The Molecular mechanism of the aberrant expression of the B-cell specific *PAX5* gene in t(8;21) AML

Debleena Ray

A Thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Institute of Biomedical Research,
School of Cancer Sciences,
College Of Medical and Dental Sciences,
University of Birmingham
October 2012

ABSTRACT

B-cell specific gene *PAX5* (Busslinger, 2004) is aberrantly expressed in t(8;21) AML (Tiacci et al., 2004) and is potentially involved in blocking myeloid differentiation. To understand the mechanism of *PAX5* deregulation in t(8;21) AML we examined the expression, chromatin structure, histone modifications and RNA Polymerase II recruitment at *PAX5* in t(8;21) AML, in non-t(8;21) myeloid precursors and in a pre-B-cell-line. Our studies show that in non t(8;21) myeloid precursors, the *PAX5* gene is poised for transcription but is repressed by polycomb complexes. This polycomb repression is alleviated in t(8;21) cells leading to *PAX5* expression. t(8;21) AML model Kasumi-1 carries an activating C-KIT mutation that leads to constitutive activation of different downstream signalling pathways (Larizza et al., 2005). Some of these signalling pathways have been shown to regulate association of polycomb complexes with chromatin (Voncken et al., 2005). Our study shows that small molecule mediated inhibition of constitutively activated JNK, MEK and P38 signalling in Kasumi-1 cells lead to a down regulation of *PAX5* expression, decrease in elongating RNA Polymerase II and H327ac with concomitant increase in H3K27me3 at *PAX5.* This suggests that deregulated MAPkinase signalling in t(8;21) AML leads to the dissociation of polycomb complexes from *PAX5* causing its activation. It also suggests a novel role of tyrosine kinase mutations in lineage specification and differentiation block in t(8;21) AML.

This work is dedicated to my parents Dr Manas Ray and Mrs Sarmistha Ray

ACKNOWLEDGEMENTS

I would like to thank Prof Constanze Bonifer for giving me the opportunity to work in her laboratory and also for excellent supervision. I have indeed learnt a lot in the last four years that I have spent in her laboratory working towards my thesis. I would like to thank Dr Hiromi Tagoh for teaching me DNaseI hypersensitive site mapping in the first six months of my study and Dr Anetta Ptasinska for her help with ChIP and various other laboratory techniques. Dr Anetta Ptasinska also provided genome-wide DNaseI-sequencing and ChIP-sequencing data prior to publication that has been used for analysis in this work. A very special thank you goes to all members of the Bonifer and Cockerill laboratory, past and present, who have inspired me with stimulating scientific discussions and have also made my stay away from home enjoyable and less lonely. I would like to thank Dr Olaf Heidenreich from the University of New Castle, UK for providing RNA from RUNX1/ETO depleted t(8;21) AML patient cells and Prof Meinrad Busslinger, Vienna Biocentre, Austria for sharing *Pax5* DHS mapping data prior to publication. I would also like to thank Leukemia Lymphoma Research (LLR) and University of Birmingham for funding my study.

Finally, I would like to thank my brother Mr Dipanjan Ray, who initially inspired me to come abroad for my studies, my boyfriend Mr Nirjhar Banerjee, who has been the source of my strength especially during the time when I was writing this thesis. Last but not the least; I would like to thank my parents Dr and Mrs Ray without whose constant support this work would not have been possible.

Table of Contents

Table of Figures

Table of Tables

List of Abbreviations

- °C - Degree Celsius
- µl - Micro litre
- AGM - Aorta Gonad Mesonephron
- AML - Acute Myeloid Leukaemia
- ATP - Adenosine Triphosphate
- B&W Buffer - Bind and Wash buffer
- BAC - Bacterial Artificial Chromosome
- B-ALL - B-cell acute Lymphoblastic Leukaemia
- BCR - B-cell Receptor
- BMI1 - B-lymphoma insertion region 1
- BMP – Bone Morphogenetic protein
- BSA - Bovine Serum Albumin
- BTM – Basal Transcription Machinery
- $CaCl_2$ – Calcium Chloride
- CBF - Core Binding Factor
- CBP - CAAT-Binding Protein
- CD - Cluster of Differentiation
- CDK - Cyclin Dependent Kinase
- cDNA - complementary DNA
- CEBP - CAAT enhancer Binding Protein
- ChIP - Chromatin Immuno-precipitation
- CLP – Common Lymphoid Progenitor

- CMP - Common Myeloid Progenitor
- CO_2 - Carbon Dioxide
- CTD - C-terminal Domain
- CTP - Cytosine triphosphate
- DamID - DNA adenine methyltransferase identification
- DEPC - Diethylpyrocarbonate
- DHS – DNaseI hypersensitive site
- DMS – Dimethyl sulphide
- DMSO – Dimethyl Sulfoxide
- DNA – Deoxyribonucleic acid
- DTT - Dithiothreitol
- EDTA - Ethylene Diamine Tetra-acetic Acid
- EGTA – Ethylene Glycol Tetra- acetic Acid
- EICE - ETS IRF Composite Element
- ESC - Extra Sex Chromosome
- ETO - Eight Twenty-one Oncogene
- EZH2 - Enhancer of Zeste
- FACS - Fluorescence activated cell sorting
- FACT - Facilitator of active transcription
- FCS - Foetal Calf Serum
- FLT3 - FMS Like Tyrosine Kinase
- GCSF - Granulocyte Colony Stimulating Factor
- GCSFR - Granulocyte Colony Stimulating Factor Receptor
- GFP - Green Fluorescent Protein

- GM-CSF- Granulocyte-Macrophage Colony Stimulating Factor
- GM-CSFR - Granulocyte-Macrophage Colony Stimulating Factor Receptor
- GMP –Granulocyte Macrophage Progenitor
- GTF- General Transcription Factor
- GTP – Guanosine Triphosphate
- H3K27ac – Histone 3 lysine 27 acetylation
- H3K27me3 – Histone 3lysine 27 tri-methylation
- H3K36me3 – Histone 3 lysine 36 tri-methylation
- H3K4me3 – Histone 3 lysine 4 tri- methylation
- H3K79me3 – Histone 3 lysine 79 tri-methylation
- H3K9ac - Histone 3 lysine 9 acetylation
- H3K9me3 – Histone 3 lysine 9 tri-methylation
- H3P30 – Histone 3 proline 30
- H3S10ph – Histone 3 serine 10 phosphorylation
- H3S28ph – Histone 2 serine 28 phosphorylation
- H4K16ac – Histone 4 lysine 16 acetylation
- HAT – Histone acetyl transferase
- HDAC – Histone deacetylase
- HP1 – Heterochromatin protein 1
- HS –Hypersensitive Site
- HSC –Haematopoietic Stem Cell
- IL - Interleukin
- JNK –Jun Kinase

- KCl – Potassium Chloride
- KD –Knock-Down
- KDM –Lysine Methyl-transferase
- LCR - Locus Control Region
- LiCl - Lithium Chloride
- LM-PCR - Ligation Mediated Polymerase Chain Reaction
- LMPP - Lymphoid Primed Multipotent progenitor
- MCSF- Macrophage Colony Stimulating Factor
- MCSFR - Macrophage Colony Stimulating Factor Receptor
- MegE - Megakaryocyte Erythrocyte
- MEP - Megakaryocyte Erythrocyte Progenitor
- $MgCl_2$ - Magnesium Chloride
- MHC - Major Histocompatibility complex
- ml - Milli Litre
- mM - Milli Molar
- MMTV - Mouse Mammary Tumour Virus
- MSK - Mitogen and Stress activate Protein Kinase
- NaCl - Sodium Chloride
- NE - New Element
- ng - Nano gram
- NHS - National Health Service
- NK - Natural Killer
- P/S - Penicillin Streptomycin
- PBS - Phosphate Buffered Saline

- PcG - Polycomb Group
- PCR - Polymerase Chain Reaction
- PI - Propidium Iodide
- PMA - Phorbol Myristate Acetate
- pmol - Pico Mole
- PRC - Polycomb Repressive Complex
- PRE - Polycomb Response Element
- PRMT - Protein Arginine Methyltransferase
- PTEFb - Positive transcriptional elongation factor b
- qRT-PCR - Quantitative Real-time Polymerase Chain Reaction
- RNA - Ribonucleic acid
- SDS - Sodium Dodecyl Sulphate
- siRNA - Small inhibitory Ribonucleic Acid
- STAT – Signal Transducer and Activator of Transcription
- SUZ12 –Suppressor of Zeste
- SWI/SNF- Switch/Sucrose Nonfermentable
- TAE – Tis-Acetate-EDTA
- TBP –TATA Binding Protein
- T_{C-} Cytotoxic T- cell
- TCR – T-Cell Receptor
- TE –Tris EDTA
- TF – Transcription Factor
- T_{H-} Helper T-cell
- T_M - Melting Temperature

- TSA – Trichostatin A
- TAF – TBP Associated Factor
- WHO – World Health Organisation

1 INTRODUCTION

1.1 Haematopoiesis

Blood is the most regenerative tissue in a living organism. Everyday about one trillion blood cells are generated in adult human bone marrow (Finch et al., 1977). The process by which this occurs is called haematopoiesis. Every mature blood cell originates from a single cell-type, called haematopoietic stem cell (hereafter referred to as HSC). Like other stem cells, HSCs are capable of 'self-renewal' (i.e. undergoing cell division to form more HSCs) and 'differentiation' (i.e. dividing to develop and mature into the various types of blood cells). HSCs express genes specific to all blood lineages at a very low level. This phenomenon is known as 'lineage priming' in HSCs (Enver and Greaves, 1998). Therefore, these cells possess the ability to differentiate and develop into all blood lineages through progressive restriction of their gene expression. HSCs sit at the apex of a hierarchy of multi-potent progenitors that progressively get restricted to a specific blood lineage. This process of 'self-renewal' and 'differentiation' of HSCs into mature blood cells is tightly controlled by transcription factor networks and signalling pathways that ensure the formation of the correct blood cells at the correct time and in correct numbers (Doulatov et al., 2012, Laiosa et al., 2006a, Orkin and Zon, 2008). Perturbation of any of these features of haematopoiesis leads to various blood cell disorders including malignancy. Haematopoiesis is therefore not only an interesting paradigm to study cell-fate decision but the understanding of haematopoiesis is crucial to the field of haematopoietic malignancies.

1.1.1 Haematopoietic stem cells: definition, origin and maintenance

Haematopoietic stem cells (like other stem cells) are defined by: 1) multipotency, which is their ability to undergo asymmetric cell division in order to give rise to different cell types and also cells that maintain its stem cell properties, 2) their ability to remain quiescent and have a long life-span but also self-renew, 3) their dependency on the niche micro-environment for quiescence and last, but not the least 4) by their ability to repopulate the entire haematopoietic system (for at least 16 weeks) of an immuno-compromised recipient in an *in-vivo* transplantation assay (Orkin and Zon, 2008, Martinez-Agosto et al., 2007). Originally, HSCs were identified by staining mouse bone marrow cells with antibodies against a combination of surface antigens (Morrison et al., 1995, Spangrude et al., 1988, Morrison and Weissman, 1994). Using this method HSCs were defined as lineage$^-$ Sca-1$^+$ckit$^+$ cells (LSK cells). The LSK cell population is further subdivided on the basis of their cell surface markers and reconstitution potential, into long-term-HSC (LT-HSC: Thy-1^{low}Flk2/Flt3$^-$), short-term-HSC (ST-HSC: Thy-1^{low}Flt3$^+$) and multi-potent progenitors (MPP: Thy-1$^-$Flt3$^+$) (Christensen and Weissman, 2001, Osawa et al., 1996, Yang et al., 2005). It is important to note that the surface markers that define a mouse HSC and a human HSC are different. Human HSCs are CD34$^+$ and FLT3$^+$ while murine HSCs are not (Civin et al., 1984, Kang et al., 2008). Murine HSCs are defined as Lin$^-$Sca-1$^+$cKit$^+$CD34$^-$CD150$^+$CD49b^{low} while human HSCs are defined as LIN$^-$CD34$^+$CD38$^-$CD45RA$^-$THY-1$^+$CD49f$^+$ cells (Bhatia et al., 1997, Conneally et al., 1997, Lansdorp et al., 1990, Doulatov et al., 2012, Notta et al., 2011, McKenzie et al., 2007).

Origin of HSCs

In mammals, during development, haematopoiesis occurs in multiple waves and in various sites (see Figure 1.1). The initial wave of haematopoiesis occurs in the extra-embryonic yolk sac and is called ‘primitive haematopoiesis (Sabin, 1920). The main purpose of ‘primitive haematopoiesis’ is the production of erythroid cells needed for the rapid development of the embryo (Orkin and Zon, 2008). A common endothelial and haematopoietic progenitor; called ‘haemangioblast’ migrates from the primitive streak of the embryo to the yolk sac contributing to the formation of blood islands (Murray, 1932, Choi et al., 1998, Fehling et al., 2003).

‘Primitive haematopoiesis’ soon gets replaced by ‘definitive haematopoiesis’ in the Aorta-Gonad-Mesonephron (AGM) followed by foetal liver, thymus, spleen and then the adult bone marrow (Medvinsky and Dzierzak, 1996, Muller et al., 1994, de Bruijn et al., 2000, Boisset et al., 2010). The origin of adult HSCs is the ‘haemogenic endothelium’ which is a common precursor for blood and endothelial tissue (Jaffredo et al., 2005b, Jaffredo et al., 2005a, Jaffredo et al., 2000, Dzierzak and Speck, 2008). A recent study involving continuous single-cell observation of mouse mesodermal cells generating blood and endothelial cells has led to the detection of haemogenic endothelium (Eilken et al., 2009) thereby resting controversies regarding the existence of the haemogenic endothelium. The emergence of HSCs involves various transcription factors and signalling pathways including *RUNX1, GFI1,GFI1B VEGF, TGFβ and NOTCH1* signalling (Dzierzak and Speck, 2008, Orkin and Zon, 2008, Chen et al., 2009, Lancrin et al., 2012).

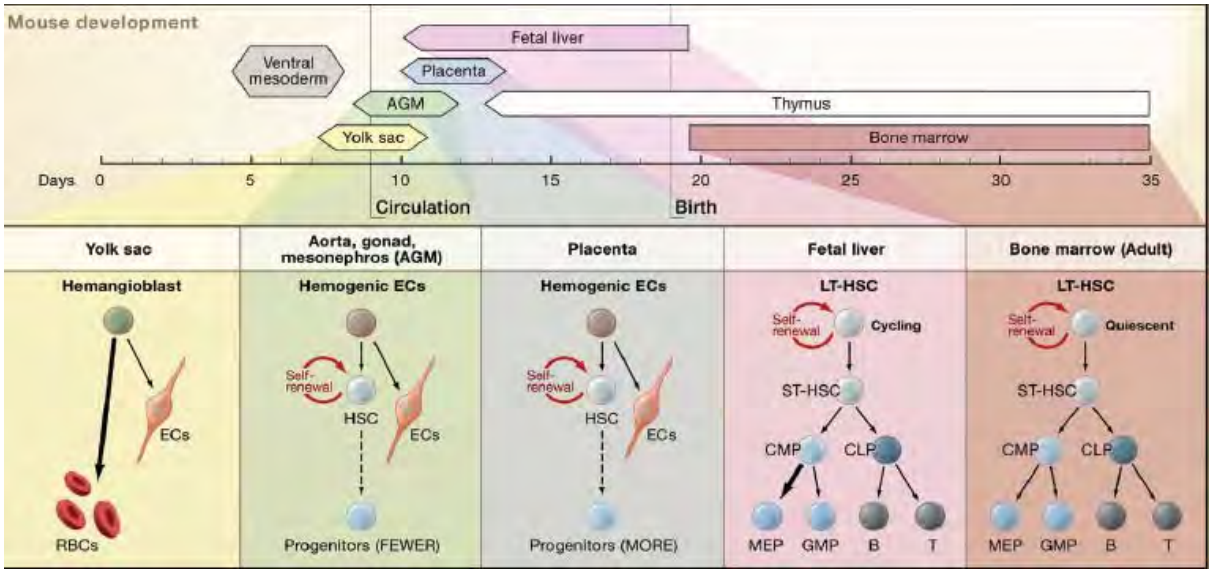


Figure 1.1: Sites of haematopoiesis during embryonic development. 'Primitive Haematopoiesis' starts in the yolk sac. This is replaced by 'Definitive Haematopoiesis' in the AGM and the foetal liver. In adults the primary site of haematopoiesis is the bone marrow microenvironment. This figure is adapted from (Orkin and Zon, 2008).

Maintenance of HSCs

Once the haematopoietic system is established the maintenance of HSC identity becomes crucial for sustained haematopoiesis. HSCs undergo asymmetric cell division to produce cells that retain their stem cell properties, remain dormant or quiescent and maintain HSC homeostasis. The other cells become differentiated into committed blood progenitors.

Like other stem cells, HSCs depend on the niche microenvironment to regulate self-renewal and differentiation in order to maintain homeostasis. The HSC niche changes as the sites of haematopoiesis change during development. Adult haematopoiesis takes place in the bone marrow (Sipkins et al., 2005,

Orkin and Zon, 2008). Therefore, the bone marrow niche microenvironment is of prime importance for HSC maintenance. Loss of BMP receptor-1 (bone morphogenetic protein receptor 1) in mouse osteoblasts results in an increased number of osteoblasts and also HSCs (Calvi et al., 2003, Zhang et al., 2003). Induction of death of osteoblasts by targeting these cells with suicide genes also perturbs haematopoiesis (Visnjic et al., 2004). Furthermore, stimulation of the parathyroid receptor leads to increase in trabecular bone and the number of HSCs (Calvi et al., 2003). The above studies, therefore, make it evident that osteoblasts play a crucial role in the maintenance of HSCs. Intravital microscopy and GFP labelled HSC transplantation experiments showed that HSCs lodge next to osteoblasts (Sipkins et al., 2005). Osteoblasts secrete N-cadherin and Notch receptor ligand. Interactions mediated by N-cadherin and TIE2/Angiopoietin anchors HSCs to niche cells and maintain HSCs in quiescent state (Arai et al., 2004, Martinez-Agosto et al., 2007).

Apart from the bone marrow micro-environment; transcription factors such as GFI-1, polycomb complex protein BMI1 (see section 1.3.6) and MEF/ELF4 control HSC self-renewal and proliferation (Eliasson and Jonsson, 2010). GFI-1 maintains HSCs in quiescent state (Hock et al., 2004), while MEL/ELF4 and BMI1 promote proliferation of the HSCs and their entry into the cell-cycle (Lacorazza et al., 2006, Oguro et al., 2006). Furthermore two recent studies have shown that LDB1 complexes regulate a transcription program required for the maintenance of HSCs by restricting its differentiation into downstream progenitors and mature blood cells (Li et al., 2011). C-MYC, on the other hand, has been shown to promote the release of HSCs from their quiescent state.

Loss of C-MYC in HSCs in the bone marrow makes these cells incapable of differentiation as their interactions with the niche cells become stronger and they remain quiescent and attached to the niche (Wilson et al., 2004). The quiescent HSCs maintain HSC homeostasis while the proliferating HSCs are the ones that contribute to haematopoietic differentiation.

1.1.2 Cellular pathways of haematopoiesis

As indicated above one of the defining features of an HSC is that it can differentiate along multiple pathways via transient amplifying cells which increasingly get restricted towards a particular cell fate option/lineage through a cascade of binary decisions.

The analysis of cell surface antigens of haematopoietic cells led to the discovery of progenitor cells. These progenitor cells are below HSCs in the haematopoietic hierarchy and have restricted lineage potential. Lineage restricted progenitors undergo cellular divisions and further lineage restrictions in order to give rise to committed and mature blood cells. This process of gradual loss in differentiation potential of a multipotent HSC is known as lineage commitment.

Cell sorting experiments aimed at identifying precursor cell types with specific differentiation potential led to the identification of a common lymphoid progenitor (CLP) that gives rise to B-cells, T- cells and NK-cells (Kondo et al., 1997), and a common myeloid progenitor (CMP) (Akashi et al., 2000). The CMP gives rise to two further progenitors: the Granulocyte-macrophage progenitor (GMP) and the

Megakaryocyte-erythrocyte progenitor (MEP). GMPs in turn give rise to neutrophils, basophils, eosinophils, macrophages and mast cells. MEPs on the other hand, give rise to megakaryocytes and erythrocytes. Based on these experimental findings, Akashi-Kondo-Weissman proposed their scheme of haematopoietic differentiation. According to this scheme of haematopoiesis, the first lineage commitment step of an uncommitted progenitor is the strict separation of lymphoid and myeloid lineages (Laiosa et al., 2006a). However this CLP-CMP model does not exclude other routes of lineage commitment. Recent experimental data describe a fraction of the primitive LSK cells with combined lymphoid and myeloid potential with little or no megakaryocyte-erythrocyte (MegE) potential. These progenitors are marked by high expression of FLT3 on the cell surface and expression of several lymphoid genes. These progenitor cells are termed lymphoid-primed multi-potent progenitors (LMPPs) (Adolfsson et al., 2005, Yang et al., 2005). The existence of LMPPs is further corroborated by using additional marker VCAM-1 (Vascular adhesion molecule-1) in combination with FLT3 (Lai and Kondo, 2006, Yoshida et al., 2006). This study subdivided the MPPS into three distinct groups: $FLT3^{lo}VCAM\text{-}1^{+}$, $FLT3^{hi}VCAM\text{-}1^{+}$ and $FLT3^{hi}VCAM^{-}$. The $FLT3^{hi}VCAM^{+}$ subgroup showed granulocyte-monocyte-lymphoid potential with little or no megakaryocyte-erythrocyte potential corresponding to the LMPPs described by Adolfsson *et al* (Adolfsson et al., 2005). This led to the proposal of an alternative haematopoietic differentiation scheme by Jacobsen and colleagues. According to this model the first step in the differentiation of HSCs/MPPs is the formation of a subset of progenitors that retain the capacity to give rise to all

haematopoietic cell types except for megakaryocytes and erythrocytes (see figure 1.2).

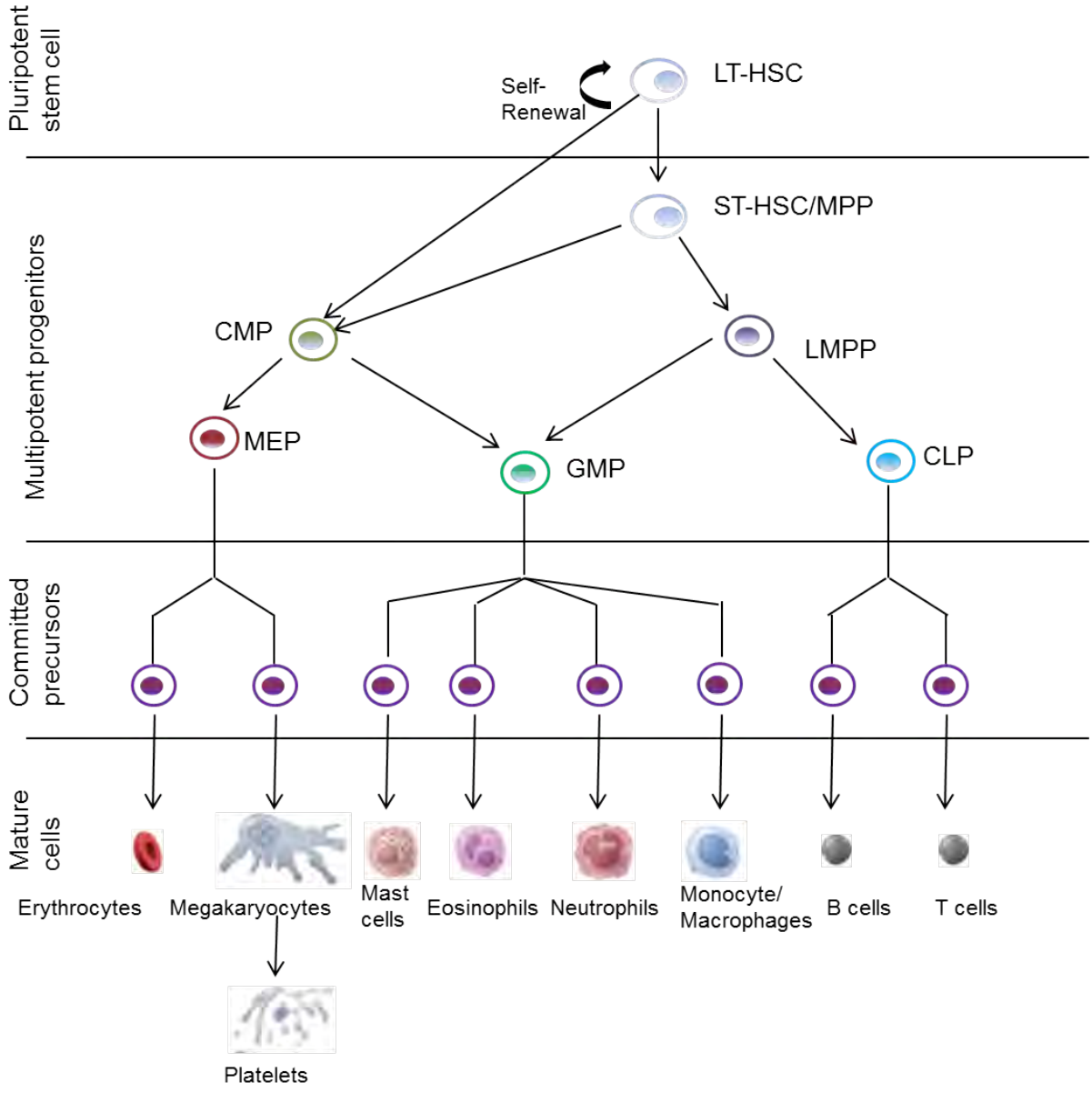


Figure 1.2: Schematic diagram of the cellular pathways of haematopoiesis. Haematopoiesis starts with a multipotent stem cell that gradually differentiates into mature blood cells via progenitor cells.

Though the studies referred to above elucidate a clear road-map of differentiation of an HSC into mature blood cells, it does not shed any light on the question as to how an individual HSC decides to enter one lineage over another. Recent studies have shown that HSCs are not a homogenous population. HSCs differ amongst themselves in the ability to self-renew and differentiate. Depending on the contribution of an individual HSC to the myeloid or lymphoid lineage, HSCs has been subdivided into lymphoid-deficient (α)/ myeloid biased (Muller-Sieburg et al., 2004), balanced (β) and myeloid-deficient (γ and δ) HSCs (Dykstra et al., 2007) (reviewed in (Muller-Sieburg et al., 2012) . This difference in differentiation potential of individual HSCs is dependent on a non-random gene expression pattern. It has been shown that TGFβ influences the maintenance one subtype of HSC versus another (see section 1.1.4) (Challen et al., 2010). The bone marrow microenvironment also has been shown to be more conducive to the α-HSC compared to the β- HSC (Benz et al., 2012). Another gene *Btaf* (known to be regulating DNA damage response) also differentially influences the maintenance of α versus β-HSCs (Wang et al., 2012). These studies are therefore slowly elucidating a molecular mechanism of how each individual HSC decides to differentiate along the haematopoietic tree. This molecular mechanism (for example the epigenetic signature) defining a particular HSC sub-type is maintained in the daughter HSCs that are produced as a result of a self-renewal divisions (Copley et al., 2012).

1.1.3 Determinants of cell fate decision in Haematopoiesis

Cell fate decisions are a complex process that is still very much an evolving field of research. It involves various molecular regulators. These include extracellular factors such as: cell-cell interactions, cytokine and other signalling pathways and also cell intrinsic factors such as transcription factors. The extracellular developmental cues finally culminate to cause a change intrinsic to the cell i.e. change in gene expression because distinct cell fate options are associated with distinct gene expression patterns.

1.1.4 Role of signalling in haematopoiesis

Extracellular factors communicate with the cell via signalling molecules. A number of signalling pathways influence haematopoiesis at various levels; from the formation and maintenance of HSCs to haematopoietic differentiation. Several evolutionarily conserved signalling pathways: Hedgehog, WNT, TGFβ/SMAD and NOTCH signalling, are involved in formation, self-renewal and maintenance of HSCs (Luis et al., 2012b). Furthermore, signalling mediated by cytokine receptors also play important role in haematopoietic lineage decisions (reviewed in (Baker et al., 2007).

There is an interesting conundrum regarding the mechanism of asymmetric division of HSCs. By asymmetric cell division HSCs divide to form two daughter cells one of which maintains the stem-cell properties and the other undergoes differentiation. One school of thought envisages that this occurs through unequal division of cell-intrinsic factors that determine cell fate. Alternatively, a second school of thought proposes that this could be due to differential

exposure to extracellular signals of the two daughter cells that are otherwise identical (Knoblich, 2008). Therefore signalling is potentially a very important component in haematopoietic stem cell maintenance as well as haematopoietic differentiation. Discussed below are the different signalling pathways that play an important role in haematopoiesis

TGFβ, NOTCH-1 and Wnt signalling pathways are involved in establishment, maintenance and differentiation of HSCs

Several signalling pathways have been shown to be of importance for the emergence, maintenance and proper functioning of HSCs. TGFβ signalling is one such conserved pathway (reviewed in (Blank and Karlsson, 2011). Neutralisation of TGFβ signalling in cultured HSCs releases the HSCs from quiescence (Soma et al., 1996, Fortunel et al., 1998, Hatzfeld et al., 1991).Therefore, the role of TGFβ in vitro is to maintain stem-cell quiescence. In-vivo, however, TGFβ knockout mice show phenotypes that are difficult to interpret. TGFβ-1 ligand knockout causes impaired reconstitution ability due to defective homing of HSCs (Capron et al., 2010) whereas the TGFβ-1 receptor knockout showed no defects in self-renewal or reconstitution ability even though the mice were under severe haematopoietic stress due to multifocal inflammation which is observed in both TGFβ ligand and receptor knockout mice (Larsson et al., 2003, Larsson et al., 2005). Therefore, TGFβ signalling is involved in immune cell homeostasis in vivo. It has also been shown that knockout of TGFβ causes increased myelopoiesis in mice suggesting that TGFβ negatively regulates myelopoiesis in vivo (Letterio et al., 1996). He *et al*. have shown that TGFβ signalling balances erythroid differentiation with

inhibition of stem cell growth (He et al., 2006). According to their model, TIF1γ (transcriptional intermediary factor-1 γ) and SMAD4 compete to bind SMAD2/SMAD3 complex. The TIF1γ/SMAD2/SMAD3 complex favours erythroid development while the SMAD4/SMAD2/SMAD3 complex inhibits HSC growth (He et al., 2006). TIF1γ has recently also been shown to play a role in the early erythroid myeloid bifurcation by regulating GATA1 and PU.1 expression (Monteiro et al., 2011).

NOTCH-1 signalling is another very important pathway for haematopoiesis. NOTCH1 is a trans-membrane receptor that undergoes cleavage after binding its ligand and translocates to the nucleus. Within the nucleus, along with its transcriptional co-factor CSL, it activates transcription of its target genes (Deftos and Bevan, 2000). Several studies have unequivocally linked NOTCH-1 signalling to definitive haematopoiesis in the AGM during embryonic development (reviewed in (Pajcini et al., 2011). Kumano *et al* analysed embryos of *Notch-1* and *Notch-2* deficient mice and found that the development of HSCs was severely impaired in the *Notch-1* deficient mice but not in the *Notch-2* deficient mice (Kumano et al., 2003). They also explanted AGM regions from wild-type mouse embryos at embryonic day 9 and 10 respectively in media with γ-secretase inhibitor and observed a differential effect on the ability of haematopoietic progenitors to proliferate in colony assays. γ-secretase is a protease complex that is an integral membrane protein. Addition of γ-secretase inhibitor inhibits the cleavage of NOTCH and prevents the release of the intracellular domain of NOTCH thereby mimicking the conditional-knockout phenotype. They found that when γ-secretase inhibitor was added on

embryonic day 9 it impaired the generation of HSCs, while there was no effect when added on embryonic day 10 (Kumano et al., 2003). This showed that NOTCH-1 signalling is important for the generation of HSCs but not its maintenance. Other studies conducted have also established that NOTCH-1 is necessary for the generation of HSCs in AGM (Yoon et al., 2008, Hadland et al., 2004, Robert-Moreno et al., 2005, Robert-Moreno et al., 2007). The role of NOTCH-1 in adult haematopoiesis, however, is still controversial. Gain of function studies showed the involvement of NOTCH-1 in the control of haematopoiesis. Addition of soluble NOTCH-1 ligand to human $CD34^{+}CD38^{-}$ LIN^{-} cord blood cells or primitive murine $LIN^{-}SCA\text{-}1^{+}CKIT^{+}$ cells in culture leads to a rapid expansion of progenitors in vitro that shows a short-term haematopoietic reconstitution potential (Karanu et al., 2000, Varnum-Finney et al., 2003). Loss-of-function studies, however, fail to establish a link between haematopoiesis and NOTCH signalling (Pajcini et al., 2011). Functional inactivation of *Notch-1*, *Notch-1* and *Notch-2* in mice does not affect HSCs (Han et al., 2002, Radtke et al., 1999, Tanigaki et al., 2002, Maillard et al., 2008). Inactivation of *Notch-1, Jagged-1*in bone marrow progenitors or stromal cells also does not affect HSCs (Mancini et al., 2005). Even though the precise role of NOTCH signalling in adult haematopoiesis is still not entirely clear, the ability to expand cord-blood cells in vitro is clinically highly relevant if put to proper usage (Pajcini et al., 2011).

Wnt signalling has also been shown to have a precisely regulated role in HSC function (Luis et al., 2012a). Retroviral over-expression of a constitutively active β-catenin in BCL-2 transgenic haematopoietic stem/progenitor cells lead to the

expansion of HSCs and an increase in repopulating capacity upon transplantation in mice (Reya et al., 2003). However, conditional over-expression of β-catenin leads to block in differentiation and a transient expansion of the HSC pool followed by exhaustion of haematopoietic cells (Kirstetter et al., 2006, Scheller et al., 2006). These seemingly counter-intuitive results were down to the precise dependence of HSC function on precise Wnt signalling dosages (Luis et al., 2012a). Wnt activity in the haematopoietic organs is rather modest (Grigoryan et al., 2008). Recent data has shown that when Wnt activity is only slightly enhanced over normal levels, HSCs engraft better. However when exposed to high levels of Wnt signalling, HSCs completely fail (Luis et al., 2011).

Loss of function experiments involving conditional deletion of β-catenin and its homolog γ-catenin failed to affect HSC function (Cobas et al., 2004, Jeannet et al., 2008, Koch et al., 2008). It was later shown that this could be explained by the fact that, the conditional deletion of these two mediators of Wnt signalling still left behind a small amount of residual Wnt activity (Luis et al., 2012a, Prlic and Bevan, 2011) and it was proposed that this residual Wnt activity was sufficient to maintain HSC function. Experiments where Wnt activity was almost reduced to zero have shown that abrogation of Wnt signalling is detrimental to HSCs (Luis et al., 2010, Fleming et al., 2008). However a quarter of the normal Wnt activity is sufficient to maintain normal HSC function. Slightly enhanced Wnt activity is beneficial for HSC functioning but high levels of Wnt activity are not well tolerated (Luis et al., 2012a). Wnt signalling has also been shown to be of importance in the regulation of the component cells of the HSC niche

(Malhotra and Kincade, 2009). Furthermore it has been shown that it plays an important role in T-cell differentiation (Xu et al., 2003). Within the haematopoietic compartment thymocytes and mature T-cells have highest Wnt activity (Weerkamp et al., 2006).

Signalling pathways therefore play a crucial role in establishment and maintenance of different haematopoietic cells. These signalling pathways interact and act synergistically or antagonistically to form a complex network that not only regulates itself but also other genes in order to regulate various haematopoietic processes.

1.1.5 Role of cytokine and cytokine receptor mediated signalling in haematopoietic differentiation

The above described signalling pathways mostly influence emergence, maintenance and functioning of HSCs. Haematopoietic differentiation, on the other hand, is influenced by cytokine signalling. Cytokines are extracellular ligands that elicit a biological response in a cell-context dependent manner via binding and activating a specific class of conserved receptors.

There are over fifty different cytokines that in some way regulate haematopoiesis. The first cytokine was identified as a biological factor that stimulates the survival, proliferation and differentiation of haematopoietic progenitors into morphologically distinct colonies and hence were called colony stimulating factors (CSF) (Metcalf and Nicola, 1984). There is wealth of data that has shown the effect of cytokines on maturing haematopoietic cells.

According to this data, cytokines were arranged in a hierarchy where cytokines acting on multi-potential cells were placed separately from the more lineage specific cytokines (see figure 1.3).

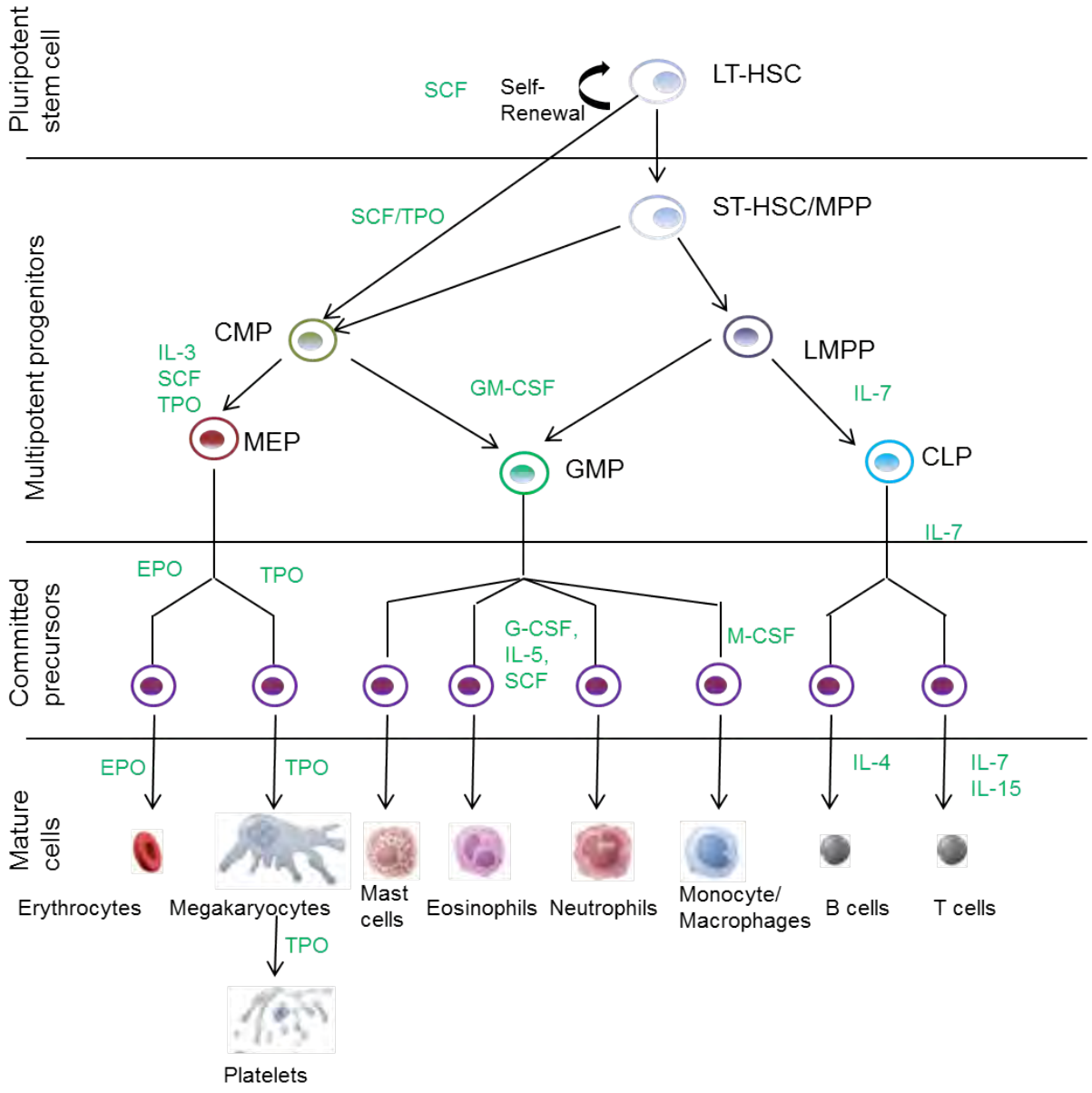


Figure 1.1: Haematopoiesis and the role of cytokines in lineage determination. The cytokines needed for cell-fate decision at the various stages of haematopoietic differentiation are indicated with green redrawn from (Robb, 2007)..

Is the role of cytokines instructive or permissive in haematopoiesis?

As described in figure 1.3, cytokines were originally thought to regulate haematopoiesis in a hierarchical fashion. There are two models explaining the role of cytokines in regulating haematopoiesis: a) an instructive role and b) a permissive role. According to the permissive model haematopoietic differentiation is primarily driven by transcription factors and cytokines which provide survival and proliferation signal for these already committed cells (Till et al., 1964). The instructive model, in contrast, envisages that cytokines play a crucial role in the cell fate decision of haematopoietic stem/progenitor cells along with important lineage determining transcription factors. There are several experiments in favour of both notions.

The phenotype of mouse models deficient of the various cytokine receptors suggests a permissive role of cytokines, as mice deficient in GM-CSF, EPO or G-CSF only show a reduction in the number of respective progenitors or mature progeny but not their complete ablation (Lieschke et al., 1994a, Lieschke et al., 1994b, Stanley et al., 1994, Wu et al., 1995). Retroviral expression of M-CSFR on erythroid cells led to the development of erythroid cells in response to M-CSF (McArthur et al., 1994, Pharr et al., 1994) which suggested that cytokine signalling did not reverse lineage choices; it only facilitated survival of cells that had determined its lineage fate through other critical determinants. EPO-R could also be replaced by prolactin receptor, growth hormone receptor and c-MPL (receptor for thrombopoietin) with the same effect (Goldsmith et al., 1998, Socolovsky et al., 1997). Further in support of the permissive role, ectopic expression of the survival factor BCL-2 in haematopoietic progenitor cell lines

led to the differentiation of these cell lines independent of cytokines (Fairbairn et al., 1993). Ectopic expression of BCL-2 *in vivo* also rescued T-cell defects and monocyte defects in IL-7 and M-CSF deficient mice respectively (Fairbairn et al., 1993). There are several *in vivo* experiments using transgenic approaches that also `suggest the permissive role of cytokines. Stoffel *et al* targeted the cytoplasmic domain of the G-CSF receptor gene (*CSF3R*) to the c-MPL locus to create a mouse with a G-CSFR-c-MPL chimeric receptor (Stoffel et al., 1999). These chimeric receptors were able to replace c-MPL functionally even though the cytoplasmic domain was that of G-CSFR. The mice did not develop thrombocytopenia or show an increase in granulocytic cells; again supporting the permissive role of cytokine signalling (Stoffel et al., 1999).

However, there are several experiments in favour of the 'instructive' role of cytokines. Expression of IL2Rβ (β-chain of the IL-2 receptor) in CLPs led to the formation of GM colonies while wild type CLPs only formed B cell colonies (Kondo et al., 2000). IL2Rβ in this case did not merely provide survival signal to the CLPs; as the ectopic expression of *Bcl-2* gene did not cause this CLPs to GM conversion observed by ectopic expression of IL2Rβ (Kondo et al., 2000). This result was interpreted such that this lymphoid to GM conversion was due to up-regulation of GM-CSFR in the IL2Rβ expressing CLPs. Therefore, the human *GM-CSFR* gene (α and β chain) was ectopically expressed in CLPs (Iwasaki-Arai et al., 2003) followed by stimulation of these cells with human recombinant GM-CSF. This also induced a similar CLP to GM conversion. However, IL2R signalling did not show an instructive role in pre-T cells or pro-B cells (Iwasaki-Arai et al., 2003). Similarly, transgenic hGM-CSFR expression in

MEPs showed only a permissive role in the commitment of MegE (Iwasaki-Arai et al., 2003). Therefore, ectopic IL2R and GM-CSFR expression shows instructive role in myeloid commitment only in cell-context dependent manner.

FLT3 is expressed in ST-HCS and MPPs and FLT3 expressing MPPs rapidly give rise to B and T-lymphoid cells (Sitnicka et al., 2003) (see 1.1.1). Mice lacking FLT3 or FLT3 ligand show severely reduced number of CLPs. IL7Rα (α subunit of the IL-7 receptor) is expressed on CLPs, B-cells and T-cells (Sitnicka et al., 2003). Mice lacking IL-7 or IL-7α show severe defects in T and B-cell differentiation. Mice lacking both FLT3 and IL-7R show total absence of B-cells in foetal and adult haematopoiesis (Sitnicka et al., 2003) It is therefore, likely that IL-7 and FLT3 signalling 'instructs' the B-cell specific transcription factors, thereby establishing the B-cell gene expression signature (Laiosa et al., 2006a). Recently Schroeder and colleagues used bio-imaging techniques to track the differentiation of individual mouse HSCs and demonstrated that M-CSF and G-CSF can instruct lineage choice (Rieger et al., 2009). Taken together, the above studies suggest that cytokines can act permissively to provide a general survival signal to cells that has already determined their cell fate option under instruction from other critical lineage determining factors (transcription factors). However, under specific cellular contexts certain cytokines have been shown to instruct cell fate option in a way that is more critical than just providing survival signal.

1.1.6 Role of transcription factors in haematopoiesis

The identity of any cell, be it stem or committed, is defined by its 'gene expression signature' i.e. the set of genes that are expressed in that particular cell at the given time. This gene expression pattern is determined by the pattern of transcription factor expression (see sections 1.1.7 and 1.1.8). Transcription factors are DNA binding proteins that bind specific sequences at the cis-regulatory elements of genes (see section 1.2) and regulate their expression. Binding of transcription factors at these elements (i.e. enhancers or promoters) of genes can either activate or repress the expression of a gene.

Transcription factors SCL (stem cell leukaemia) /TAL1, RUNX1 and GFI1 are transcription factors essential for the establishment of haematopoiesis (reviewed in (Schutte et al., 2012). *Scl* knockout mice are embryonic lethal at embryonic day 9.5 (Shivdasani et al., 1995). *Runx1* knockout mice die between embryonic day 12.5-13.5 due to block in development of definitive haematopoiesis (Wang et al., 1996b). HSCs lacking *Gfi1* undergo rapid proliferation but are unable to sustain long-term haematopoiesis and are die by embryonic day 15 (Hock et al., 2004).

A particular cell fate option is defined by the subset of these genes that are expressed in that particular cell type at that given time. Therefore spatio-temporal regulation of gene activity is essential to cellular differentiation and cell fate identity. Transcription factors interact with each other and regulatory elements of other key target genes and form a complex interaction network that determines a cell's genetic signature and hence its identity (Schutte et al.,

2012). Figure 1.4 shows a selection of network motifs found in mammalian gene regulatory networks (see figure 1.4).

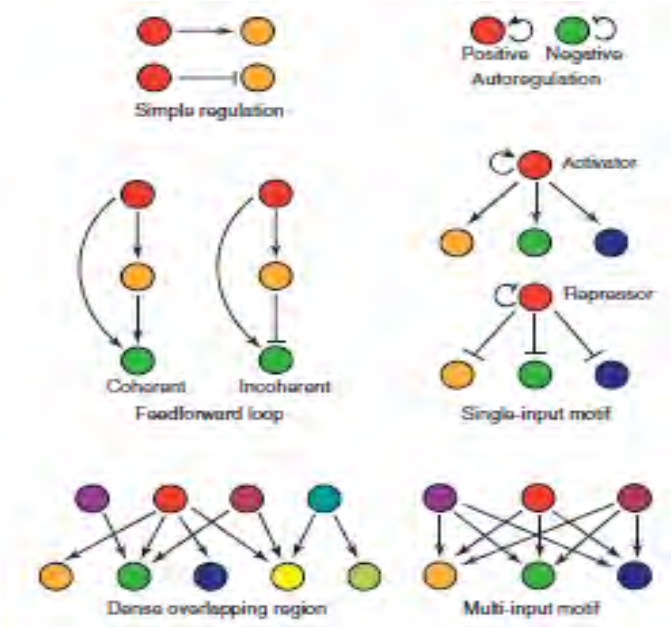


Figure 1.4: Schematic diagram showing a selection of motifs commonly found in mammalian gene regulatory network (Schutte et al., 2012). Genes form complex regulatory networks in organisms whereby one gene either activates or represses one or more genes. The above cartoon shows few examples of gene regulatory networks.

