Genomic targets of Polycomb Cbx proteins in hematopoietic stem cells and differentiated blood lineages

Marlinde Smit
Master report
University of Groningen
Master Biomedical Science
Stem Cell Biology
European Research Institute for the Biology of Ageing
Supervisors: Karin Klauke and Gerald de Haan
Date: 19-7-2014

Abstract

A proper balance between self-renewal and differentiation of hematopoietc stem cells is crucial, because impaired self-renewal can result in tissue dysfunction while excessive self-renewal can result in blood cancer (leukemia). Adult stem cells and their differentiated progeny contain identical genetic information, but their gene expression patterns differ substantially. This is accomplished by changes in chromatin compaction by DNA methylation or histone modifications, it establishes, maintains and propagates gene expression program and drives the behaviour of cellular differentiation. Polycomb group proteins (PcGs) are a specific class of epigenetic modifiers that catalyzates histone modifications to repress gene expression of target genes.

Previously, our lab showed that the balance of self-renewal and differentiation in HSCs is governed by Cbx-containing Polycomb repressive complex 1 (PRC1). Cbx7-containing PRC1 complexes induce self-renewal by repressing the expression of progenitor-specific genes, and its overexpression induces leukemia. Cbx2, Cbx4 or Cbx8 can compete with Cbx7 for integration into PRC1 and their overexpression results in differentiation and exhaustion of HSCs.

To get insight into the dynamics of Polycomb PRC1 targets during normal HSC differentiation, our aim is to compare genome wide targets of endogenous Cbx proteins in different hematopoietic cell subsets. An important technique to study protein-DNA interactions is Chromatin immunoprecipitation (ChIP), which is a very versatile tool. The basic steps including fixation, sonication and immunoprecipitation requires optimization.

Currently we have optimized a ChIP protocol for 100.000 cells with homemade buffers and tested several Cbx7 antibodies in different hematopoietic cell subsets. In the end, we aim to sequence DNA fragments bound by Cbx7, Cbx8, Cbx2 and Cbx4 in HSCs and multiple differentiated blood cells (T-cells, B-cells, granulocytes).

Introduction

Throughout our life, hematopoietic stem cells (HSCs) sustain blood cells with distinct functions, such as oxygen-transporting red blood cells, immunological white blood cells and hemostasis-mediating platelets. Most of the blood cells are short-lived and therefore HSCs and their progeny had to maintain hematopoietic homeostasis. HSCs are not only capable to differentiate into all blood cell lineages, but they also have the ability to self-renew. HSC self-renewal and differentiation are highly regulated in response to physiological conditions and it is important that there is a proper balance, because impaired self-renewal can result in tissue dysfunction while excessive self-renewal can result in blood cancer (leukemia) (Wang and Wagers, 2011; Nakajima-Takagi et al., 2013; Klauke et al., 2013; Hsu and Qu, 2013).

HSCs, separable into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), are on top of the 'Hematopoietic differentiation tree' (fig.1). Each branch point of which indicates a restriction in developmental potential. LT-HSCs maintain self-renewal potential throughout life, however ST-HSCs shows more limited self-renewal potential. Differentiation of ST-HSCs generates multipotent progenitors (MMPs), and further expansion and division results in the lymphoid lineage by generating common lymphoid progenitors (CLPs) or the myeloid lineage by generating common myeloid progenitors (CMPs). CLPs give rise to mature B-and T-lymphocytes and NK-cells. CMPs yields the granulocyte–macrophage progenitor (GMP) which will produce mature leukocytes, such as granulocytes and macrophages, and the megakaryocyte–erythrocyte progenitor (MEP) that differentiates into platelets and erythrocytes. (Sharpless and DePinho, 2007; Wang and Wagers, 2011)

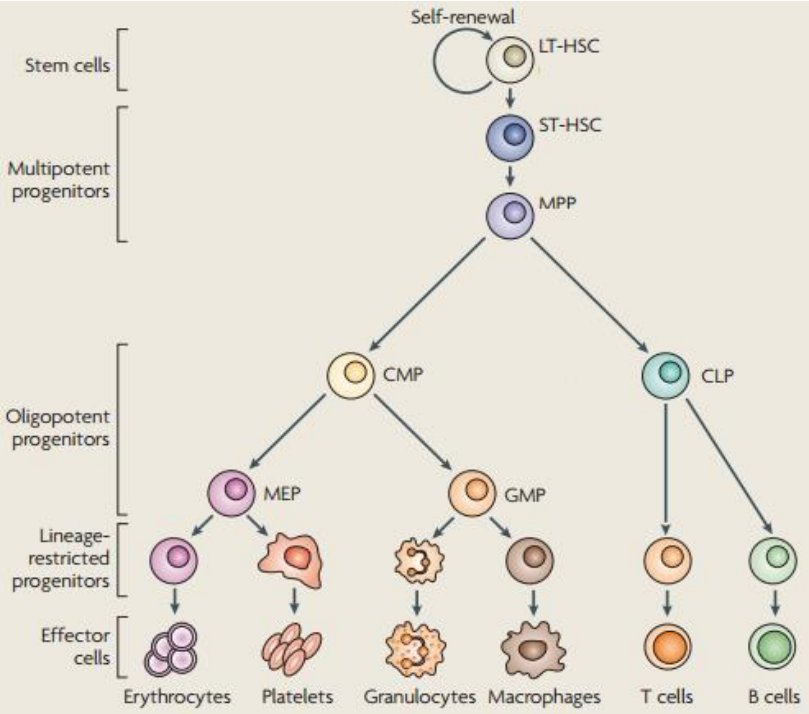


Figure 1: The hierarchy of hematopoiesis (Sharpless and DePinho, Nature reviews. Mol. Cell Biology, 2007)

Adult stem cells and their differentiated progeny contain identical genetic information, however their gene expression patterns differ substantially. This is accomplished by changes in chromatin compaction by epigenetic modifications, as DNA methylation or histone modifications, during cell differentiation (Prezioso and Orlando, 2011; Radulovic et al., 2013). Chromatin is the state in which DNA and proteins are packaged within the cell. DNA is wrapped around nucleosomes composed of histone octamers, which are the building blocks of chromatin. This provides structural stability and the capacity to regulate gene expression. Histones are subject to a large number of post-translational modifications such as acetylation, phosphorylation and methylation and can cause structural changes to chromatin (Barski et al.,2007; Kouzarides, 2007). The chromatin conformation is correlated with the expression state of genes: in a compact chromatin environment genes are less available to the transcriptional machinery than genes present in looser chromatin domains. To establish cell identity the correct set of genes must be transcribed, while other genes must be kept in a silent state (Laugesen and Helin, 2014).

Polycomb Group (PcG) proteins are transcriptional repressors that epigenetically modify chromatin and participate in the establishment and maintenance of cell fates (Sauvageau, M. and Sauvageau, G., 2010). PcGs were originally identified in *Drosophila* as regulators of anterior-posterior body patterning through the repression of homeotic (Hox) genes. The function of these proteins seemed to be highly conserved during evolution since they also showed to be important for proper development in mammals (Sauvageau, M. and Sauvageau, G., 2010; Klauke and de Haan, 2011). PcG proteins are assembled into a variety of multi-protein complexes that selectively reside in chromatin sites. The best characterized are Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) (Simon and Kingston, 2013; Ma et al., 2014). PRC2 is composed of three core subunits corresponding to *Drosophila* PcG genes: Enhancer of zeste (E(z)), Suppressor of zeste 12 (Suz12) and Extra sex combs (Esc). In mammals, PRC2 is composed of one of the two Ezh orthologs (Ezh1 or Ezh2), Suz12 and Eed. In *Drosophila,* PRC1 core complex consist of Polycomb (Pc), Polyhomeatic (ph), Posterior sex combs (psc) and Sex combs extra (Ring/Sce). Mammalian PRC1 complexes are very heterogeneous because each of the *Drosophila* subunits has several homologs in the mammalian genome and they can associate in a combinatorial fashion. These homologs are sorted into families of Cbx, Phc, Pcgf and Ring1 genes (Gao et al., 2012; Ma et al., 2014; Radulovic et al., 2013).

PRC2 catalyse the trimethylation of histone H3 on lysine 27 (H3K27me3) and Cbx proteins in the PRC1 recognize and catalyse PRC1 binding to this repressive mark. This binding of PRC1 to chromatin allows mono-ubiquitination of histone 2A on lysine 119 (H2AK119ub1), which is believed to be the final step in stable gene silencing (Simon and Kingston, 2013; Radulovic et al., 2013).

Previously it was shown that Cbx proteins are important for the balance between self-renewal and differentiation of HSCs. There are five members of the Cbx family, Cbx2, Cbx4, Cbx6, Cbx7, Cbx8 and they all can bind to H3k27me3 marks through their N-terminal chromodomain. Cbx7-containing PRC1 complexes induce self-renewal by repressing the expression of progenitor-specific genes, and its overexpression induces leukemia. Cbx2, Cbx4 or Cbx8 can compete with Cbx7 for integration into PRC1 and their overexpression results in differentiation and exhaustion of HSCs (Klauke et al., 2013). Recently Cbx7 was also identified as an embryonic stem cell (ESC) pluripotency factor. Cbx7 represses Cbx2, Cbx4 and Cbx8 in pluripotent cells to prevent differentiation and in turn Cbx7 and pluripotency genes are repressed to arrange lineage specification (Morey et al., 2012).

This suggest that different Cbx-containing PRC1 complexes repress distinct sets of genes and that alteration in the spectrum of expressed Cbx proteins will affect HSC self-renewal versus

differentiation. By studying genome wide the interaction of the Cbx proteins with the DNA, better insight into the dynamics of Polycomb PRC1 targets will be obtained.

Chromatin immunoprecipitation (ChIP) is a technique to study protein-DNA interactions and to identify target genes. The first step is crosslinking of proteins and the DNA using formaldehyde, to ensure that the chromatin structure during ChIP and isolation steps is preserved. Crosslinking is a time-critical procedure, because an excess of crosslinking can result in a decrease in the amount of protein bound to the DNA and reduced antigen availability in chromatin (Orlando, 2000; Abcam 2011). For cell lysis sodium dodecyl sulfate (SDS) can be used to dissolve cell membranes and unfold proteins. After cell lysis chromatin is fragmented by sonication in random fragments. Optimal fragmentation can be achieved by testing various sonication conditions on chromatin, followed by DNA isolation and analyzing the size of the fragments by gel electrophoresis. The presence of SDS in the sonication buffer improves sonication efficiency, but can induce foaming during sonication. Foam makes the chromatin sample inappropriate for ChIP, therefore the SDS concentration has to be diluted before sonication as well too long sonication time have to be prevented. When the crosslinking is optimal for ChIP, decrosslinking with high temperature, high SDS and high salt (NaCl) concentration is required to efficiently isolate DNA from the nuclei.

The following step is immunoprecipitation of the chromatin fragments. In ChIP antibodies are used to capture the protein of interest and the interacting DNA. Different antibodies have different affinities for their target. Low affinity antibodies may not be able to bind the protein of interest in very stringent buffers and most antibodies used for ChIP are sensitive to high SDS. Therefore different salt concentrations in wash buffers and SDS concentration in IP buffers has to be optimized. After immunoprecipitation chromatin fragments bound to the antibodies are captured using protein A/G beads, these DNA-protein-beads complexes are washed with salt buffers and reversed crosslinked. Subsequently the DNA is isolated and can be analyzed with PCR amplification with specific primers and finally sequenced to identify the DNA targets of the protein of interest. (Li et al., 2006; Haring et al., 2007; Abcam 2011). Despite the variety of DNA-bound proteins and cell types that can be examined by ChIP, the assay has been hindered by a requirement for large cell numbers (in the range of 10^6–10^7). ChIP assays for small cell numbers have been recently reported, however these are optimized for histone ChIPs or transcriptionfactor ChIPs, which is different than perform ChIPs with Polycomb proteins. (Attema et al., 2007; Dahl and Collas, 2008)

To get insight into the dynamics of Polycomb PRC1 targets during normal HSC differentiation the aim of this study was to compare genome wide targets of endogenous Cbx proteins in different hematopoietic cell subsets. We have optimised a chromatin immunoprecipitation (ChIP) protocol for 100.000 cells with homemade buffers and tested different Cbx7 antibodies in distinct hematopoietic cell subsets. In the end the aim of this project is to sequence DNA bound by Cbx7, Cbx8, Cbx2 and Cbx4 in HSCs and multiple differentiated blood cells (T-cells,B-cells, granulocytes).

Materials and methods

Cell culture

Murine myeloid cell line (32D cells) were cultured in Gibco® RPMI 1640 medium containing 10% Fetal Bovine serum (FBS) heat inactivated, 50ug Il-3, penicillin and streptomycin at 37˚C in 5% CO2.

Transduction

Phoenix eco cells were transfected with vectors SF,Cbx7-flag and Cbx8-flag in DMEM without serum and the next day medium was replaced with RPMI. 32D cells were transferred to retronectin-coated wells and virus-supernatant produced in RPMI was added. GFP+ cells were sorted 24 h later.

