Velenga, E., and Schuringa, J. J. (2015). Nonredundant and locus-specific gene repression functions of PRC1 paralog family members in human hematopoietic stem/progenitor cells. *Blood* 121, 2452-2461.
- van der Lugt, N. M., Domen, J., Linders, K., van Roon, M., Robanus-Maandag, E., te Riele, H., van der Valk, M., Deschamps, J., Sofroniew, M., and van Lohuizen, M. (1994). Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. *Genes & Development* 8, 757-769.
- Vissers, J. H., van Lohuizen, M., and Citterio, E. (2012). The emerging role of Polycomb repressors in the response to DNA damage. *J Cell Sci* 125, 3959-3948.
- Vonlanthen, S., Heighway, J., Altermatt, H. J., Gugger, M., Kappeler, A., Borner, M. M., Lohuizen, M. v., and Betticher, D. C. (2001). The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer* 84, 1372-1376.
- Wahlestedt, M., Norddahl, G. L., Sten, G., Ugale, A., Frisk, M.-A. M., Mattsson, R., Deierborg, T., Sigvardsson, M., and Bryder, D. (2013). An epigenetic component of hematopoietic stem cell aging amenable to reprogramming into a young state. *Blood* 121, 4257-4264.
- Xie, M., Lu, C., Wang, J., McLellan, M. D., Johnson, K. J., Wendl, M. C., McMichael, J. F., Schmidt, H. K., Yellapantula, V., Miller, C. A., *et al.* (2014). Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 20, 1472-1478.
- Yap, K. L., Li, S., Munoz-Cabello, A. M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M. J., and Zhou, M. M. (2010). Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. *Mol Cell* 38, 662-674.

## ACKNOWLEDGMENTS

Almost five years ago I paused my clinical education as a medical doctor in hematology and oncology in Tübingen, Germany and started the challenging adventure of scientific research at the ERIBA in Groningen, The Netherlands. This would never have been possible without the support of many people who I would like to express my deep gratitude.

First of all, I would like to thank **Gerald**, who gave me the opportunity to execute my research project in his lab and later on to accept me as a Ph.D. student.

Initially, we met at a Conference of Hematopoietic Stem Cell in Tuebingen, Germany, where we had our first informal talk about the chance to join his lab and be transformed from a clinician to a scientist. His very positive and kind manner convinced me to meet the challenge and come to Groningen. From day zero on he supported me to bring the project alive, he backed me up when applying for a Grant of the German Cancer Aid and he always gave me highly appreciated advice and comments during the long process of executing the project. Thereby he still provided me the necessary scientific freedom to develop the project in my manner. Whenever I had a question, his door was always open for discussions. After my project grant ran out, he gave me the option to sign into the Ph.D. program and to continue my project in Groningen for more than two further years by covering salaries and bench fees. I highly appreciated the various possibilities to join national and international conferences and thereby to be introduced to other scientists in the field of experimental hematology, which was essential for my professional development and network.

**Lenja**, thank you very, very much for supervising me in my daily work and thereby supporting me in developing this project further. I learned a lot from you, especially “critical thinking” and data analysis. It was great to know that there is an open door, where I can walk in and discuss in short time intervals big and small questions. I appreciated your creative and innovative thoughts about scientific problems a lot and enjoyed to learn how to approach them. Thank you very much also for being my co-promotor.

I also would like to acknowledge the members of the assessment committee, **Prof. J. Gil**, **Prof. G. Huls**, and **Prof. C. Lengerke** for taking the time to assess this thesis.

**Hein**, thank you very much for taking time to discuss with me a lot of practical aspects of my work including transplants and *in vitro* experiments. You taught me how to analyze CFUs and LTC-ICs. A big part of the project would not have been possible without your support. I wish you in your new department all the very best and hope that we can stay in touch in the future.

**Karin**, I would like to give thanks to you for supporting me already during the application for my research grant and especially during the first year of my stay in Groningen. You taught me so many research techniques and the huge step from working in a hospital towards executing research projects would have been much more demanding without your advice.