It has been proposed that key lineage determining decisions rely on a few 'master regulators' or 'lineage determining' transcription factors. These transcription factors activate target genes that are specific to a particular lineage and repress other genes that are required for alternative lineages. A few examples of such crucial transcription factors include CEBPα, PU.1, GATA1 and PAX5 which are discussed in sections 1.1.7, 1.1.8 and 1.4 in greater details with respect to lymphopoiesis/B-cell development and myelopoiesis. The study of single gene locus to elucidate regulatory elements complemented with

genome-wide ChIP-sequencing experiments are slowly but steadily revealing a comprehensive understanding of the various transcription factors and their target genes (building complex gene regulatory networks) for haematopoietic differentiation (Schutte et al., 2012).

1.1.7 Myelopoiesis

The generation of myeloid cells (i.e. erythroid, megakaryocytic, granulocytic and monocytic cells) in the bone marrow is defined as myelopoiesis. Myelopoiesis involves several binary cell-fate decisions mediated by key transcription factors that play a crucial role in the development of mature myeloid cells (see figure 1.5)

The first major 'branching' in the 'haematopoietic tree' occurs with the bifurcation of the megakaryocyte-erythrocyte progenitors (MEPs) from the lympho-myeloid lineage option (see figure 1.1 and section 1.1.2). The megakaryocyte-erythrocyte vs. the lympho-myeloid cell fate outcome of a multipotent progenitor is determined by the interplay between two critical transcription factors PU.1 and GATA-1 (Laiosa et al., 2006b). PU.1 (along with C/EBP proteins) favours the lympho-myeloid outcome while GATA-1 (and its interaction with Friend of GATA-1 i.e. FOG-1) favours the megakaryocyte-erythrocyte fate option (Scott et al., 1994, Orkin et al., 1998).

MEP^{E26} are avian progenitors that are transformed with an E26 virus expressing the *Myb-Ets* oncogene. When this oncogene is inactivated these cells differentiate to avian erythrocytes and thrombocytes but they can also be

stimulated to form myeloblasts through the activation of the RAS or PKC pathway (Graf et al., 1992). Ectopic expression of GATA-1 in myeloblasts induces the formation of MEP^{E26} cells (Kulessa et al., 1995). Ectopic expression of PU.1 in MEP^{E26} cells, on the other hand, causes reprogramming of these cells to form myeloblasts (Nerlov and Graf, 1998). Ectopic expression of GATA-1 in GMPs and C-$KIT^{+}CD34^{+}$ progenitor cells with restricted GM potential leads to the formation of MegE colonies under culture condition in significant numbers (Iwasaki et al., 2003, Heyworth et al., 2002). In zebrafish models, reducing the dosage of GATA-1 by morpholinos causes conversion of erythroid cells to myeloid cells; while lowering the dosage of PU.1 induces a switch between myeloid and erythroid lineages (Galloway et al., 2005, Rhodes et al., 2005). These experiments reveal that the dosage or stoichiometry of two key transcription factors can determine the cell fate option of an early haematopoietic progenitor.

GATA-1 and PU.1 determine MegE vs. lympho-myeloid cell fate option via antagonising each other's transactivation activity through protein-protein interaction (Rekhtman et al., 1999, Nerlov et al., 2000, Zhang et al., 2000). They bind to each other to form a PU.1: GATA-1 complex. This complex antagonizes PU.1 through hindering the interaction of PU.1 with c-JUN (Zhang et al., 1999). On the other hand this complex blocks the transactivation activity of GATA-1 by recruiting Rb protein to the GATA-1 target genes (MegE genes) followed by chromatin remodelling and repression of these genes (Stopka et al., 2005).

Once a multipotent progenitor (MPP) resolves the lympho-myeloid vs. megakaryocyte-erythrocyte cell fate option it either forms a 'Megakaryocyte-Erythrocyte Progenitor' (MEP) or a 'Lymphoid-primed multipotent progenitor' (LMPP). The MEPs further need to get resolved to an erythrocytic or a thrombocytic cell fate option. This happens via the counteraction of EKLF and FLI-1 (Starck et al., 2003). High FLI -1 expression drives thrombocytic outcome. EKLF on the other hand drives erythrocytic outcome of the megakaryocyte-erythrocyte progenitor (Starck et al., 2003). LMPPs also further get resolved into either myeloid or lymphoid linage. The LMPPs when resolved into the myeloid cell fate gives rise to granulocytes and macrophages via a common granulocyte macrophage progenitor (GMP) (see section 1.1.2 and figure 1.1). It has been shown that the dosage of PU.1 expression is critical for the determination of B cell vs. macrophage development in the context of LMPPs (DeKoter and Singh, 2000). Myeloid cells are specified by a high dosage of PU.1 expression (DeKoter and Singh, 2000, Laslo et al., 2008).

GMPs are primarily regulated by PU.1 and CEBP/ α (Laslo et al., 2008). PU.1 and CEBP/α co-operatively regulate genes that specify the myeloid lineage such as *Csf1r, Csf3r* and C*sf2r* (Tenen, 2003, Friedman, 2002). Mice deficient in PU.1 completely lacks Myelomonocytic cells (DeKoter et al., 1998). Conditional deletion of *Pu.1* in adult bone marrow causes complete loss of CMPs and GMPs (Iwasaki et al., 2005, Dakic et al., 2005). Mice deficient in *C/ebpα* lack neutrophils and eosinophils and conditional deletion in adult bone marrow leads to the absence of GMPs and reduction in the number of CMPs (Zhang et al., 2004b, Zhang et al., 1997). Conditional deletion of *C/ebpα*

specifically in GMPs does not show any phenotype (Zhang et al., 2004b). This indicates that while C/EBPα is absolutely necessary for the development of GMPs it is not needed for terminal differentiation and maturation of granulocytes and macrophages (Laiosa et al., 2006a)

GMPs get resolved into several different mature myeloid cell types: mast cells, neutrophils, eosinophils, basophils (together referred to as granulocytes) and macrophages (see figure 1.1). PU.1 and C/EBPα are needed for the development of both macrophages and neutrophils. For the development of eosinophils and basophils C/EBPα co-operates with GATA-2 (Laslo et al., 2006, Laslo et al., 2008).

It has been suggested that the relative concentration of PU.1 and C/EBPα determines macrophage vs. neutrophil development (Dahl et al., 2003). PU.1 is expressed at low levels in GMPs and this regulates a gene expression that is mixed for both macrophages and neutrophils (Dahl et al., 2003). At higher PU.1 concentration this mixed gene expression pattern gets refined to a gene expression pattern that specifies macrophages via the regulation of crucial secondary cell-fate determinants such as EGR-1/2 and GFI-1 (Dahl et al., 2003). EGR1 and 2 upregulates myeloid specific genes while down-regulating the neutrophils specific genes. GFI-1 on the other hand down-regulates macrophage specific genes and activates neutrophil specific genes in conjunction with C/EBPα (Dahl et al., 2003, Laslo et al., 2008) thereby establishing a complex gene regulatory circuit (see figure 1.5).

CSF-1 receptor (*Csf1r*) expression in differentiating myeloid precursors is absolutely crucial for the development of macrophages (Dai et al., 2002). Krysinska *et al*. showed by induction of PU.1 in a *Pu.1*$^{-/-}$ progenitor (that does not express CSF-1R) that the *Csf1r* gene gets activated in two distinct steps. Low doses of PU.1 keeps the *Csf1r* promoter primed but this does not allow robust expression of CSF-1R protein. With increased PU.1 expression EGR-2 is up-regulated and this along with PU.1 reorganizes the chromatin at the *Csf1r* intronic regulatory element (FIRE) and allows expression of CSF-1R (Krysinska et al., 2007).

The development of eosinophils and basophils depend on C/EBPα and GATA-2 (Laslo et al., 2008). The order of induction of C/EBPα and GATA-2 determines the eosinophil or basophil cell fate option. Sequential induction of these two transcription factors in CLPs which normally does not express either of these transcription factors shed light on the order of induction of these two transcription factors required for the specification of eosinophil or basophil. When C/EBPα was induced followed by GATA-2 eosinophils were generated but if GATA-2 was expressed followed by C/EBPα, then basophils were generated (Iwasaki et al., 2006).

Myelopoiesis therefore involves several transcription factors that dictate along with cytokines and signalling the cell-fate choices at every branching point within the haematopoietic hierarchy. Figure 1.5 shows the complex interaction of transcription factors at various branching points during myelopoiesis (see figure 1.5).

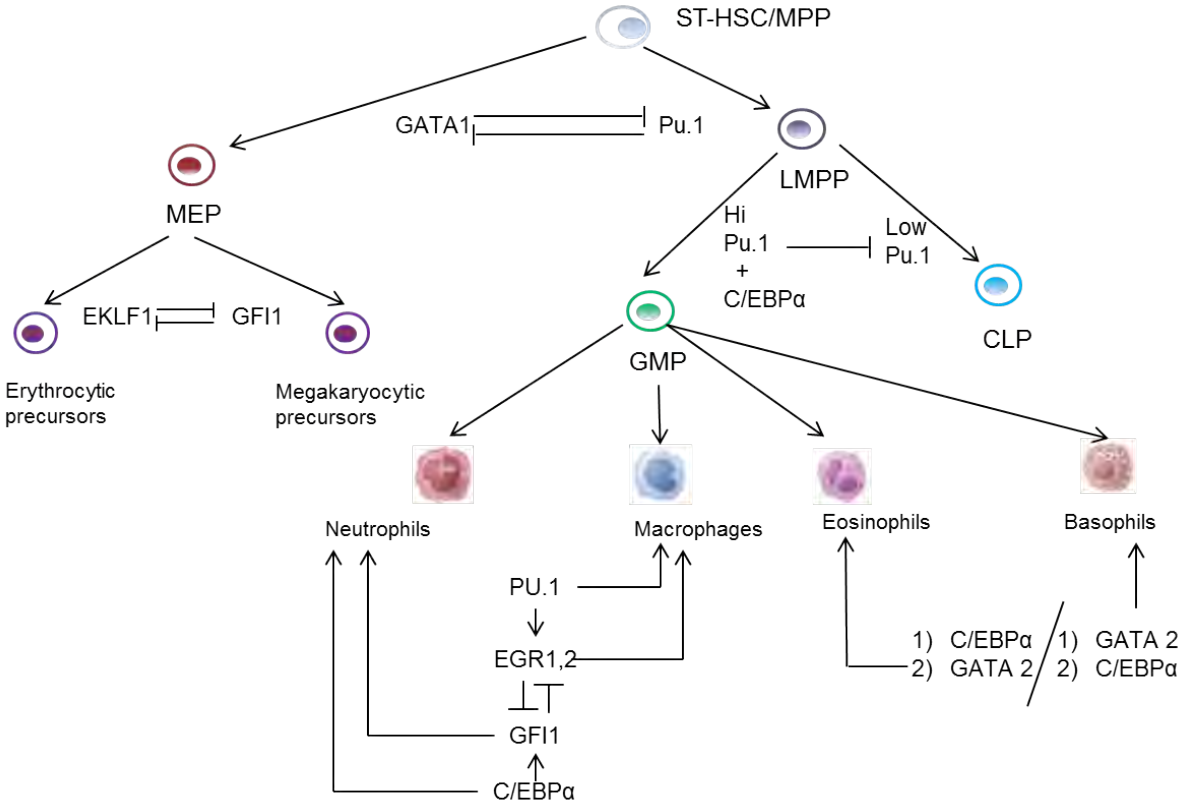


Figure 1.5: A complex network of transcription factors make binary lineage choices to regulate myeloid cell fate (Laslo et al., 2008) The scheme shows the pathways of myelopoiesis along with the various transcription factors that determine the cell-fate choices within the myeloid linage.

1.1.8 Lymphopoiesis

Lymphopoiesis is the generation of lymphocytes (i.e. B cells, T cells and Natural killer (NK) Cells) in the bone marrow and thymus. As discussed in section 1.1.2 HSCs are heterogeneous and some of these HSCs are pre-destined to be lymphoid cells and have a non-random/distinct gene expression pattern that specifies this fate (Dykstra et al., 2007). However the first definite branching out of the lymphoid lineage from the erythro-myeloid lineage occurs at the step when LMPPs give rise to common lymphoid progenitors (CLPs) that are

capable of giving rise to B-cells, T-cells and NK cells (see figure 1.1). CLPs were originally identified by Kondo and Weismann as a Linaege^{-}SCA-1^{low} c-KIT^{low}IL7Rα^{+} population within the adult bone marrow (Kondo et al., 1997). Other than CLPs, an early lymphoid progenitor (a possible predecessor of the CLP) has also been described to exist in small numbers within the LSK population (Igarashi et al., 2002). Furthermore a T-cell restricted progenitor called the Early T cell progenitor (ETP) has also been described (Allman et al., 2003, Schwarz and Bhandoola, 2004).

It is largely perceived that an HSC resembles a myeloid progenitor more than a lymphoid progenitor in terms of its gene-expression pattern. However with gradual loss in self-renewal capacity genes such and *Flt3* and *Notch-1* get up-regulated from LT-HSCs to LMPPs with the concomitant down-regulation of *Gata-1* and *Gata--2* (Christensen and Weissman, 2001, Sitnicka et al., 2002, Kikuchi et al., 2005, Forsberg et al., 2005, Laiosa et al., 2006a). FLT3 is required for the generation of CLPs (Sitnicka et al., 2002). FLT3 and its ligand together with PU.1 upregulates IL7Rα which in turn up-regulates EBF-1 that is essential for B cell development (Kikuchi et al., 2005).

The IKAROS family of proteins is essential for development of lymphocytes (Georgopoulos et al., 1992, Georgopoulos et al., 1994, Wang et al., 1996a). HSCs from *Ikaros*-null mice lack FLT3 and have impaired ability to repopulate and to differentiate into lymphocytes (Nichogiannopoulou et al., 1999). *Ikaros*-null mice lack all B cells, NK cells and fetal T-cells (Allman et al., 2003, Wang et al., 1996a). However, in *Ikaros*-null mice myeloid differentiation is unaffected (Nichogiannopoulou et al., 1999). The IKAROS family of proteins are therefore

very important transcription factors that specify lymphoid lineage in very early haematopoietic progenitors (Yoshida et al., 2006). These proteins are thought to target lineage-determining genes by chromatin remodelling via interactions with NuRD and SWI/SNF complexes (Kim et al., 1999, O'Neill et al., 2000).

Natural Killer cell lineage

NK cells are large granular lymphocytes that arise from the CLP. They form a crucial part of innate immune system. These are cytotoxic cells that respond to viral infections etc. by secretion of interferons. Mice deficient in *Ikaros* lack NK-cells (Wang et al., 1996a). *Pu.1* deficient mice show a reduced number of NK-cells (Colucci et al., 2001) and mice deficient in *Id2* show a defect in the transport of NK cells to the periphery (Yokota et al., 1999, Ikawa et al., 2001). These experiments imply that IKAROS, PU.1 and ID proteins play important roles in the development of NK cells (Laiosa et al., 2006a).

T cell development

T cells are lymphocytes that play a very important role in cell-mediated immunity. These cells arise in the bone marrow and mature in the thymus and hence the name ‘T-cells’. One of the defining features of the T-cell is the presence of a fully re-arranged T cell receptor (TCR) on the cellular membrane. The T-cell receptor plays crucial role in antigen recognition and T cell activation and commitment to $CD4^+$ or $CD8^+$ cell fate. There are various subcategories of T-cells based on their surface markers and their immune functions.

T-helper cells (T_H) cells are usually $CD4^+$ cells that recognise antigen presented by the MHC II complex. Upon antigen binding these cells undergo activation and become effector T_H cells which produce cytokines and stimulate the activity of B-cells (see next section) and cytotoxic T-cells (T_C). T_C cells are usually $CD8^+$ and they bind antigen presented on the MHC I complex and also divide to become effector T_C cells that kill infected or malignant cells. Upon activation T_H and T_C cells not only divide to form respective effector T cells but they also form memory T-cells. Memory T cells can either be $CD4^+$ or $CD8^+$. These cells persist for long after an infection has been resolved. They are capable of rabidly expanding on re-exposure to the same infection/antigen in future and hence they provide an immune memory to the system (Smith-Garvin et al., 2009).

T cells arise from the CLPs or the ETP. ETPs arise from LMPPs due to increased NOTCH signalling. NOTCH1 has been shown to down-regulate B cell specifying gene program to promote T-cell development at the expense of B cells (Hoflinger et al., 2004). Inactivation of NOTCH1 or CSL (transcriptional partner of NOTCH1, see section 1.1.4) blocks T-cell development at the earliest T-cell progenitor stage. Mice deficient in NOTCH1 also show a similar block in T-cell development (Radtke et al., 1999, Han et al., 2002). Expression of constitutively activated NOTCH1in fetal-liver derived haematopoietic progenitors or culturing of these progenitor cells on OP9 stroma that constitutively secretes the NOTCH1 ligand Delta1, induces T-cell development form these progenitors at the expense of B cells and NK cells (Schmitt et al., 2004b, Schmitt et al., 2004a). It has also been shown that a transcription factor called LRF inhibits NOTCH1 signalling in the bone marrow. Inactivation of *LRF* in HSCs, leads to

the precocious generation of T-lineage progeny directly from HSCs; due to up-regulation of NOTCH1 signalling (Maeda et al., 2007). These experiments together indicate that NOTCH1 signalling is crucial for T-cell development.

Apart from NOTCH1, the transcription factor GATA3 is also very important for T-cell development. Mice lacking GATA-3 show a block in T cell development at an early stage (Ting et al., 1996). E2A and HEB are very important for T cell receptor rearrangement (Tremblay et al., 2003). E2A and HEB form a heterodimeric complex that activates genes required for TCR rearrangement and signalling.

B-cell development

B cells are an important part of the adaptive immune system. They are generated in the bone marrow. B-cells are the precursors of plasma cells that produce antibodies and hence provide humoral immunity complementing the cell-mediated immunity provided by T-cells.

B cells arise from common lymphoid progenitors via a pro-B-cell stage ($CD34^+$ $CD19^+$ $CD10^+$), large and small Pre-B-cells ($CD34^-$ $CD19^+$) through to mature IgM^+ B-cells (LeBien, 2000). The VDJ recombination of the heavy chain of the immunoglobulin occurs at the pro-B cell stage and requires RAG-1 and RAG2 proteins (Jung et al., 2006). The expression of the pre-B cell receptors (pre-BCR) with a fully rearranged immunoglobulin heavy chain and a surrogate light chain is typical of the pre-B cell stage. Signalling through pre-BCR leads to the

VJ recombination of the immunoglobulin light chain leading to the formation of a fully functional B-cell receptor (BCR) (Jung et al., 2006).

B-cell development requires the orchestrated functioning of several transcription factors including IKAROS, PU.1, E2A, EBF1 and PAX5 (Laiosa et al., 2006a). As indicated earlier IKAROS is essential for lymphocyte development and the lack of IKAROS in vivo leads to complete loss of B cells (Georgopoulos et al., 1994). PU.1 concentration has been shown to be of critical importance for the specification of B cell vs. macrophage cell fate in LMPPs (DeKoter and Singh, 2000). A low level of PU.1 is required for B cell development while macrophage development requires 4-5 fold higher PU.1 concentration (Laslo et al., 2008) . This lineage specific regulation of PU.1 concentration is achieved through two distinct auto-regulatory loops at the PU.1 locus in B cells and macrophages. In B cell precursors, transcription factors such as E2A and FOXO1 activate the -14kb upstream regulatory element which is also bound by PU.1 and drives an intermediate level of PU.1 expression needed for B cell development (Leddin et al., 2011). In myeloid progenitors CEBPα along with PU.1 binds the -14kb URE and activates a myeloid specific enhancer at -12 kb which is bound by PU.1 activated genes such as EGR1 and drives high levels of PU.1 expression (Leddin et al., 2011).

Mice deficient in PU.1 shows impaired B cell differentiation (DeKoter et al., 2002, Medina et al., 2004). However, PU.1 is not strictly required for B cell formation (Ye et al., 2005). Conditional deletion of *Pu.1* in pro-B cells does not impair B- cell formation (Ye et al., 2005). Experiments show that PU.1 activates the *Ebf* gene via IL7-R signalling (Kikuchi et al., 2005). However EBF can be

induced by E47: an isomer of E2A, or other members of the E-box family of transcription factors (Bain et al., 1994). IL7 receptor signalling regulates the earliest events of B cell development (Miller et al., 2002). Stimulation with IL7 is sufficient to induce B cell differentiation in CLPs under culture condition. *IL7*$^{-/-}$ or *IL7-R*$^{-/-}$ mice show a block in B cell differentiation at the pre-pro-B-cell stage. This defect can be rescued by re-expression of EBF (Dias et al., 2005). Thus IL7 signalling controls B cell development through activation of *Ebf* (Laslo et al., 2008).

EBF1 or early B cell factor 1 is essential for the development of B cells. Loss of EBF1 blocks B cell development at the pro-B cell stage prior to the initiation of BCR rearrangements (Lin and Grosschedl, 1995). EBF1 is activated by E2A and also by PU.1 via IL7-R signalling (Kikuchi et al., 2005, Dias et al., 2005). It has also been shown that E2A and EBF along with FOXO1 (either as E2A and EBF or as E2A and FOXO1) bind cis- regulatory elements of target genes (transcription factors, signalling and survival factors) in order to specify B-cell fate (Lin et al., 2010).

One of the most important targets of EBF is the master regulator of B cell commitment: *PAX5* (Decker et al., 2009). PAX5 is reviewed in greater detail in section 1.4. PAX5 up-regulates B-cell specific genes such as *CD19* and *Mpo1* (Cobaleda et al., 2007) and down regulates lineage inappropriate genes such as *Csf1r* (Tagoh et al., 2006b). PAX5 also down-regulates *Blimp1* which is responsible for terminal differentiation to plasma cells (Cobaleda et al., 2007). In mice lacking PAX5, B-cell development gets stalled at the pro-B cell stage (Urbanek et al., 1994, Nutt et al., 1997). Under culture conditions these pro-B

cells can be differentiated into other haematopoietic lineages but not B-cells (Nutt et al., 1997). Furthermore deletion of *Pax5* in B cells leads to the aberrant up-regulation of myeloid genes such *Csf1r*, *Csf2r* and *CSf3r* and also T cell transcription factor gene *Notch 1* (Nutt et al., 1999, Nutt et al., 1998, Souabni et al., 2002). Taken together these results indicate that PAX5 is essential for commitment to B cell lineage and also for the maintenance of B-cell identity. Figure 1.6 illustrates the main transcription factors influencing binary lineage choices in lymphopoiesis (see figure 1.6).

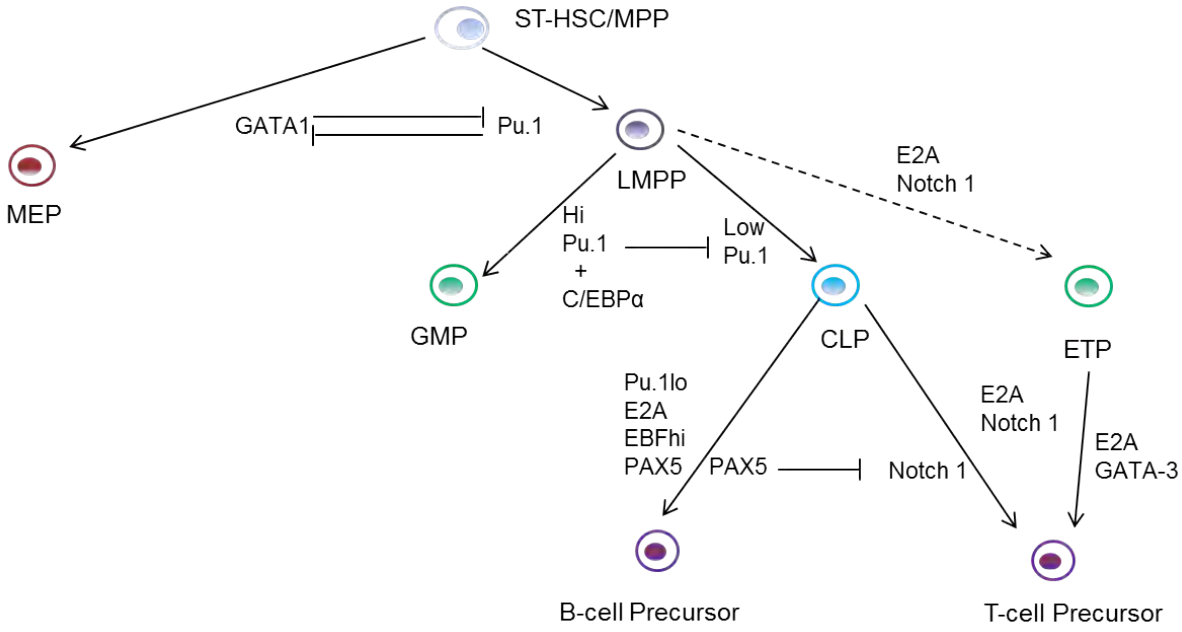


Figure 1.6: Schematic diagram showing the role of transcription factors in making binary cell fate choices during lymphopoiesis. The scheme shows the pathways of lymphopoiesis along with the key transcription factors that determine the cell-fate choices within the lymphoid lineage.

1.2 Eukaryotic Transcription and its regulation

The eukaryotic genome is very large and highly complex. Many are estimated to have between 20,000 and 25,000 genes. In a given cell at a given time only a subset of this vast and complex genome is transcribed. To understand cell differentiation in the context of haematopoiesis or any other differentiation pathway it is very important to understand eukaryotic transcription and how it is regulated in a spatio-temporal manner. The complexity of the eukaryotic genome makes the mechanism controlling its spatio-temporal regulation also highly complex. To understand the regulation of eukaryotic genes, we first need to understand the structure of a typical eukaryotic gene along with its cis-regulatory regions and also how these genes are organized to form a compact structure such as the eukaryotic chromosome.

1.2.1 The structure of a typical Eukaryotic gene

The basic behaviour of a gene was described about a century ago by Gregor Johann Mendel. According to Mendel's definition, genes are "some factors" that pass on from parent to off-spring in an unchanged manner. Genes exist in two alternative states called 'alleles'. Diploid organisms have two sets of chromosomes; one is inherited from the father and the other from the mother. Genes behave in a way similar to chromosomes; i.e. one allele of each gene is inherited form either parent. Therefore the corollary to this is that genes are carried on chromosomes.

Frederick Griffith's transformation experiment with *Streptococcus pneumonia in* 1928 (Griffith, 1928) followed by Hershey and Chases experiment in 1952 involving infection of bacteria with bacteriophages containing radio-labelled DNA and protein components provided the first evidence that 'DNA and not 'protein' is the genetic material (Hershey, 1952). Therefore a gene may be defined as a sequence of DNA that codes for a messenger RNA (by a process called 'transcription') that in turn codes for a functional protein (translation) and this meaningful protein coding sequence of DNA is inherited as part of a chromosome from parent to off-spring.

Only about 2% of the vast eukaryotic genome is accounted for by protein coding sequence. Each eukaryotic gene consists of transcriptional regulatory regions that can either be directly upstream (i.e. at the 5' end) or may be scattered over large distances. These regulatory regions are called 'cis'-regulatory elements and they are recognised and bound by 'trans'-acting transcription factors during transcription. Broadly there are two types of cis-regulatory elements: promoters and long-range regulatory elements (reviewed in (Lee and Young, 2000). Recently genome-wide profiling of 125 diverse cell and tissue types from the Stamatoyannopoulos lab has identified a vast number of cis-regulatory elements which include experimentally verified enhancers and promoters and also a large number of novel elements which have been inked to their respective genes, various chromatin features and transcription (Thurman et al., 2012).

Promoters

A gene promoter is defined as a cis-regulatory element that is required for initiation of transcription or increases the frequency of transcriptional initiation when placed close to the transcription initiation site. A gene promoter consists of the 'core promoter' and 'proximal promoter elements'.

Core promoter element: The core promoter element is about 100 bps long and consists of the transcription initiation site (that binds the RNA Polymerase II complex). About 24%-32% of eukaryotic promoters contain an AT-rich sequence ~ 20-30bps away from the transcription start site. This is called the TATA box and serves as the binding site for TBP (Novina and Roy, 1996). However most gene promoters are TATA-less and may contain an initiator sequence (Inr) or contain neither of these two motifs (Smale et al., 1998). On-going research is aimed at identifying more motifs that better describe promoter elements.

Proximal promoter element: The proximal promoter element is present 5' of the core promoter element and the transcription start site. These elements increase the frequency of transcription initiation when placed in proximity of the transcription start site. Proximal promoter elements bind transcription factors that influence transcription. These elements often have recognition sites for CAAT-binding proteins (CBP) or CAAT-enhancer binding protein (CEBP) or Sp1 that recognises the GC box (Novina and Roy, 1996, Neph et al., 2012). The binding of these transcription factors stabilises the basal transcription machinery

(see section 1.2.2) and enhances the slow rate of transcription that is achieved by the basal transcription machinery and the core promoter alone.

Long range regulatory elements

As indicated in the beginning of this chapter, eukaryotic genes are regulated in a complex lineage specific manner. To achieve this complex regulation, cis-regulatory elements other than promoters are required. The long range regulatory elements: enhancers, silencers, insulators and locus control regions; serve this purpose.

Enhancers: Enhancers are DNA sequence elements that are themselves incapable of transcription, but are able to dramatically increase the rate of transcription from a promoter element. Enhancers can be at great distances from the gene promoter and be upstream, downstream or intronic and be in either orientation (Blackwood and Kadonaga, 1998). Enhancer elements bind transcription factors and co-activators that form a complex called the 'enhanseosome' (Yie et al., 1999). The activator proteins bound on an enhancer act via chromatin remodelling so as to make the chromatin more active (see section 1.2.5). The 'enhanseosome' also interacts with basal transcription machinery (see section 1.2.2) at the core promoter by looping in order to activate distant promoters (Su et al., 1990). Enhancer activity is tissue specific (i.e. enhancers get activated by specific transcription factors in specific cell types only) and hence it accounts for tissue specific expression of the genes that they activate (Lee and Young, 2000).

Silencers: Silencers are very similar to enhancers, except for the fact that they repress the activity of a promoter rather than increasing it. These elements can also act at large distances and at any orientation. Silencers bind proteins that compact the chromatin (see section 1.2.3) further thereby inhibiting transcription (Lee and Young, 2000, Baniahmad et al., 1990).

Insulators: The presence of enhancers and silencers acting on promoters at large distances raises the question as to how the activity of the cis-regulatory elements is restricted to the appropriate promoter. One of the classic examples of an insulator sequence is in the β-globin locus. The β-globin locus is under the control of a locus control region (LCR) (see next section) that stimulates β-globin expression in erythroid cells. In order to prevent this LCR from stimulating flanking genes in the region, the β-globin gene is flanked by two insulator sequences.

An insulator is effective in blocking enhancer/silencer or LCR activity only when bound to CTCF proteins (Phillips and Corces, 2009, Farrell et al., 2002) A CTCF bound insulator blocks chromatin remodelling or looping actions of enhancers/ silencers from propagating over large distances thereby restricting the actions of these regulatory elements to the appropriate genes (Farrell et al., 2002, Burgess-Beusse et al., 2002) and reviewed in (Lee and Young, 2000).

Locus Control regions: LCRs or locus control regions were originally characterised in the mid-1980s in the β-globin gene cluster (Grosveld et al., 1987). The β-globin gene cluster consists of four functional β-globin genes and one pseudogene. A region of DNA sequence 10-20 kb upstream of this cluster

was shown to confer high levels of expression from this gene cluster both in vivo and in transgenic experiments (Grosveld et al., 1987) and reviewed in (Bulger and Groudine, 1999, Li et al., 1999). The deletion of the β-globin LCR leads to a complete lack of expression of any of the β-globin genes in the cluster even though the other enhancers and promoters of the genes remain intact (Fang et al., 2005, Bender et al., 2000). This is the cause of the lethal condition known as Hispanic thalassemia wherein no haemoglobin is produced (Bender et al., 2006). Since the entire β-globin locus is under the control of this regulatory element it is called the 'locus control region'. Following characterisation of the LCR in the β-globin locus, similar locus control regions have been defined in the α-globin cluster, the major histocompatibility complex locus and the CD2 and lysozyme genes (Li et al., 1999).

1.2.2 The basal transcription machinery

Transcription is the process by which the information encoded within a protein coding gene is converted into RNA. The process of transcription invariably starts at the 'transcription start site' which is a part of the core promoter element (see section 1.2.1) with the assembly of an apparatus that is known as the 'basal transcription machinery' The basal transcription machinery of protein encoding genes primarily consists of: a) the 12-subunit RNA polymerase II complex b) the general transcription factors and c) co-activators/mediators reviewed in (Lee and Young, 2000, Fuda et al., 2009). All protein coding genes in eukaryotes are transcribed by the RNA polymerase II enzyme. The basal transcription machinery for these genes assembles at the core promoter in a step-wise manner. The general transcription factor (GTF) TFIID complex which

consists of TBP (TATA binding protein) and TAFs (TBP associated factors) binds the DNA at the TATA box or the initiator sequence within the core promoter element in the presence of another factor called the TFIIA.

The TFIID/TFIIA/DNA complex is recognised by another GTF, TFIIB. This binds TFIID at the side opposite to that of TFIIA. The binding of TFIIB facilitates the recruitment of the RNA polymerase II enzyme in association with another factor: TFIIF. This complex is known as the initiator complex. TFIIB has also been shown by structural analysis to be of importance in holding the DNA in the correct orientation within the RNA polymerase II complex thereby allowing transcription to occur (Lee and Young, 2000).

The eukaryotic RNA polymerase II enzyme is complex consisting of 12 subunits of RPB1-12. The largest subunit of the RNA polymerase II contains a C-terminal repeat domain (CTD) consisting of 52 (in human) repeats of Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Corden, 1990, Young, 1991). The phosphorylation state of the serine residues at the CTD repeats is closely related to the functional status of the RNA polymerase II complex. RNA polymerase II is recruited to the gene promoters in a un-phoshorylated state as part of the initiator complex as described above. As transcription initiates the polymerase II complex gets phosphorylated by two specific kinases at serine 5 followed by serine 2 residues of the CTD repeats (Dvir et al., 1997).

The recruitment of TFIIF to the initiator complex facilitates the recruitment of two other factors namely TFIIE and TFIIH. Of these two factors, TFIIH is of particular importance as this possesses the CDK7 subunit that has the kinase

activity that is needed to phosphorylate serine 5 residue on the CTD repeats of the RNA polymerase II (Dvir et al., 1997, Lee and Young, 2000). Mutations in the kinase subunit of TFIIH affect CTD phosphorylation both in vivo (Akoulitchev et al., 1995, Tirode et al., 1999) and in vitro (Makela et al., 1995). The phosphorylation at serine 5 allows transcriptional initiation to occur. After being phosphorylated the RNA polymerase II complex transcribes about 20-30 base pairs and pauses. Thereafter pTEFb (positive transcription elongation factor b) is recruited which possess the CDK9 kinase and cyclin T subunits (Lis et al., 2000, Price, 2000) and mediates the phosphorylation of the RNA polymerase II CTD repeat at the serine 2 residue. This phosphorylation step releases the paused RNA polymerase II complex and allows effective transcriptional elongation (Price, 2000). TFIIF moves along with the RNA polymerase II enzyme, however TFIIA and TFIID remains attached to the promoter and facilitates subsequent recruitment of TFIIB and RNA polymerase II for repeated cycles of transcription (Lee and Young, 2000). Figure 1.7 illustrates the assembly of the basal transcription machinery at a typical eukaryotic protein coding gene promoter (see figure 1.7).

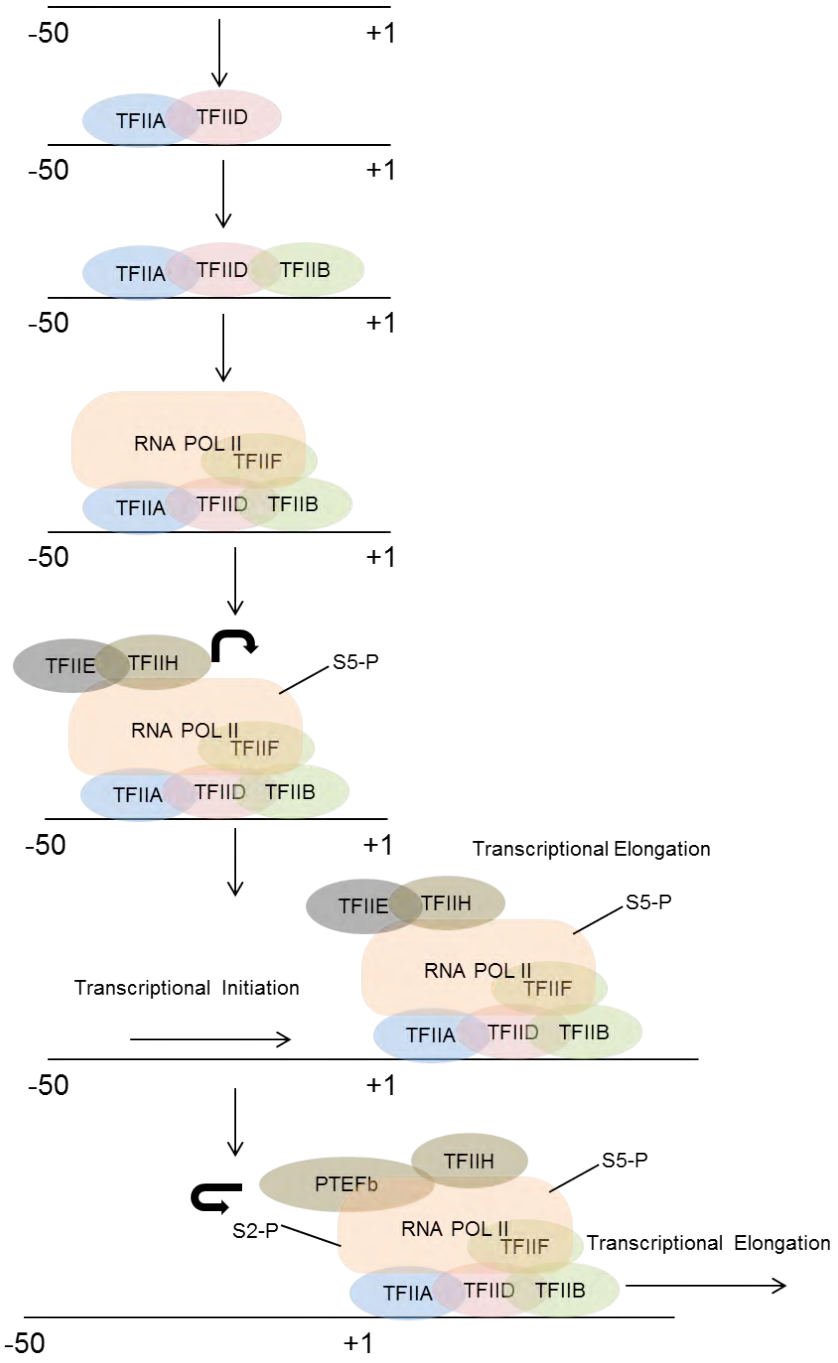


Figure 1.7: The Basal transcription machinery. A cartoon showing the step-wise assembly of the basal transcription factors at a typical eukaryotic gene and also the steps leading to transcriptional initiation and elongation.

1.2.3 Chromatin Structure

If the DNA that constitutes this large human genome were to be extended as a two dimensional double helix, it would be of an enormous length. However, this DNA is contained within the nucleus of a cell which is a rather tiny structure with an average diameter of 6µm. Therefore, DNA within a living cell does not exist as a two-dimensional double helix, but as a compact structure that is neatly packaged into the nucleus. In fact DNA exists in complex with certain nucleo-proteins (histones) and forms a compact structure called chromatin (reviewed in (Cockerill, 2011, Bonifer and Cockerill, 2011).

Nucleosomes are the building blocks of chromatin and consists of ~146 base pairs of DNA double helix wrapped around a symmetrical histone octamer containing two molecules each of H3,H4,H2A and H2B each (Richmond et al., 1984, Bakayev et al., 1977). High resolution structural analysis using X-ray crystallography has confirmed that H3-H4 heterodimers form a tetramer that loads onto 60 base pairs of DNA; this is followed by two H2A-H2B hetero-dimers associating with the H3-H4 tetramer above and below respectively to form an octamer core (see figure 1.8). The DNA double helix wraps around the surface of this octamer and the genome exists as regularly spaced nucleosomes with a DNA repeat length of 180-200 base pairs (Albright et al., 1980, Simpson et al., 1978, Simpson, 1978). The amino-terminal tails of the core histone octamer, however, remains unstructured and exposed. These amino terminal tails undergo post-translational modifications that have been shown to have far reaching effects on chromatin structure and the activity of neighbouring genes (see section 1.2.4). The 200 base pairs spaced

nucleosomes loaded onto DNA can be loosely described as a ‘beads on string structure’ (Thoma et al., 1979). which constitutes the first stage of DNA compaction. This ‘bead on string’ structure is further folded onto itself to form more compact structures termed ‘solenoids’ (Allan et al., 1982). Histone H1 plays an important role in the formation of such higher order chromatin structures (Thoma et al., 1979). Most nucleosomes recruit histone H1 to ‘seal off’ the core histone octamer. The core histone octamer along with histone H1 and ~166 base pairs of DNA is called a ‘chromatosome’ (Albright et al., 1980). Under physiological condition of ion- concentration, chains of nucleosomes, with or without histone H1; folds into the 30nm fibre (Marsden and Laemmli, 1979). However, the exact nature of this structure is still an evolving field of research (Cockerill, 2011).

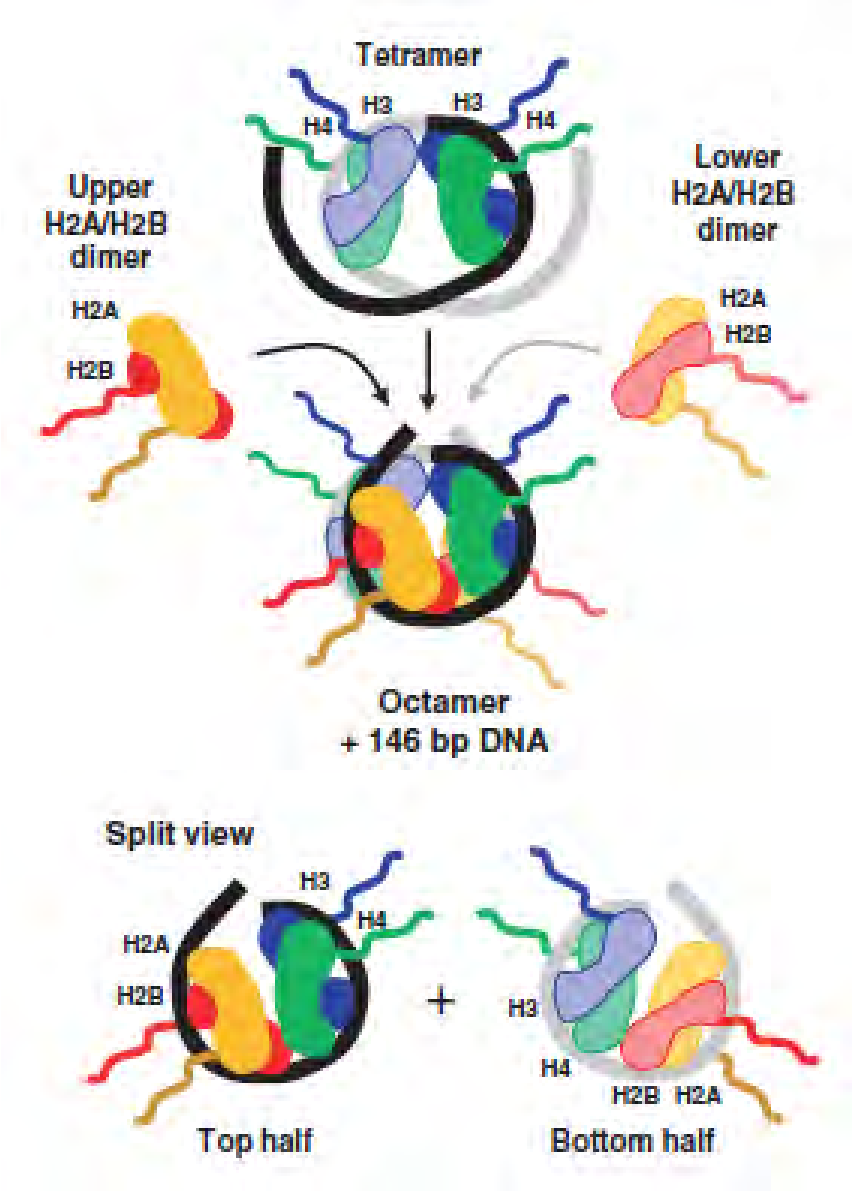


Figure 1.8: The structure of the nucleosome core. This cartoon diagram shows the assembly of histone on DNA to form nucleosome core histone octamer particle (Cockerill, 2011).

1.2.4 Chromatin modifications

The N-terminal tails of the histone proteins of the core nucleosome particle are subject to a variety of post-translational modifications. To date a number of different types of histone modifications (namely: acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation and proline isomerisation) have been identified at various amino acid residues (lysine, arginine, serine, threonine and proline) of the histone tails. Table 1.1 lists the various modifications at the various possible histone tail residues (see table 1.1).

Studies over the last decade have identified the enzymes and the particular catalytic domains of these enzymes, responsible for the various chromatin modifications (Sterner and Berger, 2000, Zhang, 2006, Nowak and Corces, 2004, Shilatifard, 2006, Cuthbert et al., 2004, Hassa et al., 2006, Nelson et al., 2006). Similarly enzymes that erase most of these modifications have also been identified n (Kouzarides, 2007). These modifications are highly dynamic, i.e. they appear and disappear from the chromatin very quickly on receiving the appropriate stimuli (see section 1.2.9). However, extensive effort has been made by way of chromatin immuno-precipitation (ChIP) (Solomon et al., 1988) and chromatin immuno-precipitation coupled with high-throughput sequencing (Barski et al., 2007) to acquire a global view of the chromatin modifications and their biological functions.

The functional consequences of histone modifications are twofold: a) establishment of a chromatin environment and b) conduction of DNA based biological tasks wed in (Kouzarides, 2007). In this context chromatin environment means maintenance of 'euchromatin' (accessible chromatin) and 'heterochromatin' (condensed inaccessible chromatin) and maintaining distinct boundaries between these two states (see section 1.2.6). DNA based biological tasks on the other hand refer to transcription of a gene, repair of damaged DNA, DNA replication or chromatin condensation.

Histone Modifications	Residues
Acetylation	H3(K4, K9, K18, K23, K27, K36, K56, K64, K79, K115,K122) H4(S1, K5, K8, K12, K16, K20, K31,K77, K79, K91) H2A(S1, K5,K9, K13, K36, K95, K99,K119) H2B(K5, K11, K12, K15, K20, K24, K46, K85, K108, K116, K120)
Methylation (Lysine)	H3 (K4, K9, K14, K18, K23, K27, K36, K37, K56, K64, K79, K122) H4 (K12, K20, K31, K79) H2A(K5, K13, K15, K74, K75, K99) H2B (K5, K23, K43, K46, K57, K108)
Methylation (Arginine)	H3 (R2, R17, R26) H4(R3, R92) H2A(R29, R77) H2BR99
Phosphorylation	H3 (T3, T6, S10, T11, S28, Y41, T45, T80, T107, T118) H4 (S1, S46, Y51, Y88) H2A (S1, T120, S122) H2B (S6, S14, S32, S36)
Ubiquitination	H2AK119 H2BK120
Proline isomerisation	H3(P30, P38)

Table 1.1.1: Chromatin modifications. This table lists the various histone modifications on the different histone particles along with the corresponding residues modified (Kouzarides, 2007).