Crosslinking and sonication

Cells were crosslinked with 1% cold Formaldehyde (Sigma, solution 36,5-38%) at various temperatures and a various time intervals, as indicated. Cell lysis was performed with SDS buffer (100mM NaCL, 50mM Tris-HCL, 5mM EDTA, 0.2% NaN_3, 0,5% SDS containing protein inhibitor cocktail complete EDTA-free (Roche Diagnostics)) and samples were frozen in -80. Sonication was performed with Bioruptor (Diagenode) as indicated at various time intervals, 30ON/30OFF in 300 ul IP buffer (66.7mM Tris-HCl, 100mM NaCl, 5mM EDTA, 0.2% NaN_3, 1.67% Triton-X-100 and 0.33% SDS containing protein inhibitor cocktail complete EDTA-free (Roche Diagnostics)). Fragmented chromatin was centrifuged 30 minutes 17000xg 4˚C and supernatant was transferred to new tubes after which ChIP was performed.

Chromatin immunoprecipitation

ChIP assays using Pierce protein A/G magnetic beads (Thermo Scientific) was carried out according to a standard protocol in IP buffer (66.7mM Tris-HCl, 100mM NaCl, 5mM EDTA, 0.2% NaN3, 1.67% Triton-X-100 and 0.33% SDS containing protein inhibitor cocktail complete EDTA-free (Roche Diagnostics)) in a myeloid cell line (32D). Complexes were washed 4x in 150mM wash buffer (1% Triton X-100, 0.1% SDS, 150mM NaCl, 2mM EDTA at pH 8.0 and 20mM Tris-HCl at pH 8.0 containing protein inhibitor cocktail complete EDTA-free (Roche Diagnostics)) and the final wash step in TE buffer. Five microgram of rabbit-anti-Cbx8 (LAST , Helin, K., Copenhagen), rabbit-anti-Cbx7 (Millipore,07-981), rabbit-anti-Cbx7 (Abcam, Chip-grade, 21873), rabbit-anti Cbx7 (HcHa-1, Peters,G., London) rabbit-anti-H3K27me3 (Upstate, 07-449) and IgG from rabbit serum (Sigma, I8140) was used per immunoprecipitation. Samples were reverse crosslinked in 1% SDS, 200mM NaCl in TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) 65 ˚C 1000rpm for overnight or 4hr. DNA was isolated with Qiaquick PCR purification kit (Qiagen) and the concentration was measured with Qubit dsDNA HS assay kit (Life Technologies). The size of the fragmented chromatin was analyzed with gel electrophoresis (1% agarose, Invitrogen) or with use of High sensitivity DNA Chips (Agilent Technologies) loaded on the 2100 Agilent Bioanalyzer (Agilent Technologies).

Purification of primary hematopoietic cells

Bones (hind limbs, spine, pelvis, sternum) were harvested from C57BL/6 mice. After removal of soft tissue, bones were crushed with a mortar and pestle in lysis solution (NH4Cl) and passed through a 100-µm filter. Cells were washed with PBS + 0.2% BSA and then stained with B220-Pacific blue (clone RA3-6B2, Biolegend 103227, 1:1000), CD45.1-PE (clone A20, Biolegend 110708, 1:200), CD3e-APC (clone 145-2C11, eBiosciences 17-0031, 1:100), Ly-6G(Gr1)-AlexaFluor700(1:100) and

Ter119-Pe/Cy7 (clone TER-119, Biolegend 116222, 1:200). Bone marrow cells were sorted with MoFlo XDP cell sorter in granulocytes (Gr1+), T cells (CD3+), B cells (B220+) and erythroid precursor cells (Ter119+).

Real-time PCR

The immunoprecipitated DNA was isolated with Qiaquick PCR purification kit (Qiagen) with a total volume of 57 µl Elution buffer. Per reaction 1µg of genomic DNA was analysed with primers for the predictive active (Ink4a, ARF, Ink4b, HoxA9, HoxB4 and Cebpa) and inactive (Actin and Gata1) loci by real-time PCR (table 1). Per reaction 0,2 pmol primer and SYBR Green (Lightcycler 480 SYBR Green 1 Master) were used to determine the reactions with the LightCycler 480 instrument II (Roche).

Table 1: primers used for real-time PCR

Name	Forward primer	Reverse primer
Ink4a	GATGGAGCCCGGACTACAGAAG	CTGTTTCAACGCCCAGCTCTC
ARF	AAAACCCTCTCTTGGAGTGGG	GCAGGTTCTTGGTCACTGTGAG
Ink4b	CACCGAAGCTACTGGGTCTC	CTGTGGCAGAAATGGTCCTT
HoxA9	CATACTCTTGGAAGGCACAAAATTCACACG	GGTGGCCCCATCCCAATCTGG
HoxB4	GGGAGGGGTAGAGAAGGGGAAATAAACC	GCCACCCGGCCTGCGATTC
Cebpa	ATTGGAAAGTCACAGGAGAAG	GCAGCTTCGGGTCGCGAAT
Actin	AACAGCCGGAGCTACACACT	GGGCTCAGCCTATGAGTCAG
Gata1	TGGATTTTCCTGGTCTAGGG	GTAGGCCTCAGCTTCTCTGTAGTA

Westernblotting

SDS-PAGE was performed according to standard protocols. Precast mini-protean TGX gels were used (Biorad). Membranes (Nitrocellulose from Transblot Turbo RTS transfer kit, Biorad) were blocked for 30 min with PBST-5% milk and stained with the following antibodies: rabbit-anti-CBX8 (1:1000, LAST), rabbit-anti-Cbx7 (1:100, Santa Cruz, P-15) and mouse-anti-H3 (1:1000, Cell Signaling, 3638) in PBST-5%milk. For secondary antibodies anti-mouse IgG (1:10.000, GE Healthcare Life Sciences, NXA931) and ECL anti-rabbit IgG (1:10.000, GE Healthcare Life Sciences, NA934V) were used in PBST-5% milk.

Library preparation

Microplex Library preparation kit (Diagenode) was used for the preparation of eleven indexed libraries (table 2) with use of the instruction manual of the kit. The library preparation begins after the isolation of DNA bound to the target protein and then used as input DNA for library generation. DNA fragments are end-repaired and an 'A'-base added to the blunt ends of each strand, preparing them for ligation to the sequencing adapters. Each adapter contains a 'T'-base overhang on the 3'-end providing a complementary overhang for ligating the adapter to the A-tailed fragmented DNA. Final product is created and the Library samples with different indices were pooled and checked with the Agilent 2100 bioanalyzer. Size selection had to be performed with 1% agarose E-gel and E-gel Electrophoresis system (Invitrogen). DNA was isolated from the E-gel with Zymoclean Gel DNA recovery kit (Zymo research) and the concentration was measured with Qubit dsDNA HS assay kit (Life Technologies). The fragmented chromatin was analyzed with use of High sensitivity DNA Chips (Agilent Technologies) loaded on the 2100 Agilent Bioanalyzer (Agilent Technologies). Finally all of the ChIP DNA fragments can be simultaneously sequenced on an Illumina sequencer.

Table 2: Sample list with prepared indexed Libraries of the ChIP-seq samples.

Sample nr	nr of cells	antibody	Index sequence
1	2x10^6	H3K27me3	ATCACG
2	2x10^6	IgG	CGATGT
3	2x10^6	10% input	TTAGGC
4	2x10^6 tech.replic.	H3K27me3	TGACCA
5	2x10^6 tech. replic.	input	ACAGTG
7	0,5x10^6	H3K27me3	CAGATC
8	0,5x10^6	IgG	ACTTGA
9	0,5x10^6	10% input	GATCAG
10	0,1x10^6	H3K27me3	TAGCTT
11	0,1x10^6	IgG	GGCTAC
12	0,1x10^6	10% input	CTTGTA

Results

Optimal crosslinking and fragmentation protocol with 100.000 cells

The first step in this study was to optimize Chromatin Immunoprecipitation (ChIP) for low cell numbers, which makes it possible to study binding sides of different (endogenous) Cbx-containing PRC1 complexes in different hematopoietic cell subsets, including HSCs. To accomplish this, several steps in the protocol had to be optimized: crosslinking of the DNA and proteins, chromatin fragmentation and chromatin immunoprecipitation. With a murine myeloid cell line (32D cells) and homemade buffers we tested different fixation times, fixation volumes, fixation temperatures, sonication times and compared the efficiency between high cell numbers and low cell numbers. As mentioned above the SDS concentration had to be diluted before sonication, therefore the sonication steps were performed in IP buffer (0.33% SDS and 100mM NaCl)

By using pre-heated 1% formaldehyde (FA) for crosslinking, an enrichment of chromatin around 500 base pairs (bp) was obtained (fig.2A). However when using 100.000 cells, over-crosslinked DNA was formed as illustrated by figure 2B. First we increased the sonication time to even 20-25 minutes, to attempt to break the high fragments into smaller fragments. However very small fragments around 150bp (nucleosome size) were formed. Subsequently room temperature 1% formaldehyde was used to prevent over-crosslinking of the DNA, yet again too small chromatin fragments were formed even by decreasing the sonication time. (fig.3A). Finally the first step was optimized by getting an enrichment of chromatin around 500bp by crosslinking 10 minutes at 4˚C with 1% cold formaldehyde and 6 minutes of sonication(fig.3B).

After several weeks, troubles in the protocol appeared and therefore crosslinking and sonication had to be optimized again. With new formaldehyde and new buffers we finally obtained chromatin enrichment around 400-500bp, by crosslinking 10 minutes at 4˚C with 1% cold FA and sonication of 4 minutes (fig. 4). In conclusion we optimized the crosslinking of the DNA and proteins, and fragmentation of the chromatin for 100.000 cells.

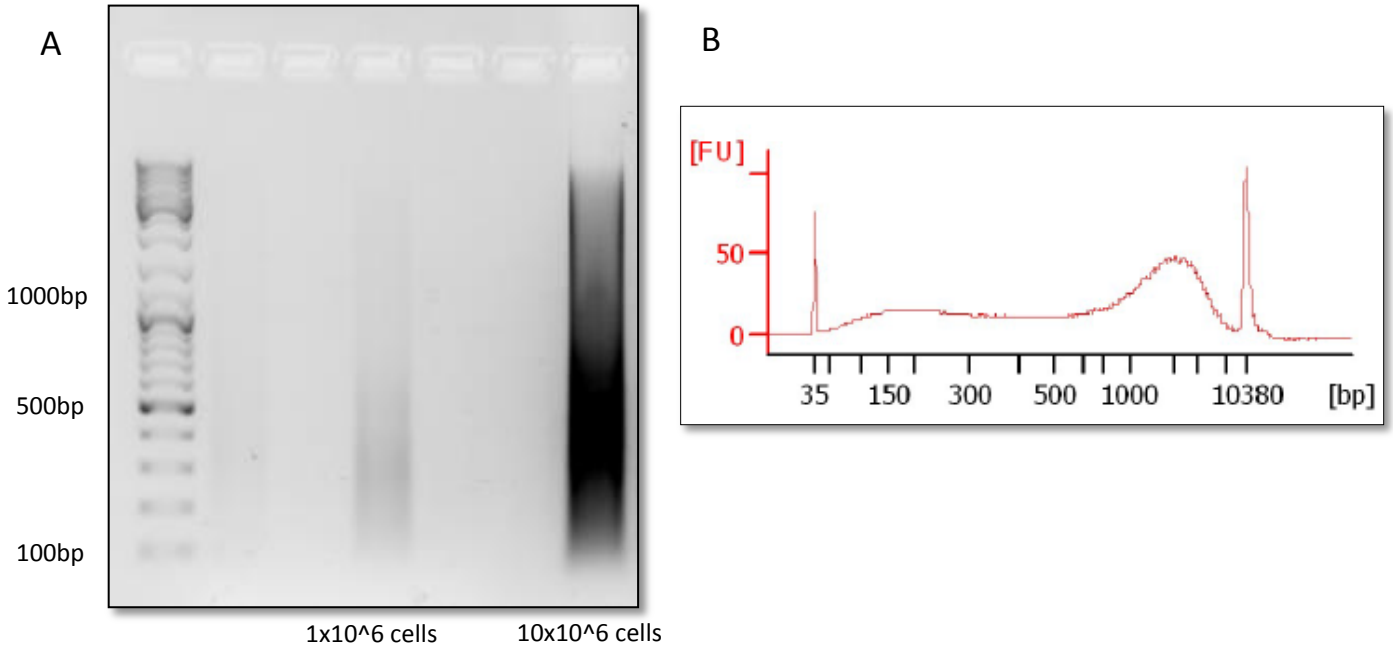


Figure 2. Analysis of crosslinked and fragmented chromatin with 32D cells. Cells were crosslinked for 8 minutes with pre-heated 1% formaldehyde and 10 minutes sonication in 500ul (high cell nr) and 300ul IP buffer (low cell nr.) (A) Enrichment of 500 base pairs with 1 million cells and 10 million cells analysed by gel electrophoresis (1% agarose). 5% aliquots from originally samples were used to load on the gel for crosslinking and sonication control and 100bp marker was used. (B) 100.000 cells analysed with 2100 Agilent Bioanalyzer.