**Susanne**, thank you very much for performing the protein-related experiments like the Chip-seq experiments with us. Your very kind and open-minded character associated with your knowledge made all collaboration very pleasant.

**Ellen**, thanks a lot for teaching me the basic knowledge of lab work including cell culture, transfection, transduction and so on. I appreciated your help very much especially on busy and long lab days, which would have been even longer without you.

Although, I joined the official animal course, I would not have been able to do the “real” animal work on a daily base without you **Bertien**. Thanks for teaching me and supporting my project regularly, not only in “mouse related objects”. Whenever necessary, I knew that I was also allowed to contact you outside of your normal working hours, for example because of sick mice or “emergency” orders- and I know that this is not self-evident. Thank you very much also for supporting me through all administrative stuff like writing DEC protocols and preparing IvD meetings.

The first lab member I met at ERIBA was **Mathilde**. Thanks’ a lot for hosting me not only in the early phase of my stay but also for teaching me molecular biology and cell culture methods and being available for a lot of questions, especially at the beginning.

**Eric**, our collaboration was always very comfortable. I am very grateful for your bioinformatic support and analysis of our big data like RNA-seq and Chip-seq. Thanks also for your assistance in all computer-related questions and in making such pretty figures in R.

I also want to thank all students (**Sonja**, **Franziska**, **Imma**, **Gemma**, and **Niek**), who I was allowed to supervise during their stay and who worked with me on that project. I hope that, at least partly, I contributed to

your professional development. Especially, I would like to mention **Sonja** who was a brilliant student and who luckily decided to join our lab for an MD/Ph.D. and is performing the last experiments of the project and is guiding the project during the revision. I wish you all the best for your clinical as well as research work and thanks for being my paronymph.

Furthermore, I would like to thank all my current PostDoc and Ph.D. student colleagues of our lab: **Mirjam, Arthur, Danielle, Alexander and Jason.**

Special appreciation also to my former Ph.D. student colleagues **Edyta** and **Sekka.** It was great to have you around and to share all joy and frustrations in the lab with you. I hope that you both will be very content at your new places and I hope that we will see each other regularly privately and on conferences.

I also would like to thank all actual and former **members** of **Prof. Landsdorp's** and **Dr. Fojier's** lab for the friendly work atmosphere, for sharing the lab and reagents, whenever someone of us run out of anything, for sharing fun as well as frustrations. Especially, I would like to thank **Bjorn** who I met the first time during the animal course. We had a lot of fun in the lab together, especially during cloning and viral work. It was also great to share great sailing trips to Greece, Spain, and Croatia with you. I am looking forewards to hopefully many more nice trips together and I wish you all the very best for your private and professional future. Thank you also very much for supporting me by being my paronymph.

Of course, I also would like to thank all members of the experimental hematology group especially **Vincent** and **Prof. JJ. Schuringa.**

Furthermore, I would like to thank all people of the management team of ERIBA by supporting us in all bureaucracy stuff: **Annet, Henk, Sylvia, Nina, Jolanda, Joke, and Peet.**

A lot of this work also would not have been possible without the help of the staff and veterinarians at the Central Animal Facility as well as the FACS facility (special thanks to **Henk, Roelof Jan, and Geert.**)

Besonders danken möchte ich meiner Familie, insbesondere meiner Frau und meiner Tochter, die mich mit viel Verständnis und Geduld immer tatkräftig unterstützt haben. Ohne Euch und Eure Hilfe wäre solch eine Arbeit gar nicht möglich gewesen.

Bedanken möchte ich mich auch bei meinen Eltern, die immer für mich da waren und mich gefördert haben.