A large volume of ChIP-sequencing data has been produced that have identified several histone modifications genome-wide and many such data sets have been analysed by various bio-informatics exercises in order to produce a consolidated functional map of histone modifications and therefore a map of the chromatin environment across the whole genome. For example Keji Zhao's group has produced ChIP-sequencing datasets for a large number of histone modifications (Barski et al., 2007). Such data sets (Barski et al., 2007, Mikkelsen et al., 2007) along with gene expression studies have helped researchers to associate certain histone modifications with actively transcribed chromatin while others with transcriptional inactivity. Studies over the years have shown that different modifications of the histone tails provide binding sites for different effector proteins that either activate or inactivate transcription and also mediate re-enforcement of the active/inactive state. For example acetylation of lysine residues (e.g. H3K9) is bound by bromodomain or PHD domain containing proteins (Jin et al., 2011, Dhalluin et al., 1999) while methylation of H3K9 recruits HP1 that in turn recruits DNA methyl-transferases which further reinforces an inactive state (Allan et al., 2012, Yun et al., 2011). This combinatorial modifications of the histone tails and the recognition of these modified histone tails by reader proteins greatly extend the potential of the information contained by the genetic code itself and this is referred to as the 'histone code' (Jenuwein and Allis, 2001).

Acetylation

Histone acetylation is always associated with active chromatin. This modification affects lysine residues on H3, H4, H2A and H2B histones in the nucleosome octamer (table 1.1.1). Acetylation of histones is carried out by enzymes broadly termed as histone acetyl transferases (HATs). Some of the well- known HATs include HAT1, p300/CBP, GCN5 (Kouzarides, 2007, Sterner and Berger, 2000). Reversal of acetylation is associated with transcriptional repression. Histone de-acetylases (HDACs) are classified into three distinct classes: class I, II and III. HDACs are part of numerous signalling pathways and repressive complexes and do not exhibit a lot of specificity for particular acetyl groups (Kouzarides, 2007, Vaquero et al., 2006). It is proposed that acetylation 'unravels' the chromatin and makes it more accessible by affecting contact between histones on adjacent nucleosomes by neutralising the charge on lysine residues (Schubeler et al., 2004). Biophysical studies indicate that inter-nucleosomal interaction is crucial for higher order chromatin structure. A more direct proof of this comes from the work of Shogren-Knaak *et al*, where they chemically ligated an acetylated histone tail (acetylated at H4K16) to a recombinant histone core particle (Shogren-Knaak and Peterson, 2006, Shogren-Knaak et al., 2006). This modification had a negative effect on the formation of the 30nm fibre. Acetylated histones have been shown to recruit bromo-domain (Dhalluin et al., 1999) and the tandem PHD domain (Zeng et al., 2010) containing proteins that facilitate chromatin activation (Shogren-Knaak et al., 2006).

Phosphorylation

Phosphorylation targets serine residues on H3, H4 and H2B and threonine in H3 (see table 1.1.1). Histone phosphorylation is mediated by serine/threonine kinases such as MSK1/2, RSK1/2, Haspin (Nowak and Corces, 2004). MSK and RSK kinases are downstream of several MAPkinase signalling pathways (Cargnello and Roux, 2011). Phosphorylation is also likely to affect chromatin structure via change in charge on amino acid residue in the histone tails (Nowak and Corces, 2004). Phosphorylation of H3S10 and H3S28 has been shown to be involved in the activation of 'immediate early genes' such as *c-Jun* and *c-Fos* by counteracting the action of HP1 and polycomb repressive complexes respectively (Macdonald et al., 2005, Lau and Cheung, 2011b, Lau and Cheung, 2011a, Gehani et al., 2010, Mahadevan et al., 1991).

Methylation

Methylation affects lysine on H3 and arginine residues on H3 and H4 (table 1.1.1). A lysine residue may be mono, di or tri methylated. Methylation is mediated by specific lysine methyl-transferases containing the SET catalytic domain or protein arginine methyl-transferases (PRMTs). SUV39H1, the MLL family of enzymes, ASH2, EZH2 are well known examples of SET domain containing lysine methyl-transferases. The arginine methyl-transferases include PRMT4/5, CARM1 (Kouzarides, 2007).

Methylation may be associated with active or repressed chromatin depending on residues modified (Sims et al., 2003, Zhang and Reinberg, 2001). Methylation of H3K9 and H3K27 (see section 1.2.10) is associated with

transcriptional repression. H3K9me3 is the mark of 'constitutive heterochromatin' that recruits chromo-domain containing HP1 (heterochromatin protein 1) that leads to further chromatin compaction and finally to DNA methylation (Munari et al., 2012, Bannister et al., 2001). Recent work has discovered that two lysine methyl-transferases PRDM3 and PRDM16 that mono-methylates H3K9 in the cytoplasm which gets converted to H3K9me3 by SUV39H1 in the nucleus to form heterochromatin. This study has shown that H3K9me1 plays a crucial role in the initiation of heterochromatin formation; depletion of *Prdm3* and *Prdm16* abrogates H3K9me1 and prevents the SUV39h-dependent H3K9me3 and de-represses satellite transcription (Pinheiro et al., 2012). H3K27me3 is the hallmark of polycomb repression (see section 1.3) and is installed by the SET domain containing EZH2 methyl-trasnferase (Beisel and Paro, 2011). In contrast methylation of H3K4 is associated with active transcription. Studies associate H3K4me3 to actively transcribed transcriptional start sites at promoters with high CpG content (Bernstein et al., 2005, Kim et al., 2005). Sites of H3K4me3 are often accompanied by other features of active chromatin i.e. acetylated histone, DNAse I hypersensitivity (see section 1.2.6), H3.3 histone variant and recruitment of RNA polymerase II (Wang et al., 2008, Hon et al., 2009a, Hon et al., 2009b, Goldberg et al., 2010). The lysine 4 residue on H3 is also subject to di and mono-methylation. H3K4me2 is the precursor to the tri-methyl mark. Orford *et al* identified a subset of gene promoters in haematopoietic progenitors containing the H3K4me2 mark instead of H3K4me3. These genes corresponded to gene promoters with low CpG content and were not expressed in progenitors but would be expressed once differentiation of these progenitors was induced (Orford et al., 2008). Thus

H3K4me2 marks promoters that are poised for activation. Enrichment of H3K4me1 along with the lack of H3K4me3 is the chromatin signature of enhancers (Heintzman et al., 2007). Using this definition Heintzman *et al* identified 55,000 putative enhancers in five different human cell types (Heintzman et al., 2009, Heintzman and Ren, 2009). H3K27ac has also been shown to be enriched at enhancers (Rada-Iglesias et al., 2011) and is a general mark of active chromatin (Creyghton et al., 2010). H3K36me3 and H3K79me3 are found in the gene body of transcribed genes (Li et al., 2007, Barski et al., 2007, Heintzman et al., 2007). Levels of H3K36me3 have been shown to correlate with the levels of gene expression (Barski et al., 2007). This indicates the likely interaction of methyl-transferase enzymes with the elongating RNA Polymerase II (Mikkelsen et al., 2007, Li et al., 2007). It is hypothesized that the methyl-transferase interacts with the transcribing RNA polymerase II enzyme and deposits the H3K36me3 mark as transcription occurs.

De-methylation of lysine residues is mediated by de-methylases which have so far been shown to have two distinct catalytic domains with two distinct de-methylation reactions: LSD1 and JmjC domains respectively (Kouzarides, 2007). LSD1 de-methylates H3K4 and represses transcription (Shi et al., 2004), while JmjC domain containing de-methylases remove H3K9me3, HK36me3 and H3K27me3 and activate transcription (Yamane et al., 2006, Tsukada et al., 2006, Cloos et al., 2006, Chen et al., 2006, Fodor et al., 2006, Agger et al., 2009). An enzyme with a methyl-arginine de-methylating activity is yet to be discovered. However, the process of de-amination has been shown to correlate with the removal of methyl-arginine modifications. This process involves the

conversion of arginine to citrulline (Cuthbert et al., 2004, Wang et al., 2004b).In vivo data has shown that mono-methylated arginines are capable of undergoing de-amination to form citrulline which prevents further methylation (Wang et al., 2004b).

Ubiquitination

Ubiquitination is another interesting modification. Ubiquitin is a 76 amino acid protein that is coupled via its C-terminal carboxyl group to the free amino group of a lysine residue on an H2A or H2B histone tail (Zhang, 2003). Ubiquitination targets H2AK119 and H2BK120 residues and this modification is mediated by BMI1/RING1B (Wang et al., 2006) and RNF20/RNF40 ubiquitin ligases respectively (Zhu et al., 2005). H2AK119ub mediated by BMI1/RING1B (see section 1.3) is a consequence of polycomb repression and leads to block of transcriptional elongation and therefore gene repression (Wang et al., 2004a, Stock et al., 2007). Though the concept of H2AK119ub1 helping in chromatin compaction has been recently challenged (Whitcomb et al., 2012, Endoh et al., 2012), largely H2AK119ub is crucial for the repression of polycomb targets (Endoh et al., 2012). On the contrary H2BK120 is an active mark. This modification promotes H3K4 and Dot1L mediated H3K79 methylation and therefore promotes active chromatin (Whitcomb et al., 2012).

'Cross-talk' between histone modifications

It is evident from the above description that a large variety of histone modifications occur at the N-terminal histone tails that recruit different effector proteins which dictate transcriptional activity or inactivity. The occurrence of

such a large variety of modifications on amino acid residues that are closely situated in space and time is likely to cause ‘cross-talk’ between these modifications. Mechanistically such communications occur at different levels. Firstly since a large variety of modifications occur at the same lysine residue some modifications are mutually exclusive. For example H3K27me3 and H3K27ac are mutually exclusive modifications of the same lysine residue (Lau and Cheung, 2011a). Secondly modifications of adjacent residues on the same histone often mask the functional effect of another modification. Phosphorylation of H3S10 masks H3K9me3 modifications hindering the binding to HP1 to the H3K9me3 mark (Fischle et al., 2005). Similarly H3K28Ph/H3K27me3 double modification leads to the reactivation of genes repressed by the H3K27me3 mark (Gehani et al., 2010). In fact, the methyl-phospho switch is hypothesized to act as a general switch from an inactive to an active chromatin state. Furthermore the catalytic activity of an enzyme may be compromised due to the modification of its substrate recognition site. Conversely an enzyme may be able to recognise its substrate more effectively due to the presence of an adjacent modification. Isomerisation of H3P30 affects the methylation of H3K36 by SET2 (Nelson et al., 2006) while the HAT GCN5 is able to recognise H3 far more effectively when H3S10 is phosphorylated (Clements et al., 2003). Histone modifications ‘talk’ to each other even when they are on separate histone tails, e.g. H2BK120ub is required for methylation of H3K4 and H3K79 (Whitcomb et al., 2012). Therefore chromatin/histone modifications form a complex dynamic network that interacts with a variety of different effector molecules (Turner, 2012). Within this network there is continuous ‘cross-talk’ between the covalent histone modifications thereby

specifying the chromatin environment and helping in activities such as transcriptional regulation, chromatin condensation etc.

1.2.5 Chromatin remodelling

Transcription requires the binding of RNA polymerase II and the assembly of the basal transcription machinery at promoters. Therefore, for transcription to occur it is important that promoters, enhancers and other cis-regulatory elements are accessible to regulatory proteins that bind to these elements in trans. As discussed in section 1.2.3 DNA is normally packaged into chromatin that forms a compact condensed structure. For regulatory proteins to access the DNA for transcription, chromatin needs to 'unravel'. This can be achieved by installing histone modifications that change charges on amino acid residues (see section 1.2.4). However, a more direct way of 'unravelling' the DNA is by disruption or repositioning of nucleosomes (Cairns, 2009).

The classical view of chromatin is that it consists of regularly spaced nucleosomes (bead-on-string structure) that folds up on itself to form higher order chromatin structures. Nucleosomes impede the access of regulatory proteins to DNA which is required for gene activation (Wang et al., 2011b). Nucleosome positioning pattern and its dynamic regulation allows regulatory factors to access underlying genetic information. Research has shown that regulatory elements (promoters and enhancers) are nucleosome depleted regions where regulatory factors can access the underlying DNA while transcribed regions have well positioned nucleosomal arrays (Bai and Morozov, 2010). It has been known for more than a decade that active genes are

enriched in nucleosomes that lack a H2A-H2B dimer of the nucleosome core particle (Baer and Rhodes, 1983). As the elongating RNA polymerase advances, it recruits facilitator of active transcription (FACT). This displaces one of the H2A-H2B dimer and partially disrupts the nucleosome. The H2A-H2B dimer is then replaced once the transcribing RNA polymerase II complex has passed (Reinberg and Sims, 2006). Histone H2A is also known to be replaced in active or poised gene promoters with a variant called the H2A.Z at the +1 or -1 positioned nucleosomes (Guillemette et al., 2005, Raisner et al., 2005, Zhang et al., 2005). In this context a nucleosome is disrupted to replace a canonical H2A with a variant H2A.Z, which is deposited in a replication independent manner by members of the SWR1 family of chromatin remodellers (Mizuguchi et al., 2004). The H2A.Z variant is differs from the canonical H2A in its amino-terminal tail sequence. Key amino acid residues of H2A.Z are changed and this alters its interaction with the H3/H4 tetramer (Cairns, 2009). Another variant histone is the H3.3 which is found at genes that are actively being transcribed or has been recently transcribed (Chow et al., 2005, Schwartz and Ahmad, 2005). Structurally H3.3 is not very different from the canonical H3.1, however, it has recently been shown that nucleosomes containing H3.3 variant is far less stable compared to nucleosomes containing the canonical H3.1 histone (Jin and Felsenfeld, 2007). Furthermore the stability of nucleosomes carrying H2A.Z depends on the histone H3 variant present. H2A.Z-H3.1 nucleosomes are (counter-intuitively) more stable than H2A-H3.1 nucleosome (Jin and Felsenfeld, 2007, Henikoff, 2008). However, a nucleosome consisting of H2A.Z and H3.3 histones is considered to be the least stable (Henikoff, 2008).

It is evident that histones of the nucleosome core particle are critical for chromatin structure and the level of its compaction. Histone H1 interacts with the linker DNA as well as with the nucleosome core and is crucial for maintaining higher order chromatin structure. Loss of H1 is sufficient to reduce the level of chromatin compaction and H1 is reduced in active genes compared to inactive genes (Garrard, 1991, Woodcock et al., 2006). A recent study has shown that H4K16ac and loss of histone H1 is required for the de-compaction of the 30-nm fibre *in vitro* (Robinson et al., 2008).

Chromatin remodelling is mediated by specialised chromatin remodellers that are classified into three families: ISWI, SWI/SNF and SWR1 (SWR 1 deposits the H2A.Z variant and has been discussed above). These chromatin remodellers use the energy from ATP hydrolysis to reorganise the nucleosomes by sliding/evicting or disrupting them ii (Cairns, 2009) . So far we have discussed chromatin remodelling in the context of 'unravelling' the chromatin in order to facilitate transcription. However, chromatin remodelling can do the contrary as well. Except for NURF and ISWI1B, all other members of the ISWI family of chromatin remodellers slide nucleosomes in order to create the equally spaced array of nucleosomes that is associated with chromatin compaction and transcriptional repression (Kagalwala et al., 2004). In fact, the ISW family of remodellers specifically act at regions that are devoid of active the H4K16ac marks (Corona et al., 2002). The SWI/SNF family of remodellers, unlike ISWI, is capable of both sliding and evicting nucleosomes. They function to disorganise nucleosomes and activate genes (Narlikar et al., 2002, Saha et al., 2006). The SWI/SNF complex consists of bromo domain containing proteins (SWI2/SNF2

subunit) (Awad and Hassan, 2008) and can recognise and bind to acetylated histone tails and further relax chromatin compaction through nucleosome repositioning (Narlikar et al., 2002, Cairns, 2009). So far no specific DNA sequence binding ability of SWI/SNF has been identified. However, several studies have identified different mechanisms by which the SWI/SNF complex is targeted to specific nucleosomes. SWI/SNF has been shown to interact with the glucocorticoid receptor and other steroid receptors, the basal transcription machinery, GAGA factors and certain DNA structures that target this complex to appropriate target promoters (Cairns, 2009).

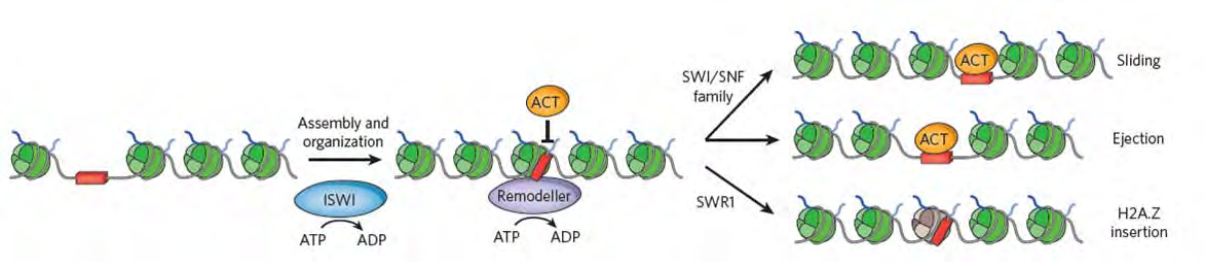


Figure 1.9: Cartoon diagram showing mechanisms of action of ISWI, SWI/SNF and SWR 1 family of chromatin remodellers (Cairns, 2009). ISWI family of chromatin remodellers slide histones while the SWI/SNF family of histone remodellers slide or eject histones. SWR 1 family of histone remodellers insert H2A.Z histone variants.

1.2.6 Active and Inactive Chromatin

In the context of assembly of basal transcription factors, histone modifications and chromatin remodelling; ‘active’ and ‘inactive’ chromatin has been referred to a number of times. It is therefore important to clearly define the scientific meaning and the basic features of ‘active’ vs. ‘inactive’ chromatin. The bulk of the human genome consists of tightly packed nucleosomes where the DNA sequence is hidden. This corresponds to mitotic chromosome or chromosomes in centromeres or telomeres. These regions of tight chromatin compaction show very little transcriptional activity and are termed ‘heterochromatin’ and are what has so far in the text been referred to as ‘inactive’ chromatin. Regions of the chromatin that is less tightly packed and have a more dispersed appearance under the electron microscope are termed ‘euchromatin’. Euchromatin shows high transcriptional activity (Lee and Young, 2000)

In *Drosophila* cells chromatin interaction map of 53 broadly selected chromatin associated proteins was generated using DamID. This DamID profile of 53 proteins showed that the genome can be segmented into five distinct groups that are defined by unique yet overlapping combinations of proteins. This study subdivided the genome into five different types of chromatin denoted as: ‘green, yellow, red, blue and black’ chromatin. ‘Green’ and ‘blue’ chromatins are inactive and are defined by HP1 and polycomb binding respectively. They identified a third kind of repressive chromatin ‘black’ chromatin that is distinct from the classical heterochromatin and is depleted in HP1, SU(VAR)3-9 and polycomb proteins. This class of chromatin is defined by a previously unknown combination of proteins. Furthermore, this study also subdivided the

euchromatin into two distinct groups ‘red’ and ‘yellow’ chromatin mainly on the basis of disparate levels of H3K36me3. Active genes within the ‘red’ chromatin exhibited a lack of the H3K36me3 mark which is a mark of active transcriptional elongation (Filion et al., 2010).

Cis-Regulatory regions of active genes are hypersensitive to DNAseI digestion

It has long been recognised that actively transcribed genes have cis-regulatory regions accessible to the transcription factors and the basal transcription machinery. This makes these regions hypersensitive to digestion with nucleases such as DNaseI (Stalder et al., 1980, Lawson et al., 1980, Smith et al., 1984). DNaseI is an endo-nuclease that cleaves DNA in a sequence independent manner (some preference to cut adjacent to pyrimidines) at phosphor-diester bonds to yield 5’ phospho-polynucleotides and free hydroxyl groups. Controlled DNaseI digestion and identification of double strand cuts by Southern blotting or high-throughput sequencing is a an important tool used to identify cis-regulatory regions of genes by way of identifying DNaseI hypersensitive sites (DHSs) (Tuan and London, 1984).

To visualise such sites chromatin is treated with increasing concentration of DNaseI enzyme *in vivo.* Following this treatment, the genomic DNA is isolated, purified and subject to restriction digestion with desired restriction enzymes. This DNA is then electrophoresed on an agarose gel and double strand cuts made by DNaseI is identified by southern blotting using probes against regions (usually cis-regulatory elements of genes) of interest (Cockerill, 2000).

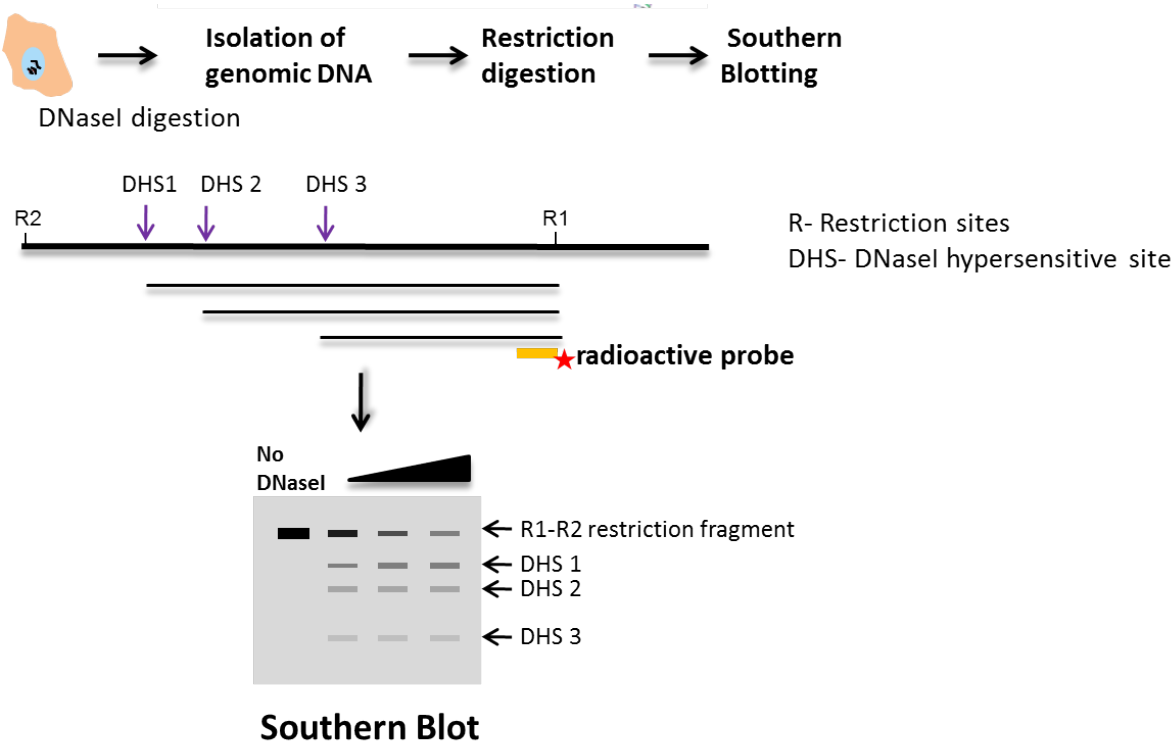


Figure 1.10: DNaseI hypersensitive site mapping. A cartoon diagram showing the method of identifying cis-regulatory elements of a gene by determining DNase I hypersensitive sites by Southern blotting.

1.2.7 Bivalent and poised chromatin

Although under most circumstances genes that have DNase I hypersensitive sites (DHSs) and have active histone modifications such as H3K4me3 are transcriptionally active, there are some exceptions where the DNA is DNase I hypersensitive and the histones carry both active H3K4me3 and inactive H3K27me3 mark (Herz et al., 2009). Such domains are termed 'bivalent domains'. Originally 'bivalent domains' were identified in mouse and human embryonic stem cells (Pan et al., 2007, Zhao et al., 2007, Bernstein et al., 2006) and later on even in haematopoietic stem and progenitor cells (Cui et al., 2009). Genes that are bivalently marked are repressed but poised for transcription.

Developmentally important transcription factors were shown to fall under this category (Herz et al., 2009). It was observed that when these stem cells/ progenitors were subjected to differentiation the bivalent marks were resolved and either the active H3K4me3 or the inactive H3K27me3 mark persisted (Cui et al., 2009, Mohn et al., 2008).

1.2.8 Role of transcription factors in regulation of transcription initiation

The initiation of transcription is regulated by the interaction of key transcription factors with response elements such as promoters and enhancers. Transcription factors are regulatory proteins that bind cis-regulatory elements of genes in trans. Transcription factors include: basal transcription factors that interact with the RNA polymerase II complex to from the basal transcription machinery (BTM) (see section 1.2.2), transcription factors that recognise short DNA sequence response elements (TF binding sites) and orchestrates the regulation of transcription via changes in chromatin and recruitment of BTM and a third class of factors termed as co-activator/co-repressors. Co-activator/co-repressors themselves do not bind the DNA but mediate protein-protein interactions with transcription factors and the BTM or chromatin modifying enzymes and chromatin re-modellers (Lee and Young, 2000).

Recognition of short DNA sequence response elements by transcription factors (TFs) is the key to transcriptional initiation (Georges et al., 2010, Li et al., 2008). These TF binding sites or response elements most often are present in gene promoters and enhancers (Gondor and Ohlsson, 2009). These TF binding sites generally occur in clusters. Transcription factors along with their co-activators

bind these response elements and form very large protein assemblies. The current paradigm entails that a transcription factor bears a special DNA binding domain that recognises a specific set of DNA sequences (Pan et al., 2010). To date several DNA binding domains in transcription factors have been identified such as: helix-loop-helix, homeobox, helix-turn-helix and leucine zipper domain (Lee and Young, 2000).

However, this current paradigm completely overlooks the complexity added by posttranslational modifications of transcription factors and the co-activators/repressors. So far only a subset of all binding sites for a given transcription factor has been defined experimentally. Computer algorithms have therefore been designed to scan the genome and predict TF binding elements. These algorithms typically construct weighted binding matrices for transcription factors based on experimentally defined binding consensus sequences (Erill and O'Neill, 2009, Lyakhov et al., 2008, Mahony and Benos, 2007, Mahony et al., 2007, Veprintsev and Fersht, 2008). Pan *et al* has reviewed the current evolving mechanisms of transcription factor-binding sequence interaction and selection (Pan et al., 2007).

So far from our discussion of eukaryotic transcription it is clear that chromatin is unravelled via the dynamic interplay between transcription factors, chromatin remodellers/modifiers and the basal transcription machinery. However, the question arises that what comes first. Several studies have shown FOXA and GATA family of transcription factors to possess this ability to bind to chromatin prior to its activation and initiating the process of chromatin and transcriptional

activation (Smale, 2010). These factors are termed ‘pioneer factors’ and in a way serve a crucial function in certain circumstances (Zaret and Carroll, 2011).

1.2.9 Signalling communicates with chromatin to regulate transcription

The activation of a gene is a sequential process and is under the control of transcription factors and the epigenetic regulatory machinery which in turn, receive instructions from outside signals. The extracellular/environmental cues that cells receive from its surroundings are translated into meaningful information as a cascade of changes within the cytoplasm via signal transduction. These changes are finally translated to the nucleus by transcription factors which recruit chromatin modifying enzymes that modulate the local chromatin thereby activating or repressing transcription of a gene. Genes, involved in development and differentiation, are largely regulated via this mechanism (Mohammad and Baylin, 2010).

In sections 1.1.4 and 1.1.5 role of Wnt, NOTCH, TGFβ and cytokine/growth factor signalling in haematopoiesis has been discussed (sections 1.1.4 and 1.1.5). Similar signalling pathways are also involved in maintenance and differentiation of embryonic stem cells (ESCs) (Marson et al., 2008, Xu et al., 2008, Bray, 2006, Kopan and Ilagan, 2009) and function by affecting key downstream transcription factors and chromatin modifying enzymes. A large number of chromatin modifying enzymes (histone methyl-transferases, DNA methyl-transferases, histone acetyl-transferases, histone de-acetylases, histone de-methylases) that establish such heritable epigenetic states are regulated by

signal transduction (Richly et al., 2010). Transcription factors are also under the control of signalling molecules.

Hormone receptors, such as those that bind retinoic acid or oestrogen (RAR and ER) act as DNA-binding transcription factors in the nucleus (Sun et al., 1998). Upon hormone stimulation they translocate to the nucleus and function as DNA-binding transcription factors that mediate activation/repression of target genes . Activation of mouse mammary tumour virus (MMTV) promoter occurs through glucocorticoid and progesterone receptors or through progestin activated ERK signalling (Vicent et al., 2010). The activation of MMTV and other progesterone receptor target genes is coupled with the activity of HATs (Vicent et al., 2010). Signal transducer and activator of transcription (STAT) proteins are another class of transcription factors that translates cellular signals into changes in transcriptional state of target genes within the nucleus. STAT4 has been shown to promote active chromatin, while STAT6 are known to associate with transcriptionally inactive chromatin (Wei et al., 2010, Korzus et al., 1998). STAT3 provides survival signal in cells by affecting genes involved in apoptosis. In ES cells STAT3 is activated by LIF signalling and in turn it activates the *Klf* and *Sox2* genes which are important in the maintenance of pluripotency in the stem cells (Niwa et al., 2009). NOTCH effector RBP-J interacts with KDM5a de-methylates H3K4me3 and mediates repression of target genes (Liefke et al., 2010). TGFβ signalling influences SMAD proteins that activates or represses target genes in a cell-context dependent manner (Massague et al., 2005).

Signalling also impacts the balance between polycomb complexes and the opposing trithorax group (reviewed in detail in section 1.3). It has been

established that posttranslational modification of both polycomb and trithorax proteins mediate its association/dissociation to the chromatin at target loci. The signalling mediated modification of polycomb group proteins is discussed in greater detail in section 1.3. During muscle differentiation trithorax group associated Ash2L methyl-transferase mediating tri-methylation of H3K4 are recruited to target genes by Mef2d. This interaction between Ash2L and Mef2d is mediated by the phosphorylation of Mef2d by P38 kinase which is a component of the mitogen activated protein (MAP) kinase pathway (Rampalli et al., 2007).

There are several examples where signalling molecules directly affect histones in order to alter gene expression. Phosphorylation of H3S10 is mediated by signalling pathways such as ERK2, RSK2, MSK1, JNK or P38 (He et al., 2003, Zhong et al., 2000, Zhong et al., 2001a, Zhong et al., 2001b, Soloaga et al., 2003). The H3 serine 10 phosphorylation by Aurora B kinase is of prime importance in activation of genes that are methylated at H3K9 and are bound by heterochromatin protein 1 (HP1) (Fischle et al., 2005). Recently it has been shown by ChIP-sequencing that during differentiation of stem cells into neurons, JNK binds to a large set of active promoters (Tiwari et al., 2012). The same study identified H3S10 to be a direct substrate of JNK and showed that promoters bound by JNK carried phosphorylated H3S10. Small molecule mediated inhibition of JNK reduced the H3S10 phosphorylation at the target genes (Tiwari et al., 2012). Similarly, other studies have shown that MSK1 (mitogen and stress activated protein kinase-1) phosphorylates H3S28 that

mediates de-repression of polycomb target genes (Gehani et al., 2010, Lau and Cheung, 2011a).

Signalling molecules also directly modify transcription factors. c-Jun is a member of the AP1 complex and is an immediate early response gene that is activated in response to mitogen, stress signals and differentiation signals. Jun kinase (JNK) is known to phosphorylate the AP1 complex (Baker et al., 1992). Plasma cell differentiation is mediated by BLIMP-1 (B-lymphocyte induced maturation protein-1) upon stimulation with antigen. In mature B-cells, prior to differentiation into plasma cells, *BLIMP-1* is repressed by PAX5. It has recently been shown that upon antigen stimulation, B- cell receptor mediated activation of ERK1/2, phosphorylates PAX5. This phosphorylated PAX5 is unable to repress *BLIMP-1* leading to the activation of *BLIMP-1* which drives plasma cell differentiation via concomitant repression of PAX5 (Yasuda et al., 2012).

The above studies therefore make it evident that signalling ‘talks’ to transcription factors and also to chromatin in order to activate or repress genes. Signalling pathways communicate the ‘environmental cues’ from the surroundings to within the cell and alter gene regulation. This communication of extracellular information into the nucleus is of prime importance in development and cell fate choice. Even though the decision of an individual cell to activate/repress a particular gene seems to be ‘stochastic’, signalling appears to be the immediate cause for a cascade of cytoplasmic and nuclear events that lead to the activation/repression of a gene

1.2.10 Repression of eukaryotic transcription

For correct development to occur in a timely manner genes need to be activated but genes not required in a specific lineage need to be repressed. Therefore the mechanisms governing the repression of a gene is just as important as the mechanisms governing its activation.

Repression of transcription involves targeting of the repression machinery to the DNA sequence/gene that is to be inactivated; this is followed by chromatin changes and then the maintenance of the repressed chromatin state through cell divisions to propagate the silenced state through generations (Beisel and Paro, 2011). Just like activator complexes, repressor complexes bind DNA sequence elements: ‘silencers’ and ‘promoters’ to mediate long range silencing of appropriate genes. There are several co-repressor proteins that are recruited to specific DNA sequences through protein-protein interaction with DNA-binding transcription factors. Groucho/Tup1 and Sir family of co-repressors are important repressor proteins that mediate long range gene silencing (Courey and Jia, 2001). Groucho/Tup1 family of co-repressors functionally interact with histone de-acetylases that establish a repressive chromatin environment (see section 1.2.4) (Chen et al., 1999, Choi et al., 1999, Brantjes et al., 2001, Watson et al., 2000, Wu et al., 2001). There are a number of co-repressors with histone de-acetylases activity. HDAC1 has been extensively characterised (Struhl, 1998). It is a part of repressive complexes such as Sin3A and NuRD (Knoepfler and Eisenman, 1999) and has also been shown to functionally interact with Groucho co-repressors. The SIR family of co-repressors also have histone de-acetylases activity and these proteins mediate long range repression

(Lustig, 1998, Grunstein, 1998). Several studies that show that co-repressors such as Groucho function not only by means of their HDAC activity but also via their interaction with the basal transcription machinery (Yu et al., 2001, Zaman et al., 2001). Apart from the recruitment of co-repressors by transcription factors, that directly mediate histone de-acetylation or talk to the BTM, there are additional mechanisms of gene silencing involving tri-methylation of the H3K9 and H3K27 residues respectively.

SET domain containing SUV39H1 mediates the tri-methylation of the H3K9 residue. This modification is identified and bound to by a ‘chromo-domain’ containing protein called heterochromatin protein-1 (HP1). Once bound this further recruits more SUV39H1 and mediates the propagation of the H3K9me3 mark leading to the formation of heterochromatin (Lomberk et al., 2006). SUV39H1and HP1 both recruits DNA methyl-transferases (Lomberk et al., 2006) and H3K9me3 mark is most often succeeded by DNA methylation. Therefore once HP1 mediated heterochromatin is established the locus is then methylated at the DNA level and is repressed for long-term.

Another parallel mechanism of gene repression involves polycomb repressive complexes. Similar to the HP1 mediated repression; polycomb repression requires a ‘chromodomain’ mediated recognition of H3K27me3 residue. It is also believed that following polycomb mediated repression; HP1 mediated heterochromatin formation takes over which in turn gives way to DNA methylation. Therefore there exists a hierarchical or step-wise recruitment of repression machineries for the silencing of a locus. In the next section we shall discuss polycomb repressive complexes; their mechanisms of recruitment to

target genes and mechanism of polycomb-mediated gene repression in greater detail (see section 1.3).

1.3 Polycomb complexes

The polycomb group of proteins (hereafter referred to as PcG proteins) were initially identified in *Drosophila melanogaster.* These proteins play an important part in regulating the homeotic (*Hox*) gene in *Drososphila* (Lewis, 1978). Mutations in the genes encoding PcG proteins lead to abnormal body patterning of flies due to aberrant expression of *Hox* genes in incorrect spatial territories (Struhl, 1981, Nusslein-Volhard et al., 1985, Zink and Paro, 1989). ChIP-sequencing experiments in the years that followed have identified hundreds of other genes that are regulated by PcG proteins both in flies (Schwartz et al., 2006) and mammals (Boyer et al., 2006, Lee et al., 2006). PcG protein targets include key transcription factors and signalling components such as the HOX cluster WNT, PAX and FOXO families important in development (Beisel and Paro, 2011).

1.3.1 Polycomb repressive complexes

Research over the last decade has identified two principal classes of repressive complexes formed by these PcG proteins: PRC1 (polycomb repressive complex 1) and PRC 2 (polycomb repressive complex 2). At its core the PRC2 complex consists of the 'SET domain' containing catalytic subunit called Enhancer of *Zeste* in flies and EZH2 in humans. To augment the catalytic activity of EZH2 another subunit called the SUZ12 (Suppressor of Zeste) along with ESC (extra sex comb in *Drsosphila*) or EED (embryonic ectoderm development in

mammals) is required. Figure 1.11 shows the general composition of the PRC1 and PRC2 complexes. The PRC1 complex is highly diverse in its composition. Multiple paralogues of each PcG genes exist allowing different combinations of subunits for the formation of the PRC1 complex. Broadly, in humans PRC1 consists of an ubiquitin ligase subunit called RING1B/RING1, a chromo-domain containing CBX subunit (with many paralogues) that recognises the H3K27me3 residue, BMI1 (paralogues include Mel18 and a number of other PCGF proteins) and PH1 subunits that augment the catalytic activity of RING1B respectively (Simon and Kingston, 2009, Eckert et al., 2011).

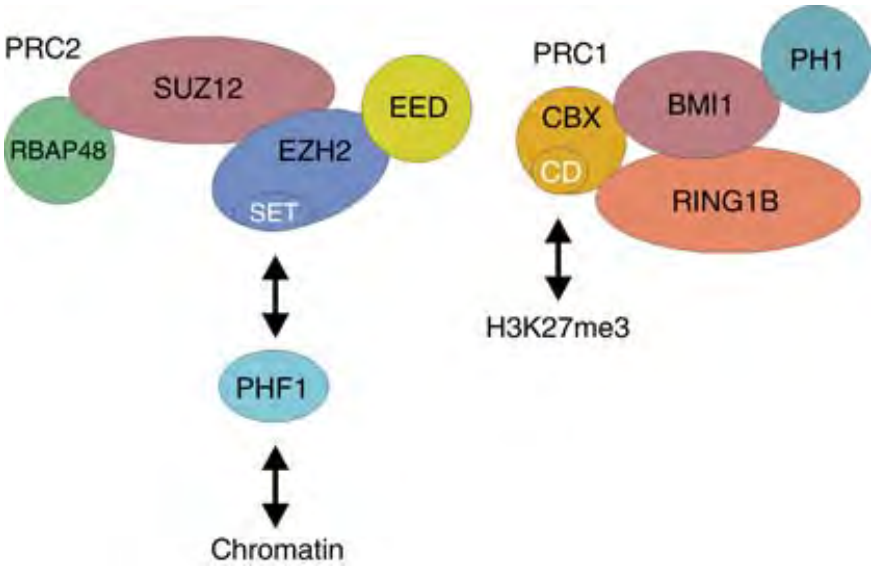


Figure 1.11: Cartoon illustrating the general composition of PRC2 and PRC1 complexes (Eckert et al., 2011). Polycomb proteins form two repressive complexes. PRC2 mediates H3K27me3 via the EZH2 domain which is read and bound by the PRC1 complex which in turn allegedly blocks transcriptional elongation.

1.3.2 Recruitment of PRC2 to target genes

In *D*. *melanogaster* polycomb repressive complexes are recruited at specific DNA sequences called polycomb response element (PRE) (Muller and Kassis, 2006, Ringrose and Paro, 2007) by pleiohomeotic (PHO) (Mohd SArip, A, mol cell boil 2002) and the synergistic action of GAGA factors (Mahmoudi, t nucleic acids res, 2003). However in mammals an equivalent of a *Drosophila* PRE has so far not been identified. Genome wide ChIP sequencing analysis has identified a large number of PcG target genes (Schwartz et al., 2010, Schwartz et al., 2006, Bracken et al., 2006). Computational motif analysis at these target sites, however, has failed to reveal an enriched DNA motif that could be defined as the mammalian 'PRE'. However, PcG complexes preferentially bind to un-methylated CpG island promoters (Ku et al., 2008). Two recent studies have reported elements functioning as mammalian PREs (Sing et al., 2009, Woo et al., 2010). One has identified a 3-kb long element in the mouse *MafB* gene that contains a GAGA-factor binding-site and two palindromic YY1 binding-sites. This element is highly similar to the Drosophila PRE (Sing et al., 2009). YY1 has been shown to function as a PcG protein in Drosphila in vivo (Atchison et al., 2003). In the second study a 1.8-kb element has been identified in the human *HOXD11* and *HOXD12* genes (called D11.12). This contains a cluster of four YY1 binding sites (Woo et al., 2010). A number of proteins have been implicated in targeting PRCs to its target genes in mammals. Recently a number of simultaneous studies identified JARID2 to associate with PRC2 at PcG target genes in mouse ES cells (Peng et al., 2009, Landeira et al., 2010, Li et al., 2010, Pasini et al., 2010, Shen et al., 2009). The AT-rich DNA sequence recognised by JARID2 is implicated in targeting PRC2 complex. Apart from this

several studies have identified long and short non-coding RNAs that recruit PcG proteins. One of the best known examples of this is in the inactivation of X chromosome. The long nc-RNA 'Xist' is required for X-inactivation (Brown et al., 1992, Zhao et al., 2008). Xist has been shown to co-immuno-precipitate with the PRC2 component EZH2 (Pandey et al., 2008, Maenner et al., 2010). PRC1 can also be directed to inactivate X-chromosome by CBX7 protein in a RNA dependent manner (Yap et al., 2010). Furthermore, more recently transcription of short stem-and-loop non-coding RNA has been shown to interact with SUZ12 subunit of the PRC2 complex and mediate repression of target genes in a manner similar to that of Xist (Kanhere et al., 2010).

1.3.3 Mechanism of transcriptional repression mediated by polycomb repressive complexes

The current accepted mechanism of PRC mediated gene silencing involves EZH2 mediated tri-methylation of H3K27 residue. This modification serves as a docking site for the PRC1 complex (Simon and Kingston, 2009). This was shown by the knockdown of the H3K27me3 de-methylase 'UTX' in human cells which resulted in increase of the H3K27me3 mark and a concomitant increase in PRC1 deposition at target genes (Lee et al., 2007). In another study, the viral methyl-transferase v-SET was used to tri-methylate H3K27 replacing PCR2. While loss of PRC2 led to loss of PRC1 targeting, v-SET mediated methylation of H3K27 restored PRC1 recruitment at target promoters (Mujtaba et al., 2008). The chromodomain containing CBX protein (Polycomb in *Drosophila*) is capable of recognising the H3K27me3 mark and has affinity to bind this mark (Kaustov et al., 2011). However, there are a large number of genomic loci that recruit the

PRC2 complex but not the PRC1 complex (Min et al., 2003). There are also a few examples of gene loci that show PRC1 accumulation independent of PRC2 or H3K27me3 (Schoeftner et al., 2006, Yu et al., 2012, Pasini et al., 2007). Furthermore, although in *D. melanogaster* PRC1 accumulation peaks at PREs (Polycomb response elements –see section 1.3.2) the H3K27me3 mark is more widespread (Schwartz et al., 2006). Moreover, recently it has been shown in mouse embryonic stem cells that PRC1 is recruited by the H3K4me3 de-methylase RBP2 and mediates gene repression independent of H3K27me3 or PRC2 recruitment (Simon and Kingston, 2009, Tavares et al., 2012).

Once recruited, the PRC1 component RING1B mediates mono-ubiquitination of the H2AK119 residue (Cao et al., 2005). However, the mechanism of repression by PRC1/H2AK119ub1 is unclear. Possible repression mechanisms include blocking of recruitment of key transcription factors at promoters and enhancers of target genes, blocking of recruitment of RNA polymerase II and the basal transcription machinery or blocking the initiation or elongation of transcription.

Many studies indicate that transcription factors and PRC1 can simultaneously bind their target sites (Bracken et al., 2006, Lee et al., 2006, Breiling et al., 2001). Therefore the first scenario of blocking TF recruitment to promoters and enhancers is unlikely. RNA polymerase II too has been shown to be recruited to polycomb target sites (Bracken et al., 2006). Therefore the option that PRC1 blocks RNA polymerase II recruitment can also be ruled out. However, PRC1 has been shown to interact with the basal transcription machinery (Breiling et al., 2001, Saurin et al., 2001). A recent study using immobilized template recruitment experiments has shown that PRC1 disassembles the RNA

polymerase II pre-initiation complex by interfering with mediator proteins. However, it does not target TBP or TFIID (Lehmann et al., 2012).

ChIP studies in mouse ES cells have identifies genes that are marked by both the activating H3K4me3 and the repressing H3K27me3 marks. Such genes are called ‘bivalent genes’ (see section 1.2.7) as they recruit RNA polymerase II but it remains paused (Pol II –phospho Serine 5) (Herz et al., 2009). These genes are also marked by the H2AK119Ub1 mark (Stock et al., 2007). Depletion of RING1/ RING1B led to the depletion of the H2AK119ub1 mark followed by conversion of the paused RNA polymerase II (phosphorylated at serine 5) into elongating RNA polymerase II (phosphorylated at serine 2) and the depression of these genes (Stock et al., 2007). This led to the hypothesis that PRC1 mediated H2AK119ub1 blocks transcriptional elongation. Studies in Drosophila show classic PcG target genes contain paused polymerase II and hence support this notion (Srinivasan et al., 2008, Petruk et al., 2006). However, PRC1 component RING1B has also been shown to repress target genes via chromatin compaction independent of the H2AK119ub1 mark (Eskeland et al., 2010). It is difficult to dissect the repressing mechanism of PRC1; it may be possible that the large numbers of PcG target genes are silenced in more than one way.

1.3.4 Signalling ‘talks’ to polycomb

As outlined in section 1.3 polycomb repressive complexes target and repress genes encoding transcription factors and signalling components (Beisel and Paro, 2011) important in differentiation of stem cells and development. These genes are re-activated in response to developmental signals. Polycomb is

therefore heavily regulated by signalling. Polycomb proteins themselves undergo post-translational modifications (ubiquitination, sumoylation and phosphorylation at various residues on different sub-units) down-stream of signalling pathways which regulate their association and dissociation from the chromatin and their repressive activity (Niessen et al., 2009).

Signalling mediated phosphorylation of PcG proteins affects sub-cellular localisation, interaction within protein complexes, enzymatic activity and association/dissociation to chromatin of PcG proteins (Niessen et al., 2009). It has been shown that the PRC1 component BMI1 gets phosphorylated in a cell cycle dependent manner and its phosphorylation status inversely correlates with its chromatin association. At G1/S phase BMI1 is hypo-phosphorylated and is bound to the chromatin, whereas in G2/M phase BMI1 is hyper-phosphorylated and dissociates from chromatin (Voncken et al., 1999). MEL18 (PCGF6) has also been shown to be preferentially phosphorylated in the G2/M phase and is a substrate of CDK7 (Akasaka et al., 2002). The PRC2 component EZH2 also gets phosphorylated in a cell-cycle dependent manner. A study from Danny Reinberg's lab shows that EZH2 gets phosphorylated at threonine 345 and 487 by cyclin dependent kinase -1 (CDK1) and this facilitates its association with non-coding RNAs HOTAIR and Xist both of which have been implicated in recruitment of PRC2 to HOX genes and X-chromosome, respectively (Kaneko et al., 2010). Another study showed phosphorylation of EZH2 at threonine 350 by CDK1 and CDK 2 (Chen et al., 2010). This study also showed that over-expression of CDK1 and CDK2 repressed the EZH2 target gene *Hoxa9*, while depletion of CDK1 and CDK2 led to the expression of *Hoxa9 (Chen et al.,*

2010). In another study Wei *et al* reported that phosphorylation of EZH2 at threonine 345 inhibited the methyl-transferase activity of EZH2 (Wei et al., 2011) due to disruption of EZH2 interaction with SUZ12 and EED. However, this study contradicts results from Kaneko *et al* and Chan *et al* (Caretti et al., 2011). Extracellular signals such as AKT have been shown to phosphorylate serine 21 residue of EZH2. This modification reduces EZH2's affinity for H3 therefore impedes EZH2's methyl-transferase activity (Cha et al., 2005). More recently P38α has been discovered to phosphorylate EZH2 at Thr372 which mediates *Pax7* repression (Palacios et al., 2010). EZH2 in fact is phosphorylated at a large number of residues by diverse signals and the functional consequences of these modifications are largely cell context dependent. Carreti et has reviewed the diversity and functional consequences of the 'Phospho-EZH ion' comprehensively (Caretti et al., 2011)(see figure 1.12). Interestingly it was shown that the opposing trithorax group of proteins are also phosphorylated in a cell-cycle dependent manner (Rampalli et al., 2007). This indicates that phosphorylation plays an important part in regulating the trithorax-polycomb balance at developmental genes.