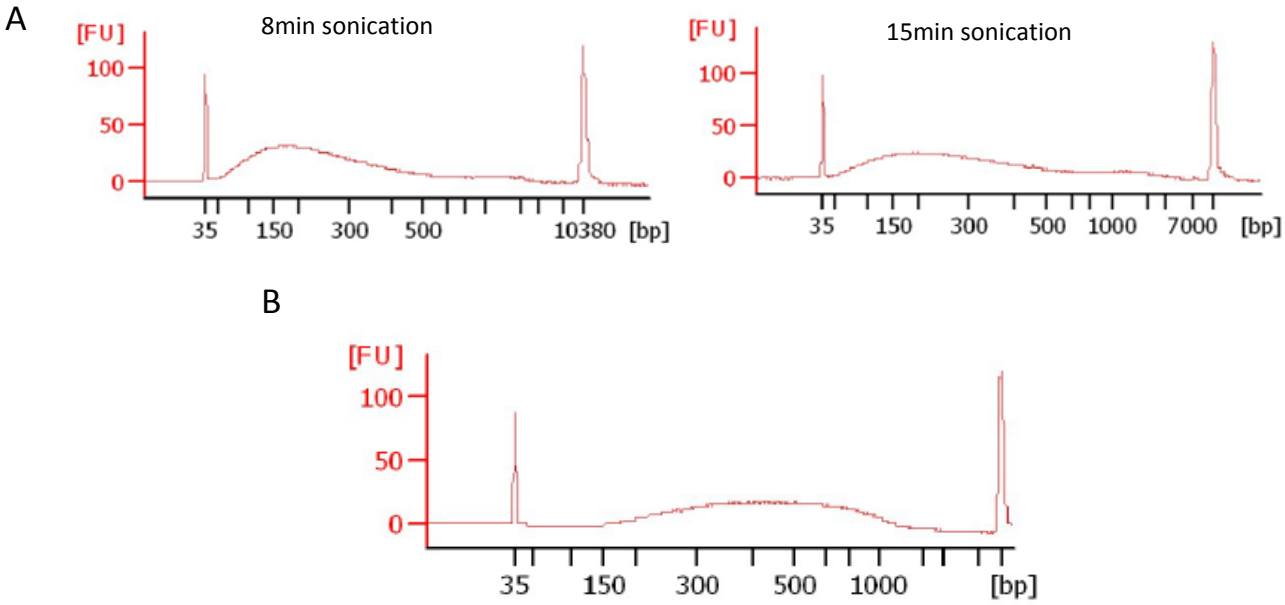


Figure 3. Sheared chromatin from 100.000 cells analysed with 2100 Agilent Bioanalyzer. (A) cells were crosslinked with room temperature 1% FA, resulted in too small chromatin fragments even by changing the sonication time; left image: 8min sonication and right image: 15 minutes sonication (both 30ON/30OFF) (B) DNA fragments around 500 bp were formed by crosslinking 8 minutes at 4˚C with 1% formaldehyde and sonication of 6 cycles 30ON/30OFF.

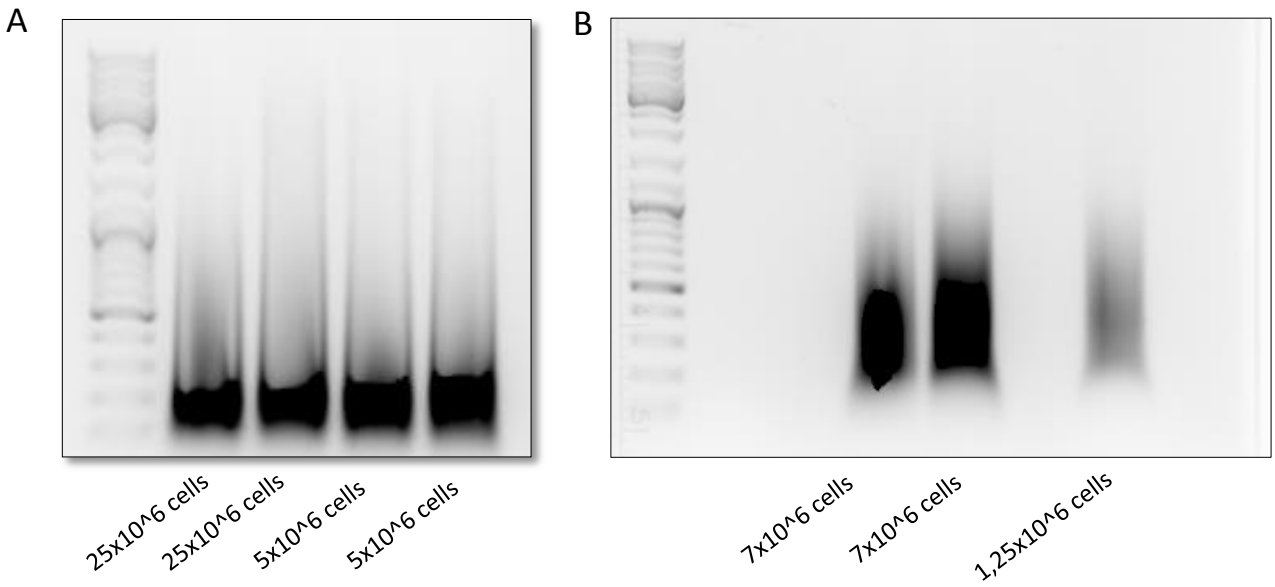


Figure 4. Second optimalisation of Crosslinking and fragmentation. Sheared chromatin was analysed by gel electrophoresis (1% agarose) with 100bp marker. 5% aliquots from originally samples were used to load on the gel for crosslinking and sonication control. (A) Very small fragments were formed from 25 million cells and 5million cells. (B) enrichment of 400-500bp by crosslinking 10 minutes at 4˚C with 1% cold FA and sonication of 4minutes 30ON/30OFF.

Optimal Chromatin immunoprecipitation protocol for 100.000 cells

After improving the crosslinking and sonication protocol, the second step was to optimise the immunoprecipitation of the chromatin with homemade buffers. The immunoprecipitated DNA was analyzed with primers for different loci with Real-Time PCR and the ChIP-qPCR data is analysed relative to input as this includes normalization for both background levels and input chromatin going into ChIP (Abcam 2011). Antibody against H3K27me3 was used as positive control, because genome-wide ChIPs showed that Cbx-PRC1 colocalizes with H3K27me3 to bind to chromatin (Simon and Kingston, 2013). H3K27me3 protein is very abundant and the anti-H3K27me3 (Upstate 07-449) is a good working antibody which is used in a lot of studies (Bracken et al., 2007; Gao et al., 2012; Klauke et al., 2013; Pemberton et al., 2014). As background control anti-IgG was used. First the antibody-protein complexes coupled to the beads were washed with both 500mM buffer (high salt) and 150mM buffer (low salt), which resulted in low input of all the samples and high background with 100.000 cells (Fig.5). Therefore the next step was to use only low salt wash buffer. Since some antibodies may not be able to bind the protein of interest in very stringent buffers, with the result that specific antibody binding can be eliminated during the wash steps. Washing only with low salt buffer resulted in an increased input of the low cell numbers, however not the input of the high cell numbers and the background was still high (fig.6). The following step was to use more beads and antibody with high cell numbers and to improve the wash step of the protocol, by short-spin the samples between the wash steps. The result was the same pattern of the different positive and negative loci between high and low cell numbers using anti-H3K27me3, as well low background (IgG) (fig.7). In conclusion we have to crosslinking 10 minutes at 4˚C with 1% cold FA, sonicate for 4 minutes in IP buffer (0,33% SDS and 100mM NaCl), antibody-binding in IP buffer and we have to wash the antibody-protein-beads complexes with low salt buffer.

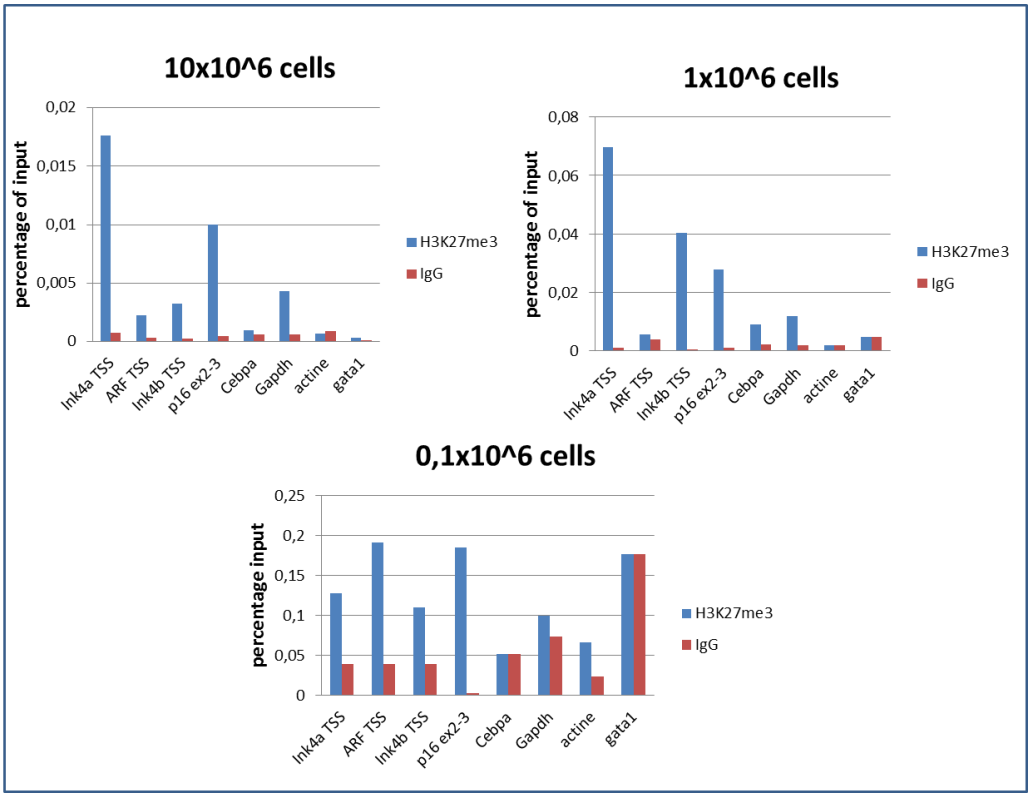


Figure 5. Washing with high salt and low salt buffers. Chromatin Immunoprecipitation was performed with homemade buffers, using high salt and low salt in the wash buffers. Sheared chromatin from 10 million cells, 1 million cells and 100.000 cells was used. ChIP was performed with positive control H3K27me3 and background control IgG in 32D cells. Quantitative PCR with following primers for different loci were used: Ink4a, ARF, Ink4b, p16 ex2-3, Cebpa ,Gapdh, Actin and Gata1. The data is normalized for 10% of input.

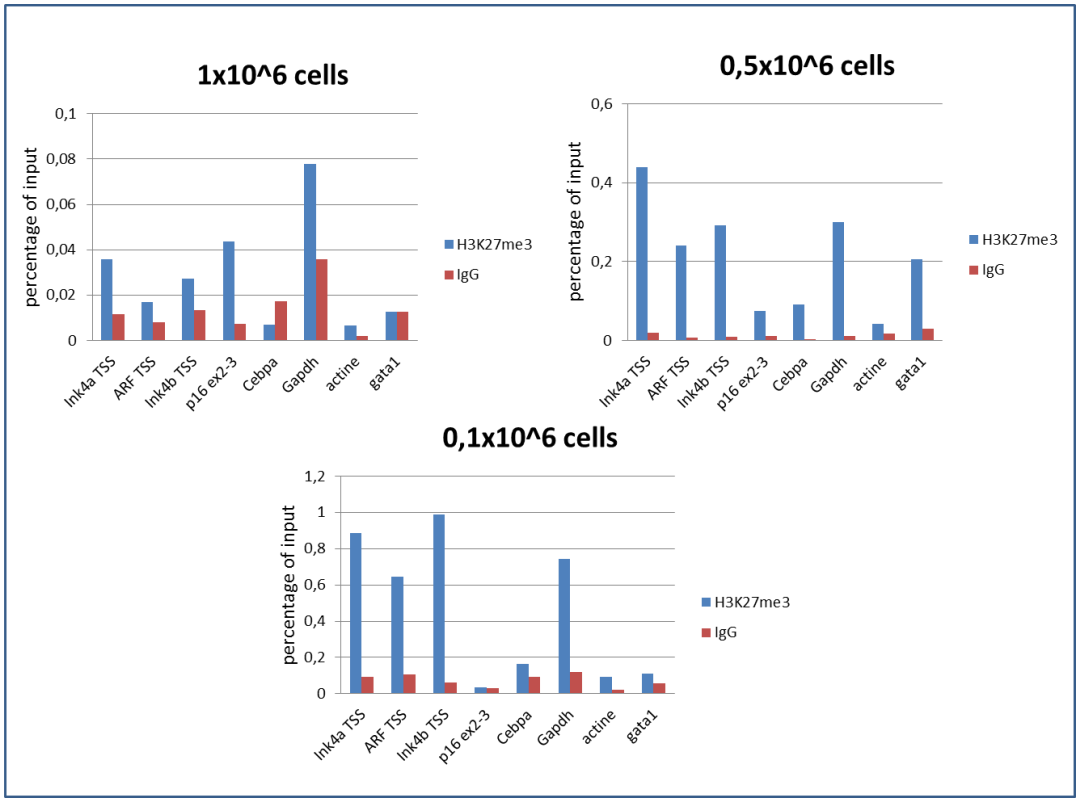


Figure 6.Washing only with low salt buffers. ChIP analysis of 1million, 500.000 and 100.000 32D cells. ChIP was performed with homemade buffers. H3K27me3 was used as positive control and IgG as background control. For Quantitative PCR the following primers of different loci were used: Ink4a, ARF, Ink4b, p16 ex2-3, Cebpa ,Gapdh, Actin and Gata1. The data is normalized for 10% of input.