## CV

### Personal data:

**Name:** Johannes Jung  
**Date and place of birth:** December 1981, Freiburg im Breisgau, Germany  
**Nationality:** German

### Education:

June 2009 Final examination  
August 2004 Medical preliminary education  
2002 – 2009 Studies of Medicine, University Freiburg, Germany  
Clinical Electives in the final clinical year abroad: Kings College London, UK and Cantonal Hospital Lucerne, Switzerland  
2001 – 2002 Studies of technical management, Technical University Karlsruhe, Germany

### Clinical Education:

Since 01.01.2018 Resident in the Department of Hematology, Oncology and Stem Cell Transplantation University Hospital Freiburg, Germany  
15.06.2009 – 30.06.2013 Resident in the Department of Oncology, Hematology, Clinical Immunology, Rheumatology and Pneumology, University Hospital Tuebingen, Germany  
01.10.2009 – 30.07.2013 Clinical investigator (Study doctor) of various AML studies of the German-Austrian Acute Myeloid Leukemia Study Group (AMLSG)

**A**

## Scientific Education:

- 01.08.2013 – 31.10.2017      PhD student  
European Research Institute for the Biology  
of Ageing, University Medical Center  
Groningen, The Netherlands  
Laboratory of Ageing Biology and Stem  
Cells, Prof. de Haan  
Project: The role of CBX proteins in benign  
and malignant human hematopoiesis
- 01.04.2005 – 31.09.2006      Medical thesis  
„Identification of a homozygous deletion in  
the AP3B1 gene causing Hermansky-Pudlak  
syndrome, type 2 (HSP2)“  
Supervisor: Prof. Dr. Peter  
Daily supervisor: Prof. Dr. Grimbacher  
Department of Rheumatology and Clinical  
Immunology  
University Hospital Freiburg, Germany  
Grade: summa cum laude
- 01.09.2004 – 31.09.2006      Research Assistant  
Lab of molecular genetics of  
immunodeficiencies  
Prof. Dr. Grimbacher  
Department of Rheumatology and Clinical  
Immunology  
University Hospital Freiburg, Germany

## Grants and awards:

18.05.2018	Paul Basset Award of the European Cancer Center (University Freiburg, Basel and Strasbourg).
Since 01.01.2018	EXCEL-Scholarship (Excellent Clinician Scientists in Freiburg- Education for Leadership), 3 years funding
26.08.2017	1st Prize, New Investigator PhD Student Award (Dirk van Bekkum Award) of the International Society for Experimental Hematology (ISEH)
24.08.2017	Travel grant of the International Society for Experimental Hematology (ISEH)
14.10.2016	Best Abstract Annual Meeting of the German, Austrian and Swiss Societies of Hematology and Oncology, Leipzig
01.08.13 – 30.02.2015	Post-Doc Grant of the German Cancer Aid, Bonn, Germany
2007	Scholarship of the Archdiocese Freiburg



## **Publications:**

### **Do hematopoietic stem cells get old?**

**Jung J**, Buisman S, de Haan G

*Leukemia*. 2016 Nov 11. doi: 10.1038/leu. Editorial

### **Hematopoiesis during development, aging, and disease.**

**Jung J**, Buisman S, de Haan G

*Exp Hematol*. 2016 Aug;44(8):689-95. doi: 10.1016/j.exphem.2016.05.007. Review.

### **Death Receptor (DR)-Expression on AML-Blasts Correlates with Unfavorable Prognosis**

Joerg Uwe Schmohl, Tina Nuebling, **Johannes Jung**, Gunnar Blumenstock, Tanja Kroell, Helmuth R. Salih, H.Schmetzer.

*Anticancer Res*. 2015 Jul;35(7):4043-52.

### **Screening of functional and positional candidate genes in families with common variable immunodeficiency**

Salzer U, Neumann C, Thiel J, Woellner C, Pan-Hammarström Q, Lougaris V, Hagen T, **Jung J**, Birmelin J, Du L, Metin A, Webster D, Plebani A, Moschese V, Hammarström L, Schaffer A, Grimbacher B.

*BMC Immunol*. 2008 Feb 7;9:3.

### **Identification of a homozygous deletion in the AP3B1 gene causing Hermansky-Pudlak syndrome, type 2**

**Jung J\***, Bohn G\*, Allroth A\*, Boztug K, Brandes G, Sandrock I, Schäffer AA, Schilke R, Welte K, Grimbacher B\*, Klein C\*.

*Blood*. 2006 Jul 1;108(1):362-9. (\*contributed equally to this study)