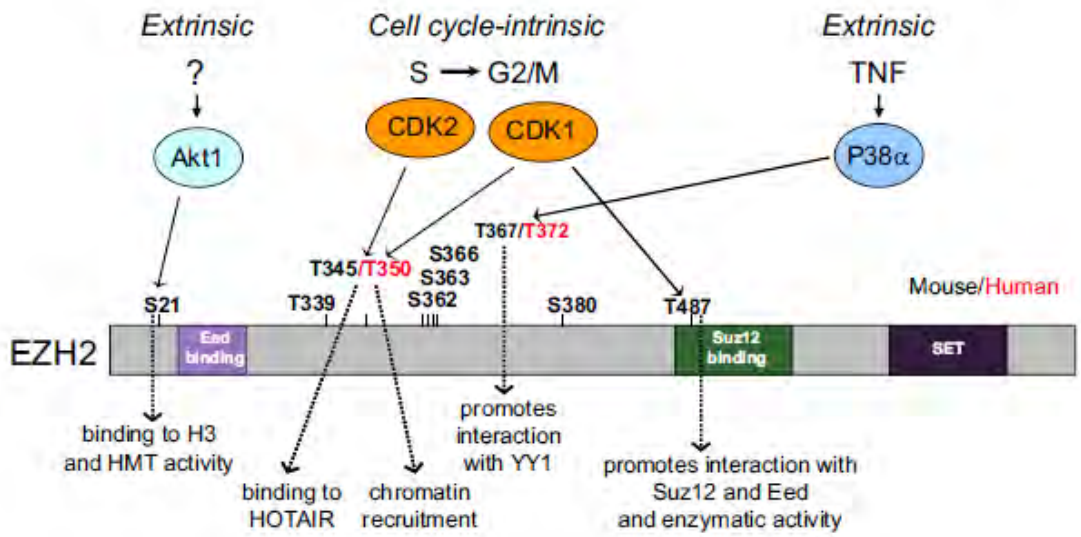


Figure 1.12: Cartoon illustrating the various signalling pathways mediating phosphorylation of EZH2 protein (Caretti et al., 2011). The SET domain containing EZH2 protein is phosphorylated at a number of residues as indicated by several cell-cycle dependent kinases and MAP kinases. This phosphorylation of EZH2 regulates the binding of EZH2 to its target genes.

1.3.5 Counteraction of polycomb repression during development

As polycomb represses genes that are often important developmental regulators, repression needs to be counteracted in a timely manner to allow differentiation and development to occur. A large proportion of modifications (phosphorylation in particular) of PcG proteins is mediated by various signalling pathways leading to the de-repression of their target genes (see section 1.3.4). This ensures that the developmental genes are de-repressed only in response to an extracellular signal; cell-cycle dependent genes are de-repressed in a cyclic order while immediate early response genes are briefly de-repressed in response to stress (Sawarkar and Paro, 2010).

Several studies have identified mechanisms by way of which polycomb repression is counteracted. In myoblasts several genes are under polycomb repression and are de-repressed during myogenesis. The homeo-box TF Six4 along with along with the histone de-methylase UTX binds these genes and mediates H3K27me3 de-methylation. This is followed by binding of MyoD (master regulator of muscle differentiation and development) and Mef2d. The final activation signal is provided by P38 MAPkinase that phosphorylates Mef2d which then recruits the trithorax component Ash2L which is part of the MLL H3K4 methyl-transferase complex. H3K4me3 counteracts polycomb mediated repression and leads to the activation of PcG target genes (see section 1.2.9) (Rampalli et al., 2007). Stress-induced signalling mediated by MSK1 which is downstream of MAPkinase signalling directly phosphorylates H3Serine 28 residue that masks the H3K27me3 mark. This double mark is not recognised by PRC complexes which fail to bind target sequences. This leads to the de-repression of immediate early response genes (Gehani et al., 2010). In a parallel study they showed that H3S28phosphorylation actually leads to the removal of H3K27me3 mark which gets replaced by the opposing H3K27ac mark leading to the de-repression of immediate early genes repressed by polycomb (Lau and Cheung, 2011a). In mouse ES cells NODAL signalling activates SMAD2/3 which then recruits JMJD3 to *Nodal* and *Brachury* loci where JMJD3 de-methylates H3K27me3 mark allowing the activation of *Nodal* and *Brachury* (Dahle et al., 2010). Signalling also mediates direct phosphorylation of PcG proteins (see section 1.3.4). As discussed above phosphorylation of EZH2 dictates it association and dissociation from the chromatin. JNK, MEK and P38 MAPkinases converge at a downstream kinase

called MAPKAP3 (3pK) (Ludwig et al., 1996). This has been shown to phosphorylate BMI1 mediating its dissociation from the chromatin (Voncken et al., 2005). In *Drosophila* activated JNK signalling has been shown to transcriptionally down-regulate PcG proteins leading to de-repression of target genes (Lee et al., 2005). Furthermore, senescence inducing stress signals, NFκB dependent stress signals, and signals from the IL4/STAT6 pathway induces transcriptional up-regulation of the histone de-methylase *JmjD3*. This leads to increased JMJD3 occupancy at target genes, followed by their de-repression (Agger et al., 2009, Barradas et al., 2009). Therefore a large number of developmental signals may contribute to the de-repression of polycomb target genes. This is an active field of research and new mechanisms are being discovered constantly.

1.3.6 Role of polycomb group proteins in haematopoiesis

PcG proteins repress a large number of genes in embryonic stem cells that are important for development. Timely de-repression of these genes in response to various developmental signals allows proper development (sections 1.3.1, 1.3.4 and 1.3.5). In haematopoiesis PcG proteins play an important role in self-renewal and differentiation of HSCs (Oguro and Iwama, 2007, Konuma et al., 2010). Like ES cells, also HSCs have been shown to contain polycomb target genes with bivalent chromatin marks (Cui et al., 2009, Weishaupt et al., 2010). Several loss and gain of function studies have dissected the role of polycomb in haematopoiesis. PRC1 genes have been shown to be highly expressed in the LSK cell population (Osawa et al., 1996). Expression of PRC1 genes positively correlate with the self-renewal potential of HSCs (Iwama et al., 2004). Loss of

BMI1 in mice shows no defect in embryonic haematopoiesis but slowly the mice develop pancytopenia and the bone marrow is replaced with adipocytes. BMI1$^{-/-}$ HSCs show defects in long-term self-renewal and colony forming capacity (van der Lugt et al., 1994, Oguro et al., 2006). Loss of Ring1b on the other hand causes hypo-cellular bone marrow with hyper-proliferating immature cells. PRC2 complex proteins are also highly expressed in HSCs (Cales et al., 2008). Heterozygocity for Eed null allele causes myelo-proliferative and lympho-proliferative disorders in mice (Lessard et al., 1999). Loss of EZH1in mice causes no HSC defect but loss of EZH2 in mice causes a block in B cell development with impaired heavy chain re-arrangement and T cell development stops at the double negative stage (Su et al., 2005, Su et al., 2003). Forced expression of BMI1 leads to marked expansion of HSC numbers *ex vivo* and increased repopulating capacity *in vivo* (Iwama et al., 2004) and over expression of EZH2 preserves the long-term repopulating potential of HSCs in serial transplantation assays (Kamminga et al., 2006). Taken together, loss and gain of function experiments establish that polycomb proteins play a crucial role in the maintenance of HSC self-renewal and pluripotency.

1.4 PAX5

PAX5, also known as the B-cell specific activator protein is a member of the PAX family of transcription factors (Cobaleda et al., 2007). Expression of PAX5 within the haematopoietic system is restricted to the B-lineage. It is expressed from the pro-B cell stage up to mature B cell stage and then down-regulated in terminally differentiated plasma cells (Cobaleda et al., 2007). The defining feature of the PAX5 family of proteins is the paired-box DNA binding domain (Czerny et al., 1993). Like other PAX family transcription factors, PAX5 has an N-terminal paired box DNA-binding domain, a partial homeo-domain that interacts with TBP and other members of the basal transcription machinery as well as a C-terminal trans-activation domain that interacts with other proteins to determine PAX5's transcriptional regulatory activity (activator/repressor) in a context dependent manner (Eberhard and Busslinger, 1999) (Figure 1.12 and see section 1.4.1). The trans-activatoion domain can either interact with co-activators such as histone acetyl-transferases or co-repressors such Groucho, depending on the genomic context (Eberhard and Busslinger, 1999, Cobaleda et al., 2007).

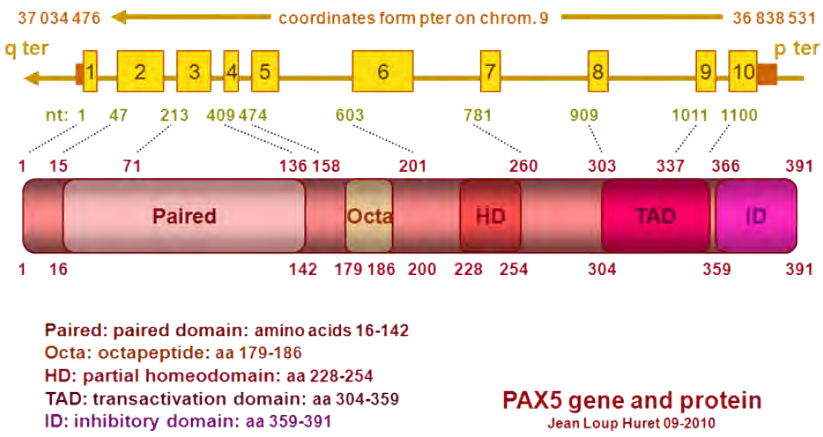


Figure 1.13: A cartoon diagram of the PAX5 protein. The figures shows the ten exons interspersed by introns of the *PAX5* gene and the PAX5 protein with its paired box DNA binding domain and the trans-activation domain (from Atlas of genetics and cytogenetics in oncology and haematology)

1.4.1 Role of PAX5 in B-cell commitment

The entry of committed lymphoid progenitors into the B-lineage is determined by three key transcription factors: E2A, EBF and PAX5 (Medvedovic et al., 2011, Busslinger, 2004) (see section 1.1.8). The role of PAX5 in B-lymphopoiesis was established by studying *Pax5* knockout mice. In the bone marrow of adult *Pax5*$^{-/-}$ null mice B-lymphopoiesis proceeds only up to pro-B-cell stage(Urbanek et al., 1994, Nutt et al., 1997). These *Pax5* null pro-B cells express E2A and EBF and can be propagated in culture in the presence of IL7 (Nutt et al., 1997). However, they are not committed to the B-lineage (Nutt et al., 1999). This established PAX5 and not E2A or EBF as the master regulator of B-cell commitment. On substituting IL7 with appropriate growth factors *Pax5*$^{-/-}$ pro-B cells gave rise to macrophages, granulocytes, osteoclasts and natural killer cells (Nutt et al., 1997). Upon transplantation these *Pax5*$^{-/-}$ pro-B cells home to the bone marrow and differentiate to every lineage other than B cells (Schaniel

et al., 2002b, Schaniel et al., 2002a). Therefore these *Pax5* null pro-B cells were not committed to the B-lineage and retained a latent multi-lineage potential. They could only differentiate to mature B-cell upon retroviral restoration of *Pax5* expression (Rolink et al., 1999, Rolink et al., 2002). PAX5 is therefore indispensable for the commitment of lymphoid progenitors to B-cells.

PAX5 tailors the gene expression pattern of uncommitted progenitors according to the requirement of B-cell lineage. It up-regulates genes that are needed for B-lineage commitment and down-regulates genes that are B-lineage inappropriate (Cobaleda et al., 2007). A large number of B-cell specific genes have been reported to be up-regulated by PAX5 (Busslinger, 2004) such as *CD79a* (signal transducing chain Igα), *CD19* and *CD21* (co-stimulatory receptors), *CD72* (inhibitory co-receptor) and *Blnk* (Fitzsimmons et al., 1996, Nutt et al., 1997, Nutt et al., 1998, Horcher et al., 2001, Kozmik et al., 1992, Walter et al., 2008). Furthermore PAX5 is known to activate the *Lef1* gene and maintain *Ebf1* expression (Roessler et al., 2007). cDNA microarray analysis has identified a large number of PAX5 target genes from which a major percentage are up-regulated by PAX5. These genes include secondary transcription factor genes (*Irf8*, *SpiB*, *IKZf3*) that reinforce the B-cell gene expression program (Pridans et al., 2008). Recently PAX5 has been shown to maintain two distinct gene expression patterns in early and late B-lymphopoiesis (Revilla et al., 2012).

The developmental plasticity exhibited by *Pax5*$^{-/-}$ pro-B cells indicates that PAX5 not only up-regulates B-cell specific genes but also down-regulates B-lineage inappropriate genes. PAX5 is known to down-regulate the macrophage colony

stimulating factor-1 receptor gee (*Csf1r*) and *Notch 1* gene required for macrophage development and T-cell development (Tagoh et al., 2006b, Nutt et al., 1999, Souabni et al., 2002). A comprehensive picture of PAX5 repressed genes appeared from the micro-array analysis that identified 110 such genes (Delogu et al., 2006). A large number of these genes are cell surface receptors and intracellular transducers involved in signalling of other haematopoietic lineages, for example the genes encoding FLT3 receptor, the SCA1 protein, the RAMP1 subunit of CGRP receptor in addition to *Csf1r* and *Notch1* (Holmes et al., 2008). Recently, a study by McManus *et al* has elucidated the mechanism of PAX5 mediated up-regulation and down-regulation of its target genes. They identified PAX5 target genes by streptavidin ChIP-chip analysis in pro-B-cells expressing biotinylated PAX5. Using PAX5-deficient and wild type pro-B cells they measured histone modifications at target loci and discovered that PAX5 induces active chromatin at up-regulated targets and removes active chromatin at repressed targets. PAX5 directly recruits chromatin remodellers, histone modifying enzymes and the basal transcription machinery to its target genes (McManus et al., 2011).

1.4.2 Regulation of murine *Pax5*

PAX5 is expressed from a 190 kb locus containing 10 exons and intervening introns (figures 1.13 and 1.14). It is transcribed from two distinct promoters (Busslinger et al., 1996) upstream of exons 1A and 1B. Expression of *Pax5* has been reported in the mid-hindbrain boundary of mid-gestation embryos of mouse in addition to B-lymphocytes (Adams et al., 1992). The expression in this developing brain region is driven from the proximal promoter while in B cells

both the distal and the proximal promoters are active (Busslinger et al., 1996). A study from Busslinger's laboratory provides great insight into the mechanisms of activation and regulation of the *Pax5* gene in mice. They identified a novel regulatory element in the fifth intron of the gene through deletion analysis and characterized this regulatory element as a B-cell specific intronic enhancer for *Pax5.* This enhancer region contains a DNase I hypersensitive region (see section 1.2.6) with four individual hypersensitive sites (HS A-D). HS A and HS B were found to be B cell specific while HS-C was present in thymocytes in addition to B cells. This enhancer region was found to contain functional binding sites for transcription factors PU.1, IRF8, IRF4 and NF-κB. They also characterized the promoter region of the gene and identified 8 hypersensitive sites (HS1-8). HS 4 and HS 8 coincide with the 5' end of exons 1A and 1B respectively and are the minimal promoter elements of the gene. HS 1-3 lie upstream of exon 1A, HS 6-7 fall within the intron between exons 1A and 1B (Figure 1.13). EBF1 that was previously implicated to be an upstream regulator of *Pax5* was shown to strongly bind to HS 7 in the promoter region. They also showed that the promoter region remained repressed by polycomb protein complexes prior to pro-B- cell stage in early lymphopoiesis and then gets activated through chromatin remodelling by EBF1 binding (Decker et al., 2009).

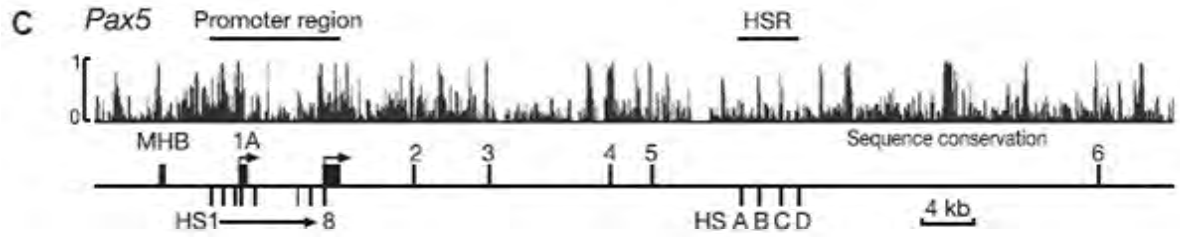


Figure 1.14: Schematic diagram showing the regulatory regions of the mouse *pax5* locus (Decker et al., 2009). The figure shows mammalian sequence conservation at the *Pax5* locus. Six of the ten *Pax5* exons are indicated with black bars. The two transcription start sites are indicated with arrows. The positions of the promoter and enhancer hypersensitive sites identified at the mouse *Pax5* locus are indicated below the horizontal line with thin black bars.

1.5 Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (hereafter referred to as AML) is a heterogeneous disorder characterized by a large number of distinct chromosomal translocations and other abnormalities. It is defined by >20% myeloblasts in the bone marrow. Pathologically, the initial stages show diffuse bone marrow infiltration with myeloblasts while later stages are marked by circulating leukemic blasts. Until recently, morphologically AML was classified under the FAB (French-American-British) classification system based on the stages of differentiation observed. A newer classification method devised by the World Health Organisation (WHO) takes into account factors other than morphology (Vardiman, 2010, Vardiman et al., 2002, Vardiman et al., 2009). One of the sub groups under this classification is ‘acute myeloid leukaemia with recurrent genetic abnormality’. Table 1.2 lists the AMLs that fall under this sub group (Table 1.2).

Acute Myeloid Leukaemia with recurrent genetic abnormality WHO classification
Acute Myeloid leukaemia with t(8;21)(q22;q22) (*AML1/ETO*)
Acute Myeloid Leukaemia with abnormal bone marrow Eosinophils t(16;16)(p13;q22) (*CBFβ/MYH11*)
Acute pro-myelocytic leukaemia t(15;17) (q22;q12) (*PML/RARα)*
Acute Myeloid Leukaemia with 11q;23 (MLL) abnormalities

Table 1.2: Table lists the acute myeloid leukaemia with recurrent genetic abnormalities as classified by WHO (Vardiman et al., 2009).

1.5.1 Acute Myeloid Leukaemia with t(8;21) (q22;q22) translocation

The t(8;21) is one of most common and widely studied chromosomal translocation associated with core-binding factor (CBF) complex leukaemia. The core-binding factor complex consists of a member of the RUNX transcription factor family along with its non-DNA binding partner CBFβ (Wang et al., 1996c). In t(8;21) AML, *RUNX1* (also called *AML1*) is fused to the entire ETO gene (also called RUNX1T1 or MTG8) (Erickson et al., 1992, Miyoshi et al., 1993) Figure 1.14). RUNX1 is a DNA-binding transcription factor that acts as a transcriptional activator and is of prime importance for haematopoiesis (Lichtinger et al., 2010, Bonifer and Bowen, 2010) and the specification of HSCs from haemogenic endothelium (Chen et al., 2009, Dzierzak and Speck, 2008). ETO on the other hand is a transcriptional repressor. This translocation (like other CBF complex translocations) disrupts the normal function of the CBF complex (Tonks et al., 2007). Morphologically, the leukemic blasts of AML with t(8;21) translocation show variable degree of differentiation. It therefore falls under the category of AML with maturation (M2) under the FAB classification (Miyoshi et al., 1991).

Under most circumstances the RUNX1/ETO fusion protein binds RUNX1 target genes and functions as a transcriptional repressor (Lam and Zhang, 2012, Okumura et al., 2008, Peterson et al., 2007b, Tonks et al., 2007). It has been shown that ectopic expression of RUNX1/ETO interferes with the normal function of RUNX1 (as a dominant negative mutant) and redirects certain gene expression programs (Tonks et al., 2007). A number of studies have shown that RUNX1/ETO recruits histone de-acetylases to its target genes and deregulates important myeloid transcription factors such as *CEBPα* (Zhang et al., 2004a, Gelmetti et al., 1998, Lutterbach et al., 1998, Pabst et al., 2001). Ptasinska *et al* has comprehensively investigated the epigenetic mechanism of RUNX1/ETO functioning (Ptasinska et al., 2012b). They performed ChIP-sequencing analysis of RUNX1 and RUNX1/ETO in Kasumi-1 cells (t(8;21) model cell line) and in primary t(8;21) patient cells. Moreover, they depleted RUNX1/ETO by siRNA in Kasumi-1 cell line and measured the effect on gene expression and transcription factor binding. They identified a large number of high confidence RUNX1 and RUNX1/ETO binding sites which were enriched in RUNX1, ETS and E-box binding motifs. This indicates that RUNX1 and RUNX1/ETO are primarily recruited to its target via DNA binding and also indicates interaction with ETS and E-box proteins. Depletion of RUNX1/ETO increased RUNX1 binding sites and also led to the formation of large number of de novo RUNX1 binding sites. Hence, RUNX1/ETO depletion leads to re-localisation of RUNX1 binding. They also showed that depletion of RUNX1/ETO reprogrammed the epigenome with complex changes in RNA polymerase II occupancy and H3K9acetylation at target sites along with inhibition of the leukemic gene expression program and concomitant induction of myeloid differentiation

(Ptasinska et al., 2012b). In a more recent study from Stunnenberg's lab they have identified chromatin accessible regions in t(15;17) and t(8;21) AML and have measured DNA methylation and several histone modifications (H2A.Z ac, H3 ac, H3K9me3, H3K27me3) along with p300 localisation. Using unsupervised clustering, they defined six distinct classes of chromatin accessible regions based on the measured chromatin features mentioned above. They identified that RUNX1/ETO (and also PML/RARα) localises at accessible chromatin sites that have p300 and hypo-acetylated histones (Saeed et al., 2012). A study from Stephen Nimer's lab has attributed the leukemogenecity of RUNX1/ETO to p300 mediated acetylation of RUNX1/ETO itself which helps in sustaining self-renewal and leukaemia in mouse models (Wang et al., 2011a)

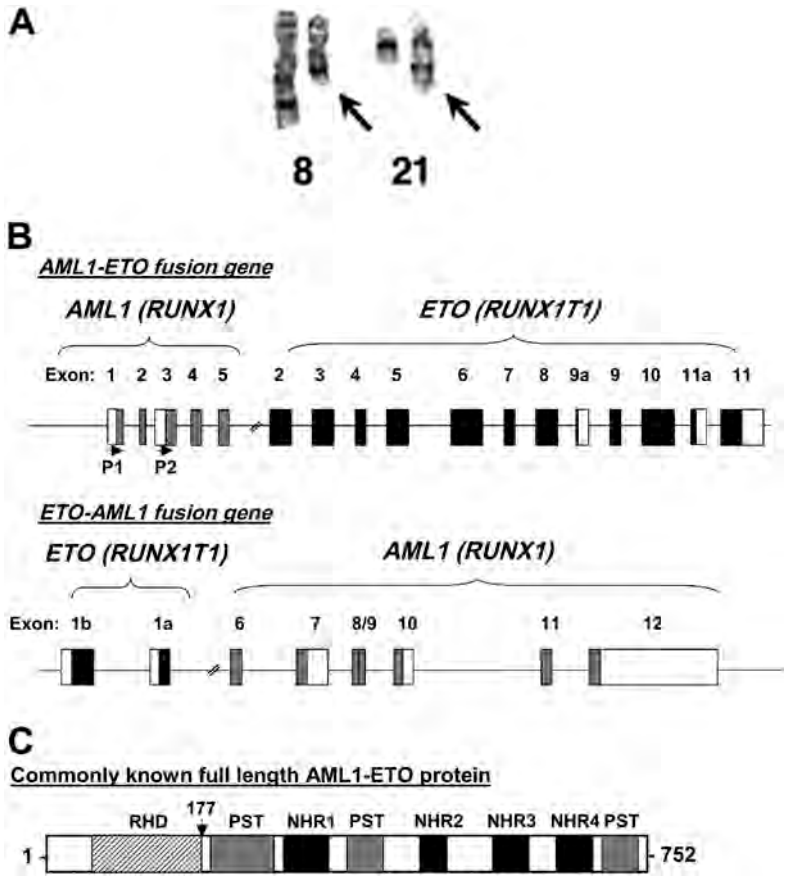


Figure 1.15: Cartoon diagram showing fusion of RUNX1 and ETO. A) Shows Chromosome translocation at chromosomes 8 and 21. B) Cartoon diagram showing RUNX1 (AML1)/ETO and ETO-RUNX1 (AML1) fusion genes respectively. C) Cartoon diagram showing the full length RUNX1/ETO fusion protein with the various domains from RUNX1 and ETO respectively (Peterson et al., 2007a).

1.5.2 t(8;21) AML requires a co-operating secondary mutation for leukemogenesis

Heterozygous *RUNX1/ETO* knock- in mice die at embryonic day11.5 (Yergeau et al., 1997, Okuda et al., 1998) and fail to establish haematopoiesis. Several transgenic RUNX1/ETO mouse models have been established and all of these mice remain healthy without any leukaemia (Rhoades et al., 2000, Yuan et al.,

2001, Fenske et al., 2004, Buchholz et al., 2000, Higuchi et al., 2002). Retroviral transduction with RUNX1/ETO followed by bone marrow transplantation in mice also failed to induce leukaemia in the recipient mice. Even though RUNX1/ETO functions to restructure the epigenome to a large extent (Ptasinska et al., 2012b), RUNX1ETO alone is unable to establish leukaemia in model organisms.

t(8;21) patients invariably present other mutations and lesions. Additional cytogenetic aberrations that co-appear with t(8;21) include loss of one X-chromosome in females and Y-chromosome in males, deletion of chromosome 9q or the trisomy of chromosome 4 or 8 . In addition to chromosomal aberrations t(8;21) patients show mutations in growth factor receptors, transcription factors and proto-oncogene: *CKIT, FLT3, PU.1*, and *RUNX1* (Peterson et al., 2007a). Table 1.3 lists the various chromosomal and genetic aberrations that co-appear with t(8;21) translocation in AML patients. Occasionally t(5;12) and t(9;22) also appear in patients with t(8;21) (Peterson et al., 2007a).

A large number of studies have classified mutation in transcription factors and tyrosine kinase receptors (C-KIT, N-RAS and FLT3) separately. The consensus in the field is that tyrosine kinase receptor mutations provide a proliferative advantage to leukemic stem/progenitor cells while chromosomal translocations such as RUNX1/ETO (or PML/RARα) and direct mutations in transcription factor genes (PU.1, RUNX1) influence the differentiation block observed in leukemic blasts.

Mutations	Frequency (in %)
One X-chromosome loss in female	35%
Y-chromosome loss in males	56%
9q deletion	18%
Trisomy 4	11%
Trisomy 8	6%
Other cytogenetic abnormalities	16%
FLT3/ITD	7%
FLT3 D853 activating mutation	3%
cKIT N822K activating mutation	19%
cKIT D816	12.3%
N RAS	8.5%
PU.1	5.3%
RUNX1	3.8%

Table 1.3: List of cytogenetic and genetic mutations that co-appear in t(8;21) AML patients (Peterson et al., 2007a).

Wang *et al* screened 54 patients with t(8;21) AML and identified 11 different *C-KIT* mutations in 48% of the patient cohort (Wang et al., 2005). Furthermore in a different study by Schessl *et al* 135 RUNX1/ETO positive AML patients were screened and they too identified activating mutations in receptor tyrosine kinases (FLT3, N-RAS and CKIT) as the most common secondary mutations in t(8;21) patients (Schessl et al., 2005). In a subsequent paper Wang *et al* produced a transduction and transplantation mouse model of t(8;21) AML. Co-expression of RUNX1/ETO and hybrid C-KIT N822K mutation caused fatal

leukaemia in mice (Wang et al., 2011c). RUNX1/ETO has also been reported to co-operate with FLT3 length- mutation (FLT3-LM) to produce leukaemia in bone marrow transplantation mouse models. These studies further confirm the two-hit model stating that aberrations in both transcription factors and signalling molecules are required for the pathogenesis of leukaemia. t(8;21) patients after remission often shows the persistence of RUNX1/ETO but mutations such as activating C-KIT or FLT3 are undetectable (Wang et al., 2005, Schessl et al., 2005). Furthermore RUNX1/ETO and t(8;21) lesion is present in newly born children long before the onset of the disease (Wiemels et al., 2002). These evidences strongly suggest that t(8;21) is an initiating lesion that requires a 'second-hit' for leukaemia to occur. These secondary mutations mostly are abnormalities of signalling molecules that provide proliferative advantage to leukemic stem/progenitor cells.

1.6 PAX5 and malignancies

PAX5 has been implicated to be a tumour suppressor and an oncogene in B-cell leukaemia (Medvedovic et al., 2011). Loss of both alleles of *Pax5* in mouse has identified *Pax5* as a tumour suppressor (Cobaleda et al., 2007). However, heterozygous mutations (mono-allelic somatic mutations and point mutations in *PAX5* has been reported in 32% B-ALL (acute lymphoblastic leukaemia) (Mullighan et al., 2007). BCR-ABL1 positive B-ALL carries heterozygous *PAX5* mutations (Mullighan et al., 2008). Heterozygous *Pax5* mutation has also been shown to co-operate with other oncogenic lesions (*Stat5b-CA* $Pax5^{+/-}$) to produce B-ALL in mouse models (Heltemes-Harris et al., 2011).

Recurrent translocation t(9;14) leading to the IGH-PAX5 mutation has been reported in B- cell non-Hodgkin lymphoma (Busslinger et al., 1996, Iida et al., 1996, Morrison et al., 1998). This translocation places the entire *PAX5* gene under the control of the *IGH* promoter leading to strong up-regulation of the translocated *PAX5* allele causing B-cell leukaemia (Morrison et al., 1998). Other *PAX5* translocations have been reported in paediatric B-ALLs. 15 different translocation partners of PAX5 have been identified which include transcription factors (ETV6, FOXP1,PML, DACH1 DACH2), chromatin regulators (NCOR1, BRD1), protein kinases (JAK1,HIPK1) nuclear pore sub unit (POM121), extracellular matrix protein (ELN) and proteins of unknown function (GOLGA6,AUTS2,CO20orf112) (Medvedovic et al., 2011, Coyaud et al., 2010, Nebral et al., 2009)

1.6.1 *PAX5* is aberrantly expressed in AML with t(8;21) translocation

Although PAX5 is the master-regulator of B-cell lineage (see section 1.4.1) it has been shown to be aberrantly expressed in t(8;21) AML. One of the defining features of t(8;21) AML is a mixed lineage phenotype. It co-expresses myeloid genes along with theB-lineage surface marker CD19 (Walter et al., 2010, Tiacci et al., 2004). Tiacci *et al* reported *PAX5* expression in t(8;21) AML patient blasts by RT-qPCR and showed expression of *PAX5* to be more common in t(8;21) AML than previously reported by immuno-histochemistry experiments. They also noticed that *PAX5* expression in AML selectively clusters with t(8;21) AML patients and suggested that this might be a reason the expression of B-lineage genes in this type of leukaemia (Tiacci et al., 2004). In a later study Valbuena *et al* investigated 28 AML patients with t(8;21) and identified PAX5 expression by

immuno-histochemistry in blast cells of all of them, thus, further confirming the selective association of PAX5 expression with t(8 21) translocation (Valbuena et al., 2006). Gibson *et al* also reported selective correlation of *PAX5* expression in t(8;21) myeloid blasts but they also reported rare PAX5 expression in AML with 11q23 abnormality and AML with slight differentiation (Gibson et al., 2006).

1.6.2 *PAX5* expression has functional consequence in t(8;21) AML

Aberrant expression of PAX5 is has functional consequence as it has been implicated to be one of the factors that suppress myeloid differentiation and maintain leukemic progenitors in t(8;21) AML. PAX5 has been shown to repress *Csf1r*, a factor that is required for macrophage differentiation, during B-cell commitment (Tagoh et al., 2006b). Therefore, PAX5 is likely to do the same in t(8;21) AML thereby contributing to the myeloid differentiation block. Ectopic expression of PAX5 in bone marrow stem/progenitor cells leads to the formation of bi-phenotypic $B220^{+}GR1/MAC1^{+}$ cells that could be maintained in culture. Although these cells remained cytokine dependent they acquired the capacity for sustained generation of myeloid progenitors. The $PAX5^{+}B220^{+}$ $GR1/MAC1^{+}$ bi-phenotypic progenitors expressed both myeloid genes and PAX5 target B-lineage genes at the single cell level (Anderson et al., 2007). In a separate study ectopic expression of PAX5 by retroviral transduction in cytokine stimulated human $CD34^{+}$ cord-blood cells seeded on HESS-5 cells in the presence of SCF and GCSF resulted in the generation small myeloid progenitors that co-expressed CD33 and CD19 with blocked myeloid differentiation (Sekine et al., 2008). These studies strongly suggest that aberrant expression of PAX5 in t(8;21) AML accounts for its bi-phenotypic

features. Walter *et al* showed a positive correlation between *CD19* expression and *PAX5* expression in t(8;21) patient blasts while this correlation does not exist between *EBF1* and *CD19* expression (Walter et al., 2010). This strongly suggests that PAX5 and not EBF1 is of importance for the up-regulation of B-cell genes such as *CD19* in t(8;21) AML. They showed that *CD19* exists in poised state in myeloid progenitors; in t(8;21) AML PAX5 binds *CD19* at the enhancer and promoter to remodel the chromatin structure at the promoter leading to the aberrant up-regulation of CD19 in t(8;21) AML (Walter et al., 2010)

1.7 Aims and Objectives

PAX5 is aberrantly expressed in t(8;21) AML and is of functional consequence in this type of leukaemia (see sections 1.6.1 and 1.6.2) but the mechanism of *PAX5* deregulation in this type of leukaemia was unknown. The primary objective of this study was therefore to investigate the molecular mechanism of aberrant up-regulation of *PAX5* in t(8;21) AML. To understand this mechanism we first needed to have a clear understanding of the regulation of the *PAX5* gene in normal B cells. However, while the regulation of the *Pax5* gene in mice is well understood the human *PAX5* gene had not been studies. To this end, we set out to elucidate the mRNA expression, chromatin structure, histone modifications and RNA Polymerase II occupancy at the *PAX5* locus in human B-cells and compare it to non t(8;21) myeloid precursors and t(8;21) AML. The working hypothesis of the experiments performed was that an upstream transcription factor activates and maintains *PAX5* expression in t(8;21) AML.

The ultimate aim of our study was to achieve better understanding of the pathways that are responsible for mixed-lineage phenotype of t(8;21) AML and how they contribute to the differentiation block.

2 MATERIALS AND METHODS

2.1 Cell Culture

Human cell lines Ramos, Nalm-6, Kasumi-1, SKNO-1, HL60, KG 1 and HeLa were grown in the following way:

Cell Type	Culture Medium	Culture Technique
Ramos	RPMI 1640 (Sigma) supplemented with 10% heat –inactivated FCS (GIBCO), Penicillin and streptomycin (P/S) and L-Glutamine (GIBCO).	The suspension of cells were maintained at $2x10^5$ cells/ml and the medium was changed every two days
Nalm-6	RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FCS, P/S and L-Glutamine (GIBCO).	The suspension of cells were maintained at $2x10^5$ cells/ml and the medium was changed every two days
Kasumi-1	RPMI 1640 (Sigma) supplemented with 20% heat-inactivated FCS, P/S and L-Glutamine (GIBCO).	The suspension of cells were maintained at $4\text{-}5x10^5$ cells/ml and the medium was changed every 2-3 days
SKNO-1	RPMI 1640 (Sigma) supplemented with 20% heat –inactivated FCS (GIBCO), 1% Pen/Strep, L-Glutamine (GIBCO) and recombinant human GM-CSF growth factor (Peprotech) at 5ng/ml final concentration.	The suspension of cells were maintained at $4\text{-}5x10^5$ cells/ml and the medium was changed every 2-3 days

HL-60	Iscove's modified Dulbecco's media (Sigma) supplemented with 10% heat – inactivated FCS, P/S and L-Glutamine (GIBCO).	The suspension of cells were maintained at $3x10^5$ cells/ml and the medium was changed every 2 days
KG-1	Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% Heat-inactivated-FCS , L-Glutamine and P/S	The suspension of cells were maintained at 4 $x10^5$ cells/ml and the medium was changed every 2 days
HeLa	RPMI 1640 (Sigma) supplemented with 10% Heat –Inactivated FCS, P/S and L-Glutamine.	Adherent cells were resuspended with 1x Trypsin/ scraped with sterile plastic scraper on confluence and split 1 in 5

Table 2.1: **Culture Condition used to grow human cell lines**. All cell types were incubated at 37° C in an incubator having humified air supplemented with 5% CO_2.

2.2 Growing primary cells from t(8; 21) patients

Patient material was obtained with consent from the NHS ethics approval committee (Leeds Teaching Hospital NHS Trust) as part of a parallel study in the lab (Ptasinska et al., 2012a). 2x 10^6 frozen cells isolated from the bone marrow of a t(8;21) patient who had relapsed after recovery was thawed and cultured in RPMI media (Sigma) supplemented with 20% heat-inactivated FCS (GIBCO), Penicillin/streptomycin cocktail (GIBCO) and L-Glutamine (GIBCO) for two hours at 37°C with humified air and 5% CO_2. The cell suspension was divided in two halves and one half of the cells were treated with 30µl of sterile Dimethyl Sulfoxide (DMSO)-Hybri-Max® (Sigma) and the other half was treated with 10µM each of JNK, MEK and P38 small molecule inhibitors.

2.3 Treatment of cells with protein kinase inhibitors

2x10^6 cells (for RNA extraction) or 2x10^7 cells (for extracting chromatin for ChIP) were resuspended in RPMI (Sigma) supplemented with 20% FCS (GIBCO), Penicillin/Streptomycin (GIBCO) and L-Glutamine (GIBCO) and treated with 10µM (final concentration) of AKT inhibitor (Akt Inhibitor IV, sc-203809, Santa cruz Biotechnologies), JNK inhibitor (P600125 Sigma), P38 inhibitor (SB20219 Sigma), MEK inhibitor (PD98059 Sigma), STAT3 inhibitor (Static:sc202818 Santa Cruz Biotechnologies) or MSK1 inhibitor (H89 dihydrochloride hydrate Sigma Aldrich) and was harvested at various time points depending on the experiment (see section 3.7). The small molecule inhibitors were dissolved in sterile dimethyl sulfoxide (DMSO)-Hybri-Max® (Sigma) and stored at a stock concentration of 10mM.

2.4 Treatment of Kasumi-1 cells with HDAC inhibitor Trichostatin A (TSA)

2 x10^6 cells were resuspended in RPMI media (Sigma) supplemented with 20% FCS (GIBCO). The cells were treated with 20ng/ml (final concentration) of Trichostatin A (Sigma Aldrich) dissolved in molecular biology grade absolute Ethanol (Sigma) and incubated for 2 hours at 37°C with 5% CO_2.

2.5 siRNA mediated knockdown of genes in Kasumi-1 cell line

2-5x10^7 Kasumi-1 cells were transfected with 200nM siRNA in RPMI media with (20%FCS, P/S and L-Glutamine) using a Fischer 3500 electroporator (Fischer, Heidelberg, Germany) at 350V with an electrical pulse of 10msec (Heidenreich, 2009). The transfections were done in a 0.4cm, sterile Sigma-Aldrich™ electroporation cuvette, (Ptasinska et al., 2012a). The siRNAs used were: C-KIT siRNA (h): sc-29225 (Santa Cruz Biotechnology, Inc.) and 3pK siRNA (h): sc-39105 (Santa Cruz Biotechnology, Inc.). The siRNAs were resuspended according to the manufacturer's protocol with nuclease free water provided by the manufacturer. The resuspended siRNA was stored at -20°C (short-term) or -80°C (Long-term) in individual aliquots. Following transfection the transfected cells were seeded in pre-warmed RPMI media (Sigma) supplemented with heat-inactivated serum (GIBCO) and incubated at 37°C with 5% CO_2 until harvested for experiments as indicated in sections 3.9.3 and 3.10.

2.6 Differentiation of HL-60 cells to macrophage like cells with phorbol myristate acetate (PMA)

3 x 10^5 HL-60 cells were differentiated by culturing in media containing PMA (Sigma) dissolved in DMSO at the final concentration of 50 ng/ml. Cells were incubated at 37°C, 5% CO_2 for 3 days. Differentiation was confirmed by visual inspection under the microscope.

2.7 Annexin/ PI staining

10^5 cells were counted and centrifuged at 300 x g for 5 minutes. The resulting pellet was resuspended in Annexin Buffer and stained with Annexin V and PI for 5 to 10 minutes at room temperature according to the kit manufacturer's instructions (Annexin V (FL) sc-4252, Santa Cruz Biotechnologies, Inc.). The stained cells were analysed by flow cytometry (see section 3.7).

2.8 Flow Cytometry

2 x 10^5 cells were counted and centrifuged at 300 x g at room temperature for 5 minutes in BD Falcon™ round bottomed snap-cap polypropylene FACS tubes. The resulting pellet was washed with 1ml of buffer containing 0.5% BSA and 2mM EDTA (FACS buffer) and resuspended in the residual buffer after discarding most of the supernatant. To the cell suspension 2µl of primary antibody : human Anti IgG-FITC MACs Miltenyi # 130-093-193 (Isotype control for Annexin V stained cells; see section 3.7), human Anti IgG-PE MACs Miltenyi # 130-093-193 (Isotype control for CD117 stained cells), human CD117 (A3C6E2)-PE MAC Miltenyi # 130-091-734, was added and incubated for 30

minutes at 4°C. The cells were then washed in 2 mls of FACs buffer and resuspended in 500µl of FACs buffer.

The stained cells described above and the Annexin/PI stained cells (see section 3.7) were analysed by Flow cytometry (CyAn FACs analyser, DAKO) using Summit 4.3 (Beckman Coulter, INC) software according to the manufacturer's protocol.

2.9 Extraction of RNA

Approximately 5 x10^6 cells were centrifuged at 300 x g and lysed in 1ml of TRIZOL solution (Invitrogen) and incubated for 5minutes at room temperature. To the lysate, 200µl of chloroform (Sigma) was added and incubated at room temperature. This was followed by centrifugation for 15 minutes at 12000 x g to separate the organic phase and the aqueous phase. The aqueous phase was transferred to a fresh tube and to this 500µl of 2-propanol was added and incubated at 4ºC for 10 minutes to allow precipitation of RNA. The precipitated pellet was washed with 70% ethanol (made in DEPC treated water: added 0.1% DEPC to sterile water and autoclaved) and resuspended in 20µl of DEPC treated water. Genomic DNA was removed by treatment with RQI RNase-free DNaseI (Promega) at 37°C for 1 hour according to the manufacturer's protocol. To the DNaseI treated samples 250µl of TRIZOL and 50µl of chloroform was added, vortexed and centrifuged at 12000 x g at 4ºC for 5 minutes. The top aqueous layer was transferred to a fresh tube and to this 700µl of chloroform was added and centrifuged again at 12000 x g for 5 minutes at 4ºC to allow separation of the aqueous and the organic phases. The aqueous layer was

transferred to a fresh tube and to this 150µl of 2-propanol was added and incubated at 4°C for 10 minutes to precipitate the RNA. The precipitated RNA pellet was washed with 70% ethanol (prepared in DEPC treated water) and air dried to remove any traces of ethanol. This washed and dried pellet was resuspended in 20µl of DEPC treated water. The isolated RNA was quantified using NanoDrop 1000 spectrophotometer™ (Thermo scientific) according to the manufacturer's instructions.

2.10 cDNA Synthesis

1µg of RNA was annealed to 1µl of oligo(dT) primers (50µM) (Invitrogen) by incubating at 70°C for 5 minutes followed by 4ºC for 5 minutes. Annealed to oligo (dT) primers, 4µl of 5 x first strand synthesis Buffer (Promega), 5µl of 2mM dNTP, 1µl of 200U/µl M-MLV Reverse Transcriptase (Promega), 0.5µl of 400U/µl of RNase out (Invitrogen) were added to the RNA and incubated at 37°C for 1 hour followed by 95°C for 5 minutes in a T300 thermocycler (Biometra).

2.11 Real-Time PCR

Real-Time PCR was performed using of SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK), 100nM primers (final concentration) under standard conditions in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). 0.25µl of 10µM (working dilution) primers were added to 2.5µl of diluted cDNA or ChIP template, to this 2.5µl of nuclease free water and 5µl of SYBR® Green PCR master mix was added to make up a total reaction volume of 10µl. Custom primers for Real-Time PCR were

designed (Table 3.2) using Primer Express™ and obtained from Sigma Aldrich. Expression of each gene was calculated relative to the expression of *TBP* or *GAPDH*. Amplification efficiencies of the different primers were determined by serial dilution of cDNA (for gene expression analysis) or genomic DNA templates (for ChIP analysis). cDNA standard for gene expression analysis was prepared using cDNAs obtained from Ramos, Nalm-6, Kasumi-1, HL-60, HL-60+PMA, HeLa and KG-1 in equal proportions.

Prime Name	Forward	Reverse
RT-PAX5 (8/9)	CTGGACAGGGCAGCTACTCAG	CTGAGGGTGGCTGTAGGGACT
RT-TBP	CAGGAGCCAAGAGTGAAGAACA	AGCTGGAAAACCCAACTTCTGT
RT GAPDH	CCTGGCCAAGGTCATCCAT	AGGGGCCATCCACAGTCTT
RT RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
RT 3pK	AGGAGATGACCAGTGCCTTG	AGGAGCCGGTTGTTAGAGGT
RT UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
RT CKIT	CACCGAAGGAGGCACTTACAC	GGAATCCTGCTGCCACACA
RT JMJD3	GGAGGCCACACGCTGCTAC	GCCAGTATGAAAGTTCCAGAGCTG
RT INK4A	GAAGGTCCCTCAGACATCCCC	CCCTGTAGGACCTTCGGTGAC

Table 2.2: Table showing sequences of primers used for quantitative Real-time PCR analysis to measure gene expression.

2.12 DNaseI Hypersensitive site mapping

A bacterial artificial chromosome (BAC) construct containing the *PAX5* coding region (BAC CTD 2230P4 ResGen™) was prepared using Qiagen Plasmid Plus Maxi kit (Qiagen) according to the manufacturer's protocol.

2.12.1 Designing and preparation of probes for Southern blotting

Probes for Southern blotting were designed to hybridise on either side of the chosen restriction fragments (See figures 3.2.and 3.3). The probes were 500-600 base pairs long to ensure proper signal from Southern blots. Furthermore probe sequences were designed so as to avoid complementarity to repeat elements within the human genome.

The probes were prepared by standard PCR reaction using 1µg of *PAX5* BAC CTD 2230P4 (Resgen™) preparation as template, plus 25 mM dNTP mix, 5µl of 10µM primers (forward and reverse), $MgCl_2$ (3mM) and 1U Taq DNA Polymerase (recombinant) (Invitrogen) in a 50µl reaction. Cycling conditions used for PCR were: 96ºC for 2 minutes, 96ºC for 30 seconds, 52-65 ºC for 30 seconds, 72ºC for 60 seconds for 20 cycles followed by a final extension of 72ºC for 15 minutes in T300 thermocycler (Biometra). The primers used for the PCR reactions were designed using Primer3 version 0.4.0 ™. The names and sequences of the primers used are listed in table 2.3.