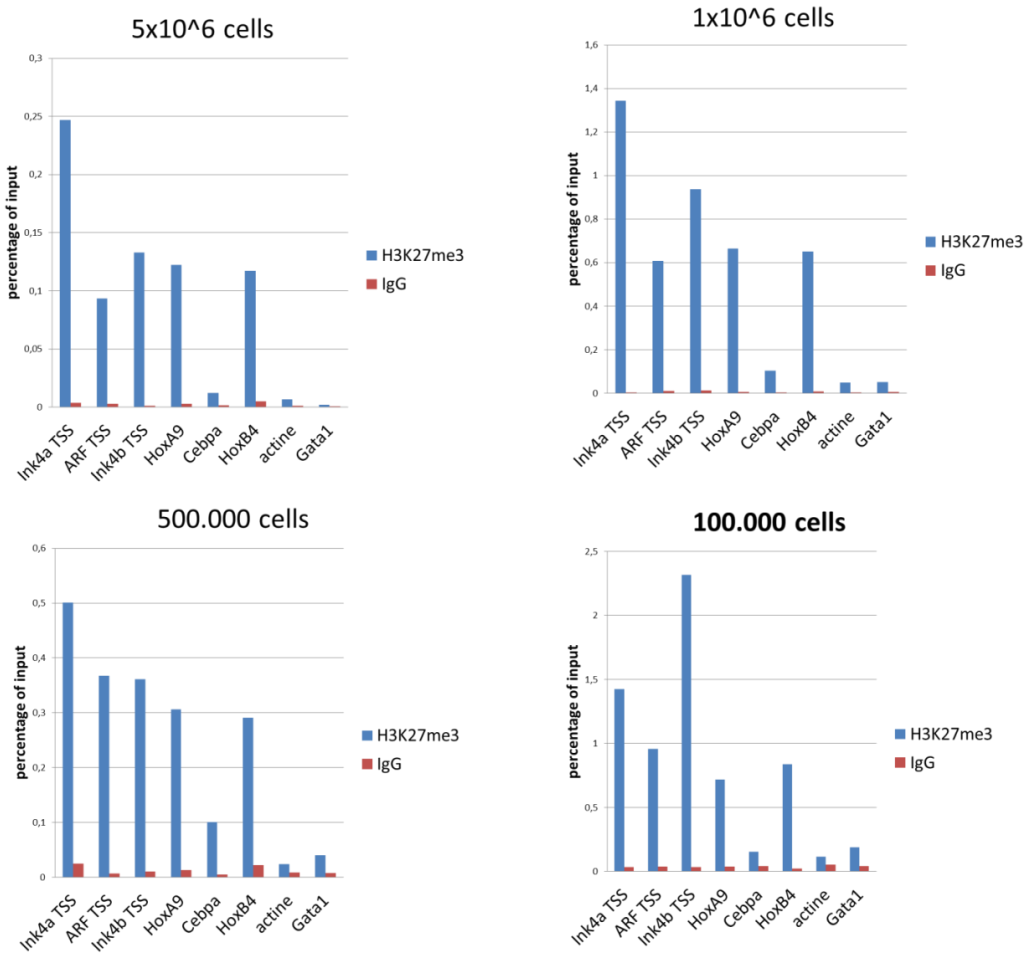


Figure 7. Optimised ChIP protocol for 100.000 cells. ChIP was performed with homemade buffers, using sheared chromatin from 5 million cells, 1 million cells, 500.000 cells and 100.000 cells. Postive control H3K27me3 and background control IgG were used. RT- PCR was performed with primers for different loci: Ink4a, ARF, Ink4b, HoxA9, Cebpa, HoxB4, Actin and Gata1. The data is normalized for 10% of input.

Preparing ChIP samples for deep sequencing

With ChIP-sequencing (ChIP-seq) it is possible to identify genome wide target genes. Therefore the following step of this study was to verify the ChIP data with deep sequencing, to validate that we do not loose genomic targets when using low cell numbers. After analysing new ChIP samples with 2 million, 500.000 and 100.000 cells with Quantitative PCR for two positive and two negative loci (fig. 8), indexed Libraries were prepared with the MicroPlex Library Preparation kit (diagenode) for sequencing. Quantitative PCR was used again to check the library samples for the same positive and negative loci (fig.9). After pooling the Libraries with different indices, a higher fragment "bump" was shown on the bioanalyzer as illustrated by figure 10A. Subsequently size selection was performed to get an enrichment between 200 and 600 bp (fig.10B). These data showed that the sample was well prepared for deep sequencing. We are currently analyzing the sequencing data, therefore the data are not included in this report.

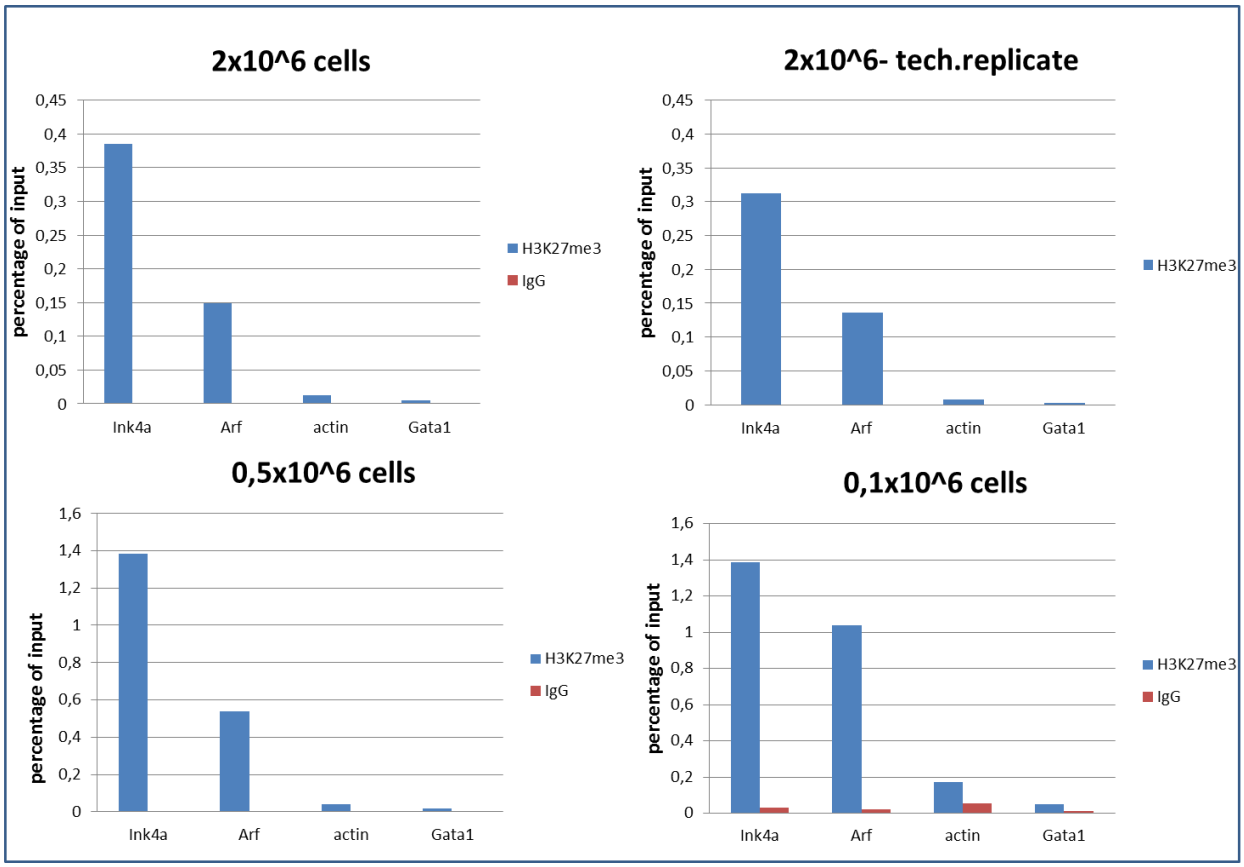


Figure 8. ChIP samples analysed with RT-PCR for two positive and two negative loci. H3K27me3 was used as positive control and IgG as background control. Technical replicate is split after crosslinking and before sonication, using only the positive control and input. ChIP was performed with 2 million, 500.000 and 100.000 cells. The data is normalized for 10% of input.

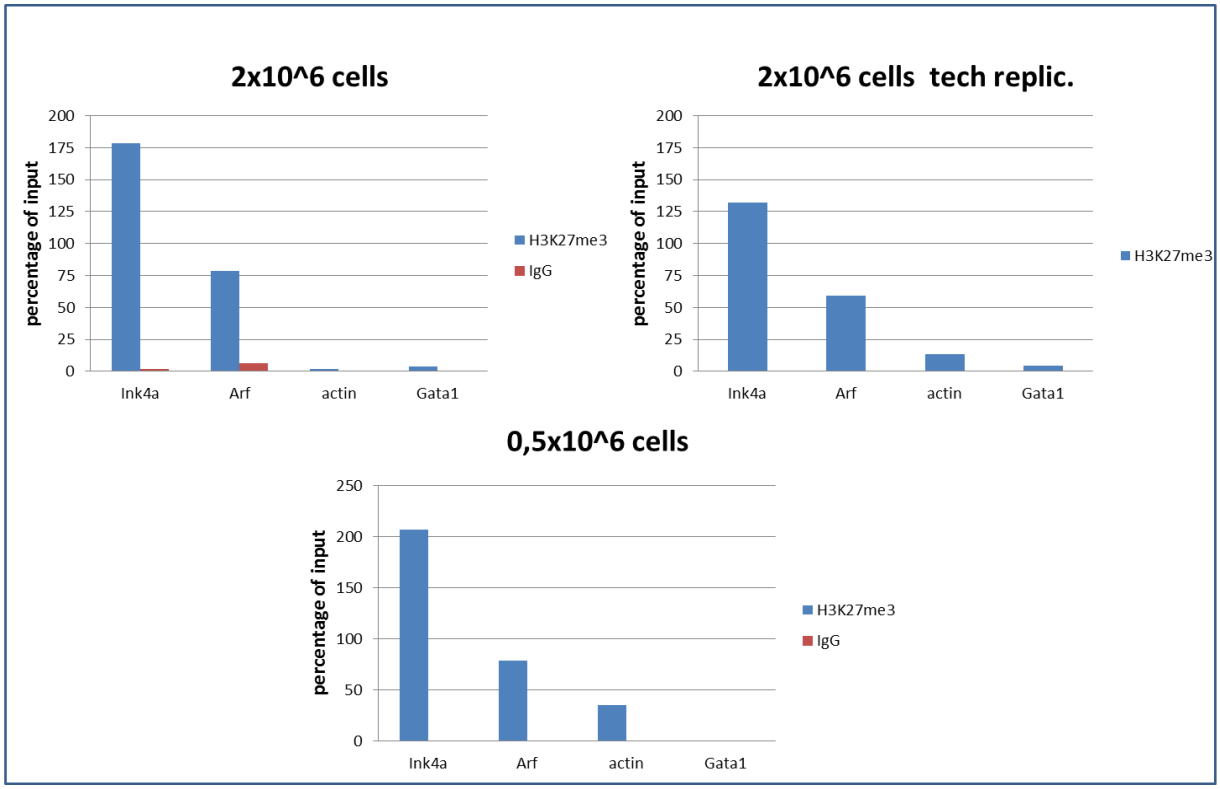


Figure 9. Library samples analysed with RT-PCR for two positive and two negative loci. Library prepared from 2 million cells plus technical replicate, and 500.00 cells are shown. The library of 100.000 cells is not shown because of using different amplification cycles for the input and the controls. The data is normalized for 10% of input.