Primer Name	Forward	Reverse
P1	TGATAGGATCCAACCACAGAGGGT	TGATGGAATTCAGGGCTAGTCACTGTCATG TTATC
P2	TAGGTCAGAATTCGGTCTAGGCTGG GCCGTTTTCCGT	CACCGCGGATCCCTGGACCTCCTTCA
Pa	CCCTGAAGGATTCTAATCATTTAATC AATAAAG	CTGAAGAATTCTGGCTCCCTGCTTAGTGTT GACCTGACC
Pb	CTTACGGATTCTTCCCACTCTTTTAC ACTTCTCTTAA	CTTTACGAATTCAGACTTTCTGCCCAAAACA CAGTGGCAA

Table 2.3: Table showing sequences of primers used to prepare probes for Southern blotting

2.12.2 *In vivo* DNaseI treatment

10^8 cells were harvested and washed in ice cold PBS followed by DNaseI buffer (11mM KPO4 pH 7.4, 108mM KCl, 22mM NaCl, 5mM MgCl2, 1mM $CaCl_2$, and 1mM DTT) and centrifuged at 50g for 10 minutes at 2ºC. The resulting pellet was resuspended in 1 ml of DNase buffer containing 1mM ATP.100µl of this cell suspension was treated with 104µl of buffer containing 80µl of DNaseI buffer 4µl of 10% NP-40 and 20µl of water containing varying concentrations of DNaseI enzyme (Worthington). See table 2.4 for the various concentrations of DNaseI enzymes used for the different cell lines. This reaction was incubated at room temperature for exactly 6 minutes. The DNaseI reaction was then stopped by lysing the nuclei with 200 µl of lysis buffer (100mM Tris pH 8.0, 40 mM EDTA, 2% SDS, 500µg/ml ProteinaseK) and incubated overnight at 55°C. The samples were then subjected to two rounds of Phenol-chloroform-isoamyl extractions followed by an extraction with chloroform. The purified samples

were then treated with RNaseA (50µ/ml) at 37°C for 1 hr. This was followed by another round of phenol-chloroform and chloroform extractions. The DNaseI treated samples were precipitated in ethanol and the resulting pellet was washed in 70% ethanol and resuspended in 100µl of 1xTE buffer.

Cell Lines	The range of units of DNaseI used
Nalm-6	0 U, 3.12 U, 6.25 U, 12.5 U
Kasumi-1	0 U, 0.78 U, 1.56 U, 3.12 U
SKNO-1	0 U, 0.78 U, 1.56 U, 3.12 U
HL-60	0 U, 3.12 U, 6.25 U, 9.36 U
HeLa	0 U, 6.25 U, 9.36 U, 12.5 U

Table 2.4: The units of DNaseI enzymes used for in-vivo DNaseI treatment of each of the human cell lines.

2.12.3 Southern Blotting

20µg of the DNaseI treated samples were restriction digested according to the manufacturer's protocol with BamHI (for the *PAX5* enhancer), EcoRI/AseI (for the proximal *PAX5* promoter) or MfeI (for distal the *PAX5* promoter) (New England Biolabs) in the appropriate buffers supplied by the manufacturer (See figures 3.2 and 3.3). The digested samples were then electrophoresed on a 0.8% agarose gel in 1X TAE with 0.5mg/ml of ethidium bromide at 50mV overnight to ensure good resolution. The gel was then incubated for 10 minutes with depurination buffer (0.25M HCL), rinsed with distilled water and soaked in denaturation buffer (1.5M NaCl and 0.5M NaOH) for 30 minutes in order to denature the DNA into single strands to allow binding to the membrane. The denatured DNA was then transferred onto a nylon membrane, positively charged with quaternary amine groups (Zeta-probe membrane, Bio-Rad) by capillary transfer overnight in denaturation buffer (Figure 3.1). Following transfer the membrane was rinsed in 2x SSC (0.3M NaCl, 0.015M sodium citrate pH 7.0) to neutralise the high alkaline pH of the denaturation buffer and then the DNA was cross-linked to the membrane using a Stratalinker® UV cross-linker (Stratagene) and baked at 80°C for 1-2 hours. The cross-linked membrane was pre-hybridised with denatured herring testes DNA (0.5mg/ml) in Rapid Hyb-buffer® (Amersham Pharmacia) for 30 minutes at 65°C. Following pre-hybridisation the membrane was hybridised with P^{32} labelled and denatured probes at 65°C for 2 hrs.

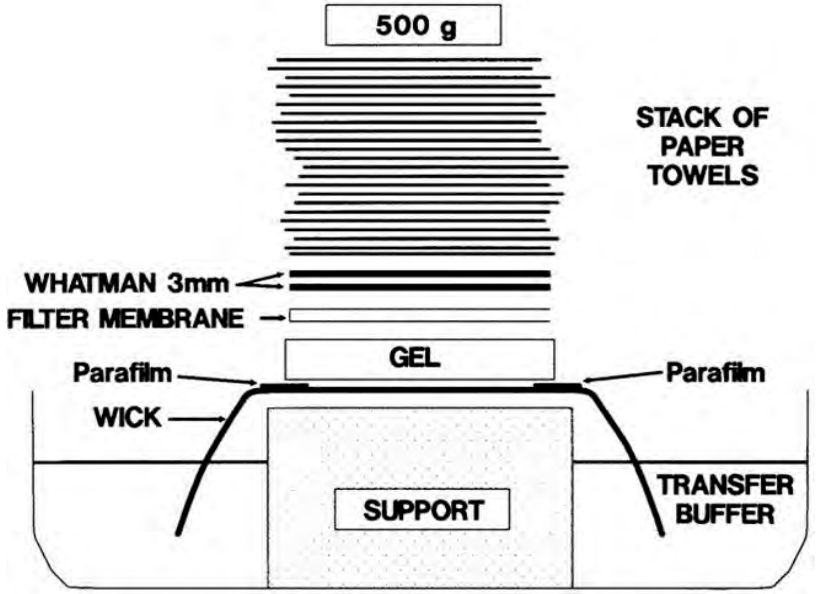


Figure 2.1: Schematic diagram showing assembly of gel for capillary transfer in Southern blotting. The gel is placed over a support which has a paper wick dipped into the transfer buffer contained within a tray. The nylon membrane is placed directly on the gel. To facilitate transfer of DNA from the gel to the membrane layers of Whatman paper and paper towels are placed above the membrane. The whole assembly is held together by placing a weight of about 500gms on top. The figure is adapted from Roberts *et al*; October 2006, Current problems in Cardiology, Vol 31, Issue 10.

2.12.4 Labelling probes

Probes were labelled with αP^{32} CTP and αP^{32} ATP (Perkin Elmer) using Megaprime DNA labelling kit (Amersham) using the manufacturers protocol.

2.12.5 Washing the membrane and visualisation of the Southern Blot

Following hybridisation, the membrane was washed thrice with low stringency buffer (2 x SSC, 0.1% SDS) and once with high stringency buffer (0.1x SSC, 0.1% SDS) if required at 65ºC in order to remove any non-specifically bound

probe from the membrane. The washed membrane was then exposed overnight to imaging screen-K (BIO-RAD) and scanned with Molecular imager PharosFX™ system (BIO-RAD).

2.12.6 Mapping and analysing DNaseI hypersensitive sites

The cis-regulatory regions of the murine *Pax5* gene were mapped by Decker *et al.* (Decker et al., 2009). Using this information, corresponding regions on the human *PAX5* gene were identified as possible intronic enhancer and proximal and distal promoters. Convenient restriction fragments were chosen at the possible enhancer and promoter regions of the human *PAX5* gene within which the DNaseI sites were likely to be contained. For the possible enhancer region a BamHI fragment was chosen while for the proximal promoter an EcoRI/AseI fragment and for the distal promoter an MfeI fragment was chosen. Positions of the probes binding on each of these fragments were known.

The DNaseI treated samples were first electrophoresed alongside a DNA ladder. The distance migrated by the various bands of the DNA ladder from the well were measured. The logs of the distances travelled by the fragments were plotted against the size of each of these fragments to obtain a standard curve. Then the distances travelled by the fragments detected in the lanes containing DNaseI treated samples were measured. Using the standard curve, the sizes of these fragments were determined. As the positions of the probes within each restriction fragment were known, the position of the DNaseI hypersensitive sites relative to the respective restriction sites could be calculated. Once this was done restriction enzymes close to each of the DNaseI hypersensitive sites were chosen. Human genomic DNA was digested with each of these restriction

enzymes to create a custom DNA ladder and this was once again electrophoresed and blotted alongside DNaseI treated samples. Using the method described above a standard curve was plotted and the exact positions of the DNaseI hypersensitive sites were determined. The position of the restriction site relative to the *PAX5* HS4 transcription start site was known and hence the position of each of the DNaseI hypersensitive sites relative to the HS4 transcription start site was calculated.

2.13 Chromatin immuno-precipitation assay (ChIP)

2.13.1 Cross linking using formaldehyde and sonication

$2X10^7$ cells were harvested by centrifugation at 300 x g at room temperature for 5 minutes. The cell-pellet was resuspended in 10mls of media. To this, formaldehyde (16% stock solution, Pierce) was added to a final concentration of 1% and incubated at room temperature for 10 minutes. This reaction was quenched by adding freshly prepared 2M Glycine (Sigma) solution in phosphate buffered saline (PBS) to a final concentration of 0.4M. The in-vivo cross-linked cells were harvested by centrifugation at 300 x g at 4°C for 5 minutes. The pellet was then washed twice with ice-cold PBS. The cells were then resuspended in 10 ml of ice cold Buffer A (10mM HEPES, pH 8.0, 10mM EDTA, 0.5mM EGTA, 0.5mM EGTA, 0.25% Triton X-100, 1:1000 dilution of protease inhibitor cocktail (Roche) and 0.1mM PMSF (Sigma) and incubated at 4°C for 10 minutes with rotation in order to isolate intact nuclei. The nuclei obtained were centrifuged at 500xg at 4°C for 5 minutes. Following centrifugation the nuclei were resuspended in 10 ml of ice-cold Buffer B (10mM HEPES, pH 8.0, 200mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.01% Triton X-100, PIC 1:1000

dilution and 0.1mM PMSF) and incubated at 4°C f or 10 minutes with rotation. The pellet was then resuspended in 600µl of ice-cold ChIP buffer (25mM Tris-HCL, pH 8.0, 150mM NaCl, 2mM NaCl, 2mM EDTA,1% Triton X-100, 0.25% SDS, PIC 1:1000 dilution and 0.1mM PMSF). This was then sonicated using Bioruptor™ (Diagenode) at 240W for 15 cycles of 30sec on and 30 sec off at 5°C to obtain average fragments of length 400-500bp. The resulting sonicated chromatin was then centrifuged at 16,000xg at 4°C for 10 minutes. The pellet was discarded and the supernatant was diluted with 1200µl of 3x ChIP Buffer (25mM Tris-HCl, pH 8.0, 150mM NaCl, 2 mM EGTA, 1% Triton X-100, 7.5% Glycerol, PIC 1:1000 dilution and 0.1mM PMSF.

2.13.2 Immuno-precipitation

For each immuno-precipitation reaction 15µl of Protein G beads (Dyanbeads™) were washed twice with phosphate buffer (pH 8.0) and incubated for 2 hours at 4°C with BSA (0.5% final concentration), 2µg of antibody and phosphate buffer to make up a total volume of 10µl. To this, 20-25µg of sonicated chromatin was added and incubated for 2 hours at 4ºC with rotation. Thereafter, the Protein G beads conjugated to the antibody and the pulled down chromatin was separated from the unbound chromatin using a DynaMag™-2 (Invitrogen) magnetic separator and washed once with 1 ml of wash Buffer 1 (20mM Tris-HCl pH 8.0,150mM NaCl, 2mM EDTA, 1% Triton-X-100, 0.1% SDS), twice with 1 ml of wash Buffer 2 (20mM Tris-HCl pH 8.0, 500mM NaCl, 2mM EDTA, 1% Triton-X-100, 0.1% SDS), once with 1ml of LiCl Buffer (10mM Tris-HCl pH 8.0, 250mM LiCl, 1mM EDTA, 0.5% NP-40).5% Na-deoxycholate) and twice with TE/NaCl Buffer (10mM Tris-HCl PH 8.0, 50mM NaCl, 1mM EDTA). The pulled down

chromatin was then eluted in 100µl of elution buffer (100mM NaHCO3 and 1% SDS) on a shaker at room temperature for 15 minutes and reverse cross-linked using 1µl of proteinase K (50mg/ml) at 65ºC overnight. Following reverse cross-linking, the immuno-precipitated DNA was purified using an Agencourt AMPure XP PCR purification kit (Beckman Coulter Genomics) using the manufacturers protocol. The purified DNA was then analysed by quantitative Real-Time PCR (see section 2.11) using the primers listed in table 2.6.

Antibody	Vendor	Catalogue Number
H3K4Me3	Millipore	04-745 Clone MC 315
H3K9ac	Abcam	Ab 4441
H3K27me3	Abcam	Ab 6002
H3K27ac	Abcam	Ab 4729
Pol II	Abcam	Ab 817
Pol II serine 2 Phos	Abcam	Ab 5095
H3	Abcam	Ab1791
EZH2	Millipore	07-689

Table 2.5: Table showing the antibodies used for ChIP experiments.

Primer Name	Forward Sequence	Reverse Sequence
PAX5 HS 1	GGGTTTCTGGGATTCTAGGG	CGGGGAGTCTAGGCTCAGAT
PAX5 HS 2	ACACACGCAAACTCTCACCA	GTGGGGGATCGGGTTATATT
PAX5 HS 3	GCAATAGTCAGGACCCCAAC	CGACGTTCCGGTTTACTCC
PAX5 HS 4	TCTTGTGATGTTGGCGAGAA	GAAGGCACCGTGAAATGATTA
PAX5 HS 5	CTGGCTTAGGTCCCCATGT	GAGGCCTACTGTCCCTGCTA
PAX5 HS 6	TAGTTTCCTTTCGCGGTCTC	GTGATGCGCTTTCTTTCTCC
PAX5 HS 7	GCGGATCATCACTCATGAAA	CAAGAACGACGCTGAGGATT
PAX5 HS 8	AGCGCGTTTGTCAATGAAC	GCCTGATTAGCGGACACCT
PAX5 HS B	ATGGGGTGGTGTACTGGTG	CCCAAACTGATTCCCTGAAC
PU.1 (P)	CTGCCGCTGGGAGATAG	CGGCCAGAGACTTCCTGTA
CSF1R	AGAAGAGGTCAGCCCAAGGA	AGGGATCGGGACACTGGAC
IVL	GCCGTGCTTTGGAGTTCTTA	CCTCTGCTGCTGCCACTT
TBP	CTGGCGGAAGTGACATTATCAA	GCCAGCGGAAGCGAAGTTA
GAPDH	CGCCGCCATGTTGCA	GGAAGGCCTAAGCAAGATTTCA
CHR9	GGATTTCCTGGGGAATGTCT	CGAGGCTTTGAGGAAGAATG

Table 2.6: Table showing sequences of primers used for amplifying DNA pulled down by ChIP by Real-Time PCR analysis

2.14 In-vivo DNaseI and DMS foot-printing

2.14.1 *In vivo* DNaseI treatment

Nalm-6, Kasumi-1, SKNO-1, HL-60, primary t(8;21) patient cells and HeLa cells were treated *in vivo* with DNaseI enzyme following the protocol detailed in section 2.12.2.

2.14.2 *In situ* DMS treatment

2 x 10^7 cells (Nalm-6, Kasumi-1, SKNO-1 HL-60 and HeLa) were harvested and washed twice with phosphate buffer saline (PBS) and resuspended in 0.2 % dimethyl sulphate (DMS) (Sigma)/PBS and incubated at room temperature for 5 minutes. The DMS treated cells were centrifuged at 300xg at room temperature for 5 minutes. The DMS/PBS mixture was discarded safely in 3M sodium acetate solution to neutralise the DMS. The cell pellet was washed three times in ice-cold PBS. After each wash, the PBS was discarded safely in 3M sodium acetate solution. The cells were thereafter resuspended in lysis buffer 2 (150mM NaCl, 10mM EDTA, pH 8) containing 600µg/ml of proteinase K and to this an equal volume of lysis buffer 1 (20mM Tris-HCL, pH 8.0, 20 mM NaCl, 20mM EDTA, pH 8.0, 1% sodium dodecyl sulphate (SDS) was added. The cell lysate with ProteinaseK was incubated over-night at room temperature. Thereafter 100µg/ml RNase A was added and incubated at 37ºC for 1 hour. The samples were then purified using phenol-chloroform-isoamyl alcohol (see section 2.12.2) and precipitated with 1 volume of isopropanol and 1/10 volume of 5M NaCl and 20µg of glycogen at -20ºC overnight. The samples were then

centrifuged at 13000 x g and the DNA pellet was rinsed with 70% ethanol, air dried and resuspended in 99µl of 0.1 x TE.

2.14.3 Preparation of DMS treated genomic control DNA

50-100µg of genomic DNA isolated from Kasumi-1 cells was precipitated with ethanol and resuspended carefully in 100µl of nuclease free water (HyClone Molecular Biology grade water, Thermo Scientific). This was gently mixed with 100µl of 2X DMS buffer (100mM sodium cacodylate, pH 8.0, 2mM EDTA). To this 1µl of DMS was added and incubated at room temperature for 3 minutes. The reaction was stopped with 30µl of DMS stop buffer (1.5M sodium acetate, pH 7.0, freshly prepared 1M 2-mercaptoethanol) and 750µl of pre-chilled 100% ethanol and allowed to precipitate for 30 minutes at -80ºC. The DNA was then centrifuged at 15000xg for 5 minutes at room temperature and rinsed with 70% ethanol and resuspended in 99 µl of 0.1 x TE by gentle pipetting.

2.14.4 Piperidine treatment of *in* vivo DMS treated samples and genomic DNA control

To the 99 µl of DMS treated samples prepared as described in sections 2.14.2 and 2.14.3, 1 µl of piperidine (10 M) (Fischer scientific Ltd.) was added and mixed gently and incubated at 90 ºC for 10 minutes and then transferred to ice-water. To this 300 µl of water was added and samples were extracted using 2 volumes of iso-butanol. The DNA solution was transferred to fresh tubes and precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate. The DNA was allowed to precipitate at -20 ºC for at least 30 minutes. The

precipitated DNA was then resuspended in 0.1 x TE to a final concentration of 1 µg/µl.

2.14.5 Primer Design

Primers were designed using the Oligo™ 1.5 primer designing software. Primers were designed according to the conditions described in Grange *et al (Grange et al., 1997)*.

2.14.6 Primers optimisation

The best annealing temperature for each of the primers designed were determined by performing an LM PCR using G reaction /naked DNA control (see section 2.14.3) and the test primer set. For each test primer three different annealing temperatures were used, whereby the annealing temperatures was increased by 2-3 ºC each time, the highest temperature tried being T_m-3 ºC. Table 3.7 lists the sequence of primers used for *in* vivo foot printing along with their annealing temperatures (table 2.7).

Primer Name	Sequence
HS8 Fa1	ACAGCCGCGATCCACTA
HS8 Fa2	CCTTAGTCCAGCGCGTTTGTCA
HS8 Fa3	TCCAGCGCGTTTGTCAATGAACGTG
HS8 Fb1	CCCATTCTCTCCCATTGCCCAGGTG
HS8 Fb2	TAATCAGGCGGAGCCTGTGGGCT
HS8 Fb3	GGAGCCTGTGGGCTGCTTAGCCAATG

Table 2.7: Table showing sequence of two sets of primers ('a' and 'b') used for in vivo DNaseI and DMS footprinting at HS8 of the distal *PAX5* promoter.

2.14.7 Ligation mediated polymerase chain reaction

Primer extension

5µl of reaction mixture was prepared by combining: 1 µg of DNaseI/DMS treated DNA template, 20µM biotinylated primer, 0.25 µl of 25mM dNTP, 0.25 µl DMSO, 0.5µl of 10X Thermo Pol buffer (New England Biolabs), 1.00µl of VentR® exo$^-$ DNA polymerase (New England Biolabs), 1.00µl of Nuclease free distilled water to a total volume of 5µl. The reaction was then incubated in a T300 thermocycler (Biometra) at 95ºC for 15 minutes, annealing temperature as determined by primer optimisation for 20 minutes, 72ºC for 20 minutes respectively.

Preparation of LP25-21 linkers

20µM LP25 and LP21 oligonucleotides were mixed with nuclease free water (HyClone Molecular Biology grade water, Thermo Scientific) and annealed by incubating the oligonucleotide mixture at 95º C for 15 minutes and then gradually cooled down to 4ºC.

Ligation of LP25-21 linkers to the primer extension product

primer extension LP25-21 linker was ligated to the primer extension product accordingly by combining: 4.25µl of ligation buffer (676 µl of 50%PEG, 82.8mM Tris, pH 7.5, 23mM MgCl2, 56mM DTT, 2.8mM ATP, 0.14mg/ml BSA), 2.00µl of LP25-21 Linkers, .50µl of T4 DNA Ligase (Epicentere®), 0.50µl of ddH_2O and 5µl of the primer extension product to make up a total volume of 12.25µl. The mixture was then incubated at 16ºC overnight.

Capture of primer extension products ligated to LP25-21 linkers

15µl of M-280 Dyanbeads (Dynabeads® M-280 Streptavidin, Invitrogen) were washed with 2x bind and wash buffer (B&W buffer) (10mM Tris pH 7.5, 1mM EDTA, 2M NaCl). Thereafter it was resuspended in 12.5µl of 2X B&W buffer. To the resuspended beads 12.5µl of ligated products were added and incubated for 2 hours at room temperature with rotation. Thereafter the beads were separated using a magnetic separator. The supernatant was discarded and the beads were washed twice with 150µl of 2X B&W buffer and once with 150µl 1X

TE. The beads were then resuspended in 10µl of 0.1 X TE and the DNA was released from the beads by heating the suspension at 95º C for 10 minutes.

PCR Amplification with second nested primers and LP-25

PCR amplification reaction mixture was prepared by combining: 1 µof 10mM dNTP, 1.25 µl of 20µM primer (second nested primer), 1.25 µl of 20µM LP25 primer, 1.50 µl of DMSO, 10.00 µl of 5X Phusion GC buffer (Finnzymes), 0.50 µ of 2U/µl Phusion® Hot start High-fidelity DNA polymerase (Finnzymes), 24.50 µl of ddH2O and 10µl of DNA captured by Dynabeads to make up a total volume of 50 µl. The reaction mixture was then amplified in a T300 thermocycler (Biometra) at 98ºC for 30 seconds, 98ºC for 10 seconds, annealing temperature of primer for 10 seconds, 72ºC for 30 seconds for 22 cycles.

Labelling of third nested primer with P^{32} isotope

40pmol of the third nested primer was incubated with 10 X PNK buffer (70mM Tris-HCl,10mM $MgCl_2$ and 5mM DTT), 10 units of T4 Kinase (New England Biolabs) and 1.85MBq of [γ-^{32}P] ATP (Perkin Elmer) at 37ºC for 60 minutes. To this 40µl of distilled water was added and the entire volume of the labelled primer was purified using an illustra MicroSpin™ G25 column (GE Healthcare) according to the manufacturer's protocol. The labelling PCR was performed by combining: 10µl of 10mM dNTP, 2.00µl of 20µM labelled ^{32}P-primer, 0.15µl of DMSO, 0.80µl of 5X Phusion GC buffer (Finnzymes), 0.25µl of 2U/µl Phusion® Hot start High-fidelity DNA polymerase (Finnzymes), 0.70µl of ddH20 and 10µl of amplified PCR product to make up a total volume of 14µl. The reaction

mixture then incubated in a T300 thermocycler (Biometra) at: 98 ºC for 30 seconds, 98 ºC for 10 seconds, annealing temperature of primer for 10 seconds, and 72 ºC for 30 seconds for 6 cycles in a T300 thermocycler (Biometra).

2.14.8 Electrophoresis and visualisation

The labelled and amplified samples were then electrophoresed in a 6% polyacrylamide gel in a Life Technologies model S2 sequencing gel electrophoresis apparatus for 1.5 hours at constant 80 Watts. The gel was then dried using a Double-up Gel dryer Vacuum pump system (Biorad). The gels were then exposed overnight to imaging screen-K (BIO-RAD) and scanned with molecular imager PharosFX™ system (BIO-RAD) (Tagoh et al., 2006a).

2.15 Protein isolation and western blotting

Whole cell protein was extracted from cells using RIPA buffer (1% NP-40, 0.1% DSD, 50mM Tris-HCl pH 7.4, 0.5% sodium deoxycholate, 1mM EDTA). 10^7 cells were harvested by centrifugation at 300 x g for 5 minutes, resuspended in RIPA buffer and incubated at 4°C for 30 minutes followed by centrifugation at 16000 x g at 4°C for 15 minutes. The resulting pellet was discarded and the supernatant was transferred to fresh tube and used for western blotting or stored at -20°C for use in future.

For western blotting the protein was electrophoresed in a 10% polyacrylamide gel (Resolving gel: Bis-acrylamide 29:1 5mls, 1.5M Tris-HCl pH 8.8 3.75ml, 10% SDS 0.15ml, 10% APS 60µl, TEMED 15µl, Water 6mls and Stacking gel:

bis-acrylamide 29:1 680µl, 1M Tris-HCl pH 6.8 500µl, 10% SDS 40µl, 10% APS 24µl, TEMED 12µl and water 2.72 ml) at 100 Volts until the dye from the sample loading buffer (2x sample buffer: 10mg Bromophenol blue. 30mls of 10% SDS, 10ml Glycerol, 12.5ml 'Upper Tris' and water to bring the volume up to 50mls) had run three- quarters through the gel. The gel electrophoresis was performed in Mini-PROTEAN tetra electrophoresis system (Biorad) according to the manufacturer's protocol using a reservoir buffer for the electrophoresis (10x reservoir buffer: 30g Tris Base, 144g glycine, 100ml 10% SDS and water up to 1litre). The protein standard used to determine molecular weight of proteins of interest was Precision Plus™ protein standard all blue.

After electrophoresis the proteins on the gel was electro-blotted on to a nitrocellulose membrane (Thermo scientific, Pierce) using Mini-Trans blot cell (Biorad) according to the manufacturer's protocol at 120 Volts for 1 hour at 4 °C. After transfer the membrane was blocked using 5% skimmed milk in PBS for 30 minutes and incubated in 5 ml of primary antibody (3pK (C-4) sc-365148 Santa Cruz Biotechnologies, INC) at 1:1000 dilution over-night at 4°C with constant rotation. The membrane was then washed 3 times for 5 minute with PBS-T (PBS+00.1% Tween-20). The membrane was then incubated in 20 ml of secondary HRP-conjugated anti-mouse antibody at 1:10000 dilutions for 1 hour at room temperature. Following incubation with secondary antibody the membrane was washed three times for 5 minutes with PBS-T and visualised using Super-Signal® west Pico Chemiluminescent substrate (Thermoscientific, Pierce) according to the manufacturer's protocol.

3 RESULTS

3.1 *PAX5* is aberrantly expressed in t(8;21) AML

PAX5: the master regulator of B cell development and commitment is aberrantly expressed in patients with acute myeloid leukaemia carrying the t(8;21) translocation (see section 1.6.1) (Tiacci et al., 2004, Valbuena et al., 2006). Kasumi-1 and SKNO-1 cell lines are well-established models of acute myeloid leukaemia with t(8;21) translocation (Asou et al., 1991, Matozaki et al., 1995). In order to compare levels of *PAX5* expression between human B-cell lines and t(8;21) AML cells, we measured *PAX5* expression by q-RT-PCR in the following cell types: Nalm-6 (pre-B cell line established from human ALL), Ramos (B lymphocyte established from Burkitt's lymphoma), Kasumi-1 and SKNO-1 (cell lines established from t(8;21) AML patients) and also primary cells from two t(8;21) patients (patient #1: CD34+ cells from peripheral blood of t(8;21) patient, patient#2 : $CD34^+$ cells from the bone marrow of t(8;21) patient after relapse). HL-60 (pro-myeloblastic cell line established from human acute myeloid leukaemia) and KG-1 (myeloblastic cell line established from human acute myeloid leukaemia) were used as non t(8;21) myeloid precursor controls for comparison with t(8;21) AML. HeLa, an epithelial cell line was used as a non-haematopoietic control. Our experiments showed that *PAX5* is robustly expressed in B-lymphoid cell lines. In non t(8;21) myelobasts (HL-60 and KG-1) and also in HeLa *PAX5* expression could not be detected. t(8;21) AML cell lines and t(8;21) patient cells showed aberrant *PAX5* expression albeit at a level much lower compared to B lymphoid cells (figure 3.1)

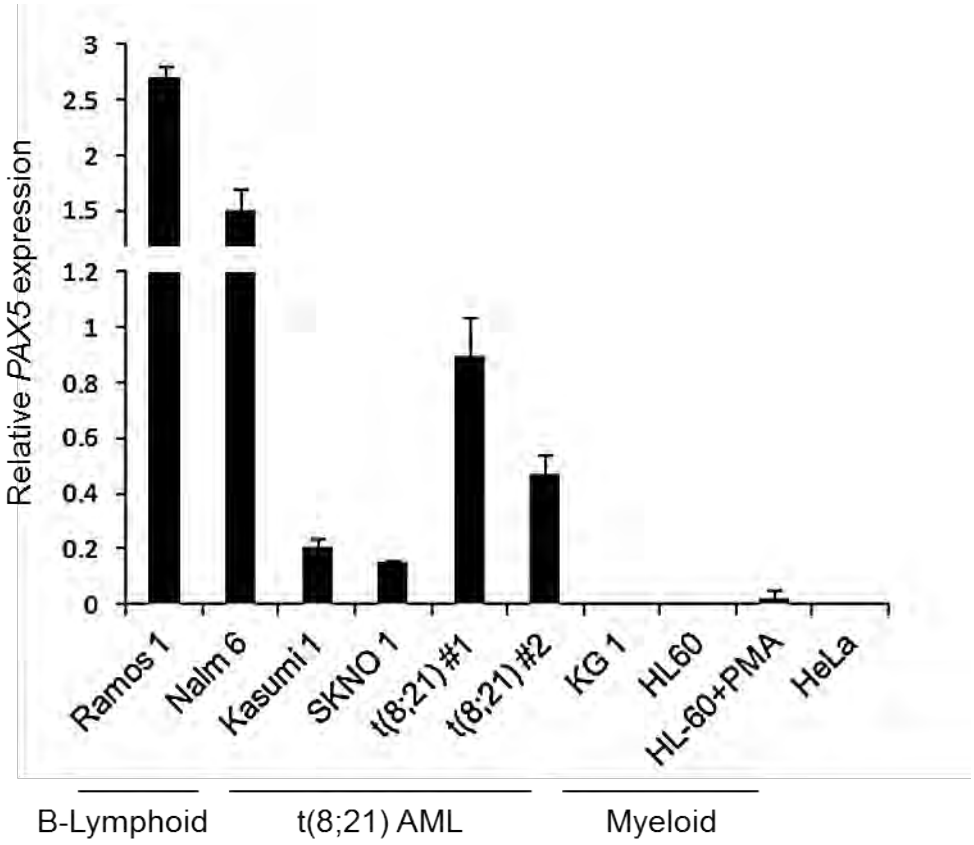


Figure 3.1: **Relative *PAX5* expression in B-lymphoid, t(8;21) AML and non-t(8;21) myeloid precursor cell lines**. Quantitative real-time PCR analysis showing *PAX5* expression relative to *TBP* expression in Ramos (B-cell line), Nalm -6 (pre-B cell line), Kasumi-1 and SKNO-1 t(8;21) AML cell lines and primary cells from two t(8;21) patient samples (t(8;21)#1 and t(8;21)#2 respectively),KG1, HL-60 (myeloid cell lines without t(8;21) translocation and HL-60+PMA (HL-60 cell line differentiated to macrophage like cells by treatment with PMA) and HeLa (epithelial cell line). The y-axis represents average relative *PAX5* expression from at least three independent experiments; the error bars represent standard deviation between three independent experiments. For the patient samples the error bars represent variability between duplicate q-PCR measurements

3.2 *PAX5* is organized in an open chromatin conformation and carries active histone marks in human B-lymphoid cells

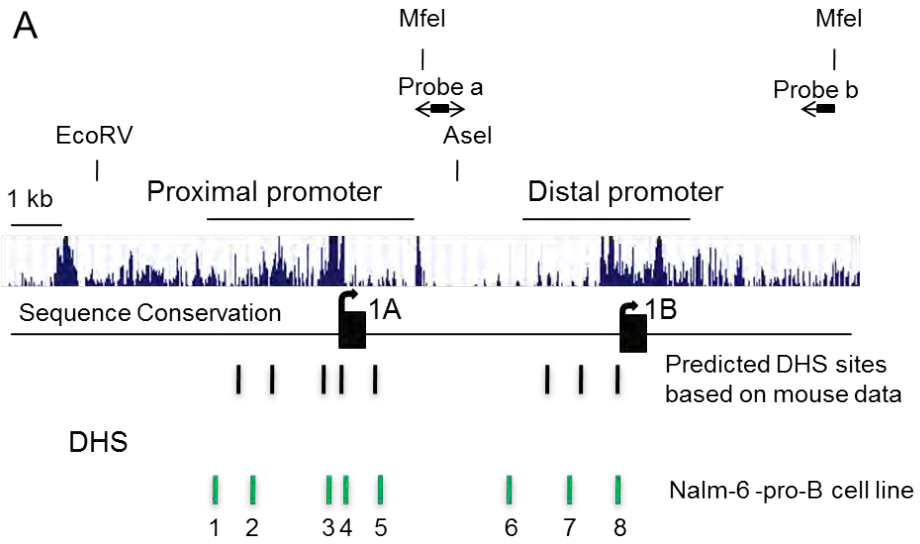
3.2.1 DNase I hypersensitive site mapping at the PAX5 proximal and distal promoters in Nalm-6 pre-B cell line

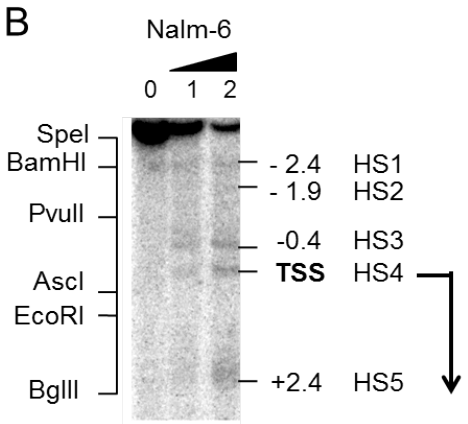
In order to study the molecular mechanism of *PAX5* de-regulation in t(8;21) AML we needed a clear understanding of transcriptional regulation of *PAX5* in normal human B cells. The murine *Pax5* locus has been well characterised by Decker *et al* in mouse B lymphoid cells (Decker et al., 2009) (and also see section 1.4.2). However, such a characterisation of the human *PAX5* locus was missing from the relevant literature. To this end we set out to characterise the cis-regulatory elements of the human *PAX5* locus in B-lymphoid cells using DNase I hypersensitive site mapping (referred to as DHS mapping hereafter).

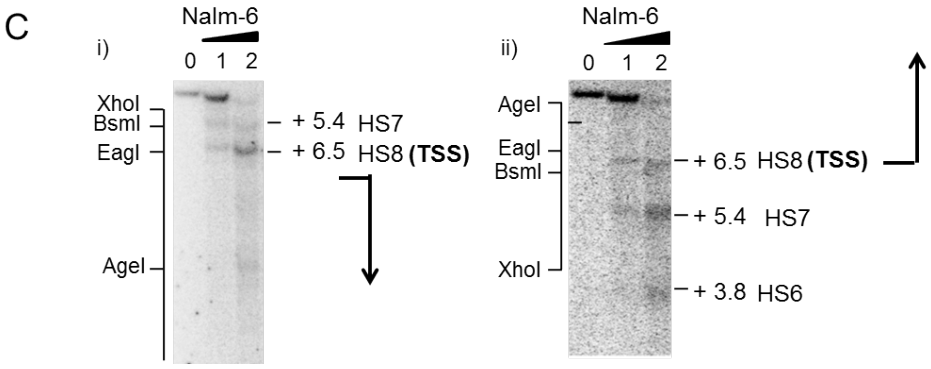
The positions of the DHSs at the murine *Pax5* proximal and distal promoters were known (Decker et al., 2009). Using this as a reference, we predicted the positions of the corresponding human DHSs and designed a strategy for mapping the human *PAX5* promoter DHSs by Southern blotting (figure 3.2 A).

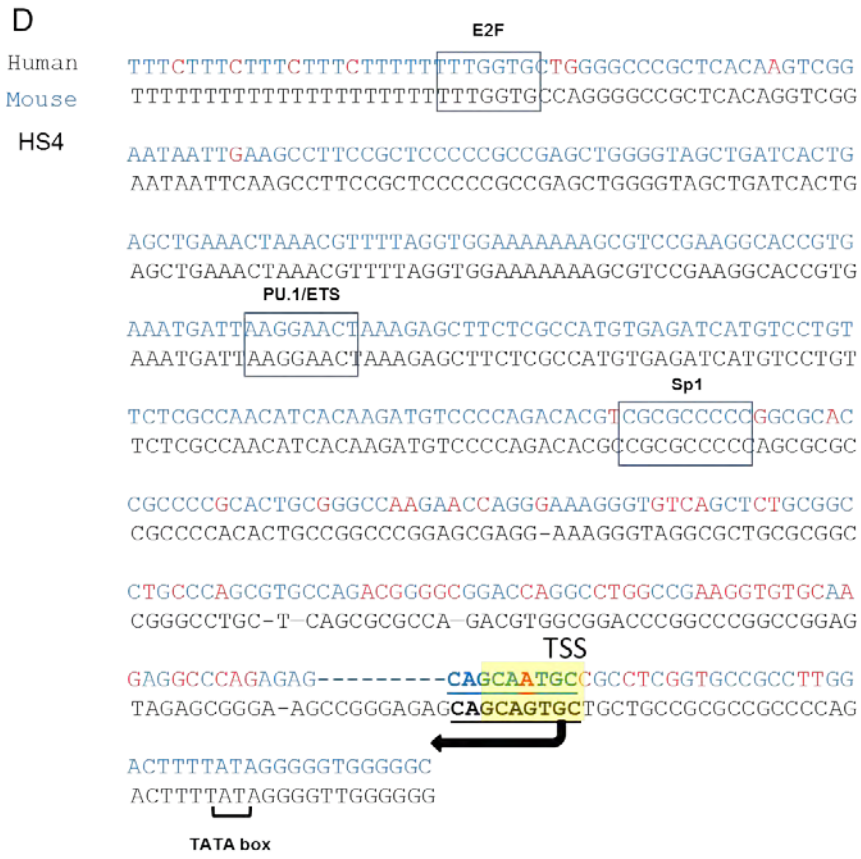
The proximal promoter at exon 1A was mapped using ‘Probe a’ within an EcoRV/ AseI restriction fragment as indicated in figure 3.2 A. This promoter element contained a cluster of five hypersensitive sites labelled as HS 1 to 5 (figure 3.2 A and B). The distal promoter at exon 1B was mapped within an MfeI restriction fragment using ‘Probe a’ and ‘Probe b’ from either end of the MfeI fragment (figure 3.2A). This promoter element contained a cluster of three hypersensitive sites labelled as HS 6 to 8 (figures 3.2 A and C i and ii). The

positions of HS 1, HS 2, HS 6 and HS 7 were shifted from their corresponding murine hypersensitive sites (green bars in figure 3.2 A). However, the positions of HS 4 and HS8, which are the minimal promoters carrying the two transcription start sites respectively, were conserved between human and mouse. Furthermore comparison between human and mouse sequences at HS 4 and HS 8 shows 84.5% and 91% sequence conservation respectively (figures 3.2 D and E).






C
Nalm-6
i)
0 1 2
XhoI
BsmI
EagI
AgeI
+ 5.4 HS7
+ 6.5 HS8 (TSS)
Nalm-6
ii)
0 1 2
AgeI
EagI
BsmI
XhoI
+ 6.5 HS8 (TSS)
+ 5.4 HS7
+ 3.8 HS6


D
HS4
Human
Mouse
E2F
PU.1/ETS
Sp1
TSS
TATA box

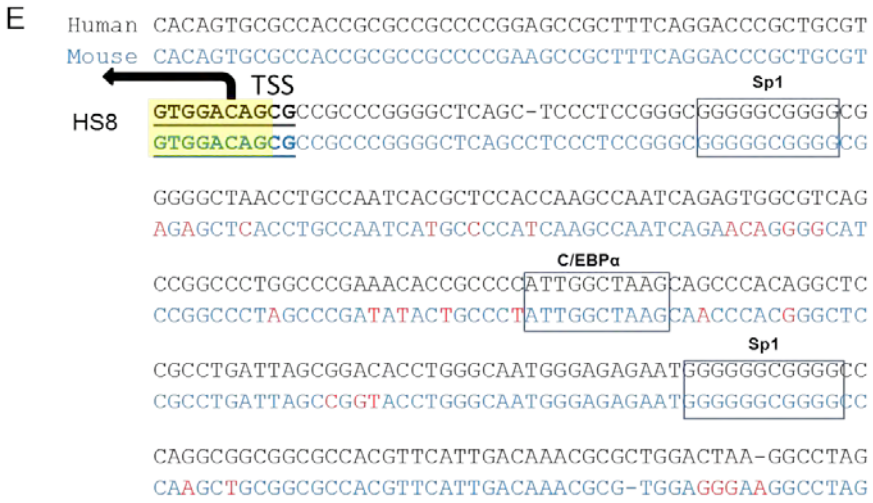


Figure 3.2: DNase I hypersensitive site mapping of *PAX5* proximal and distal promoters in pre-B cell line (Nalm-6). A) Schematic representation of the strategy used to map DNAse I hypersensitive sites (DHS) by Southern blotting at the proximal and distal *PAX5* promoters. The proximal promoter DHSs were mapped using probe Pa within the EcoRV /AseI restriction fragment while the distal promoter was mapped within the MfeI restriction fragment using probes 'Probe a' and 'Probe 'b. The positions of the hypersensitive sites predicted from the already characterised mouse *Pax5* promoter are indicated as black bars, while the hypersensitive sites mapped in the human pro-B Nalm-6 cell line are indicated with green bars B) Southern blot showing DNAse I hypersensitive sites at the proximal *PAX5* promoter mapped using probe Pa. C) Southern blot showing DNase I hypersensitive sites at the distal *PAX5* promoter mapped using probe 'Probe b' (i) and Pa (ii) respectively. Each of the hypersensitive sites in B and C are labelled with distance in kilo base pairs mapped from HS 4 TSS. D) Comparison of mouse and human sequences at HS 4 transcription start site. E) Comparison of mouse and human sequences at HS 8 transcription start site. The human sequence is in black and the mouse in blue. The differences in sequences are marked in red. The conserved transcription factor binding sites (as defined by MatInspector) are indicated with black boxes.

3.2.2 DNase I hypersensitive site mapping locating the PAX5 intronic enhancer in Nalm-6 pre-B cell line

A B-cell specific enhancer with a hypersensitive region consisting of four DHSs (HS A to HS D) was described at the fifth intron of the murine *Pax5* gene by Decker *et al* (see section 1.4.2). This paper demonstrated that the expression of *Pax5* in mouse B cells largely depended on this intronic enhancer element (Decker et al., 2009). It was therefore important to identify the corresponding enhancer element in human B cells at the *PAX5* locus.

We predicted possible positions of the human *PAX5* enhancer- DHSs based on the positions of the mouse DHSs (black bars in Figure 3.3 A). We then used this, to devise a strategy for mapping the human enhancer-DHSs by Southern blotting. This strategy involved mapping the enhancer DHSs with two probes: 'Probe1' and 'Probe 2' from either end of a BamHI fragment as illustrated in Figure 3.3 A. Our experiments showed the presence of the 4 previously described DHSs HS A, B, C and D (figures 3.3 A, B and C). In addition to these DHSs we also observed three DHSs specific to the human enhancer at +37.4, +36.8 and +36.2 kb relative to the HS4 transcription start site respectively (figures 3.3 A and B). Of the enhancer hypersensitive sites, HS B is the best characterised element in mouse and was shown to bind PU.1, NFκB and IRF4 (Decker et al., 2009). In Nalm-6 cells, HS B formed the most prominent DNase I hypersensitive site and most likely also the main enhancer element of human *PAX5*. The sequence at the DHSs of the *PAX5* enhancer is only moderately conserved. Sequence comparison between mouse and human HS B showed only a modest 58% conservation (figure 3.3 D) However, the actual binding

sites of NFκB, IRF, E-Box proteins and EICE (ETS IRF composite element) and their order are well conserved between mouse and human.

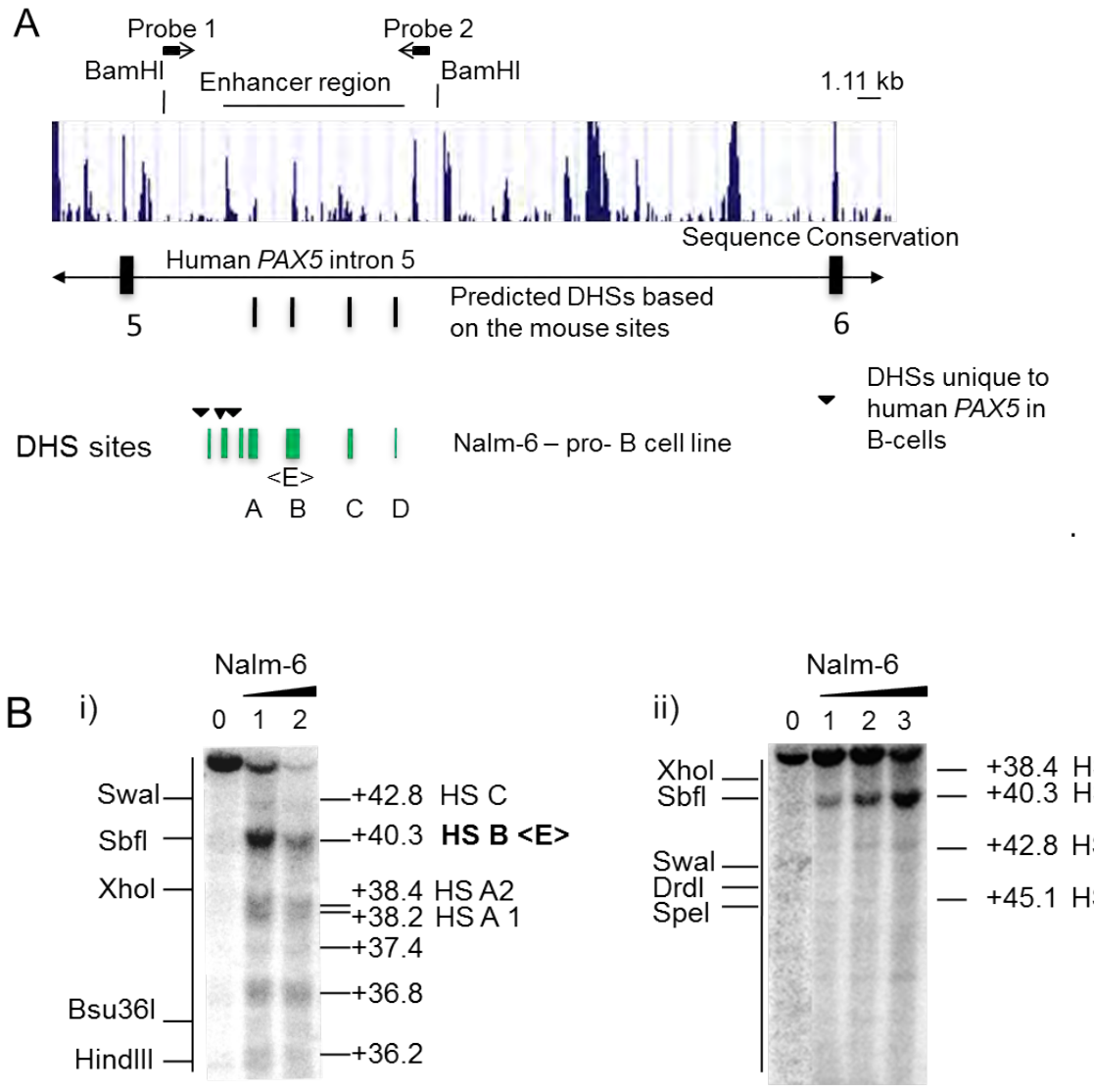


Figure 3.3: DNAse I hypersensitive site (DHS) mapping at *PAX5* intronic enhancer in pre B cell line (Nalm-6). A) Schematic representation showing mammalian sequence conservation at the *PAX5* enhancer region and the strategy used to map DHSs by southern blotting at the *PAX5* intronic enhancer. The DHSs at the *PAX5* enhancer were mapped using probes1 and 2 within a BamHI fragment as indicated in the figure. The positions of DHSs at the characterised murine *Pax5* intronic enhancer (Decker et al., 2009) is indicated with black bars, while the positions of the DHSs observed in Nalm-6 human pre-B cell line are indicated with green bars. B) Southern blot showing DNAse I hypersensitive sites (DHS) at the *PAX5* intronic enhancer mapped with Probe 1 (i) and Probe 2 (ii). The DHSs are marked with their names and the distance in kilo base pairs from HS 4 transcription start site (see figure 2A). C) Comparison of human and mouse sequences at HS B intronic enhancer. The human sequence is shown in black while that of the mouse in blue. The differences in sequences are indicated in red. The conserved transcription factor binding sites (as defined by Decker *et al* and MatInspector) are marked with black boxes.

3.2.3 *PAX5* promoter hypersensitive sites carry active histone marks and recruit RNA polymerase II in Nalm-6.

Having identified the DNaseI hypersensitive sites at the *PAX5* promoters and enhancer in a human pre-B cell line, we wanted to further characterise these DHSs. To this end we performed chromatin immuno-precipitation assays (ChIP) to examine the distribution of active and inactive histone modifications as well as RNA polymerase II recruitment.

H3K4me3 is a histone mark specifically associated with active promoters and H3K9ac is a histone modification generally associated with open chromatin (Lee and Young, 2000). ChIP-qPCR assays showed high levels of H3K4me3 and H3K9ac at the *PAX5* promoter DHSs (HS 1 to HS 8) in Nalm-6 cells (figures 3.4 A and B). H3K27me3 is an inactive histone mark and is the hall-mark of polycomb mediated transcriptional repression (Margueron and Reinberg, 2011). The *PAX5* gene which is highly expressed in Nalm-6 cells, showed no enrichment of H3K27me3 (figure 3.4 C). Furthermore, consistent with the fact that the gene is highly expressed in Nalm-6, ChIP assays showed high levels of RNA polymerase II (total and phosphorylated-serine 2 RNA polymerase II) recruitment across the *PAX5* promoter elements but not at HS B enhancer (figures 3.4 D and E). Taken together, these experiments establish that the *PAX5* locus shows an open chromatin structure, active histone marks, RNA polymerase II recruitment and a high level of transcription in the human pre-B cell line Nalm-6.

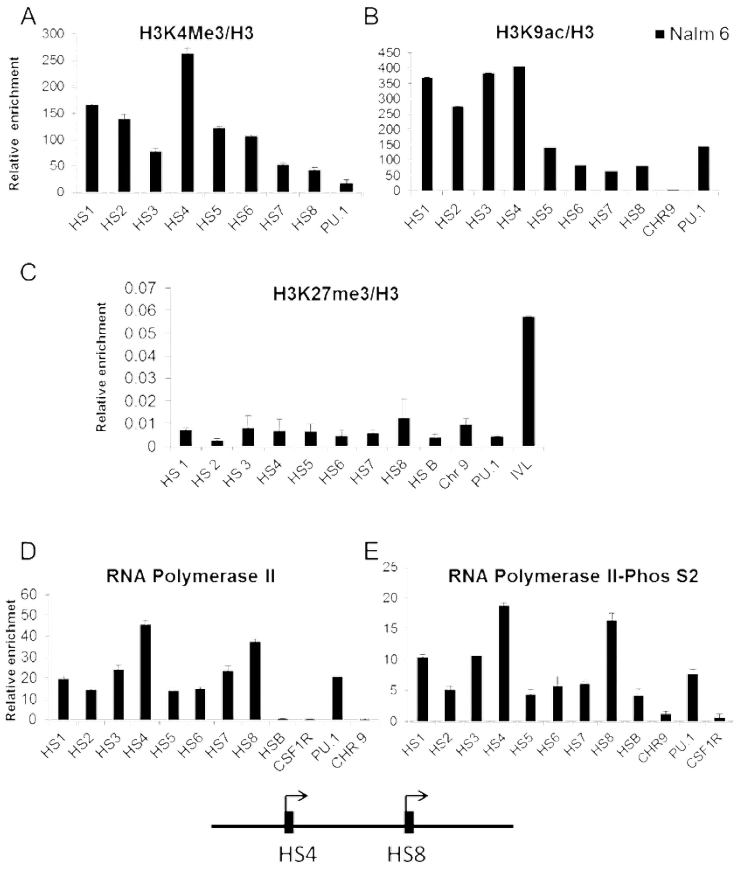


Figure 3.4: Active histone marks and elongating RNA polymerase II are present at the proximal and distal *PAX5* promoters in the Nalm-6 cell line. ChIP-qPCR showing relative enrichment of H3K4me3 (A), H3K9ac (B), H3K27me3 (C), RNA Polymerase II (D) and RNA Polymerase II phosphorylated at serine 2 residue (E) at the *PAX5* promoter hypersensitive sites HS1-8 and at the *PAX5* intronic enhancer hypersensitive site HS B. *PU.1* promoter primers are used as a positive control, *CSF1R* primers are used as a negative control and *CHR9* primers located at the 3' end of the *PAX5* gene and are used as an internal negative control. The y-axis in A, B, D and E show relative enrichment over the inactive *IVL locus* while C shows relative enrichment over input while *IVL* is used as a positive control region. H3K4me3, H3K9ac and H3K27me3 (A, B and C) is normalised to H3 ChIP. Each bar graph is representative of at least two independent ChIP experiments and the error bars represent variation between duplicate q-PCR measurements.

3.3 *PAX5* is poised for transcription in non-t(8;21) myeloid precursors

3.3.1 PAX5 proximal and distal promoters show active chromatin conformation in t(8;21) AML and also in non-t(8;21) myeloid precursors.

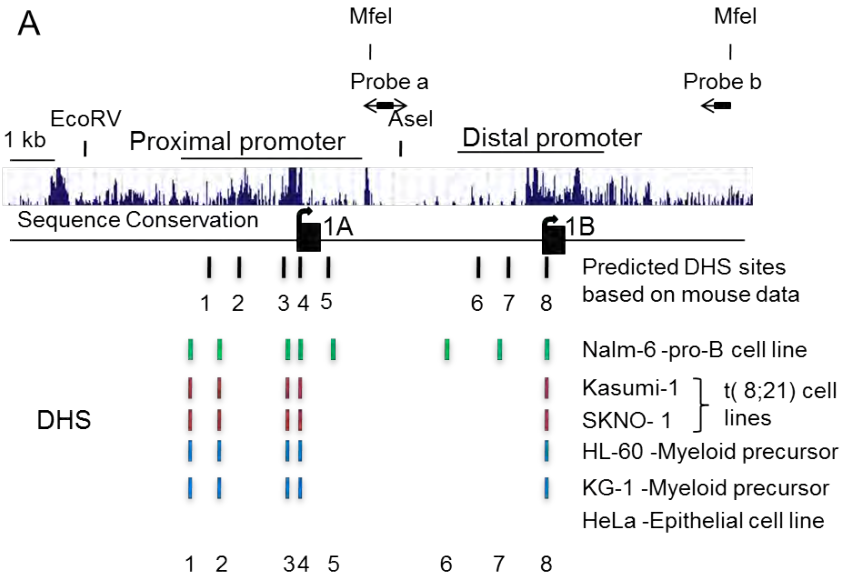
Having established the DHS pattern and histone modification patterns at the *PAX5* promoters and enhancer in human pre-B cells (see sections 3.2.1, 3.2.2 and 3.2.3), we wanted to do the same for t(8;21) AML cells. Using the same strategy as described in section 3.2.1 for DHS mapping, we found that in t(8;21) AML cells the minimal promoter sites HS 4 and HS 8 at the proximal and distal promoters respectively, remain DNase I hypersensitive (figure 3.5 A).

At the proximal promoter, four of the five DHSs identified in the pre-B cell line were hypersensitive in t(8;21) AML (HS 1 to HS 4) and at the distal promoter only one of the three DHSs (i.e. HS 8) was hypersensitive in t(8;21) AML (figures 3.5 A, B and C). Genome-wide DNase I-sequencing in cells from two t(8;21) patients and also in Kasumi-1 confirmed this pattern of DHS sites at the *PAX5* promoter cluster (figure 3.7) (Ptasinska et al., 2012a).

We used HL-60 and KG-1 (for KG-1 data not shown) as non t(8;21) myeloid precursor controls which do not express *PAX5*. However, to our surprise HL-60 and KG-1 showed exactly the same DHS pattern at the two *PAX5* promoters as t(8;21) AML (figures 3.5 A, B and C). DNase I-sequencing in HL-60 cells done by the University of Washington as part of the ENCODE project also shows the same DHS pattern at the *PAX5* locus as that observed by our DHS-Southern blotting experiments (UCSC Genome Browser). Furthermore, DNase I-sequencing performed by Ptasinska *et al* in normal CD34+ myeloid progenitors

also showed a similar DNAse I hypersensitive pattern at the *PAX5* promoters (figure 3.7) (Ptasinska et al., 2012a). $CD34^{+}$ myeloid progenitors are mobilized stem cells from the peripheral blood of healthy donors and do not express *PAX5*. HeLa (figure 3.5) and CD14 + Monocytes (figure 3.7) however showed no DHSs at the *PAX5* locus and these cell types serve as proper negative controls for the experimental procedure.

Taken together, the DHS mapping by Southern blotting and analysis of published genome-wide DNAse I-sequencing experiments establish that the *PAX5* promoters remain in an open chromatin conformation in t(8;21) AML as well as in non-t(8;21) myeloid precursors.



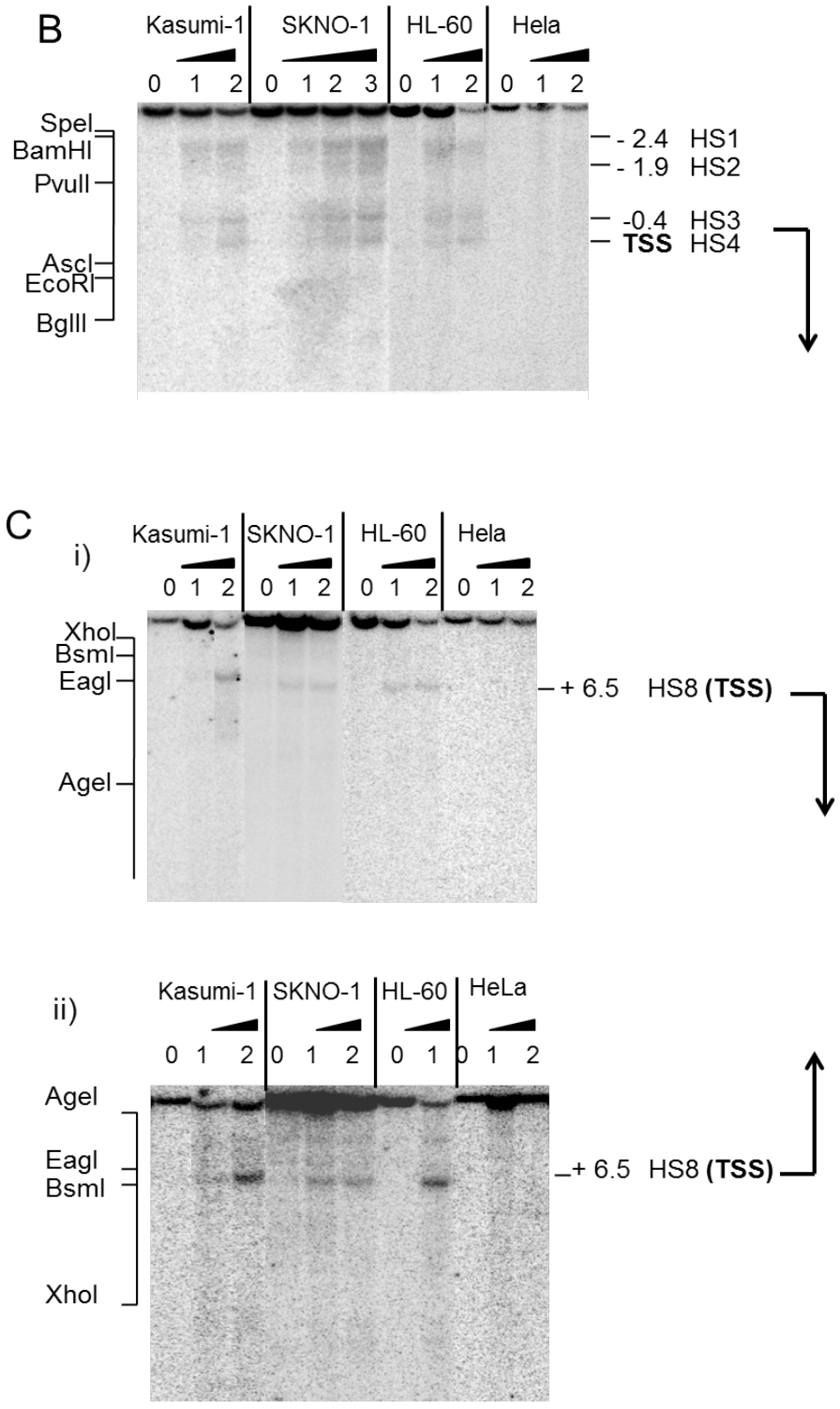


Figure 3.5: DNase I hypersensitive site mapping of the *PAX5* proximal and distal promoters in t(8;21) and non t(8;21) myeloid precursor cell lines. A) Schematic representation showing the mammalian sequence conservation and strategy used for mapping the DHSs (see figure 2A) at the *PAX5* proximal and

distal promoters. Positions of predicted DHSs based on the characterised murine *Pax5* promoter region are indicated with black bars, while the positions of the DHSs observed in Nalm-6 (human pro-B cell line), Kasumi-1 and SKNO-1 (human t(8;21) AML cell lines) and HL-60 and KG-1 (non-t(8;21) AML cell lines)) are indicated with green, red and blue bars respectively. HeLa (human epithelial cell line) showed no DNAse I hypersensitive sites at the *PAX5* promoter region. B) Southern blot showing DNase I hypersensitive sites (DHS) at the *PAX5* proximal promoter in the indicated cell lines mapped with probe 'Probe a' C) Southern blots showing DHSs at the *PAX5* distal promoter in the indicated cell lines, mapped with probes 'Probe b' (i) and 'Probe a' (ii) respectively. All the DHSs are labelled with their names and distances in kilo base pairs in relation to HS 4 transcription start site

3.3.2 *PAX5* enhancer displays an inactive chromatin conformation in t(8;21) AML

In order to determine whether the *PAX5* intronic enhancer (see section 3.2.2) was also organized in an active chromatin conformation in t(8;21) AML, we performed DHS mapping at the enhancer in these cell types. Out of the seven hypersensitive sites identified in Nalm-6 (green bars Figure 3.6 A), only HS A and HS C were hypersensitive in t(8;21) AML. However, HS A and HS C were also hypersensitive; not only in HL-60, KG-1 (myeloid precursors) but also in HeLa cells (figure 3.6). Therefore, we reasoned that HS A and HS C are ubiquitous hypersensitive sites present not only in myeloid precursor controls (HL-60 and KG-1) but also in epithelial negative control cells (HeLA)

Of the four DHSs at the intronic *PAX5* enhancer, the best characterised DHS in mouse B cells and the strongest in Nalm-6 cells was HS B (figure 3.3). HS B is completely absent in t(8;21) AML as well as non-t (8;21) myeloid controls (figure 3.6). This result was also confirmed by DNase I-sequencing experiments in Kasumi-1 and in primary t(8;21) patient samples (figure 3.7) (Ptasinska et al., 2012a, Cobaleda et al., 2007). From the experiments described above, it is reasonable to conclude that the main *PAX5* enhancer hypersensitive site (HS B) is in an inactive chromatin conformation and hence the enhancer is inactive in t(8;21) AML.

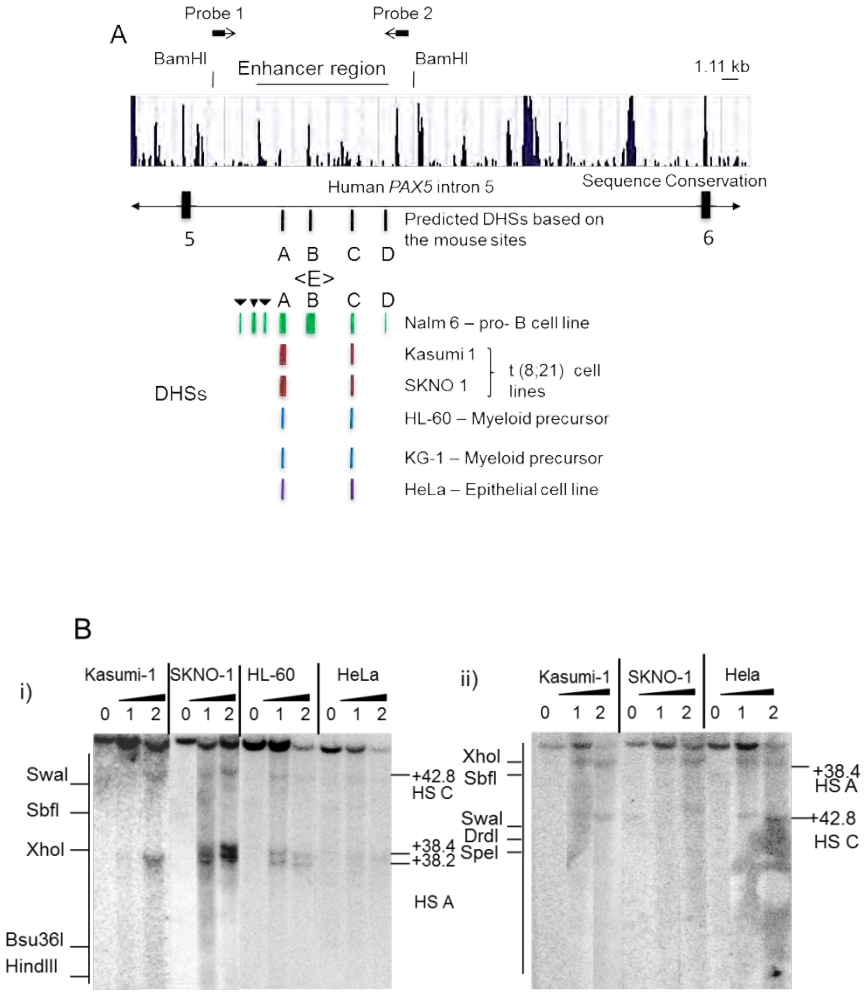


Figure 3.6: DNase I hypersensitive site mapping of the *PAX5* intronic enhancer in t(8;21) AML and non-t(8;21) myeloid precursor cell lines. A) Schematic representation showing the mammalian sequence conservation and strategy used for mapping the DHSs (see figure 3A) at the *PAX5* intronic enhancer. Positions of predicted DHSs based on the characterised murine *Pax5* enhancer is indicated with black bars, while the positions of the DHSs observed in Nalm-6 (human pro-B cell line), Kasumi-1 and SKNO-1 (human t(8;21) AML cell lines), HL-60 and KG-1 (non-t(8;21) AML cell lines)) and HeLa (human cervical cancer cell line) are indicated with green, red, blue and purple bars respectively. B) Southern blots showing DNase I hypersensitive sites (DHS) at the *PAX5* enhancer in the indicated cell lines mapped with Probe1 (i) and Probe 2(ii) respectively. All the DHSs are labelled with their names and distances in kilo base pairs in relation to HS4 transcription start site.

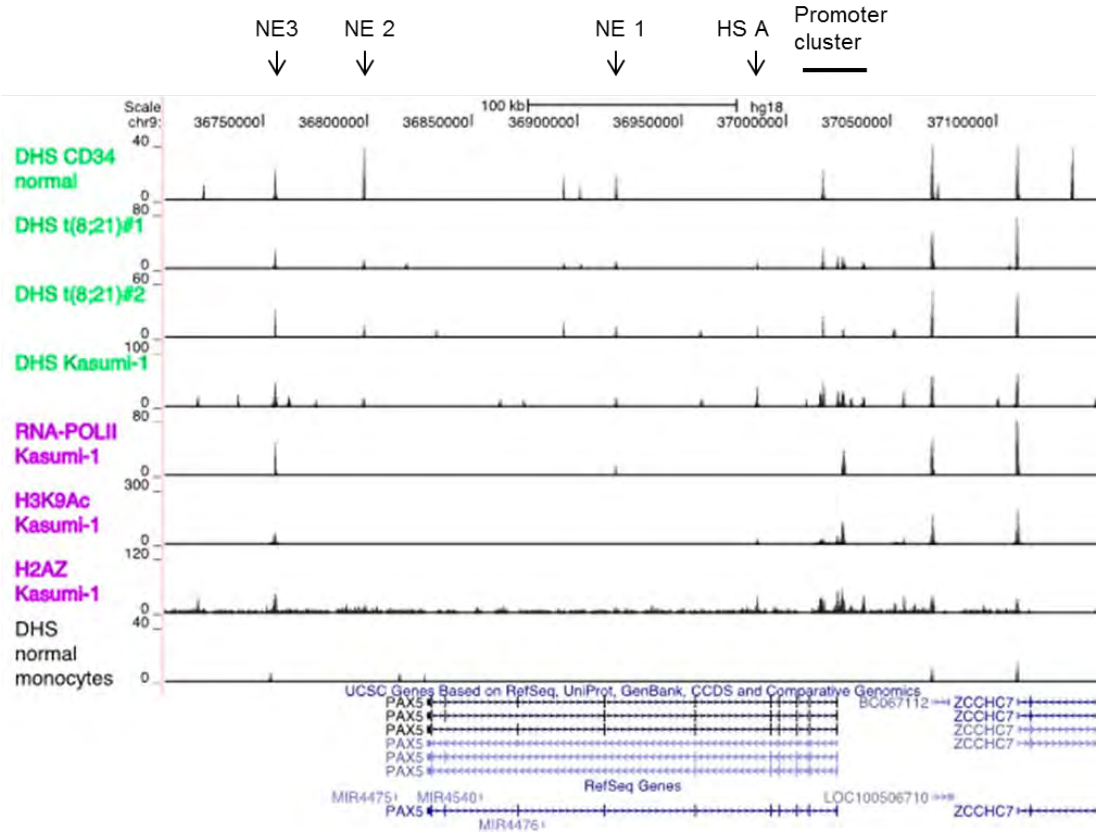


Figure 3.7: *PAX5* locus shows open chromatin conformation in Kasumi-1, primary cell from t(8;21) patients and in normal human CD34+ progenitors but not in CD4+ monocytes. Screenshot showing DNAse I-sequencing tracks at the *PAX5* locus in CD34+ progenitors from two primary t(8;21) patient samples, Kasumi-1 cell line and CD4+ normal monocytes. Along with the promoter cluster hypersensitive sites and HS A of the intronic enhancer (see figures 4 and 5); potential new elements (labelled as NE1-3) were identified with the genome- wide DNAse I -seq approach. This screen shot also shows ChIP-seq tracks showing RNA polymerase II, H3K9ac and H2AZ enrichment at the hypersensitive sites. The genome-wide data was produced as part of work done by Ptasinska *et al* (Ptasinska et al., 2012a).

3.3.3 DNase I-sequencing identifies new hypersensitive sites in the *PAX5* gene body

DNAse I sequencing in primary t(8;21) patients and in Kasumi-1 cells, identified hypersensitive sites that had not been previously characterised as *PAX5* regulatory elements. We named them as NE (new elements) 1, 2 and 3 (figure 3.7). These sites were also present in normal CD34+ myeloid progenitors. On inspection whether DHSs corresponding to NE1, NE2 and NE 3 were seen in B lineage cells (GM 12878 B lymphoid cells); we found that while NE 1 and NE 3 were present, NE 2 was not (UCSC genome Browser). The role of these hypersensitive sites in the regulation of *PAX5*, if any, is so far unknown.

3.3.4 *PAX5* promoters carry high levels of active histone marks in t(8;21) AML but not in non-t(8;21) myeloid precursors.

DHS mapping revealed that the *PAX5* locus is sensitive to DNase I digestion at the promoter elements in t(8;21) AML and also in non t(8;21) myeloid precursors. In non- t(8;21) myeloid precursors the gene, however, is transcriptionally repressed (figure 3.1), indicating that the gene may be in a poised configuration characterized by bivalent chromatin marks and a poised chromatin (see sections 1.2.7 and 1.3.2). In order to determine if there was any difference in histone modifications at these elements between *PAX5*-expressing t(8;21) cells and *PAX5*-not expressing myeloid precursors, we first carried out chromatin immuno-precipitation (ChIP) assays for active histone marks: H3K4me3 and H3K9ac. ChIP-qPCR assays showed that *PAX5* promoter

hypersensitive sites carry high levels of H3K4me3 and H3K9ac in Kasumi-1 and SKNO-1 cells but not in HL-60 cells (figures 3.8 A and B).

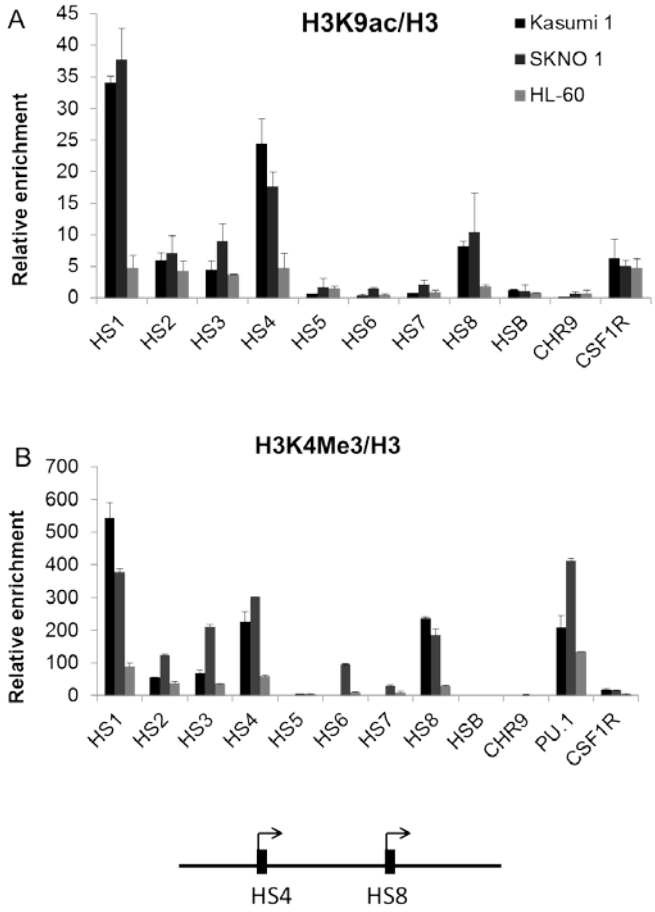


Figure 3.8: *PAX5* promoters show active histone marks in Kasumi-1 and SKNO-1 cell lines but not in HL-60. ChIP-qPCR showing relative enrichment of H3K4me3 (A) and H3K9ac (B) at the *PAX5* promoter hypersensitive sites HS 1-8 and the intronic enhancer hypersensitive site HS B. *PU.1* and *CSF1R* promoter primers are used as positive controls. CHR9 primers are located at the 3' end of the *PAX5* gene and are used as an internal negative control. The relative enrichment is calculated over *IVL*. The H3K4me3 and the H3K9me3 ChIPs are normalised to H3 ChIP. Each bar graph is a representative of at least two independent ChIP experiments and the error bars represent the variation between duplicate q-PCR measurements

3.3.5 *PAX5* promoters carry stalled RNA Polymerase II complexes in non-t(8;21) myeloid precursors

Further to the histone modification ChIPs described above, we performed ChIP-qPCR assays for total RNA polymerase II and its elongating form which is phosphorylated at serine-2 residue of the C-terminal repeat (see section 1.2.2). ChIP-qPCR showed high levels of total RNA polymerase II recruitment at the *PAX5* promoters in Kasumi-1 and SKNO-1 (figure 3.9 A). Surprisingly, also in HL-60 cells, there was substantial RNA polymerase II recruitment (figure 3.9 A). In order to explain this observation in light of the fact that *PAX5* is not expressed in HL-60 cells; we performed ChIP for the elongating form of RNA polymerase II complex. Kasumi-1 and SKNO-1 showed high levels of serine-2 phosphorylated RNA Polymerase II enrichment at the *PAX5* promoters, but HL-60 did not (figure 3.9 B). This result indicates that the RNA polymerase II that is recruited at the *PAX5* promoters in HL-60 fails to be phosphorylated at serine 2 residue and hence does not form an elongating RNA Polymerase II complex that is capable of effective transcriptional elongation.

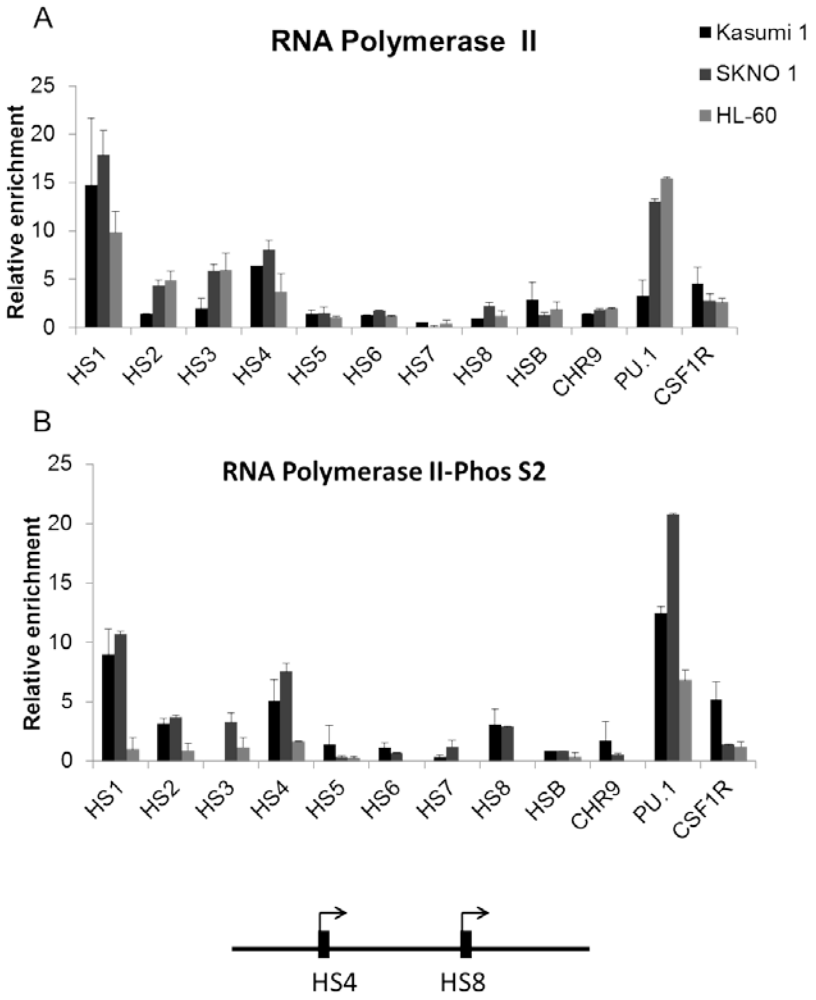


Figure 3.9: *PAX5* promoters carry paused RNA polymerase II in HL-60 (non t(8;21) myeloid precursor) but in Kasumi-1 and SKNO-1 t(8;21) cell lines RNA Polymerase II gets phosphorylated at the serine 2 residue and forms an elongating RNA polymerase II complex. ChIP-qPCR showing relative enrichment of RNA polymerase II (A) and RNA Polymerase II S2P (B) occupancy at the *PAX5* promoter hypersensitive sites HS 1-8 and the intronic enhancer hypersensitive site HS B. *PU.1* and *CSF1R* promoter primers are used as positive controls. CHR9 primers are located at the 3' end of the *PAX5* gene and are used as an internal negative control. The relative enrichment is calculated over *IVL*. Each bar graph is a representative of at least two independent ChIP experiments and the error bars represent the variation between duplicate q-PCR measurements.

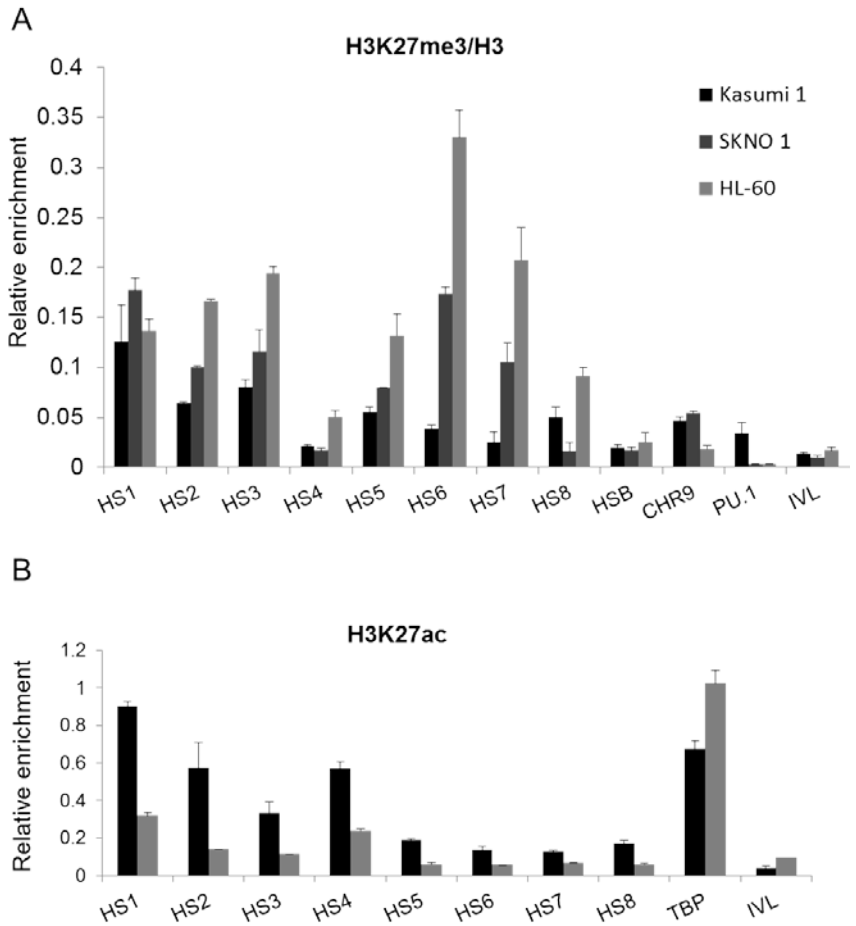
Taken together, the DNAse I hypersensitive site mapping and ChIP-qPCR data in Kasumi-1, SKNO-1 and HL-60 cells establish that in t(8;21) AML the *PAX5* locus is organized in an open chromatin structure with active histone modifications and recruits the elongating RNA polymerase II complex which leads to the transcription of the gene. In HL-60 (myeloid precursors), however, *PAX5* locus is in an open chromatin conformation with a stalled RNA polymerase II complex and the gene is transcriptionally inactive.

3.4 *PAX5* is repressed by polycomb repressive complexes in non-t(8;21) myeloid precursors

Having established that *PAX5* is poised for transcription in non-t(8;21) myeloid precursors, we examined what keeps the gene repressed in these cells. *PAX5* is a developmentally regulated lineage specifying gene(Cobaleda et al., 2007). Previous studies have reported repression of *PAX5* and other lineage specifying genes in pluripotent stem cells and in inappropriate lineages by polycomb repressive complexes (Chen et al., 2011). In order to see whether this was the case for *PAX5* repression in non-t(8;21) myeloid precursors; we performed ChIP assay for H3K27me3, H3K27ac and EZH2.

ChIP assays showed high levels of H3K27me3 in HL-60 cells, especially at the distal promoter elements. Kasumi-1 and SKNO-1 cells also showed some H3K27me3 mark, but much lower compared to HL-60 cells (figure 3.10 A). ChIP-qPCR showed much higher enrichment of EZH2 in HL-60 than in Kasumi-1 cells consistent with the H3K27me3 ChIP (figure 3.10 C ChIP performed by Anetta Ptasinska). Recent studies have reported that H3K27ac is a mark that

indicates removal of polycomb from a polycomb target gene and that this mark and H3K27me3 are mutually exclusive (Lau and Cheung, 2011a, Lau and Cheung, 2011b). Our experiments showed that Kasumi-1 cells carry high levels of acetylation at H3K27 at the *PAX5* promoter DHSs while HL-60 showed none (figure 3.10 B). Taken together, this data indicates that in HL-60 cells *PAX5* is a target of polycomb complexes that represses its transcriptional activity even though the gene is in an active chromatin conformation and recruits RNA polymerase II.



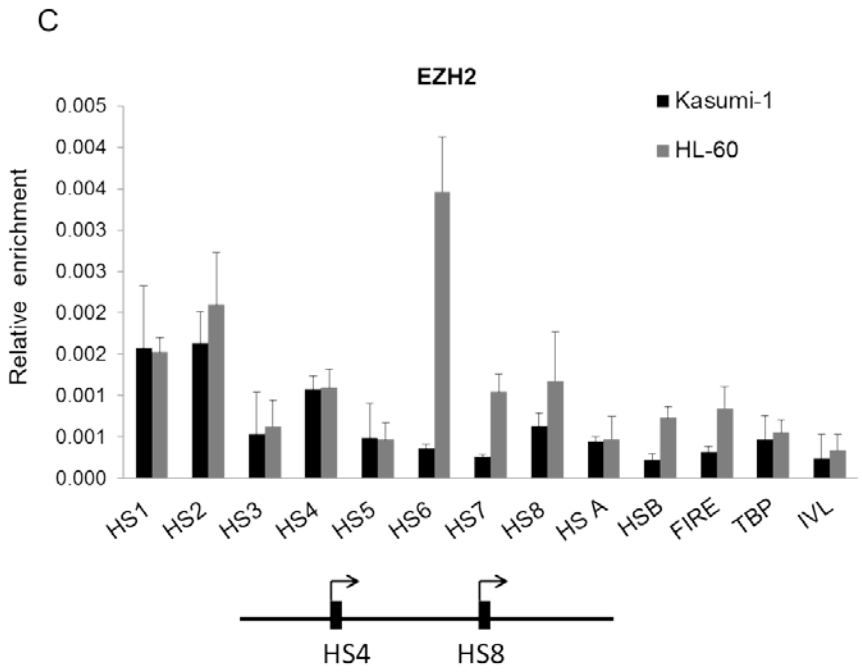


Figure 3.10: *PAX5* is a polycomb target in myeloid precursors without t(8;21) translocation. ChIP-qPCR showing H3K27me3/H3 (A), H3K27ac (B) and EZH2 (C) enrichment at the *PAX5* locus. The bars represent relative enrichment over input. Each bar graph is a representation of at least two independent experiments. The error bars represent variation between duplicate q-PCR measurements. *TBP, IVL FIRE* and *PU.1* primers are used as negative controls in A and C while in B, *TBP* promoter is used as a positive control. CHR9 serves as an internal negative control for the *PAX5* gene.

3.5 DMS and DNAse I *in-vivo* foot-printing show that *PAX5* distal promoter HS 8 display an identical chromatin fine structure and transcription factor binding pattern in B cells, t(8;21) AML and non t(8;21) myeloid precursors

Our experiments so far showed that the *PAX5* gene is poised for transcription but repressed by polycomb repressive complexes in non-t(8;21) myeloid precursors (sections 3.3 and 3.4). We hypothesized that in t(8;21) this polycomb repression is somehow relieved, leading to effective transcriptional elongation. Classically, activation of a gene requires binding of transcription factors to its cis-regulatory elements to drive the assembly of the basal transcription machinery leading to effective transcription (Lee and Young, 2000) (also see sections 1.2.2 and 1.2.8).

It is known that EBF-1 is a critical factor in the regulation of *Pax5* in mouse B cells. However the binding sites for EBF-1 were present in HS 6 and HS 7 which are not in an open chromatin conformation in t(8;21) cells (figure 3.5). Furthermore, the same study showed that the enhancer HS B binds factors such as IRF4, PU, 1 and NFκB in mouse B cells (Decker et al., 2009). HS B, too is in an inactive chromatin conformation in t(8;21) AML Hence these known transcription factors and their interactions with *PAX5* regulatory elements were unlikely to be responsible for the transcription of *PAX5* in t(8;21) AML.

We therefore set out to identify one or more unique transcription factors that may drive *PAX5* transcription in t(8;21) AML via differential binding to the *PAX5* promoter elements in B cells, t(8;21) AML and non t(8;21) myeloid precursor cells. To this end, we used *in vivo* DMS and DNase I foot-printing technique.

This technique creates lesions in the DNA of a live cell (in this case cleavage by DMS/DNAse I). The pattern of this cleavage can be compared to the pattern of cleavage/lesion created by in vitro DMS/DNase I treatment on naked DNA. This provides useful information about chromatin conformation, transcription factor binding, and nucleosome positioning and chromatin compaction at loci of interest (Tagoh et al., 2006a).

To identify transcription factors that might be responsible for *PAX5* transcription in t(8;21) AML we performed DMS and DNAse I footprinting at HS 1, HS 4 (data not shown) and HS 8. HS 4 and HS 8 are the minimal *PAX5* promoters carrying the transcription start sites, while HS 1 is a very strong hypersensitive site in t(8;21) AML which recruits a lot of RNA Polymerase II and shows the presence of high levels of H3K4me3 and H3K9ac (see Figures 3.8 and 3.9). *In vivo* foot-printing experiments at HS1, HS4 (data not shown) and HS 8 (figure 3.11) showed that the chromatin fine structure of B cells and that of t(8;21) AML and non-t(8;21) myeloid precursor controls at these hypersensitive sites was identical (figure 3.11). DNAse I and DMS foot-printing showed several foot-prints at HS 8 which, however, were the same in B cells, t(8;21) AML and non-t(8;21) AML cells but were different in HeLa (figures 3.11 A, B and C). Inspection of the sequence under the protected and hypersensitive regions in figures 3.11 A, B and C showed binding sites for transcription factors such as Sp1, WT1 and NF1. These results indicate that the *PAX5* locus not only shows open chromatin structure and recruitment of RNA polymerase II in HL-60, but also shows exactly the same chromatin fine structure and transcription factor

binding pattern as that of B cells and t(8;21) AML cells where the gene is transcriptionally active.

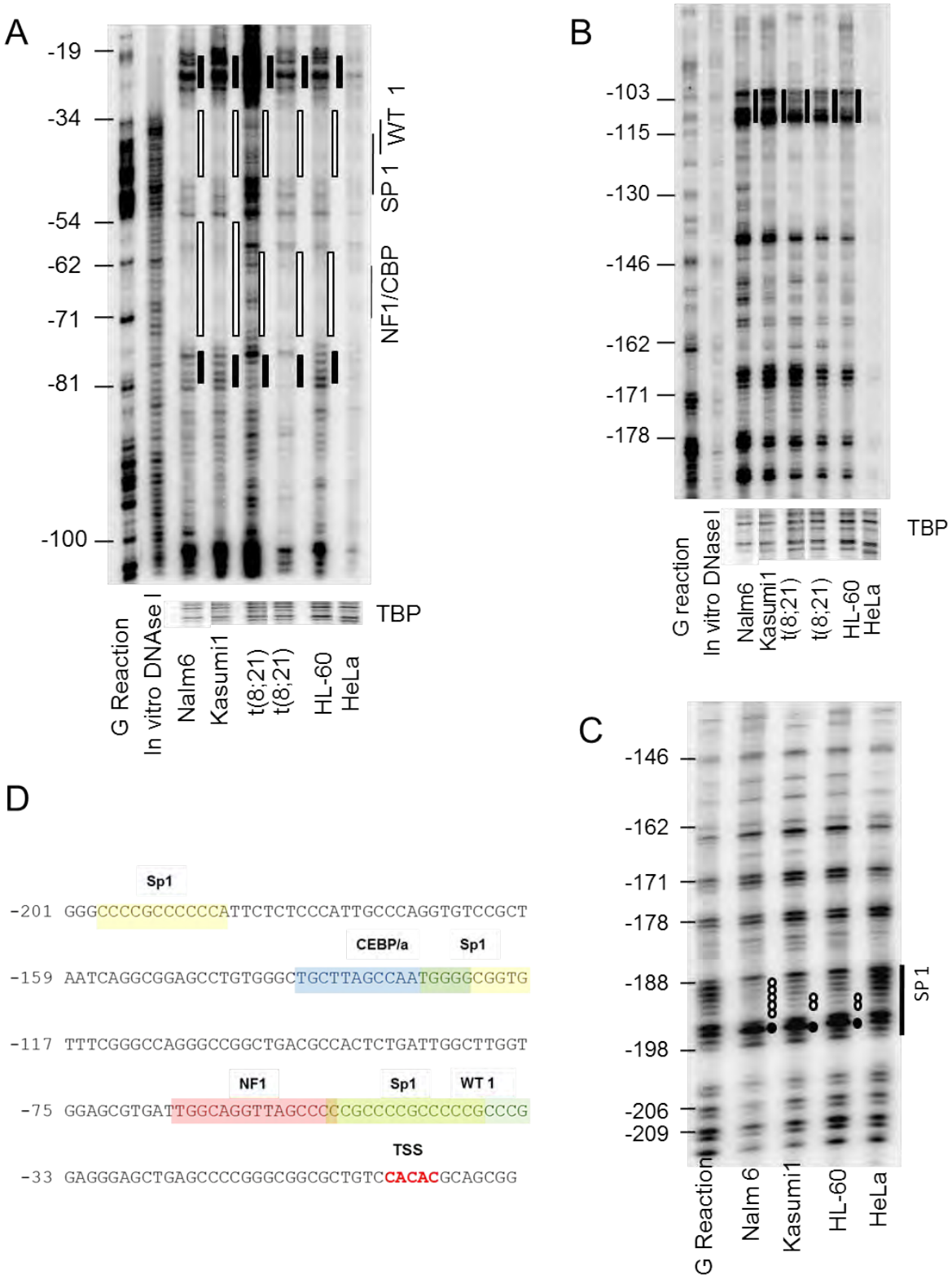


Figure 3.11: The chromatin fine structure at HS 8 *PAX5* distal promoter in B cells, myeloid precursors and t(8;21) AML is identical. DNase I footprinting (A) and (B) at *PAX5* HS 8 showing that the chromatin fine structure and transcription factor binding is identical in B cell, t(8;21) AML and myeloid precursors. The *TBP* promoter region is shown as a control for equal DNase I digestion of the samples in A and B. C shows DMS- footprinting at *PAX5* HS 8 distal promoter and protection over Sp1 binding site in B cells, t(8;21) AML and in myeloid precursors. Regions of protection are marked by hollow bars (A and B) or circle (C) while regions of hypersensitivity are marked by solid bars (in A and B) or solid dots (in C). The base pairs are labelled in relation to HS8 transcription start site as indicated in the UCSC genome browser. D shows the sequence of the *PAX5* HS 8 foot-printed in A, B and C. The possible transcription factors binding at the various regions of protection and hypersensitivity observed are indicated in the figure.

3.6 *PAX*5 is neither a direct nor an indirect target of RUNX1or RUNX1/ETO

An obvious cause of aberrant activation of *PAX5* in t(8;21) AML could be due to direct or indirect effect of RUNX1/ETO on the gene. RUNX1/ETO is the fusion protein expressed as a result of the t(8;21) translocation leading to the fusion of the genes: *RUNX1* and *ETO*. Normally RUNX1/ETO functions as a repressor, however, in certain cases RUNX1/ETO has been reported to function as an activator (Nimer and Moore, 2004, Wang et al., 2011a) (also see section 1.5.1). We therefore wanted to test whether *PAX5* was one of the few genes that were up-regulated by RUNX1/ETO directly or indirectly.

To test whether *PAX5* was a direct target of RUNX1/ETO in t(8;21) AML, we inspected the *PAX5* locus for RUNX1 or RUNX1/ETO peaks in RUNX1 and RUNX1/ETO ChIP-sequencing data produced as part of a parallel study in the

lab (Ptasinska et al., 2012a). Figure 3.12 B shows that *PAX5* was not a target of RUNX1 or RUNX1/ETO in Kasumi-1 or in cells from t(8;21) AML patients.

We next investigated whether RUNX1/ETO indirectly affects the expression of *PAX5* in t(8;21) AML. To this end we measured *PAX5* and *RUNX1/ETO* expression in primary cells from t(8;21) patients where RUNX1/ETO expression was knocked-down by siRNA (siRNA RE) designed specifically against the RUNX1 and ETO junction(Heidenreich, 2009). As a control for comparison the same cells were transfected with siRNA with a scrambled sequence (siRNA MM). We received RNA from these cells as a generous gift from Dr Olaf Heidenreich (Newcastle University, UK). We also used cDNA from RUNX1/ETO knocked-down Kasumi -1(using the same siRNA RE described above) cells generated by Ptasinska *et al* (Ptasinska et al., 2012a). qRT-PCR expression analysis showed that RUNX1/ETO knock-down had no effect on *PAX5* expression in Kasumi-1 cells or in cells from t(8;21) patients (figure 3.12 A). Therefore, we conclude that *PAX5* is not a direct target of RUNX1/ETO and its presence or absence does not influence *PAX5* expression.