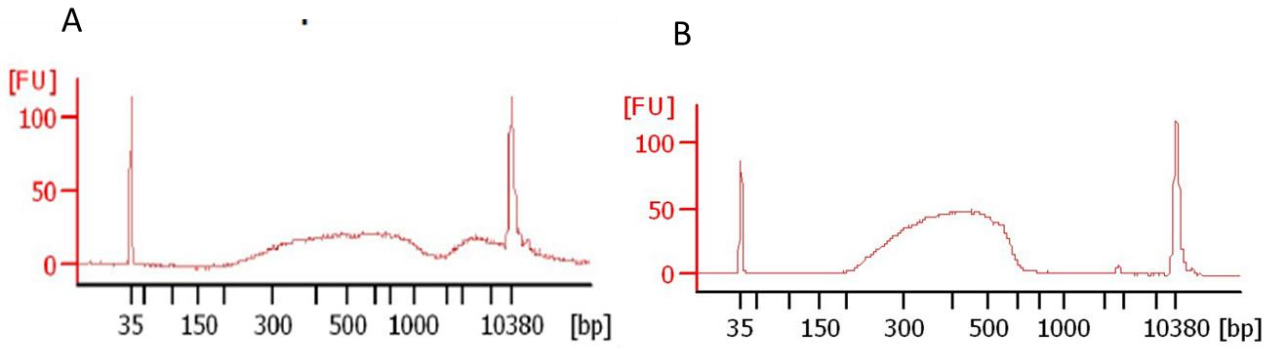


Figure 10. Library pool analysed with the Bioanalyzer. (A) High fragment 'bump' after pooling the library samples with different indices. **(B)** Library pool after size selection with 1% agarose E-gel (Invitrogen).

Testing different Cbx7 and Cbx8 antibodies

Before making ChIP samples from different hematopoietic cell subsets, several Cbx7 and Cbx8 antibodies had to be tested for endogenous expression. With Western blot we analyzed the protein expression of Cbx8 and Cbx7 in 32D cells. Cbx8 showed relatively high endogenous expression in 32D cells, however Cbx7 expression was very low (fig. 11). Consequently we used 32D cells with overexpression of Cbx7-flag to first test which Cbx7 antibody works best after which other cells will be used to test for endogenous Cbx7 ChIP. ChIP assays were performed using anti-Cbx7 (Millipore,

07-981) and anti-Cbx7 Chip-grade (Abcam). Since Cbx7 antibody (Peters, G. Londen) was working well in ChIP experiments with Polycomb proteins in human fibroblast (Pemberton et al, 2014), we also wanted to test this antibody in 32D cells. Compared to previous ChIP assays shown above Cebp-α is very low in the positive control, similar to the two negative loci Actin and Gata1. Next time we have to test more negative loci, to verify that the ChIP worked. The anti-Cbx7 from Millipore and Abcam showed the same pattern in the different loci and a relative high percentage of input, which suggest that they work. However the anti-Cbx7 (Peters, G., London) was very low in all positive and negative loci, suggesting that this antibody is not working in our 32D cell line (fig.12). The Cbx8 LAST antibody was also not working for endogenous Cbx8 in the 32D cell line, the percentage of input was as low as the background control (fig. 13). Maybe we used a wrong antibody batch, since this antibody seemed to work in 32D cells (Klauke et al, 2013). In conclusion these results suggest that we found two working Cbx7 antibodies.

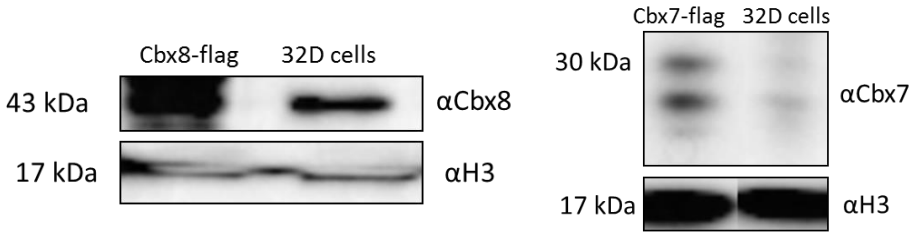


Figure 11. Protein expression of Cbx8 and Cbx7 in 32D cells. Antibodies used for detection were anti-cbx8 LAST (K. Helin, Copenhagen), anti-Cbx7 (p-15) (Santa Cruz) and anti-H3 (Cell signalling).

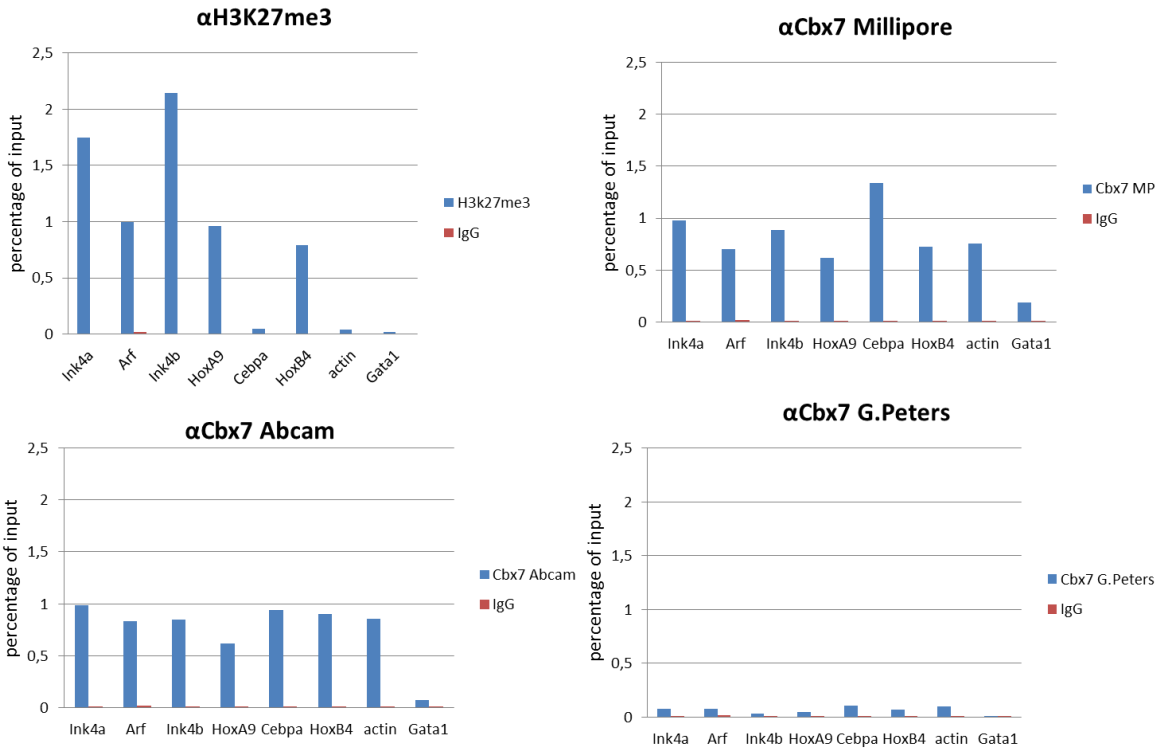


Figure 12. Different Cbx7 antibodies tested for ChIP with Cbx7-overexpressed 32D cells. The following antibodies were used: H3K27me3, as positive control, Cbx7 (Millipore), Cbx7 (Abcam), Cbx7 (Peters, G., London) and background control IgG. For Quantitative PCR the following primers of different loci were used: Ink4a, ARF, Ink4b, HoxA9, Cebpa, HoxB4, Actin and Gata1. The data is normalized for 10% of input

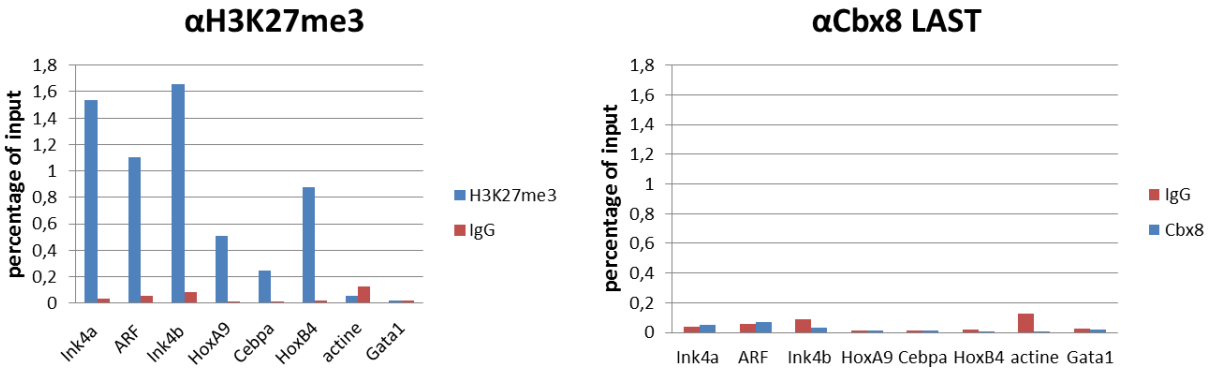


Figure 13. Cbx8 LAST antibody tested for ChIP with 32D cells. Anti-H3K27me3 was used as positive control and IgG as background control. For Quantitative PCR the following primers of different loci were used: Ink4a, ARF, Ink4b, HoxA9, Cebpa , HoxB4, Actin and Gata1. The data is normalized for 10% of input

Different gene expression of Cbx family members in distinct hematopoietic cell populations.

To compare genome wide targets of endogenous Cbx proteins in different hematopoietic cell subsets, we first wanted to analyse the expression of Cbx family members in purified hematopoietic subpopulations (fig. 14; Klauke et al.,2013). Cbx7 is highly expressed in long-term HSCs (LT-HSCs) and in differentiated lymphoid lineages (T-cells, B-cells). Cbx8 is equally expressed in various primitive haematopoietic cells. Most of the haematopoietic cell populations show high expression of Cbx4, but low expression of Cbx2. Because of the distinct expression of Cbx family members during haematopoietic differentiation, we sorted different haematopoietic subpopulations from bone marrow of mice for ChIP (supplementary fig.1). With isolated B-cells and T-cells we first wanted to test endogenous Cbx7 with the working Cbx7 antibodies shown above (supplementary fig. 2).

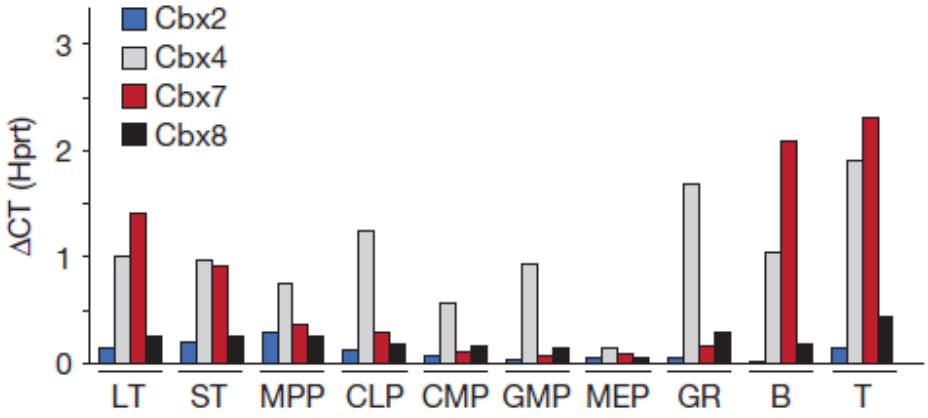


Figure 14. Gene expression of Cbx family members in different haematopoietic cell populations. abbreviations used: Long-term hematopoietic stem cell (LT), Short-term hematopoietic stem cell (ST), multipotent progenitors (MPP), Common lymphoid progenitors (CLP), common myeloid progenitors (CMP), megakaryocyte-erythroid progenitors (MEP), granulocytes (GR), B-lymphocytes (B) and T-lymphocytes (T). Bars represent the mean of 2-3 technical replicates.

Discussion

Previously our lab showed that different Cbx family members composed in the PRC1 complex results in self-renewal or differentiation of the hematopoietic stem cells (HSCs), by repressing different sets of target genes (Klauke et al.,2013), which is also found in embryonic stem cells (ESCs) (Morey et al., 2012). Therefore the aim of this study was to compare genome wide targets of endogenous Cbx proteins in different hematopoietic cell subsets, to get insight into the dynamics of Polycomb PRC1 targets during normal HSC differentiation.

A very useful technique to study DNA-protein interactions is chromatin immunoprecipitation (ChIP). ChIP is a very versatile tool, the procedure requires optimization of reaction conditions, especially when using low cell numbers (Das et al., 2004). We wanted to optimize a good working ChIP protocol for 100.000 cells. Since there were a lot of basic steps in this technique to optimize we first used a myeloid cell line, before performing ChIP in hematopoietic cell subsets. Additionally we wanted to compare high cell numbers with low cell numbers in a cell line, to validate that we did not lose important targets by performing ChIP with low cell numbers. For crosslinking of DNA and proteins we used formaldehyde, which is the most commonly used cross-linking agent (Orlando, 2000; Das et al., 2004). Formaldehyde can easily cross-link the DNA and proteins, and due to its small size it can cross-link only closely associated proteins (Klockenbusch and Kast, 2010). Although formaldehyde is a very good cross-link agent, epitopes recognized by antibodies against endogenous proteins could be destroyed by formaldehyde modification. (Haring et al.,2007; Das et al., 2004)Therefore optimal conditions for formaldehyde cross-linking had to be determined. In the beginning we obtained overcross-linked DNA from 100.000 cells by using pre-heated 37 °C formaldehyde, which results in difficulty in fragmentation by sonication and can also lead to a loss of immunoprecipitated material (Haring et al.,2007; Das et al., 2004). We found that crosslinking with lower temperature prevents overcrosslinking with 100.000 cells. The reason for that could be, because fixation is diffusion dependent, the temperature during fixation affect the fixation process. Fixation at 37 °C tends to increase the rate of the reaction (Kohja et al., 2012), however when using low cell numbers this could lead to overcrosslinking.