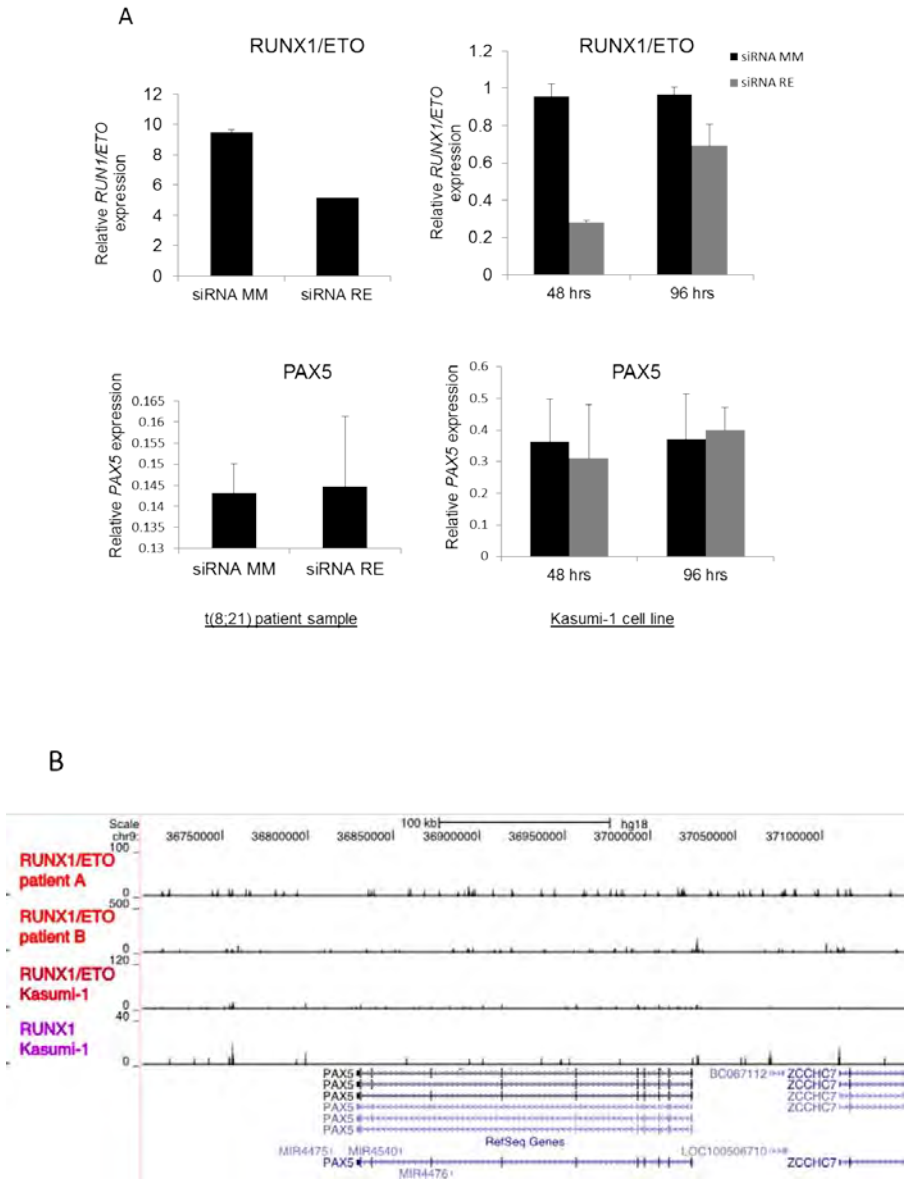


Figure 3.12: *PAX5* is neither a direct nor an indirect target of RUNX1/ETO. A) qRT-PCR showing *RUNX1/ETO* (top panel) and *PAX5* (bottom panel) expression after treatment of primary t(8;21) sample (left) and Kasumi-1 cell line (right) with siRNA MM (siRNA mismatch) and siRNA RE (siRNA RUNX1/ETO) in order to knock-down RUNX/ETO (Patient samples were a gift from Dr Olaf Heidenreich and the RUNX1/ETO KD Kasumi-1 cDNA was from Ptasinska *et al* , Leukaemia 2012. B) ChIP-seq track showing *PAX5* locus does not bind RUNX/ETO or RUNX1. ChIP-seq data from (Ptasinska et al., 2012a).

3.7 Aberrant expression of *PAX5* in t(8;21) AML requires MAPkinase signalling

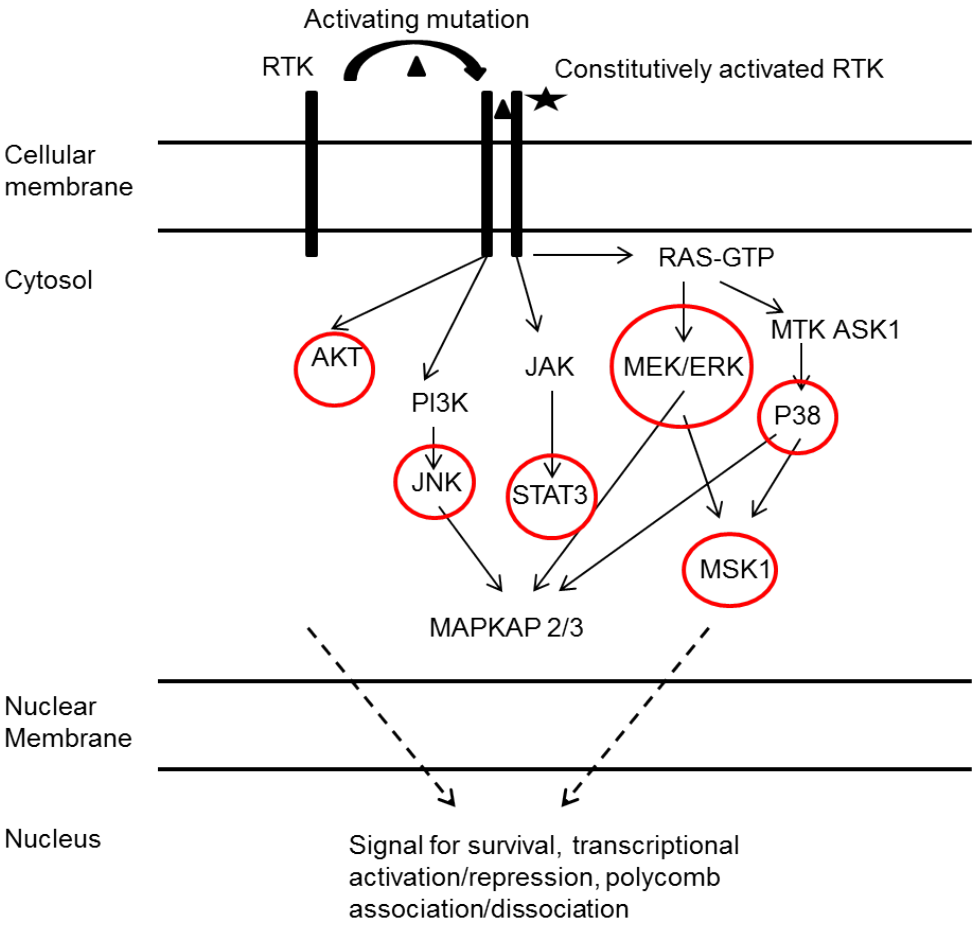
Our experiments so far demonstrate that at the *PAX5* locus, chromatin structure, RNA Polymerase II recruitment and transcription factor binding in t(8;21) AML cells and in non- t(8;21) myeloid precursors were identical. The only differences observed between t(8;21) AML and HL-60 cells were in the higher occupancy of polycomb complexes and the lack of the elongating RNA polymerase II in HL-60 cells (sections 3.3.5 and 3.4). We therefore, hypothesized that *PAX5* expression in t(8;21) AML was due to alleviation of polycomb repression and addressed the question what caused it. A large body of literature supports the idea that cellular signalling directly impacts on polycomb activity, thereby regulating its association/dissociation from the chromatin (Section 1.3.4) (Sawarkar and Paro, 2010). We therefore set out to explore whether the up-regulation of *PAX5* in t(8;21) AML cells is caused by aberrant signalling.

As described in section 1.5.2, RUNX1/ETO alone is unable to cause overt leukaemia in mouse models. A second mutagenic event is required to co-operate with the t(8;21) translocation to induce overt leukaemia (Wang et al., 2011c, Wang et al., 2005). The most common second mutagenic events that have been reported to co-operate with RUNX1/ETO are mutations in tyrosine kinase receptor genes namely *C-KIT, FLT3, RAS* and *RAF*. These receptor tyrosine kinase mutations are believed to provide proliferative advantage to leukemic cells (see section 1.5.2).

Kasumi-1 and SKNO-1cell lines are *C-KIT* mutant models of t(8;21) AML. Activating Asn822Lys mutation in the C-KIT receptor constitutively activates a number of downstream signalling pathways namely AKT, JAK/STAT3 and MAPkinase pathways (figure 3.13 A)(Larizza et al., 2005). To test whether chronic signalling is responsible for the relief of polycomb repression and *PAX5* activation, we systematically inhibited each of these pathways in Kasumi-1 cells by using small molecule inhibitors (indicated with red circles in figure 3.13 A) alone and in combination. After inhibition of each of these pathways we measured *PAX5* expression by qRT-PCR (figures 3.13 A, B C and D).

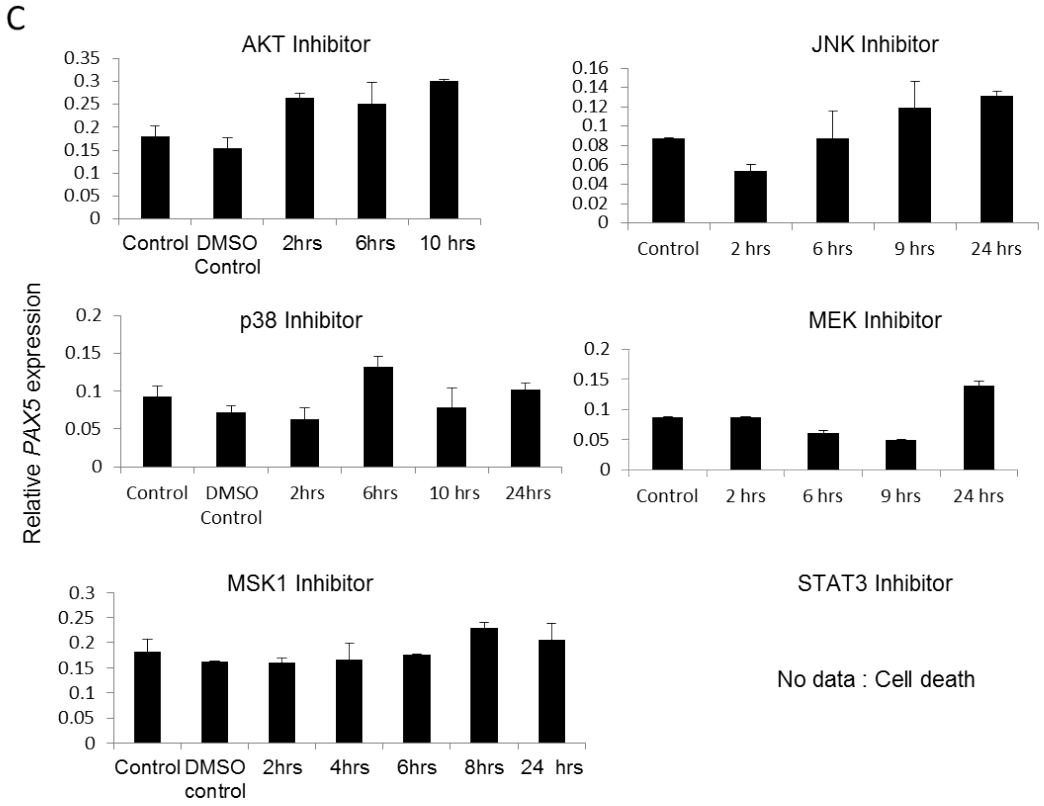
Inhibition of AKT, JNK, P38 and MSK1 signalling showed no effect on *PAX5* expression. Inhibition of MEK showed a small but variable effect on *PAX5* expression (figure 3.13 B and C) whereas inhibition of STAT3 signalling dramatically drove the Kasumi-1 cells into apoptosis within 2-3 hours of inhibitor treatment (figure 3.14 C). On treatment of Kasumi-1 cells simultaneously with JNK, MEK and P38 inhibitors for 6 hours, *PAX5* expression was down-regulated more than 2 folds. A similar level of down-regulation in *PAX5* expression was also achieved with only JNK and P38 inhibitors (figure 3.13 D). These experiments show that JNK and P38 signalling is involved in the aberrant up-regulation of *PAX5* expression in t(8;21) AML.

A



B

Pathway/s	Small molecule	Effect
AKT	Akt Inhib IV	No effect
JNK	P600125	No effect
P38	SB20219	No effect
MEK	PD98059	Meagre effect
JNK+MEK		No effect
P38+MEK		No effect
STAT3	S31-201	Cell death & apoptosis
JNK+MEK+P38		Down-regulation of *PAX5* expression
JNK+P38		Down-regulation of *PAX5* expression



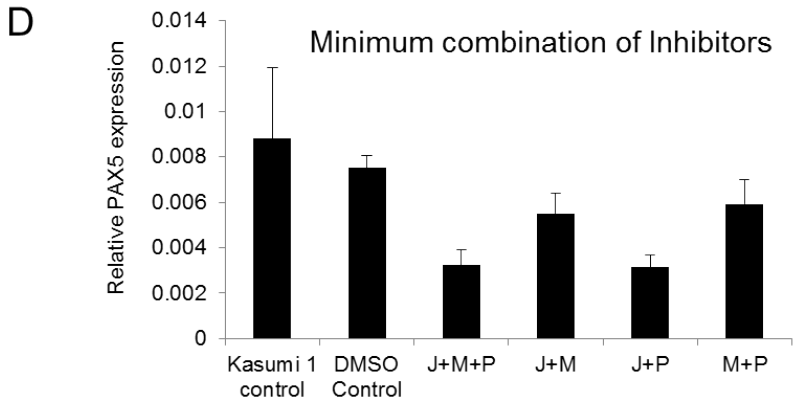


Figure 3.13: Activating receptor tyrosine kinase (RTK) mutation constitutively activates downstream pathways required for PAX5 deregulation in t(8;21) AML. A) Cartoon showing downstream signalling pathways deregulated by activating receptor tyrosine kinase mutations and the complex mutual cross-talk between these signalling pathways. B) Table

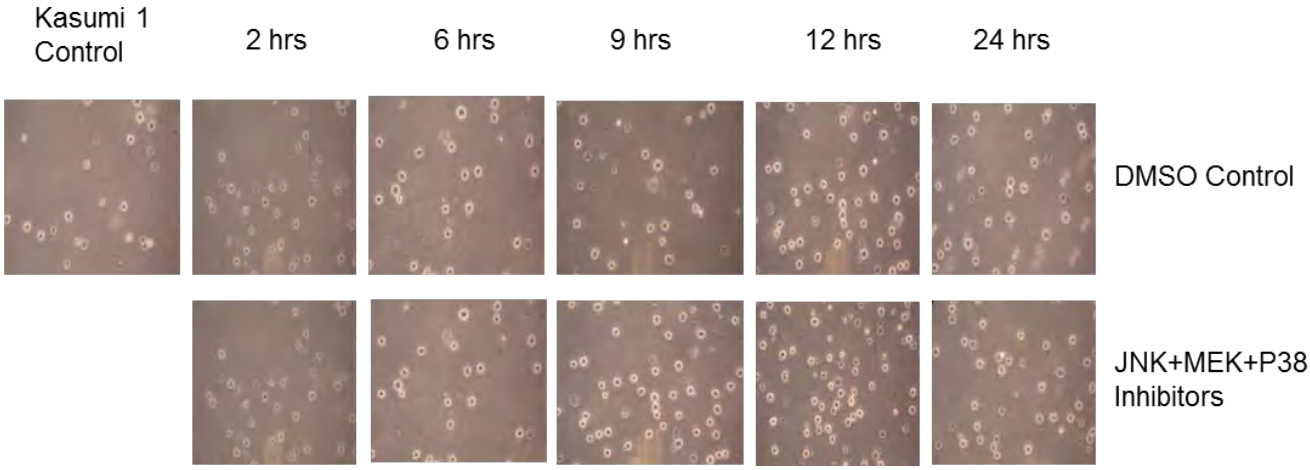
showing signalling pathways inhibited by small molecule inhibitor treatment of Kasumi-1 cells and their effects on *PAX5* expression respectively. C) qRT-PCR showing *PAX5* expression kinetics after treatment of Kasumi-1 cells with the indicated protein kinase inhibitors. D) q-RT-PCR showing various combinations of JNK, MEK and P38 inhibitors and effect on down-regulation of *PAX5* expression in Kasumi-1 cells. The bar plot shown is a single representative of two biologically independent repeats. The error bars represent variability between duplicate q-PCR measurements

3.7.1 Simultaneous inhibition of JNK, MEK and P38 signalling does not affect the survival of Kasumi-1 cells

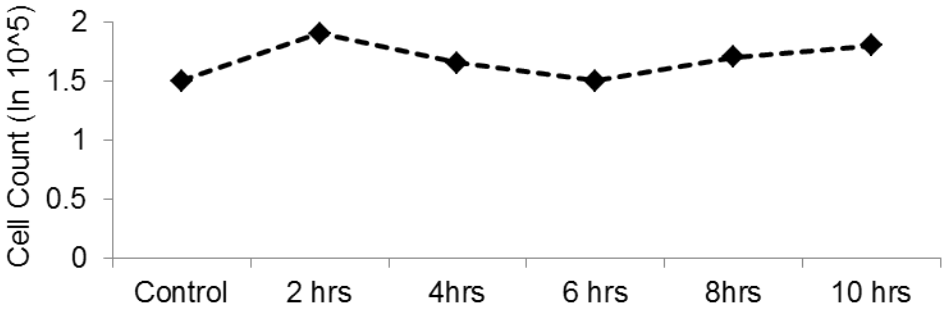
Simultaneous inhibition of JNK, MEK and P38 signalling down-regulates expression of *PAX5*. To ensure that this down-regulation of *PAX5* is not due to cell death or dramatic differentiation of the cells, we recorded the morphology of the inhibitor treated Kasumi-1 cells at various time points over a 24 hour time period. We also recorded cell survival by trypan blue staining over 10 hours and Annexin/PI staining 10 hours after treatment with inhibitors.

Photographs of Kasumi-1 cells taken over 24 hours did not show any change in morphology or viability of the inhibitor treated Kasumi-1 cells as compared to the DMSO treated control cells (figure 3.14 A). Trypan blue staining showed that the cells did not die off during these 10 hours of inhibitor treatment (figure 3.14 B). Annexin/PI staining further confirmed the fact that there was no increase in the number of apoptotic cells 10 hours post treatment with JNK, MEK and P38 inhibitors (figure 3.14 C). These results, therefore, show that inhibition of MAPkinase (JNK, MEK and P38) signalling does not affect the survival of Kasumi-1 cells.

A


Kasumi 1
Control
2 hrs
6 hrs
9 hrs
12 hrs
24 hrs
DMSO Control
JNK+MEK+P38
Inhibitors

B


Cell Count (ln 10^5)
2
1.5
1
0.5
0
Control
2 hrs
4hrs
6 hrs
8hrs
10 hrs

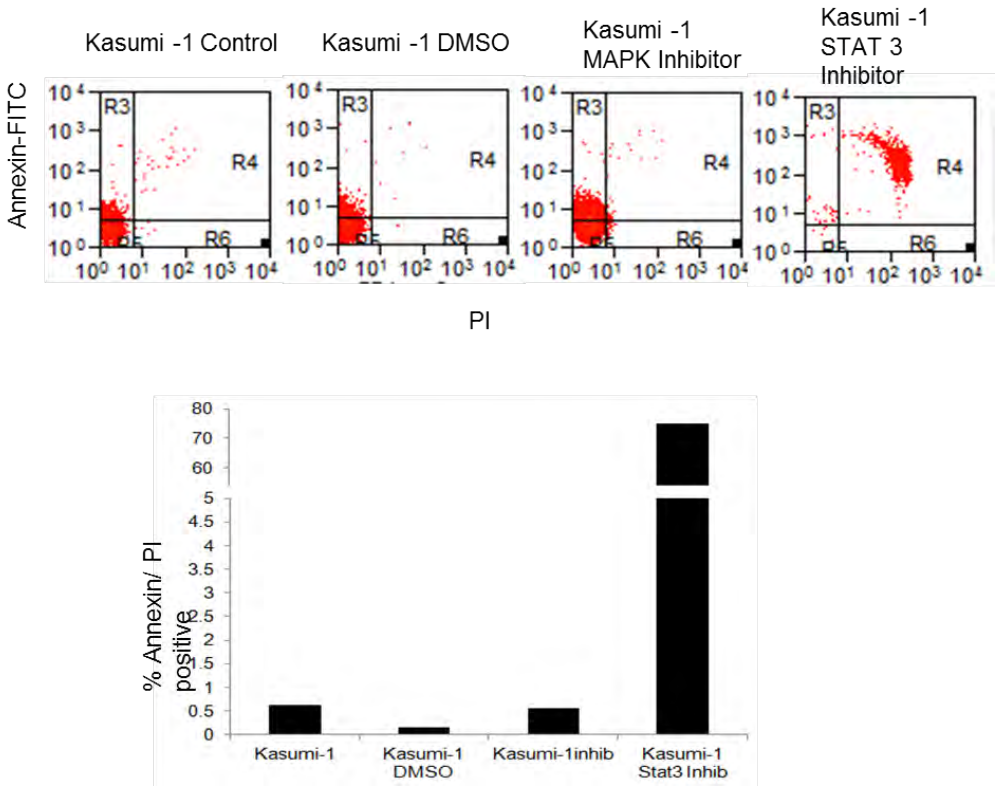


Figure 3.14: Simultaneous treatment with JNK, MEK and P38 inhibitors is not toxic and does not affect the survival of Kasumi-1 cells. A) Photographs showing DMSO control treated and inhibitor (JNK+MEK+P38) treated Kasumi-1 cells at 2, 6,9,12 and 24 hours respectively. B) Cell survival assay with trypan blue staining showing that Kasumi-1 cell do not die over 10hrs after treatment with JNK, MEK and P38 inhibitors. The x-axis shows the various time points while the y-axis represents cell count in 10^5 per ml. C) cell survival assay using Annexin/PI staining showing that Kasumi-1 does not undergo apoptosis 10 hours afters JNK,MEK and P38 inhibition. However cells undergo apoptosis 3 hours after treatment with STAT3 inhibitor.

3.7.2 Inhibition of STAT3 signalling induces apoptosis in t(8;21) Kasumi-1 cells.

Constitutive activation of STAT3 signalling has been demonstrated in many types of cancer including AML (Redell et al., 2011). Genes responsive to STAT3 signalling include anti-apoptosis genes and cell-cycle regulators. Consistent with previous reports (Redell et al., 2011), inhibition of STAT3 signalling in Kasumi-1 cells rapidly induced apoptosis. Annexin/PI staining of Kasumi-1treatment with STAT3 inhibitor showed that 75% of the cells were dead 3 hours after treatment with the small molecule inhibitor (figure 3.14 C). STAT3 inhibitors therefore could be potential therapeutic option for treating AML.

3.7.3 Inhibition of MAPkinase signalling specifically down-regulates *PAX5* in t(8;21) AML but not in Nalm-6 pre B cells

As described in section 3.7.1, simultaneous inhibition of JNK, MEK and P38 signalling did not affect the survival of Kasumi-1 cells. It also did not affect the transcription of control housekeeping genes such as *TBP*, *RPL13A* and *UBC* (figure 3.15 B and C) However *PAX5* showed ~2fold down-regulation after 4 hours of treatment and up to ~4 fold down-regulation after 6 hours of treatment which was maintained for more than 12 hours in Kasumi-1 cells. Control treatment with 30µl of DMSO did not affect the expression of *PAX5* in Kasumi-1 (figure 3.15 A)

SKNO-1 is another t(8;21) cell line with the same activating C-KIT Asn822Lys mutation. We wanted to see whether down-regulation of *PAX5* in response to inhibition of constitutively activated MAPkinases was specific to Kasumi-1 or it

was a more general phenotype in t(8;21) AML. To this end we treated SKNO-1 cells with JNK, MEK and P38 inhibitors over a period of 10 hours. Inhibition of these signalling pathways in SKNO-1 showed a down-regulation of *PAX5* expression, albeit, the down-regulation achieved in SKNO-1 was lower than that in Kasumi-1 cells. After 6 hours of inhibitor treatment *PAX5* was only down-regulated ~1.6 fold compared to *PAX5* expression in untreated or DMSO treated SKNO-1 cells (figure 3.16 A). However, *TBP* expression was unaffected by inhibitor treatment just like in Kasumi-1 cells (figure 3.16 B).

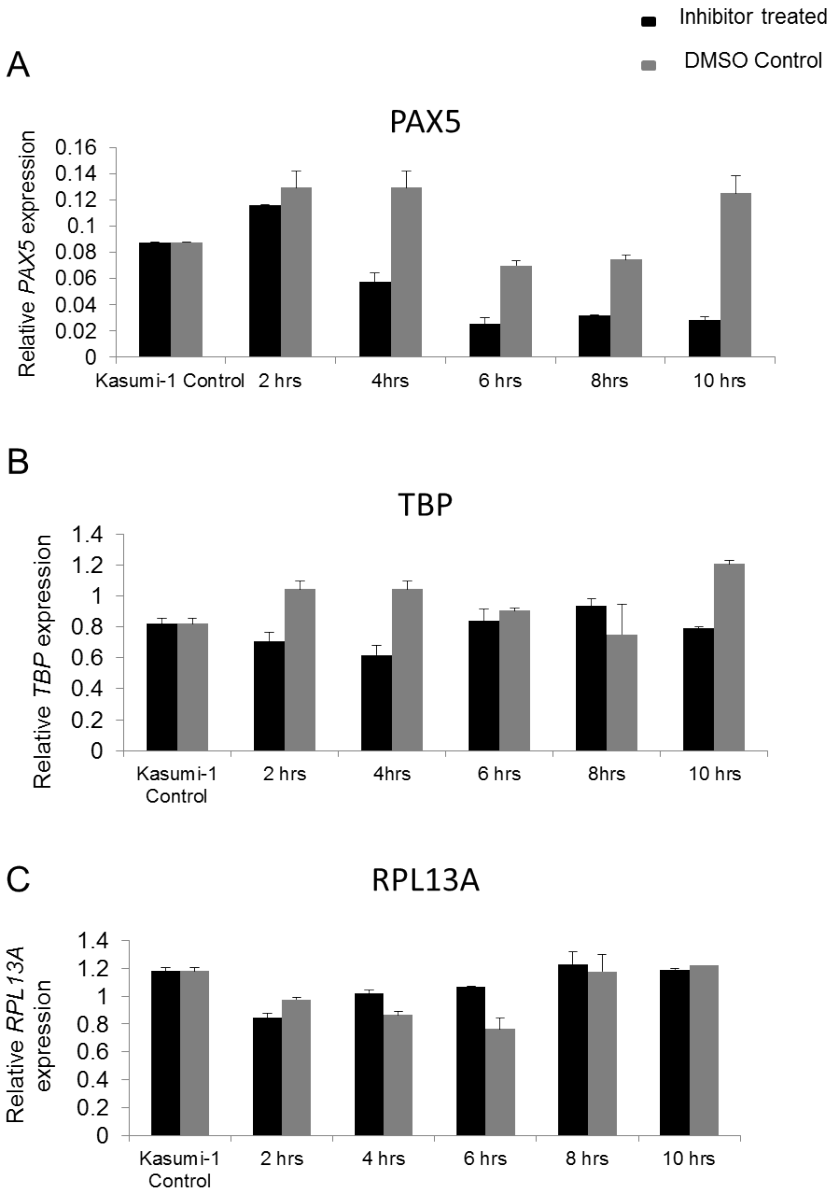


Figure 3.15: Aberrant *PAX5* expression in Kasumi-1 t(8;21) cell line requires MAPkinase signalling. qRT-PCR analysis of *PAX5* (A), *TBP* (B) and *RPL13A* (C) expression kinetics after inhibition of JNK, MEK and P38 signalling for 2,4 6,8 and 10 hours respectively. The expression plotted is relative to GAPDH expression and each bar graph shows one representative of at least three independent biological repeats. The error bars represent variability between duplicate q-PCR measurements. TBP and RPL13A are used as control genes to show that inhibition of JNK, MEK and P38 signalling does not affect transcription as a whole.

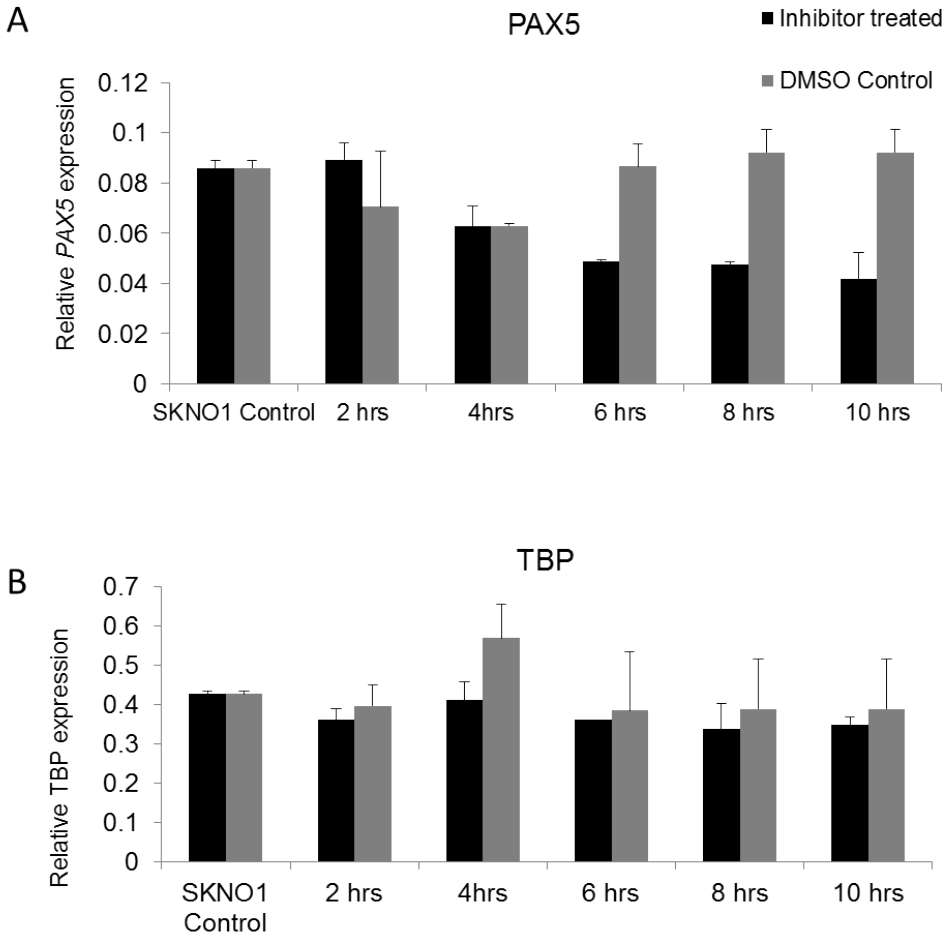


Figure 3.16: Aberrant *PAX5* expression in SKNO-1 t(8;21) cell line requires MAPkinase signalling. qRT-PCR analysis of *PAX5* (A), and *TBP* (B) expression kinetics after inhibition of JNK, MEK and P38 signalling for 2, 4 6, 8 and 10 hours respectively. Y-axis shows expression relative to *GAPDH* expression and each bar graph shows one representative of at least two independent biological repeats. The error bars represent variability between duplicate q-PCR measurements. TBP is used as a control gene to show that inhibition of JNK, MEK and P38 signalling does not affect transcription as a whole.

We furthermore cultured (frozen) cells derived from the bone marrow of a t(8;21) patient who had relapsed after remission, in RPMI media with 10% FCS

and no cytokines for 2 hours. After 2 hours in culture half the cells were treated with JNK, MEK and P38 inhibitors and half were treated with DMSO. Following treatment, the cells were incubated for another 6 hours, RNA was extracted from the cells and *PAX5* expression was measured by qRT-PCR. As seen in figure 3.17 A after treatment with inhibitors *PAX5* expression was down-regulated ~ 2 fold compared to DMSO treated cells. There was no effect of the inhibitor treatment on *TBP* expression in these t(8;21) AML cells (figure 3.17 B).

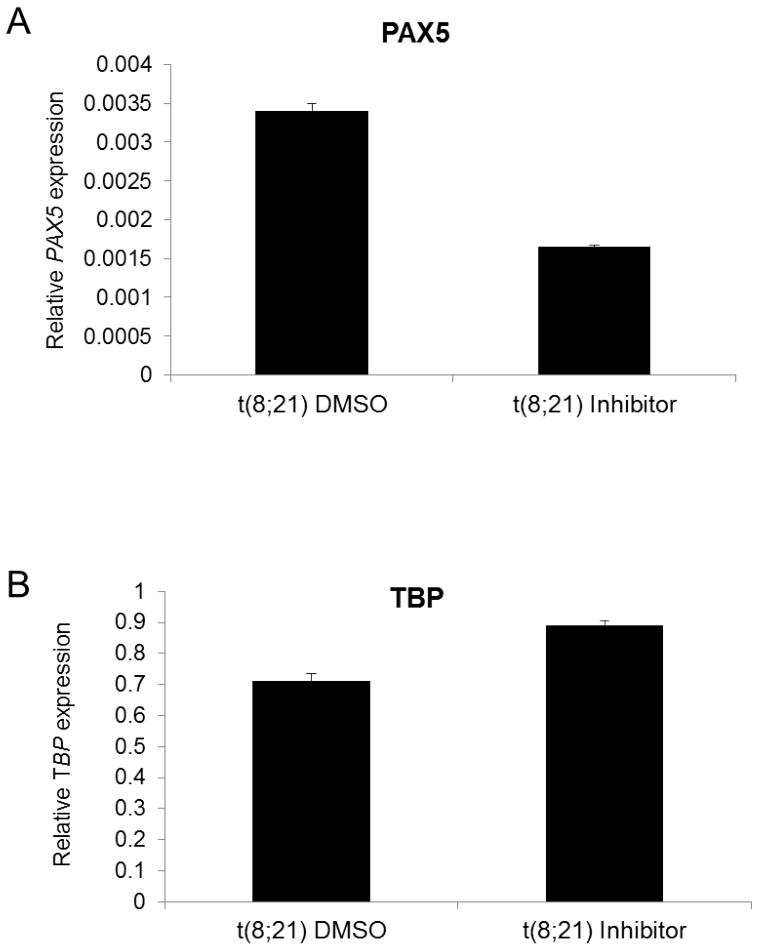


Figure 3.17: Aberrant *PAX5* expression in primary t(8;21) cells requires MAPkinase signalling. qRT-PCR analysis of *PAX5* (A), and *TBP* (B) expression after inhibition of JNK, MEK and P38 signalling for 6 hours. $CD34^+$ cell frozen from the bone marrow of a t(8;21) patient after relapse were cultured in RPMI1 media with serum for 2 hours after which JNK, MEK and P38 inhibitors were added and incubated for 6 hours. Y-axis shows expression relative to *GAPDH* expression. The error bars represent variability between duplicate q-PCR measurements. TBP is used as a control gene to show that inhibition of JNK, MEK and P38 signalling does not affect transcription as a whole.

One reason for the down-regulation of *PAX5* after signalling inhibition could be that this gene is regulated by a transcription factor that is signalling responsive. We therefore tested whether *PAX5* expression in B cells needed MAPkinase signalling. To this end, we treated Nalm-6 cells with JNK, MEK and P38 inhibitors and measured *PAX5* expression over a time course. In these cells treatment with MAPkinase inhibitors did not affect *PAX5* expression (figure 3.18). It can therefore be concluded that inhibition of MAPkinases affects *PAX5* expression in t(8;21) AML but not in pre-B cells (Nalm-6). Furthermore the down-regulation of *PAX5* in t(8;21) did not affect the survival of these cells and also did not inhibit transcription as a whole as house- keeping genes remained unaffected by the inhibition of these signalling pathways. Therefore it is possible to conclude that MAPkinase dependent *PAX5* expression is specific to t(8;21) AML.

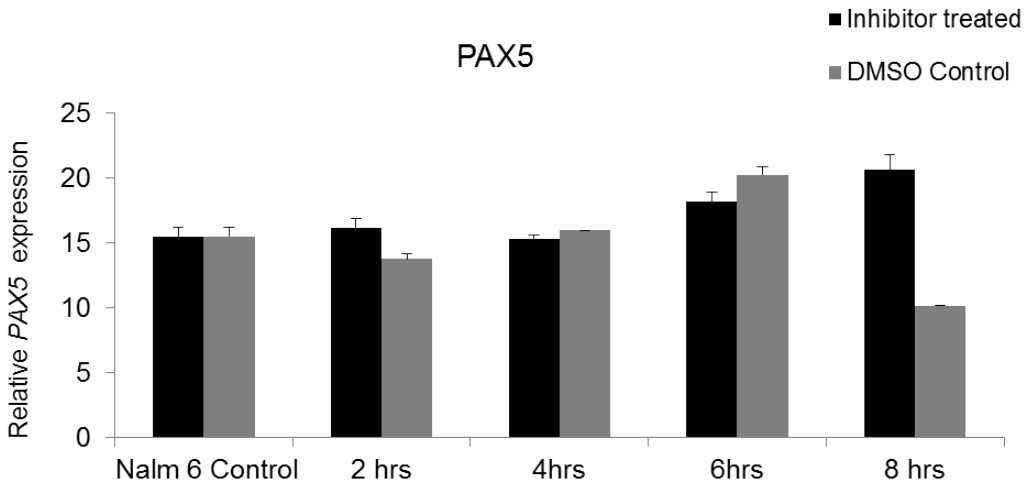


Figure 3.18: *PAX5* expression in Nalm-6 B cells is independent of JNK, MEK and P38 signalling. qRT-PCR showing *PAX5* expression kinetics in Nalm-6 pro-B cell line after simultaneous treatment with JNK, MEK and P38 inhibitors for 2, 4, 6 and 8 hours respectively. The y-axis shows *PAX5* expression relative to *GAPDH* expression. The bar graph is a representative of

two independent repeats and the error bars represent variability between duplicate q-PCR measurements

3.7.3 Inhibition of JNK, MEK and P38 signalling leads to the loss of RNA polymerase II and alteration of chromatin at the *PAX5* locus

To further investigate the molecular mechanism behind the down-regulation of *PAX5* expression in response to inhibition of MAPkinase signalling, we performed ChIP-qPCR assays for RNA Polymerase II, H3K27ac and H3K27me3 in MAPkinase inhibitor treated, DMSO treated and untreated Kasumi-1 cells 6 hours after treatment.

Treatment with inhibitors caused a significant loss of RNA Polymerase II at HS 1 and HS 4 of the *PAX5* promoter region (figure 3.19 A). This is in accordance with the fact that *PAX5* expression is down regulated after treatment with MAPkinase inhibitors.

As described in Sections 3.3.5 and 3.4, *PAX5* is poised for transcription but repressed by polycomb complexes in non-t(8;21) myeloid precursors. In order to investigate whether inhibition of signalling had any effect on polycomb occupancy at the *PAX5* locus, we performed H3K27me3 and H3K27ac ChIP experiments. We observed that after overnight treatment (~16hrs) with JNK, MEK and P38 inhibitors Kasumi-1 cells showed an overall increase in H3K27me3 (figure 3.19 C) across the *PAX5* locus. This was concomitant with the loss of the opposing H3K27ac mark that is associated with loss of polycomb from a target gene (figure 3.19 B) (also see section 1.3.5). Taken together these

results suggest that aberrant MAPkinase signalling in t(8;21) leads to the removal of polycomb from the *PAX5* gene.

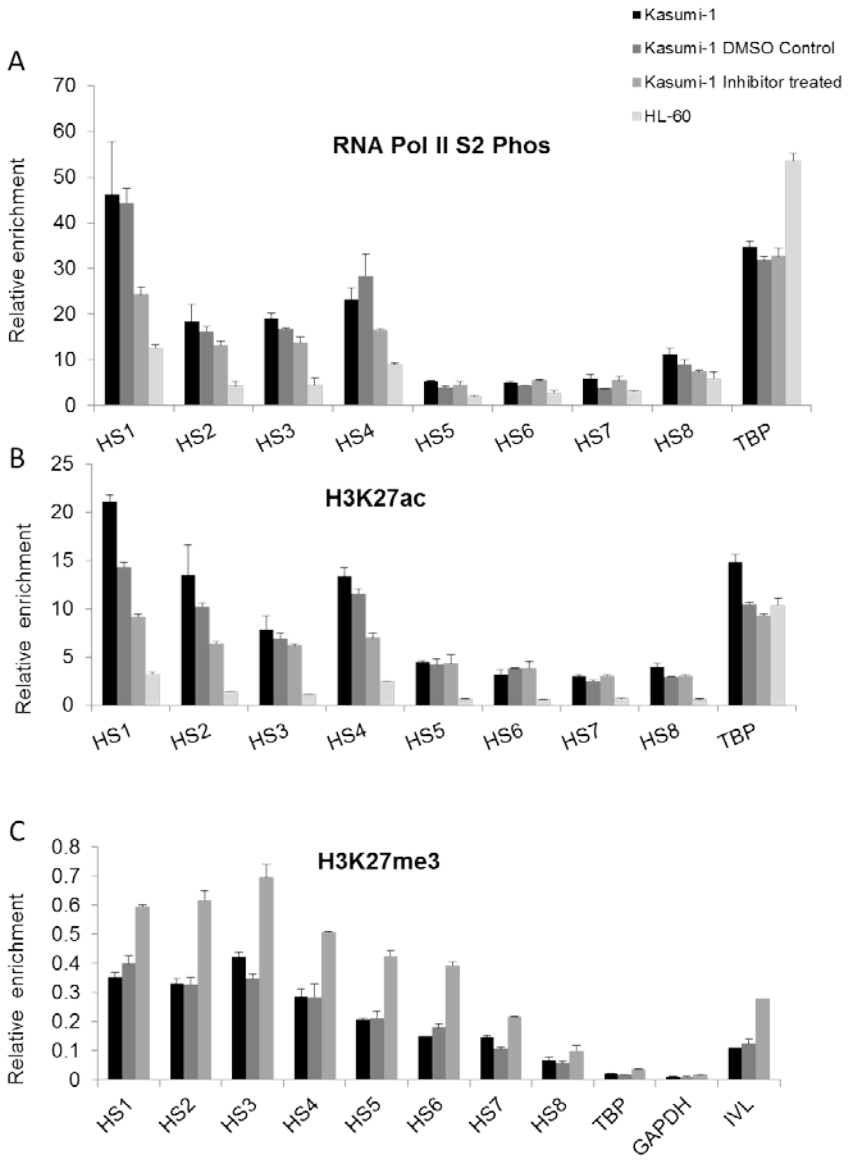


Figure 3.19: Inhibition of JNK, MEK and P38 signalling leads to loss of RNA polymerase II and alteration of chromatin at the *PAX5* locus in Kasumi-1 cell line. ChIP-qPCR showing relative RNA Polymerase II (A) and H3K27ac (B) and H3K27me3 (C) enrichment at the *PAX5* locus after 6 hours (A and B) and overnight treatment (C) with inhibitors. Y-axis shows relative enrichment to IVL (A and B) or input (C). Each bar graph is a representative of

at least two independent experiments. The error bars represent standard variability between duplicate q-PCR measurements.

3.8 The HDAC inhibitor TSA enhances *PAX5* expression in Kasumi 1 cells and counteracts down-regulation of *PAX5* after JNK, MEK and P38 inhibition

Our results so far indicate that *PAX5* is up-regulated in t(8;21) AML due the removal of polycomb by constitutively activated aberrant MAPkinase signalling. The inhibition of these signalling pathways bring polycomb back on to the *PAX5* locus thereby accounting for the down-regulation of *PAX5* expression after treatment with inhibitors. It is known that polycomb complexes recruit histone deacetylases (HDACs) to mediate repression of its target genes. Therefore we examined whether Trichostatin A (TSA): a well-known inhibitor of class I and II HDACs, can counteract the down-regulation of *PAX5* observed on inhibition of MAPkinase signalling. To this end we treated Kasumi-1 cells with TSA alone or simultaneously with JNK, MEK, P38 inhibitors. Treatment with TSA alone enhanced the expression of *PAX5* in Kasumi-1, while this was not the case for Kasumi-1 cells treated with ethanol as control. When TSA was added in conjunction with JNK, MEK and P38 inhibitors, the expression of *PAX5* was rescued up to the levels in untreated Kasumi-1 cells. Cells treated with DMSO and ethanol simultaneously as control did not show a change in *PAX5* expression (figure 3.20). This result shows that down-regulation of *PAX5* due to inhibition of constitutively activated MAPkinase signalling in Kasumi-1 cells involve histone deacetylases.

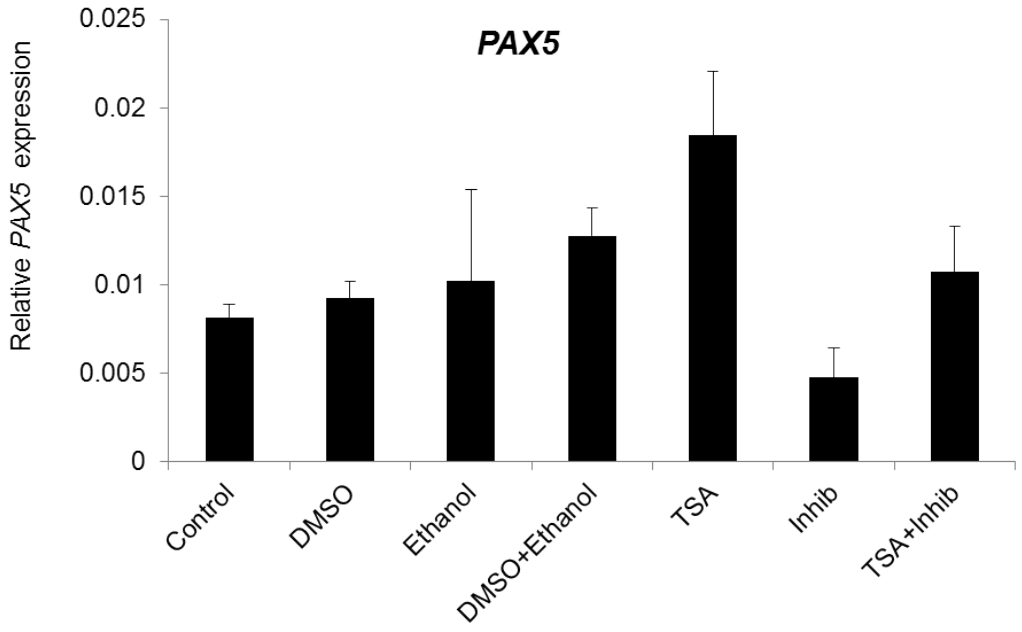


Figure 3.20: Treatment of Kasumi-1 with HDAC inhibitors enhances *PAX5* expression and rescue down-regulation of *PAX5* after JNK, MEK and P38 inhibition. qRT-PCR showing *PAX5* expression relative to GAPDH after treatment with TSA, JNK+MEK+P38 inhibitors, TSA+JNK+MEK+P38 inhibitors and for control DMSO, ethanol and DMSO + ethanol. This is a representative of two independent experiments and the error bars represent variability between duplicate q-PCR measurements.

3.9 Removal of polycomb from *PAX5* does not involve MSK1, 3pK or JMJD3

There is a large body of literature that describes the cross-talk between cellular signalling and polycomb (see sections 1.3.4 and 1.3.5 (Niessen et al., 2009). Various mechanisms have so far been described to be involved in the regulation of dissociation/association of polycomb to its target (Sawarkar and Paro, 2010). Figure 3.21 illustrates the various possible mechanisms that lead to the de-repression of a polycomb target gene.