Antibodies are the most important factor for a successful ChIP experiment. The success of an antibody in CHIP is dependent on which epitope(s) it recognizes. The antibodies we tested for Cbx7 and Cbx8 were all polyclonal antibodies. Polyclonal antibodies are preferred over monoclonal antibodies, because polyclonal antibodies recognize several epitopes of the target increasing signal levels. However Monoclonal antibodies do have an advantage, since they show much less batch-to-batch variation (Das et al.,2004; Haring et al., 2007; Abcam2011). We had to reduce the wash buffer to low salt (150mM NaCL), because washing with high salt (500mM NaCL) resulted in a low signal. Probably the reason is that some antibodies have low affinity for the protein of interest and therefore you have to test different washing conditions. On one hand you want to keep the stringency as high as possible, as reducing can increase background and affect the signal to noise ratio. However in very stringent buffers low affinity antibodies can be eliminated during the wash steps (Abcam 2011). So far we found two working Cbx7 antibodies in a myeloid cell line and we are currently optimising the ChIP protocol for these antibodies for endogenous Cbx7 in B cells and T cells isolated from the bone marrow of mice, because Cbx7 is highly expressed in these cells. Specific and effective antibodies for endogenous Cbx8, Cbx4 and Cbx2 still have to be found.

We optimized a ChIP protocol for 100.000 cells, however by deep sequencing we still have to validate our findings and it still needs to be determined if we do not lose genomic targets by using

100.000 cells. It is important to know the genomic targets of DNA binding proteins, because the interaction between proteins and DNA is essential for many vital cellular functions. The ink4a-ARF-Ink4b locus is one of the earliest identified target for Polycomb group genes (PcG) and both Cbx7 and Cbx8 bind throughout this locus. This locus encodes two members of the Ink4 family of cyclin-dependent-kinase inhibitors, $p15^{ink4b}$ and $p16^{ink4a}$ and protein $p19^{ARF}$. The three proteins have tumor suppression potential by influencing cell-cycle, cellular senescence, apoptosis and stem-cell self-renewal (Das et al., 2004; Gil and Peters, 2006; Bracken et al., 2007; Klauke and de Haan, 2011; Klauke et al., 2013). Because Ink4-ARF-Ink4b locus is identified as target gene of Cbx-containing PRC1 complex we used this as positive locus for ChIP analysis with real-time PCR. Furthermore we used C/EBPα target gene, which serves to protect adult HSCs from apoptosis and to maintain their quiescent state, as a result it is associated with loss of self-renewal and it promotes myeloid differentiation (Hasemann et al., 2014). Aditionally two Hox genes HoxB4 and HoxA9 were used as positive loci. We tested different Cbx7 antibodies in a myeloid cell line, which showed a high signal of C/EBPα. However the positive control H3K27me3 showed a low signal of C/EBPα. The reason for this could be since Cbx7 showed low gene expression in the myeloid lineage (granulocytes and common myeloid progenitors) (fig. 14), it cannot repress the C/EBPα target gene. Additionally the test was performed in overexpressed Cbx7 cells, and as a result the positive control gave a low signal by the abundance of Cbx7 in these cells.

The different gene expression of Cbx family members in distinct hematopoietic cell populations suggests that the Cbx family repress distinct set of genes. Furthermore Cbx proteins have various activities as either oncogenes or tumor suppressors. Cbx7 overexpression induces leukemia, while the other Cbx proteins including Cbx2, Cbx4 and Cbx8 induces differentiation and decreased cell proliferation (Klauke et al., 2013; Ma et al., 2014). Cbx7 is also involved in several other cancer types including prostate, lymphoma, pancreatic, colon and gastric cancer (Ma et al, 2014). To validate the unique roles of the Cbx proteins in various lineages, we want to sequence DNA bound by Cbx7, Cbx8, Cbx2 and Cbx4 in HSCs and multiple differentiated blood cells in the future. Increasing knowledge about the roles of the Cbx proteins and their targets during differentiation, might offer the potential of developing therapeutic targets.

In conclusion we have optimized a chromatin immunoprecipitation protocol for 100.00 cells with homemade buffers. Further we found two working Cbx7 antibodies, which we are currently testing in B-cells and T-cells. Whether endogenous Cbx proteins bind to different genomic loci in distinct hematopoietic cell subsets still needs to be determined.

Acknowledgements

I would like to thank Karin Klauke for her guidance, Gerald de Haan for the opportunity to work in his lab, Leonid Bystrykh for valuable scientific discussions, the Stemcell Biology group for laboratory assistance, Inge Kazemier and Nancy Halsema for assistance with the Qubit, Bioanalyzer, E-Gel and sequencing.

References

Abcam (2011) A Beginner's Guide to ChIP. Online available: docs.abcam.com/pdf/.../**A-beginners-guide-to-ChIP.**

Attema, J.L., Papathanasiou, P., Forsberg, E.C., Xu, J., Smale, S.T., Weissman, I.L. (2007) Epigenetic characterization of hematopoietic stem cell differentiation using miniChIP and bisulfite sequencing analysis. Proc Natl Acad Sci U S A. 104(30):12371-12376.

Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., Zhao, K. (2007) High-resolution profiling of histone methylations in the human genome. Cell. 129(4):823-837.

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., Hansen, K.H., Helin, K. (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev. 21(5):525-530.

Dahl, J.A., Collas, P. (2008) A rapid micro chromatin immunoprecipitation assay (microChIP). Nat Protoc. 3(6):1032-1045.

Das, P.M., Ramachandran, K., vanWert, J., Singal, R. (2004) Chromatin immunoprecipitation assay. Biotechniques. 37(6):961-969.

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., Reinberg, D. (2012) PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. Mol Cell. 45(3):344-356.

Gil, J. and Peters, G. (2006) Regulation of the INK4b–ARF–INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol. 7(9):667-677.

Haring, M., Offermann, S., Danker, T., Horst, I., Peterhansel, C., Stam, M. (2007) Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. Plant Methods. 3 :11.

Hasemann, M.S., Lauridsen, F.K., Waage, J., Jakobsen, J.S, Frank, A.K, Schuster, M.B., Rapin, N., Bagger, F.O., Hoppe, P.S., Schroeder, T., Porse, B.T.(2014) C/EBPα is required for long-term self-renewal and lineage priming of hematopoietic stem cells and for the maintenance of epigenetic configurations in multipotent progenitors. PLoS Genet. 10(1): doi: 10.1371/journal.pgen.1004079

Hsu, P. and Qu, C. (2013) Metabolic plasticity and hematopoietic stem cell biology. Curr Opin Hematol. 20(4):289-94.

Khoja, H., Smejkal, G., Krowczynska, A., Herlihy, J.D. (2012) Optimizing sample fixation and chromatin shearing for improved sensitivity and reproducibility of chromatin immunoprecipitation. Covaris Inc. online available: www.covarisinc.com

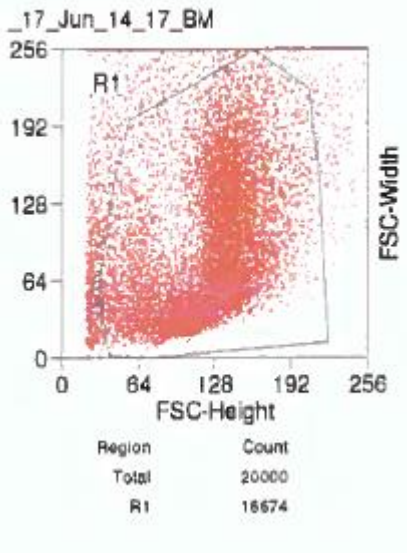
Klauke, K. and de Haan, G. (2011) Polycomb group proteins in hematopoietic stem cell aging and malignancies. Int J Hematol. 94(1):11-23.

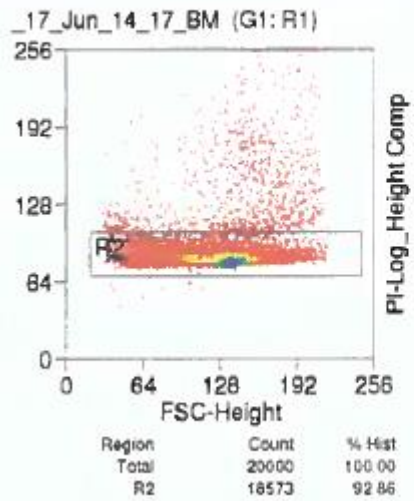
Klauke, K., Radulovic, V., Broekhuis, M., Weersing, E., Zwart, E., Olthof, S., Ritsema, M., Bruggeman, S., Wu, X., Helin, K., Bystrykh, L., de Haan, G.(2013) Polycomb Cbx family members mediate the balance between haematopoietic stem cell self-renewal and differentiation. Nature Cell Biology 15(4):353-362.

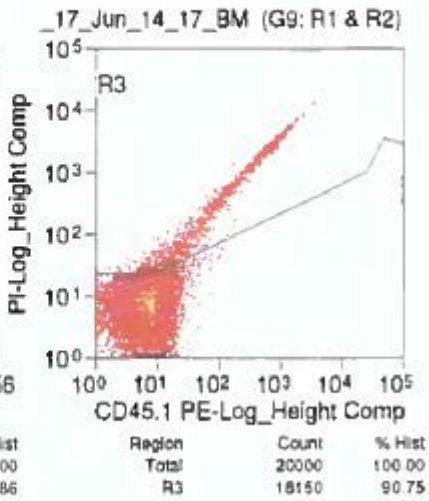
Klockenbusch, C. and Kast, J. (2010) Optimization of formaldehyde cross-linking for protein interaction analysis of non-tagged integrin beta1. J Biomed Biotechnol. 2010: Article ID 927585.

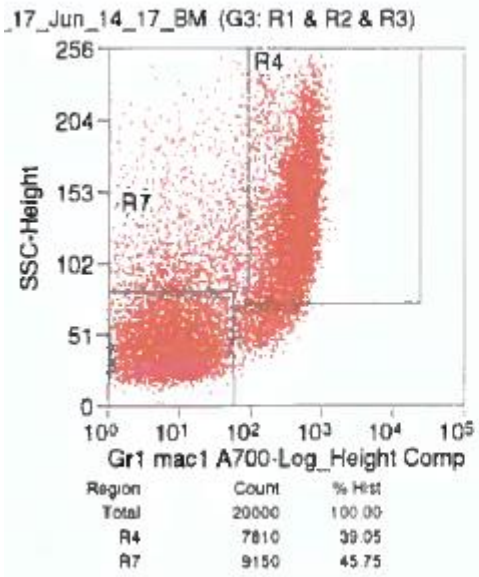
Kouzarides, T. (2007) Chromatin modifications and their function. Cell. 128(4):693-705.
Laugesen, A., and Helin, K. Chromatin Repressive Complexes in Stem Cells, Development, and Cancer. Cell Stem Cell. 14(6):735-751.
Li, C.C., Ramirez-Carrozzi, V.R., Smale. S.T.(2006) Pursuing gene regulation 'logic' via RNA interference and chromatin immunoprecipitation. Nat Immunol. 7(7):692-697.
Ma, R.G., Zhang, Y., Sun, T.T., Cheng, B. (2014) Epigenetic regulation by polycomb group complexes: focus on roles of CBX proteins. J Zhejiang Univ Sci B. 15(5):412-428.
Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S.A., Di Croce, L. (2012) Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. Cell Stem Cell. 10(1):47-62.
Nakajima-Takagi, Y., Osawa, M., Iwama, A. (2013) Manipulation of Hematopoietic Stem Cells for Regenerative Medicine. The Anatomical Record 00:00–00.
Orlando, V. (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci. 25(3):99-104.
Pemberton, H., Anderton, E., Patel, H., Brookes, S., Chandler, H., Palermo, R., Stock, J., Rodriguez-Niedenführ, M., Racek, T., de Breed, L., Stewart, A., Matthews, N., Peters, G. (2014) Genome Biol. 15(2):R23
Prezioso, C. and Orlando V.(2011) Polycomb proteins in mammalian cell differentiation and plasticity. FEBS Lett. 585(13):2067-2077.
Radulović, V., de Haan, G., Klauke, K.(2013) Polycomb-group proteins in hematopoietic stem cell regulation and hematopoietic neoplasms. Leukemia. 27(3):523-533.
Sauvageau, M. and Sauvageau, G. (2010) Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. Cell Stem Cell. 7(3):299-313.
Sharpless, N.E. and DePinho, R.A. (2007) How stem cells age and why this makes us grow old. Nat Rev Mol Cell Biol. 8(9):703-13.
Simon, J.A. and Kingston, R.E. (2013) Occupying chromatin: Polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. Mol Cell. 49(5):808-824.
Wang, L.D. and Wagers, A.J. (2011) Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nature reviews Molecular Biology 12 : 643–655.

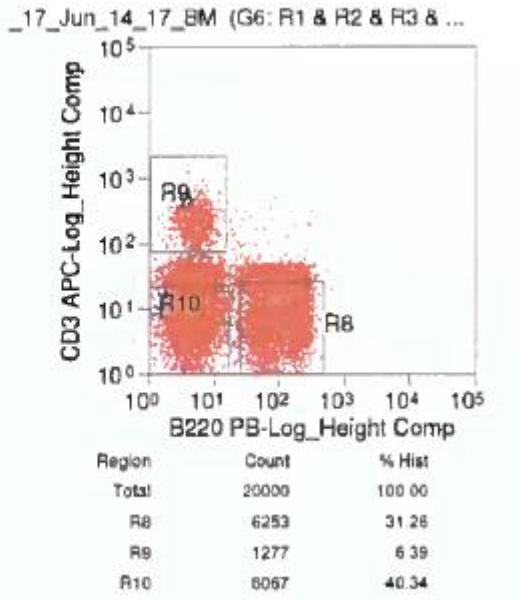
Supplementary information

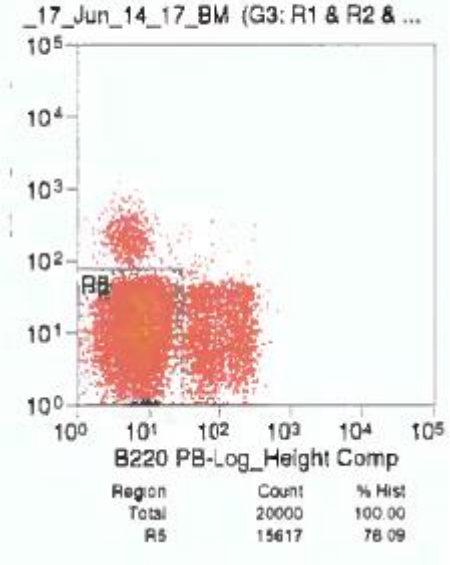












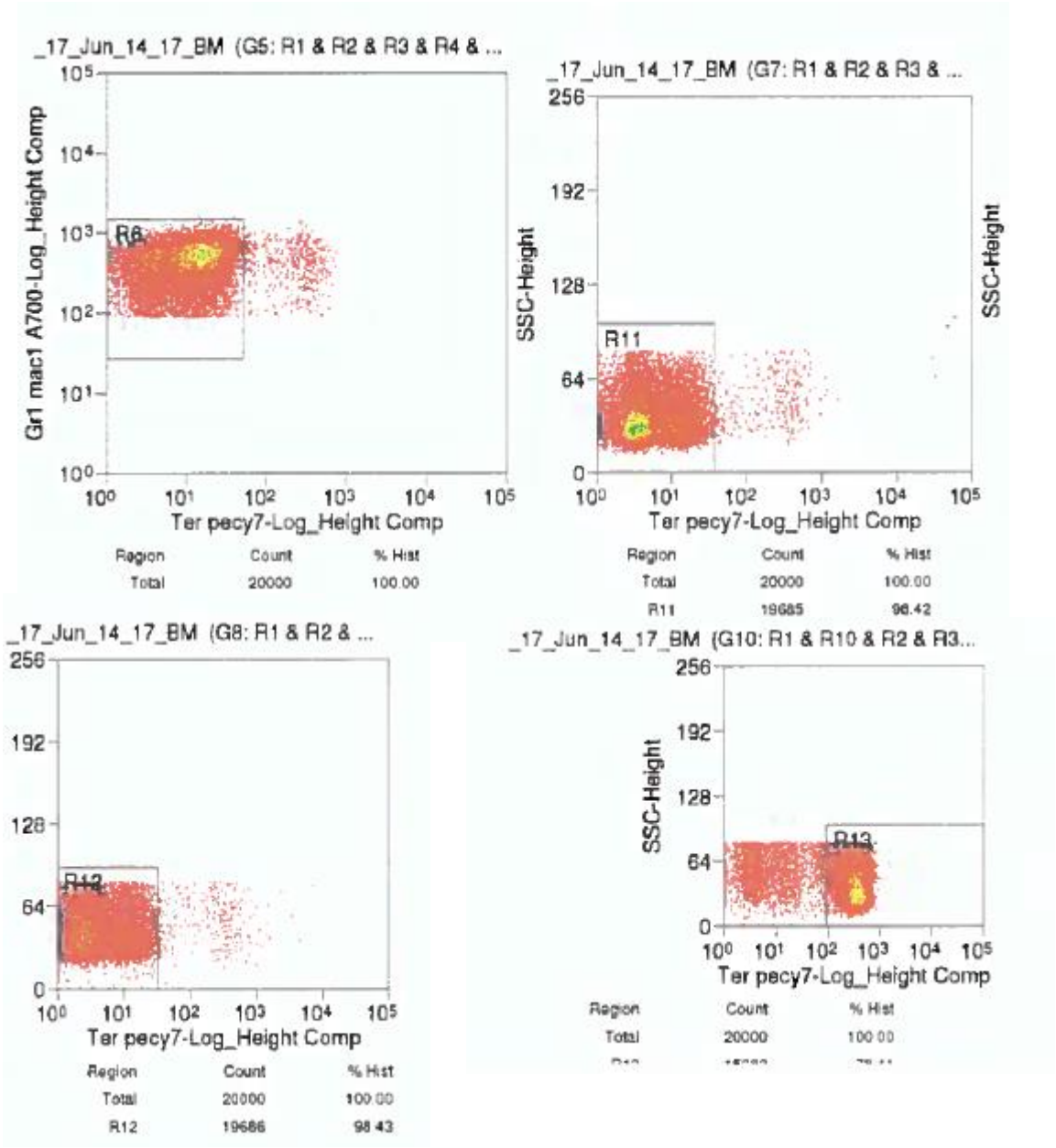


Figure S1. Facs plots of the purification of primary hematopoietic cells. Isolated cells are B cells (B220+), T cells (CD3+), granulocytes (GR1+) and erythroid precursor cells (Ter119+).

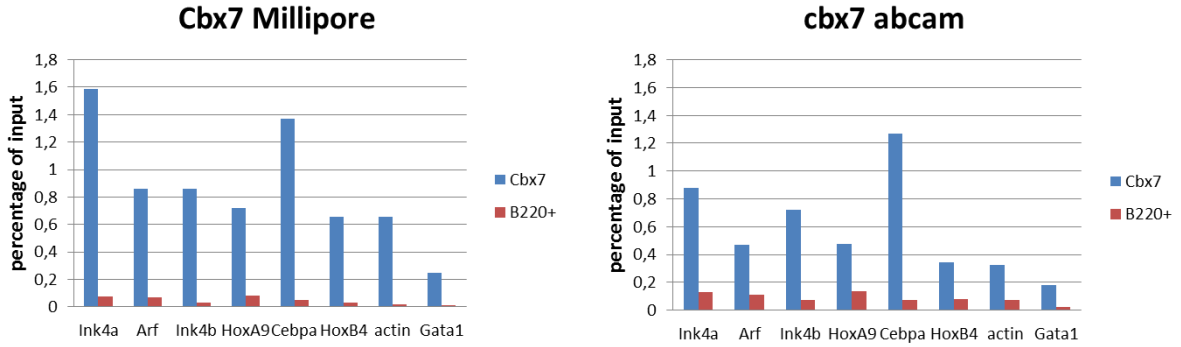


Figure S2. Cbx7 antibodies tested in B-cells. 32D cells with overexpressed Cbx7 was used as control.

Chromosome instability

Aneuploidy, which is the hallmark in 70-80% of cancer, is the state of a cell with an abnormal DNA content. Chromosome instability (CIN) is the process that leads to aneuploidy and can be classified into two groups: numerical CIN (nCIN), in which whole chromosomes are gained or lost, and structural CIN in which chromosome integrity is distorted through translocations, amplifications and deletions. The classic assay to quantify whole-chromosome aneuploidy is karyotyping. Cultured or freshly isolated cells are treated with nocodazole, which blocks the dividing cells in mitosis when the chromosomes are condensed, allowing for chromosome counting (Foijer 2010).

As in many cancer types, chromosomal instability play an essential role in leukemogenesis, by promoting the accumulation of genetic lesions responsible for malignant transformation. (Vajen et al., 2013) Previously our lab showed that Cbx7 can induce different types of leukemia (Klauke et al., 2013) and we are interested whether overexpression of Cbx7 can cause genetic instability. We first optimized a fixation protocol for metaphase spreads, with an untransduced murine myeloid cell line (32D cells). These cells have a karyotype of 40 chromosomes, which corresponds to mouse standard karyotype. Subsequently we overexpressed Cbx7 in myeloid cells and karyotype the cells by counting the number of chromosomes per cell. (Supplementary fig.3)

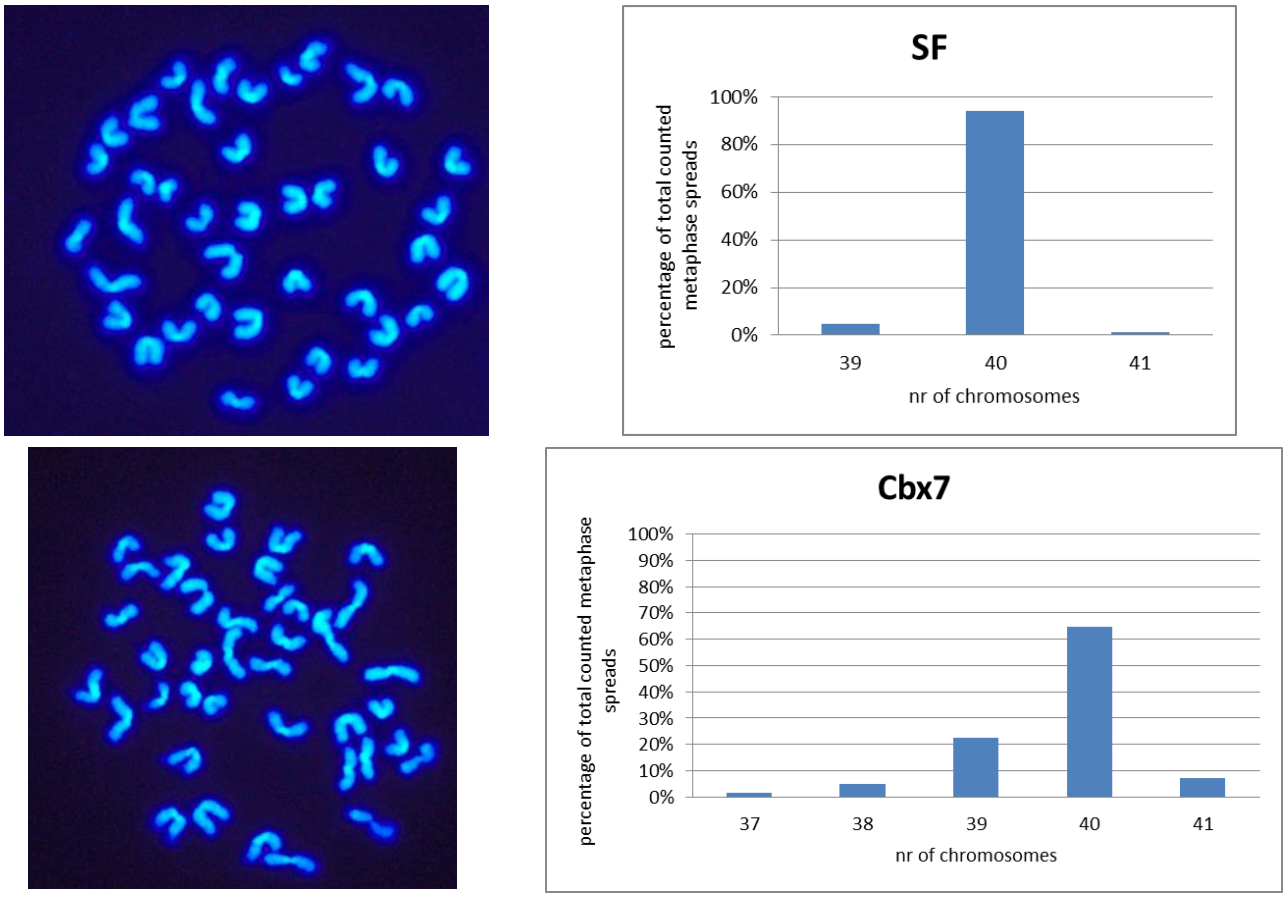


Figure S3. Karyotyping of 32D cells with empty vector and with overexpressed Cbx7. (A) 32D cells with an empty vector (SF). Left picture: metaphase spread of 40 chromosomes. Right graph: number of chromosomes counted in total of 152 cells (n=2). (B) 32D cells with overexpressed Cbx7. Left picture: metaphase spread of 39 chromosomes. Right graph: number of chromosomes counted in total of 208 cells (n=3).

Foijer, F.(2010) CINister thoughts. Biochem Soc Trans 38(6):1715-1721.

Vajen, B., Thomay, K., Schlegelberger, B. (2013) Induction of Chromosomal Instability via Telomere Dysfunction and Epigenetic Alterations in Myeloid Neoplasia. Cancers 5(3):857-874.

ChIP protocol

Optimised by: Marlinde Smit

Crosslinking and sonication

1) Harvest your cells and wash them 1x with cold PBS
2) Resuspend the cell pellet in 1% cold Formaldehyde and fixate for 10 minutes at 4˚C (coldroom) on a rotator platform.
3) Stop fixation by addition of glycine to final concentration of 0.125 M and incubate for 5 min. (For 5ml fix: add 313ul, for 1ml fix add 62,5ul)

4) Wash 2x with cold-PBS (if using 15ml tubes, transfer sample to low adherence tubes after the first wash step)
5) Resuspend pellets after final washstep **in 500ul SDS buffer +prot.inhib** (50mlSDS+ 1 tablet prot.inhib)
6) put the samples first on dry ice before you store the samples (**-80C**)
   (If you want to continue the same day, the samples have to be stored -80 for at least 30min)

7) Thaw cells from freezer in a water bath at RT (20-24C, not to warm!). Be sure that all the SDS went back in solution.
8) Centrifuge 5 min 3500rpm , remove supernatant with a pipet (carefully! You don't get a nice pellet→ leave some sample in the tube),
9) resupend pellets in **300ul IP buffer** (or 500ul IP buffer, when using high cell nr.)
   IP buffer: 30ml SDS buffer (+prot.inhib) +15ml Triton dilut. buffer

10) Sonicate samples to an average length of 500bp with Bioruptor **30ON/30OFF 3min**
    (*the tube holders should always be completely filled with tubes. Never leave empty spaces in the tube holder. Fill the empty spaces with tubes containing the same volume of distilled water.)*

11) Centrifuge sonicated chromatin **30min 4C 17000xg** (=max)→ transfer supernatant in new low adherence tubes.

Reverse crosslinken

overnight or 3,5-4hr 65C in a total volume of 120ul

(reverse crosslinken in final 1%SDS and *200mM NaCl)*
(aliquot contain: 0,33%SDS and *100mM NaCl)*

Take a 15 or 25ul aliquot (=5% off your sample) for sonication control →Transfer to a new tube and store the originally samples **-80C**

Mastermix:25ul of the 500ul sample:	Mastermix: 15ul v 300ul sample:
77,73 ulx4= 310,92 ul TE buffer	87,55ulx4 =350,2ulTE
11,67x4= 46,68ul 20% SDS	11,75 x 4 =47ul 20%SDS
5,6ulx 4 = 22,4 ul 5M NaCL	5,7x 4 =22,8ul 5M NaCL
→95ul rev xlink buffer/sample	→ 105ul rev xlink buffer/sample

12) Isolate the DNA with Qiaquick PCR purification kit (Qiagen)

Analyze the fragments with gel electrophoresis (1% agarose gel, Ladder: gene ruler (4C), Loading buffer : orange (home-made)) or when using low cell numbers with the Qubit+ Bioanalyzer.

ChIPpen

Washing of the beads (protein A/G magnetic beads (4C)) for 12 samples (preclearing+next day)

- Take 400ul beads (cut off pipet tip, beads has to be in suspension!)
- Wash **3x** with 1ml cold PBS → add PBS, turn tube couple times, short spin (not higher then 1), remove supernatant with magnetic rack
- Wash **1x** with cold IP buffer (+prot.inhib) → leave some buffer in tube→ 300ul

Make fresh **IP buffer** or use the one from the day before→ **in 50 ml tube : 30ml SDS buffer+15ml Triton dilut. +1 tablet prot.inhib.**
Store beads 4C

Add to 1ml or 500ul IPbuffer(+prot.inhib) to the samples

pre-clearing: 7ul beads/sample *(*cut off pipet tips!)
1hr incubation 4C rotating platform (cold room)

Tranfer supernatant to new tubes (low adherence) with the help of the magnetic rack
Split the samples : (don't forget the 10% input)

Store Input samples 4C

After splitting: add to 1ml or 500ul IP buffer to the samples

Add 5 ug antibody per IP (for low cell nr 3ug is also good)
H3K27me3 upstate 1ug/ul → 5ul/IP
IgG rabbit sigma 10ug/ul →0,5 ul/ IP
Cbx7 Millipore → 0,5mg/ml 5ug= 10ul/IP
Cbx7 abcam 1ug/ul → 5ul/IP
Cbx8 LAST → 1ug/ul → 5ul/IP

Incubate Overnight 4C rotating platform (cold room)

The next day: add 20ul washed beads to every sample (low cell nr less beads)
3hr incubation 4C rotating platform

Remove supernatant with the help of the magnetic rack
Wash the beads (750ul): (*short spin between the wash steps, not higher than 1000 rpm!)*

- 4x low salt (150mM washbuffer) → +prot.inhib tablet
- 1x TE buffer

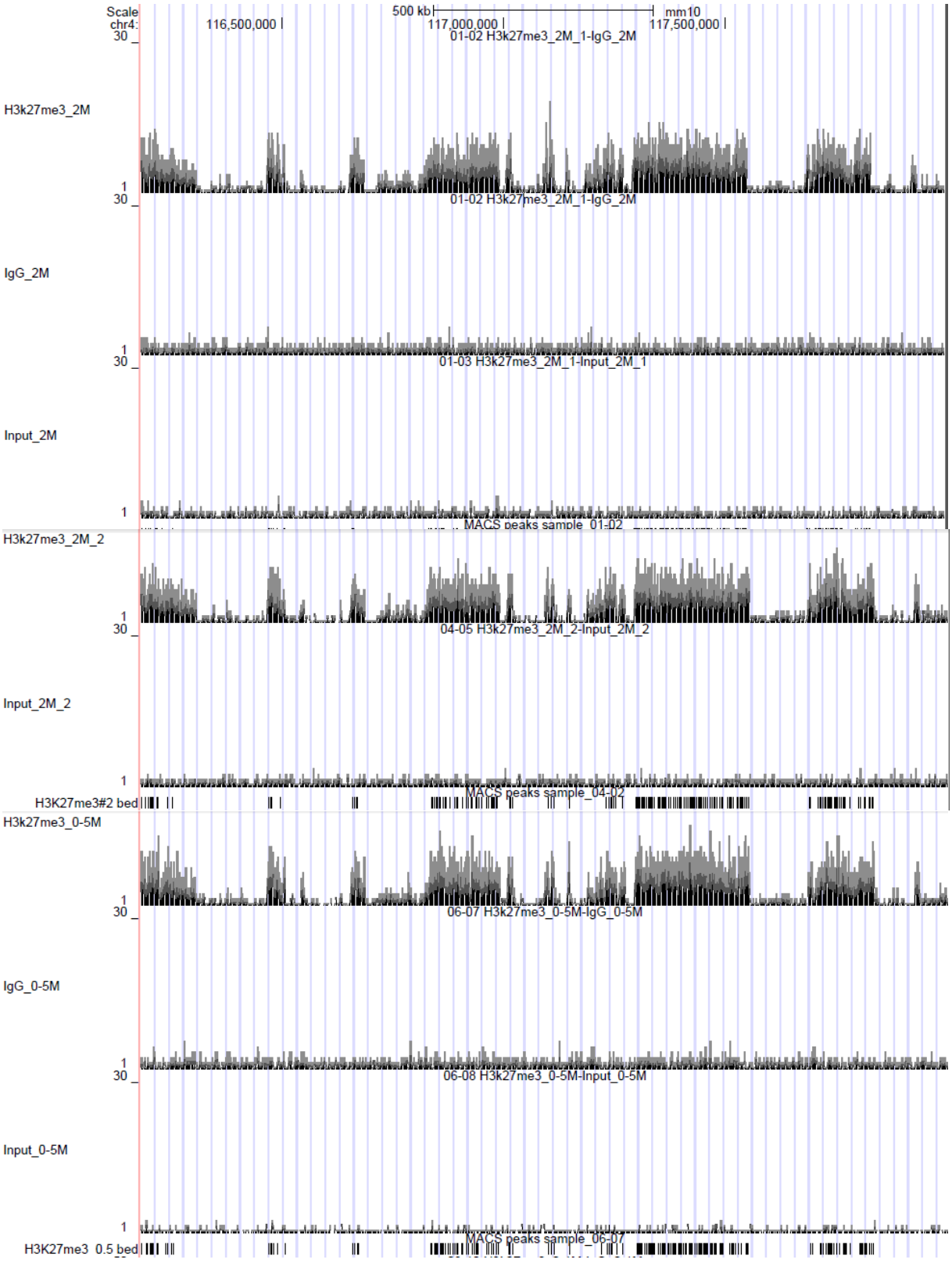
Add directly after the last wash step reverse crosslinking buffer:
2ml: 1820ul TE buffer+ 100ul SDS (20%)+ 80ul NaCL (5M) → 120ul / beadsample

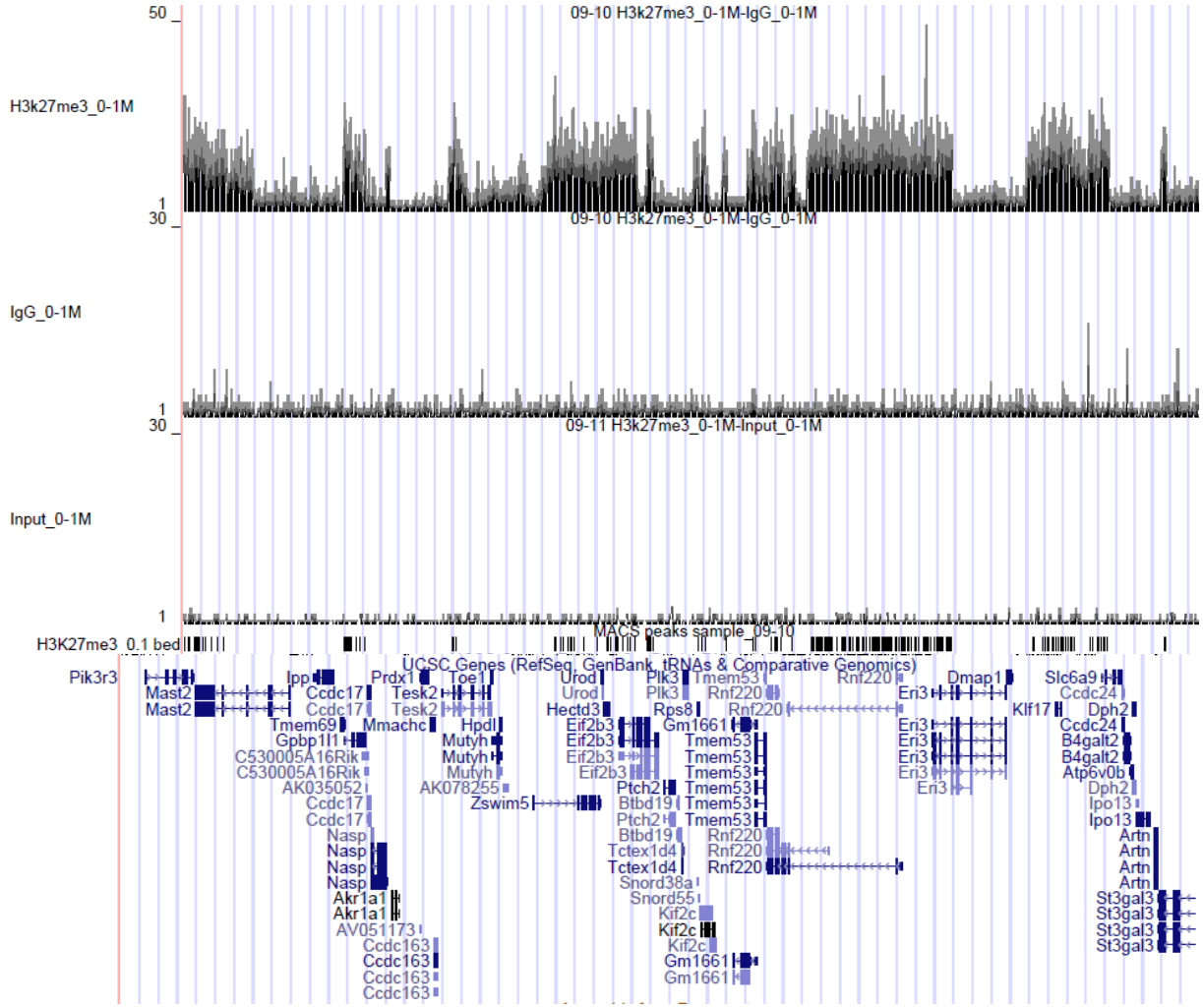
Add IP buffer to the input sample, (all input samples must have the same amount of volume)

Reverse crosslinken 4hr 65C 1000 rpm (final concentration: 1%SDS, 200nM NaCL)

→Isolate DNA: 120ul sample+600ul PB buffer
→elute: in 2x: 30ul+27ul=57ul in total
Store -20 for next day: qPCR

ChIP-seq data





ChIP-Seq data with 2 million, 500.000 and 100.000 cells shows the same pattern of H3K27me3 signal also with a low background (IgG), which suggest that the ChIP protocol we have optimised worked for 100.000 cells. The X axis represents a stretch of Chr IV and coding genes are shown below.