Signalling mediated regulation of polycomb association and dissociation to the chromatin is a highly evolving field and the mechanisms discussed in sections 1.3.4 and 1.3.5 and illustrated in figure 3.21 might only be few of many mechanisms that regulate this process. We, therefore, investigated whether some of these already known mechanisms were responsible for the de-repression of *PAX5* in t(8;21) AML.

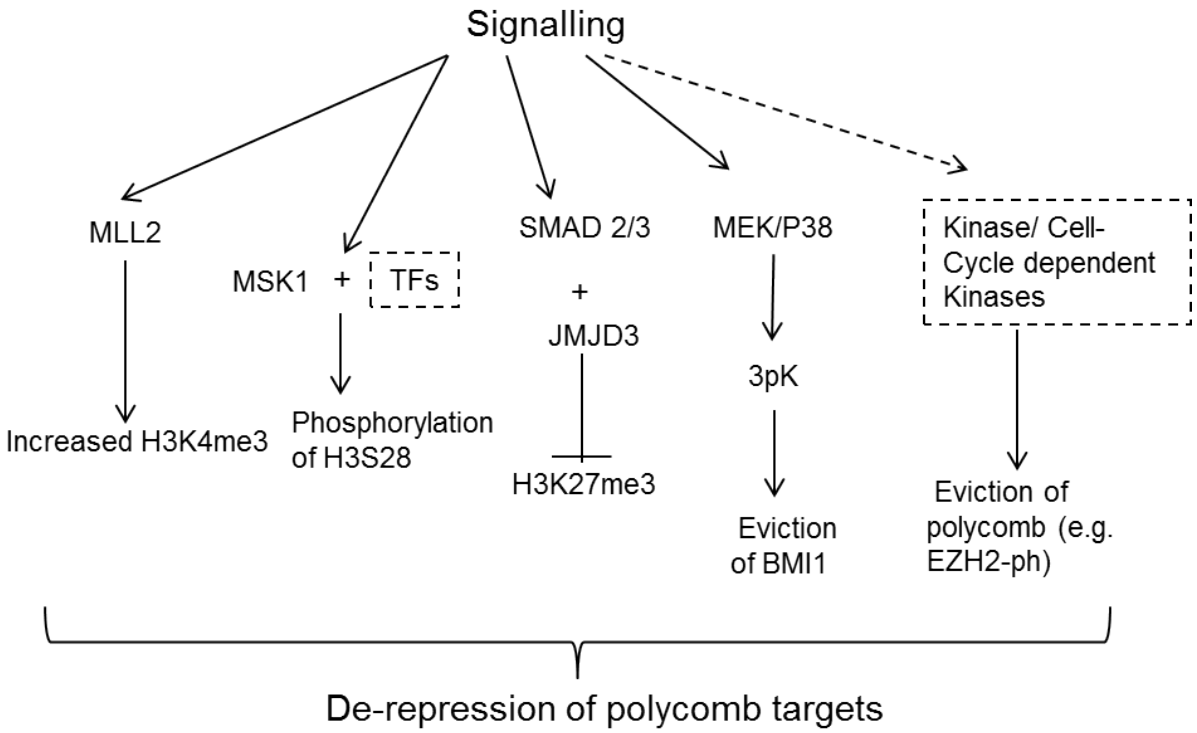


Figure 3.21 Possible mechanisms of de-repression of polycomb target genes. Schematic representation showing the various mechanisms of de-repression of polycomb target genes reported in literature (Sawarkar and Paro, 2010).

3.9.1 MSK1 is not involved in the de-repression of *PAX5* in t(8;21) AML

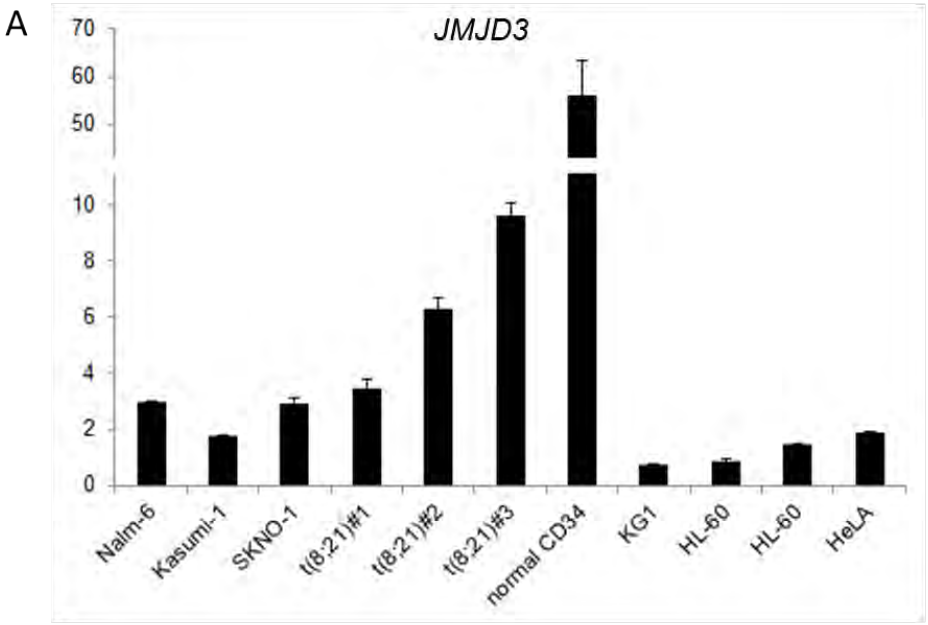
MSK1 is a protein kinase downstream of MEK/ERK pathway that has been shown to phosphorylate the H3S28 residue and is implicated in de-repression of polycomb target genes (Gehani et al., 2010, Lau and Cheung, 2011a) (see section 1.3.5). We investigated whether MSK1 was involved in the de-repression of *PAX5* in t(8;21) AML by treating Kasumi-1 cells with a specific MSK1 small molecule inhibitor (figure 3.13 A) over a time course of 24 hours. At each indicated time-point RNA was extracted from these treated cells and *PAX5* expression was measured by qRT-PCR. MSK1 treatment showed no effect on the expression of *PAX5* gene (figure 3.13 C). This indicates that MSK1 and phosphorylation of H3S28 is not involved in the de-repression of *PAX5* in t(8;21) AML.

3.9.2 Aberrant *PAX5* expression in t(8;21) AML does not involve signalling mediated up-regulation of *JMJD3* expression

JMJD3 is the histone de-methylase that specifically de-methylates H3K27 tri-methylation and thus is associated with the de-repression of polycomb target genes (reviewed in Sawarkar and Paro 2010). It contains the JmjC catalytic domain and a conserved histidine residue for cofactor binding (Swigut and Wysocka, 2007). Up-regulation of J*MJD3* has been reported in many cancers (see section 1.3.5). Therefore, we examined whether *JMJD3* was up-regulated in t(8;21) AML. q-RT-PCR analysis shows that *JMJD3* expression in Kasumi-1, SKNO-1 and cells from t(8;21) patients was considerably higher compared to non t(8;21) myeloid precursors KG-1 and HL-60 cells. However, the expression

of *JMJD3* was observed to be very high in CD34+ mobilized stem cells from the peripheral blood of normal healthy donors (figure 3.22 A).

We next tested whether the expression of *JMJD3* in Kasumi-1 cells was dependent on aberrant constitutive MAPkinase signalling. To this end we measured *JMJD3* expression after simultaneous treatment with JNK, MEK and P38 inhibitors. We also treated the Kasumi-1 cells with DMSO as control for comparison. However, *JMJD3* was down-regulated in the DMSO controls as well as in inhibitor treated samples suggesting that this was the effect of DMSO rather than the inhibitors itself (figure 3.22 B). Hence we conclude that aberrant *PAX5* expression in t(8;21) AML is not a consequence of signalling mediated *JMJD3* up-regulation.



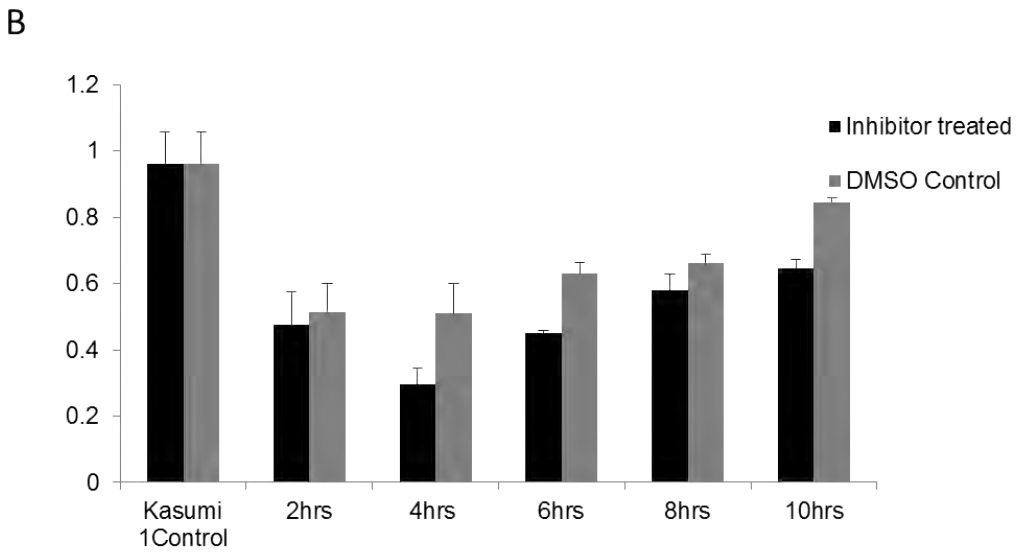


Figure 3.22: Aberrant *PAX5* expression does not involve signalling mediated up-regulation of *JMJD3*. A) qRT PCR showing *JMJD3* expression in the indicated cell lines. The error bars represent standard deviation between duplicate q-PCR measurements. B) q-RT PCR showing *JMJD3* expression kinetics in Kasumi-1 cell line after treatment with JNK, MEK and P38 inhibitors. Treatment with DMSO is done as a control. The values are an average between two independent experiments and the error bars represent variability between the values from these two experiments

3.9.3 MAPKAPkinase3 / 3pK is not responsible for aberrant up-regulation of *PAX5* in t(8;21) AML

It has been reported that loss of BMI1: a component of the PRC1 complex leads to up-regulation of *Ebf1* and *Pax5* in mouse HSC/ MPP (Oguro et al., 2010). Association of BMI1 to chromatin is governed by its phosphorylation status (Voncken et al., 2005). MAPKAP kinase 3 (3pK) is a protein kinase downstream of JNK, MEK and P38 which is known to phosphorylate BMI1 leading to its dissociation from the chromatin (see section 1.3.5). Our experiments showed that combined inhibition of JNK and P38 signalling in Kasumi-1 cells down-regulated *PAX5* expression within 4 hours of treatment. We hypothesized that this down-regulation of *PAX5* could be the result of inhibition of 3pK via the inhibition of upstream MAPkinase pathways. In order to test our hypothesis, we knocked down the expression of the *3pK* gene in Kasumi-1 cells by siRNA. We transiently transfected siRNA directed against the *3pK* mRNA and achieved ~75% down-regulation at the mRNA level within 18 hours (figure 3.23 B) and ~60% down-regulation at the protein level after 4 days of treatment with siRNA (figures 3.23 A and B). qRT-PCR analysis of RNA extracted from *3pK* depleted Kasumi-1 cells at various time-points, however, did not show an effect of 3pK depletion on the expression of *PAX5* (figure 3.23 C)

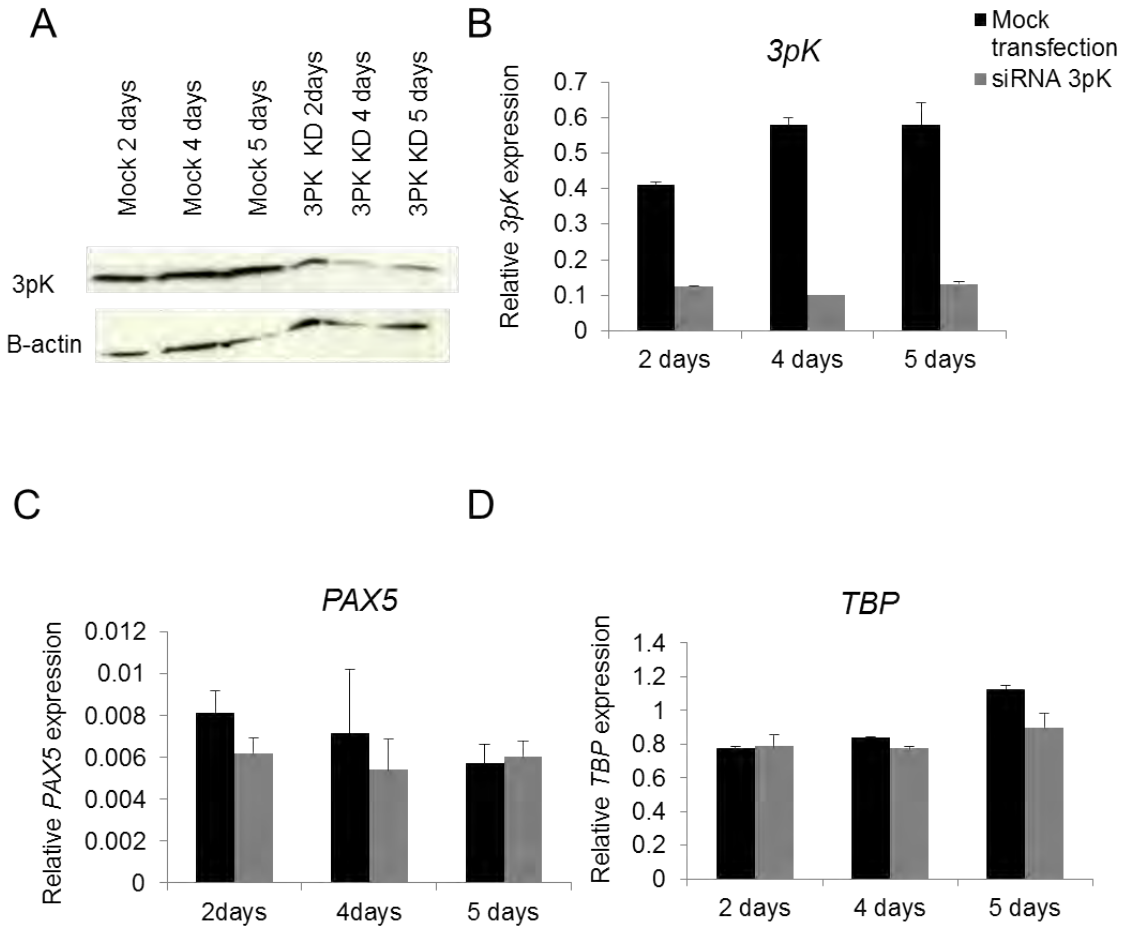


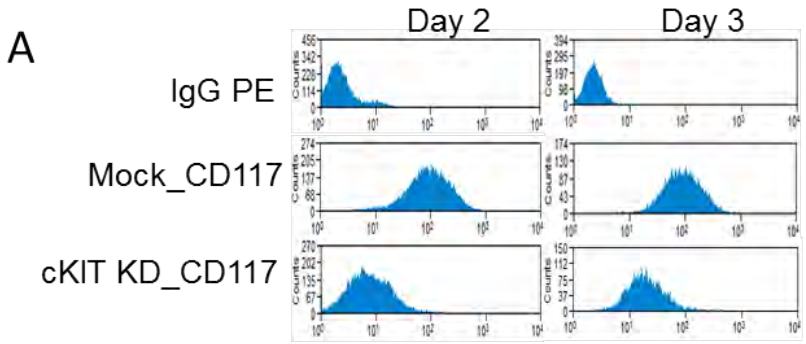
Figure 3.23: MAPKAP kinase 3 (3pK) is not responsible for the de-repression of *PAX5* in t(8;21) AML. A) Western blot showing knock down of 3pk protein after 4 days of treatment with siRNA 3pK in Kasumi-1 cells. B) qRT-PCR showing effective knock-down of 3pK in Kasumi-1 cells after 2, 4 and 5 days respectively. C and D) qRT-PCR assay showing PAX5 and TBP expression is not affected by the knockdown of 3pK in Kasumi-1 cells. The relative expression of *3pK, PAX5* and *TBP* are relative to *GAPDH.* The error bars represent variability between duplicate q-PCR measurements.

3.10 siRNA mediated knock-down of c-KIT down-regulates *PAX5* expression

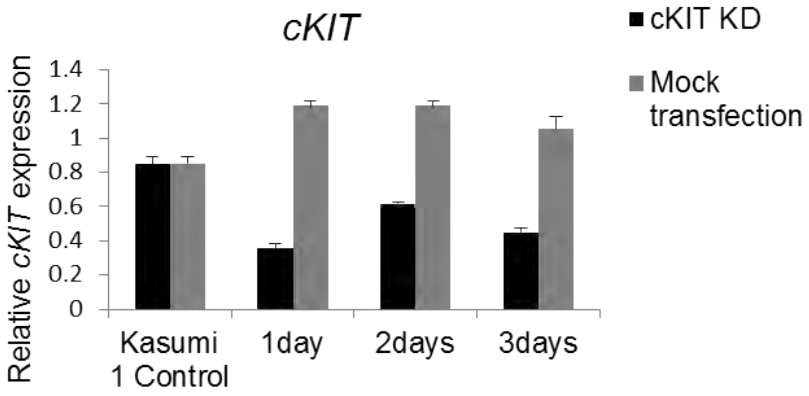
Inhibition of JNK, MEK and P38 signalling down-regulated *PAX5* expression in t(8;21) AML, whereby JNK and P38 are sufficient for down-regulation (figure 3.13 D). These signalling pathways get de-regulated as a result of activating tyrosine kinase mutations in t(8;21) AML. In Kasumi-1 and also in SKNO-1 the C-KIT receptor is rendered constitutively active due to the point mutation Asn833Lys. We therefore tested whether siRNA mediated knockdown of *C-KIT* down-regulated *PAX5* expression. To this end we transiently transfected Kasumi-1 cells with siRNA against *C-KIT* mRNA. qRT-PCR confirmed the knock-down of *C-KIT* at the mRNA level (figure 3.24 B). Flow cytometry analysis of CD117 showed the depletion of C-KIT receptor protein from the cell surface of Kasumi-1 cells two days after transfection (figure 3.24 A). Even though C-KIT provides essential survival signals, depletion of C-KIT receptor is tolerated by the Kasumi-1 cells for at least 4 days. Annexin/PI staining confirmed that depletion of surface C-KIT did not drive Kasumi-1 cells into apoptosis.

After knockdown of C-KIT, RNA was extracted from these C-KIT depleted Kasumi-1 cells and *PAX5* expression was measured. qRT-PCR analysis showed 2 fold down-regulation of *PAX5,* 3 days after C-KIT knockdown (figure 3.24 A). The same effect was not observed in control house- keeping genes such as *TBP* and *RPL13A* (figure 3.24 C). This result establishes the link between C-KIT mutation and de-regulated MAPkinase signalling as a cause for aberrant *PAX5* expression in t(8;21) AML.

A


Day 2
Day 3
IgG PE
Mock_CD117
cKIT KD_CD117
Counts

B


cKIT
Relative cKIT expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Kasumi 1 Control
1day
2days
3days
cKIT KD
Mock transfection

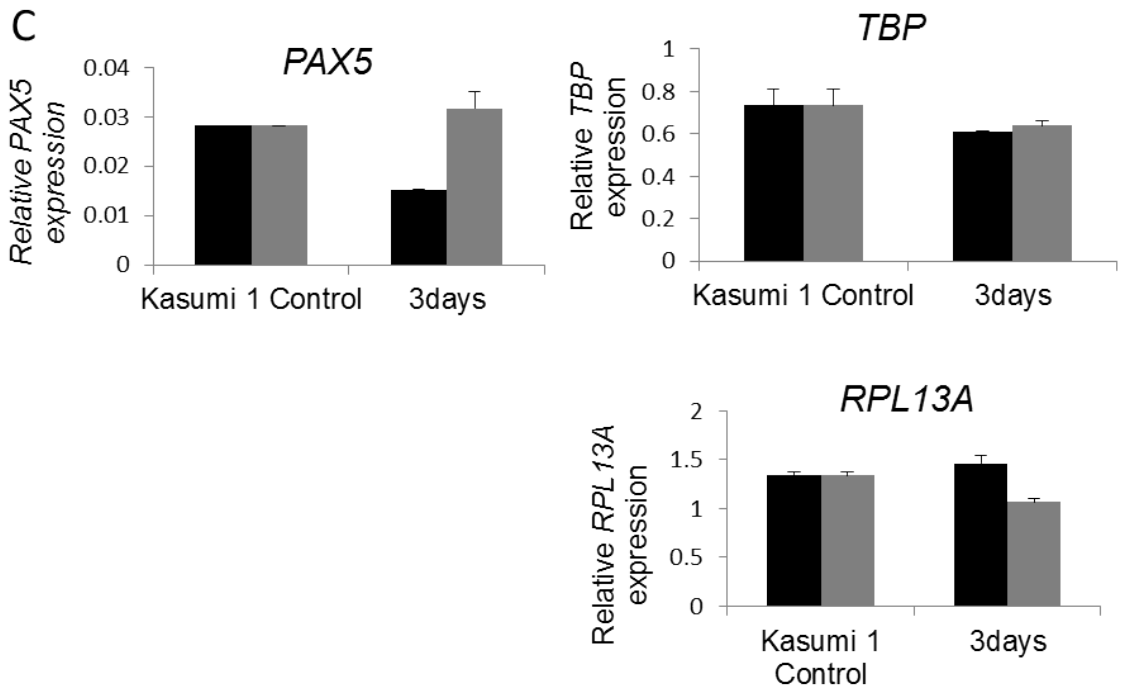


Figure 3.24: siRNA mediated knockdown of c-KIT receptor in Kasumi 1 cells down-regulates *PAX5* expression. A) Flow cytometry analysis showing loss of surface CD117 receptor (c-KIT) after siRNA mediated C-KIT knockdown in Kasumi-1 cells. The top panel shows IgG-PE staining of Kasumi-1 cells; the middle panel shows CD117 staining of mock transfected Kasumi-1 cells, while the bottom panel shows CD117 staining of C-KIT siRNA transfected Kasumi-1 cells. B) q-RT PCR showing effective knock- down of *C-KIT*. The expression shown is relative to *GAPDH.* Error bars represent variation between duplicate q-PCR measurements C) qRT-PCR showing *PAX5, TBP* and *RPL13A* expressions relative to *GAPDH*, 3 days after C-KIT knock-down. This is a representative of at least two independent experiments. The error bars represent variation between duplicate q-PCR measurements

4 DISCUSSION

4.1 PAX5 expression is lower in t(8;21) AML cells than in B-lymphoid cells

It is well known that *PAX5*: the master regulator of B-cell development is aberrantly up-regulated in t(8;21) AML (Tiacci et al., 2004, Valbuena et al., 2006) (see sections 1.6.1 and 3.1 and figure 3.1). This aberrant up-regulation of *PAX5* is of functional consequence in t(8;21) AML as it drives the expression of the B-cell specific *CD19* gene (see section 2.6.2), but the cause for *PAX5* was not known. In this study we, therefore, investigated the molecular mechanism of aberrant *PAX5* expression in this type of leukaemia. To this end we used two well-established t(8;21) AML cell lines: Kasumi-1 and SKNO-1 (Asou et al., 1991, Matozaki et al., 1995) as *in vitro* models together with primary cells from two t(8;21) AML patients. Kasumi-1 was originally established from the peripheral blood of a 7-year old Japanese boy with t(8;21) AML who had relapsed after bone marrow transplantation (Asou et al., 1991). SKNO-1 was established from a 22-year old male patient suffering from t(8;21) AML who also acquired chromosome 17 monosomy and his disease became chemo-resistant (Matozaki et al., 1995). Kasumi-1 and SKNO-1, therefore, represent very aggressive forms of t(8;21) AML. *PAX5* expression has been reported in these cell-types both at the message level and the protein level (SKNO1 only) (Tiacci et al., 2004). Several research studies investigating t(8;21) AML have used these cell lines as models (Ptasinska et al., 2012a). Consistent with previous reports, *PAX5* expression was detected in Kasumi-1, SKNO-1 and in cells from t(8;21) AML patients (figure 3.1) by q-RT-PCR analysis. However, the

expression of *PAX5* in t(8;21) was found to be considerably lower than the B-lymphoid cells (Ramos and Nalm-6) (Figure 3.1).

Several explanations are possible as why t(8;21) AML (Kasumi-1, SKNO-1 and primary cells) express *PAX5* at such a low level compared to B-lymphoid cells. Decker *et al* identified a B-cell specific intronic enhancer at the murine *Pax5* locus which they found was responsible for the robust *Pax5* expression in mouse B-cells (Decker et al., 2009). We identified the corresponding enhancer element (HS B) at the human *PAX5* locus in human pre-B cells (Nalm-6) (see figure 3.3). This strong DNase I hypersensitive site (HS B) is in an inactive chromatin conformation in t(8;21) AML cells (figures 3.6 and 3.7) (also see section 3.3.2). Transgenic lines carrying the deletion of intron 5 (that carries the enhancer element) in *Pax5-Gfp* BAC, showed only minimal expression of the transgene in mature mouse B-cells(Decker et al., 2009) . This indicates that the high levels of *Pax5* expression in mouse B-cells are dependent on the intronic enhancer element. Therefore, the lack of an active corresponding human enhancer element (HS B) in t(8;21) AML cells is likely to be one of the prime reasons for the low expression observed in these cells.

4.2 Aberrant expression of *PAX5* in t(8;21) AML is independent of *EBF1* expression

Many previous studies have shown E2A and EBF1 act genetically upstream of *Pax5* (Bain et al., 1994, Lin and Grosschedl, 1995). Using *Ebf1* $^{-/-}$ MPPs cultured on OP9 feeder cells, Decker *et al* showed that the activation of the murine *Pax5* promoter (HS1-7) at the chromatin level and also the expression of *Pax5* were dependent on the expression of EBF1 (Decker et al., 2009). However, we failed to detect *EBF1* expression in Kasumi-1 or SKNO-1 cell lines by q- RT-PCR (data not shown), although both cell lines clearly express *PAX5* and show an active chromatin conformation at the proximal (HS1-4) and distal promoters (HS8) (see section 3.3.1 and figure 3.5). Cells obtained from t(8;21) AML patients did show *EBF1* expression that was, however, much lower than B-lymphoid cells (data not shown). Moreover, previous studies from our lab demonstrated that *EBF1* expression does not directly correlate to *PAX5* expression in primary t(8;21) blasts (Walter et al., 2010). EBF1 has been shown to primarily bind HS7 and weakly HS6 (by ChIP and *in vivo* footprinting experiments) at the murine *Pax5* promoter to regulate *Pax5* (Decker et al., 2009). In t(8;21) AML these sites (HS7 and HS6) are not hypersensitive to DNase I digestion (see section 3.3.1 and figure 3.5) and hence are occluded from EBF1 binding. Taken together, the lack of active HS 7and HS 6 (which binds EBF1 in mouse B-cells),the lack of expression of *EBF1* in Kasumi-1 and SKNO-1cell lines and also the EBF1 independent formation of hypersensitive sites HS1-4 suggest that the mechanism of up-regulation of *PAX5* in t(8;21) AML does not directly involve EBF1. However, further studies are necessary to examine whether EBF1 binds any of the other promoter hypersensitive sites in

those t(8;21) AML patients that do express EBF1, before completely ruling out the involvement of EBF1 in the aberrant activation or maintenance of *PAX5* expression in t(8;21) AML.

4.3 *PAX5* is poised for transcription and repressed by polycomb repressive complexes in non t(8;21) myeloid precursors

Within the haematopoietic system, *PAX5* is exclusively expressed in the B-lymphoid lineage. However, the promoter cluster of *PAX5* has been shown to show hypersensitivity to DNase I digestion in early $CD34^+$ and $CD133^+$ progenitor cells (Cui et al., 2009), in blasts from non-t(8;21) AML patients (Ptasinska et al., 2012a) and in human pro-myelocytic leukaemia cell line (HL-60) (Encode, UCSC Genome Browser). It is known that poised genes show active chromatin, bivalent histone marks and carry a stalled RNA Polymerase II (Beisel and Paro, 2011). To study this in more detail, we used HL-60 cells as a non t(8;21) myeloid precursor control in our experiments. HL-60 cells were established from a female patient who was suffering from acute myeloblastic leukaemia with differentiation falling in M2 category under the FAB classification (Dalton et al., 1988).Therefore, HL-60 cells are very similar to Kasumi-1 cells in their differentiation stage. Our experiments show that in HL-60 cells the *PAX5* gene shows an active chromatin conformation at the promoters (see sections 3.3.1, 3.5 and figures 3.5 and 3.11) and carries bivalent histone marks (see section 1.2.7 and figures 3.8B and 3.10A) and a stalled RNA Polymerase II figure 4.9). Taken together, these results show that in HL-60 cells, *PAX5* is poised for transcription. It is interesting to note that *CD19*, another B-cell gene that is deregulated in t(8;21) AML, also shows poised chromatin structure at the

enhancer in myeloblasts (Walter et al., 2010). Using DNase I hypersensitive site mapping, *in vivo* DNAse I footprinting and histone modification ChIP experiments, Walter *et al* showed that the *CD19* enhancer exhibits a poised chromatin in myeloblasts in the absence of up-stream B-cell specific factors such as PAX5 and EBF1(Walter *et al*. 2010). Inspection of genome-wide DNAse I hypersensitive site mapping in HL-60 (UCSC genome browser) also reveals an open chromatin conformation at the *CD19* enhancer and promoter.

The *PAX* family of transcription factors are well-known targets of polycomb repressive complexes (Bracken et al., 2006). In fact, *Pax5* has been shown to be repressed by polycomb repressive complexes in early haematopoietic progenitors (HSCs and MPPs) (Bernstein et al., 2006, Mikkelsen et al., 2008), in T-cells (Wang et al., 2008) and in *Pax5* non- expressing MEFs (Mikkelsen et al., 2008). Decker *et al* showed that in *Ebf1*$^{-/-}$ and in *Tcfe2a*$^{-/-}$ MPPs which do not express *Pax5* and fail to form seven out of the eight *Pax5* promoter hypersensitive sites, the *Pax5* promoter is marked by the polycomb mediated H3K27me3 repressive mark (Decker et al., 2009). Our H3K27me3, H3K27ac and EZH2 ChIP experiments confirm this finding and provide direct evidence that indeed in *PAX5* non-expressing HL-60 cells, the *PAX5* gene is repressed by polycomb repressive complexes (figure 3.10 and section 3.4). Such poised chromatin imposed by polycomb repressive complexes is found at a number of developmentally regulated genes in early stem/ progenitor cells (Cui et al., 2009, Beisel and Paro, 2011). Deletion of *Bmi1* in mouse HSCs/MPPs leads to the untimely up-regulation of *Pax5* and *Ebf1* in HSCs/MPPs (Oguro et al.,

2010). Therefore polycomb mediated repression of *PAX5* is of paramount importance for the spatio-temporal regulation of *PAX5*.

There are several known mechanisms of polycomb mediated repression (see section 1.3.3) including H2AK119ub1 mediated block in transcriptional elongation. Our ChIP experiment measuring the elongating phosphorylated -serine2 RNA polymerase II complex indeed shows that in HL-60 cells transcription is blocked at the elongation stage (see figure 3.9B). Future experiments will test whether this block in transcriptional elongation at *PAX5* in HL-60 is due to the deposition of PRC1/H2AK119ub.

4.4 Aberrant up-regulation of *PAX5* in t(8;21) AML is not dependent on the presence of RUNX1/ETO

Our data clearly show that the presence or absence of RUNX1/ETO does not influence *PAX5* expression. Microarray analysis of RNA isolated from human $CD34^+$ haematopoietic progenitor cells transduced with RUNX1/ETO containing retrovirus also does not show deregulation of *PAX5* (Tonks et al., 2007). In a recent study Dunne *et al* describes that siRNA mediated depletion of RUNX1/ETO in primary t(8;21) patient cells down-regulates *PAX5* expression by ~10 folds within 24 hours (Dunne et al., 2012). However, we have failed to reproduce this result by knocking down RUNX1/ETO for up to 96 hours in Kasumi-1 cells using the same siRNA (gift from Dr Olaf Heidenreich, Newcastle ,UK) as this group (Figure 3.12 A). As a part of a parallel study in the lab, RUNX1/ETO knocked down Kasumi-1 cells were maintained for 10 days and RNA isolated at various time points from these cells were used for microarray

analysis (Ptasinska et al., 2012a). Inspection of this microarray data does not show a change in *PAX5* expression as a result of RUNX1/ETO depletion in Kasumi-1 cells (Ptasinska et al., 2012a). To further confirm this, we obtained RNA from RUNX1/ETO depleted t(8;21) patient cells from Dr Olaf Heidenreich and found that *PAX5* expression was unaffected as a result of RUNX1/ETO depletion (figure 3.12A). Furthermore, genome-wide RUNX1 and RUNX1/ETO ChIP-sequencing showed that *PAX5* is not a direct target of RUNX1 or RUNX1/ETO (Figure 3.12 B) in Kasumi-1 cells or primary t(8;21) AML patient cells (Ptasinska *et al*. 2012). It is important to note that, Dunne *et al* transduced foetal liver haematopoietic progenitor cells with RUNX1/ETO9a (a truncated version of the RUNX1/ETO protein that is capable of inducing leukaemia in transplantation mouse models) and observed only a small increase in *PAX5* expression (Dunne et al., 2012). They did observe a robust increase in *PAX5* expression on transducing the foetal liver haematopoietic progenitors with retroviruses containing RUNX1/ETO9a and POU4F1 (Dunne et al., 2012) and therefore suggest that RUNX1/ETO alone is unable to up-regulate *PAX5*; and requires the synergistic effects of RUNX1/ETO and POU4F1 (BRN3A) (Dunne et al., 2012).

Our results and the literature discussed above clearly show that RUNX1/ETO does not directly bind *PAX5* to regulate its expression, nor does the presence/absence of RUNX1/ETO alone affect *PAX5* expression. However, the idea of another genetic mutation, or deregulated protein (such as BRN3A) co-operating with RUNX1/ETO to up-regulate *PAX5* is a plausible hypothesis. To

prove this, direct binding of BRN3A to the *PAX5* locus needs to be shown and a mechanism of BRN3A and RUNX1/ETO co-operativity needs to be discovered.

4.5 Constitutively activated C-KIT mediated aberrant MAPKinase signalling is required for up-regulation of *PAX5* in t(8;21) AML

Full length RUNX1/ETO protein on its own is unable to cause overt leukaemia in transplantation models; it requires co-operating mutations (see section 1.5.2). A sizeable percentage of secondary mutation in t(8;21) AML are in tyrosine kinase receptors *(FLT3, c-KIT, NRAS* and *KRAS*) (Peterson et al., 2007a, Valk et al., 2004, Bullinger et al., 2004) which co-operate with t(8;21) translocation to induce leukemic transformation (see section 1.5.2). The frequency of C-KIT mutation has been reported to be as high as 48% in t(8;21) AML (Wang et al., 2005). KIT mutations are correlated with high white blood cell count (Cairoli et al., 2003, Nguyen et al., 2002), poor prognosis and highest frequency of relapse in t(8;21) AML (Park et al., 2011, Cairoli et al., 2006, Wakita et al., 2011). Kasumi-1 and SKNO-1 have been shown to carry an activating N822K point mutation in the activation domain of C-KIT receptor which leads to its ligand independent auto-phosphorylation (Beghini et al., 2002, Becker et al., 2008). Kasumi-1 carries a wild-type allele of C-KIT but the mutant allele is amplified 5-fold due a concomitant trisomy of chromosome 4 (Beghini et al., 2002). SKNO-1 is homozygous for the N822K C-KIT mutation (Becker et al., 2008). A large amount of research has elucidated the effect of activating C-KIT mutation on down-stream signalling pathways (Tsujimura et al., 1997, Timokhina et al., 1998, Chian et al., 2001, Ning et al., 2001, Linnekin, 1999). Larizza *et al* has reviewed this body of work and put forward a composite picture of deregulated

signalling pathways (which includes AKT, JAK/STAT, JNK, P38 and MEK) down-stream of constitutively activated C-KIT signalling in t(8;21) AML (Larizza et al., 2005). Western blotting experiments have clearly shown constitutively phosphorylated PI3K, AKT, STAT3 and JNK proteins in Kasumi-1 cells (Beghini et al., 2005). Though constitutively activated ERK1/ERK2 kinases were not detectable by western blotting in Kasumi-1 cells (Beghini et al., 2005), other studies have reported an effect of high concentration (30µM) of MEK1/MEK2 inhibitor on the survival and apoptosis of Kasum-1 cells (Rovida et al., 2006). Furthermore ERK1 and ERK2 (down-stream of MEK) were the first signalling pathways to be shown to be deregulated as a result of activating C-KIT mutation (Serve et al., 1995, Okuda et al., 1992, Druker et al., 1992). Stimulation of normal bone marrow cells show robust activation ERK and AKT signalling in a variety of C-KIT positive cells (Ronnstrand, 2004). It is important to note that different MAPKinases interact with each other extensively and exhibit complex 'cross-talk' (Chen et al., 2001).

Our experiments demonstrate that simultaneous inhibition of JNK, P38 and MEK (or JNK and P38 –figure 4.13D) down-regulated *PAX5* expression within 4 hours of treatment in Kasumi-1 cells (figures 3.13D and 3.15), SKNO-1 cells (figure 3.16) and in cells from t(8;21) AML patients (figure 3.16). Furthermore, within 6 hours of treatment with JNK, P38 and MEK inhibitors *PAX5* promoter hypersensitive sites lost the elongating form of RNA polymerase II and H3K27acetylation (figures 3.19 A and B) in Kasumi-1 cells. siRNA mediated knock-down of C-KIT in Kasumi-1 cells, which is known to be the causative mutation behind aberrant MAPkinase signalling in these cells, also down-

regulated *PAX5* expression (figure 3.24). This experiment directly links mutated C-KIT to aberrant *PAX5* expression in Kasumi-1 cells and makes it evident that aberrant MAPKinase signalling which is an effect of constitutively activated C-KIT, is required is required to maintain elevated *PAX5* expression in t(8;21) AML cells.

Our experiments have shown that in HL-60 cells *PAX5* is not expressed (figure 3.1), the gene is repressed by polycomb repressive complexes (figure 4.10) but the locus is poised for transcription (figures 3.6, 3.8 and 3.9). In this context it is interesting to note that unlike Kasumi-1, HL-60 cells have wild-type alleles of C-KIT and are responsive to SCF (Jalal Hosseinimehr et al., 2004). Western blotting experiments have confirmed the absence of constitutively activated MEK (Miranda et al., 2002) and P38 MAPKinases (Tian et al., 2010) in HL-60 cells. Though constitutive activation of JNK has been detected in this cell-type (Rovida et al., 2006), it clearly does not have an effect on *PAX5* expression.

Activating mutations in C-KIT forms up to 48% of the secondary mutations in t(8;21) AML. However, *PAX5* expression is detectable in a much larger percentage of t(8;21) AML patients (Tiacci et al., 2004, Valbuena et al., 2006). The other common mutations co-operating with RUNX1/ETO in t(8;21) AML affect the signalling molecules N-RAS, K-RAS and FLT3. It is important to note that activated RAS-RAF kinases activate MAPkinases (Serve et al., 1995, Okuda et al., 1992). FLT3 is structurally very similar to C-KIT. There are several studies that show that constitutively active FLT3 constitutively activates STAT5 and MAPkinases (Hayakawa et al., 2000, Williams et al., 2012). FLT3 and C-KIT mutations lead to the deregulation of the same pathways in AML, which is

the likely explanation for the finding that these mutations are mutually exclusive in AML (Care et al., 2003). Therefore the convergence of activating C-KIT, FLT3 and RAS mutations at down-stream MAPkinases could be a possible explanation for the activation of *PAX5* expression in t(8;21) AML with or without C-KIT mutation.

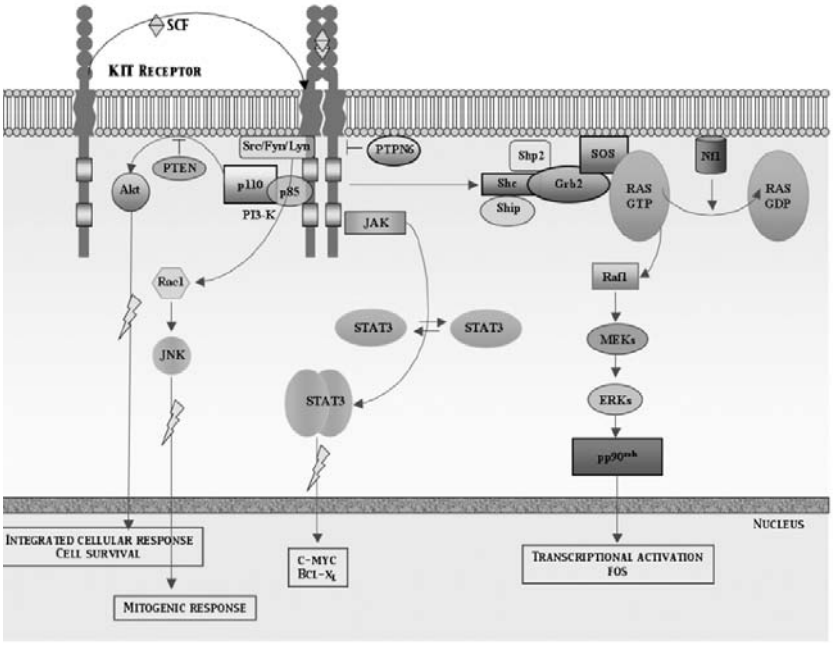


Figure 4.1: Schematic summary of constitutively activated signalling pathways down-stream of an activating C-KIT mutation (Larizza et al., 2005)**.**

4.6 STAT3 is the main survival signal in t(8;21) AML

STAT3 is a critical signalling component in haematopoietic cells (Chung et al., 2006). and plays a key role in cancer (Bromberg et al., 1999). Constitutively activated STAT3 has been detected in squamous cell carcinoma of the head and neck (Grandis et al., 1998) and also in various other malignancies including

AML (Benekli et al., 2002, Spiekermann et al., 2001, Steensma et al., 2006). STAT3 has been shown to be active in t(8;21) AML as a result of constitutively activated C-KIT signalling (Larizza et al., 2005, Beghini et al., 2005).Fibroblasts expressing constitutive STAT3 develop malignancy in culture and form tumours in mice (Garcia et al., 1997). Our experiments showed that small molecule mediated inhibition of constitutively active STAT3 signalling in Kasumi-1 cells led to complete cell death within 3 hours of treatment by driving the cells into apoptosis (figure 3.14 C) as measured by Annexin/ PI staining. This confirms previous results which also demonstrated STAT3 dependency of survival in other AML cell lines (Kasumi-1, HL-60, K562, GDM-1,THP-1 KG-1 and NB-4) representing different types of acute myeloid leukaemia and primary paediatric AML (Redell et al., 2011). Similar results have been reported in HL-60 (Zhao et al., 2011) and U937 cells with different STAT3 small molecule inhibitors (Kang et al., 2012). It is noteworthy that our experiments showed no apoptotic effect of AKT, JNK, MEK or P38 inhibition alone or in conjunction in Kasumi-1 cells (figure 4.14 A,B and C). However, Rovida *et al* reported apoptosis of Kasumi-1 cells on inhibition of constitutively activated JNK in conjunction with HDAC inhibitors (Rovida et al., 2006). Our results indicate that constitutively activated STAT3 (and not AKT or MAPKinases) is the primary survival signal in Kasumi-1 cell line.

Recent work from Dong-Er Zhang's lab has identified 19 genes as direct targets of RUNX1/ETO9a by combined microarray and ChIP-on-chip experiments in the $Lin^{-}Sca1^{-}$ c-Kit^{+} (LK) leukemic population of cells from an AML mouse model carrying RUNX1/ETO9a oncogene (Lo et al., 2012). One of these 19 genes was

CD45. Their microarray data showed that CD45 is highly down-regulated in t(8;21) AML patient samples as well as the LK cells from the leukemic mouse model. CD45 is a haematopoietic JAK phosphatase that down-regulates JAK/STAT signalling. They found that as a result of down-regulation of CD45 in RUNX1/ETO9a expressing mouse cells, JAK/STAT signalling was enhanced with higher levels of phosphorylated JAK1, JAK2 and several STAT proteins including STAT3 compared to control cells. Re-expression of CD45 suppressed JAK/STAT signalling and delayed leukaemogenesis and drove the t(8;21) positive AML cells to apoptosis (Lo et al., 2012). Therefore this study exhibited an alternative (different from C-KIT mediated) mechanism of activation of JAK/STAT signalling but it re-enforces the fact that JAK/STAT signalling is the prime survival and growth signal in t(8;21) AML. Therefore, STAT3 is clearly a lucrative therapeutic target for treating acute myeloid leukaemia.

4.7 Aberrant MAPkinase signalling in t(8;21) AML inactivates polycomb repressive complexes

Inhibition of MAPKinase signalling in Kasumi-1 cells leads to the increase of the PRC2 mediated H3K27me3 mark across the *PAX5* promoter hypersensitive sites (figure 3.19 C) with the concomitant decrease of the opposing H3K27ac mark (figure 3.19 B) and experiments conducted by So Yeon Kwon form the Bonifer lab has also confirmed the re-recruitment of EZH2 (unpublished results). The result is a down-regulation of *PAX5.* This inhibition of signalling mediated down-regulation of *PAX5* can be counteracted by the HDAC inhibitor: TSA and is consistent with the finding that Polycomb complexes recruit HDACs to mediate repression (van der Vlag and Otte, 1999). Therefore, we propose in

t(8;21) AML aberrant MAPkinase signalling de-regulates polycomb which leads to its dissociation from of its target genes one of which is *PAX5*. This could either be due to direct signalling mediated modification (phosphorylation) of polycomb proteins or via another protein (transcription factor) that itself gets modified by signalling and then regulates polycomb. These two mechanisms are currently being investigated.

A large number of mutations in polycomb group proteins have been detected in cancer including AML where genes encoding polycomb group proteins function as oncogenes or tumour suppressors (Raaphorst, 2005, Jacobs et al., 1999, Kikuchi et al., 2010, Mochizuki-Kashio et al., 2012). Our studies, however, suggest that polycomb might also become functionally de-regulated in t(8;21) AML as a result of aberrant signalling. Such de-regulated polycomb activity in turn deregulates the expression of downstream target genes which may be key factors in determining leukaemogenesis. Several studies have mechanistically linked signalling pathways to the de-repression of polycomb target genes. The immediate early response genes become de-repressed in response to MAPkinase pathway activation (Lau and Cheung, 2011a, Gehani et al., 2010). Furthermore, de-repression of polycomb targets in stem/progenitor cells form an important step in differentiation and development (Sawarkar and Paro, 2010). Therefore, the mechanism of *PAX5* de-regulation mediated by a functionally de-regulated polycomb could be a paradigm for gene de-regulation observed in several malignancies.

4.8 Aberrant up-regulation of *PAX5* by mutant C-KIT is a novel role of C-KIT signalling which may contribute to the differentiation block in t(8;21) AML

Aberrant up-regulation of *PAX5* in t(8;21) AML is a key component of the mixed lineage phenotype of t(8;21) AML. Ectopic expression of *PAX5* in myeloid precursors leads to the formation of bi-phenotypic progenitor cells with B-cell and myeloid surface markers (see section 2.6.2). PAX5 has been shown to directly up-regulate *CD19* expression in t(8;21) AML (Walter et al., 2010). In normal haematopoiesis PAX5 is a well-known repressor of myeloid specific genes such as *CSF1R* (Tagoh et al., 2006b). It is therefore likely to also repress myeloid genes in t(8;21) AML, thus contributing to the block in myeloid differentiation.

C-KIT and other receptor tyrosine kinase genes were shown to regulate haematopoiesis by mostly influencing the HSC microenvironment (Ashman, 1999). SCF/C-KIT signalling provides growth signal for multipotent progenitors (Ogawa et al., 1991, Carow et al., 1991) and crucial survival signal to HSCs (Sharma et al., 2006). C-KIT is expressed from HSCs up to pro-myelocytic progenitor stage during normal haematopoiesis (Zheng et al., 2009). Mutations in C-KIT are classically classified under the Class I mutations (such as mutations in *RAS, FLT3, PDGFRβ, CSF1R*) that are thought to provide survival and growth advantage to leukemic cells which carries another mutation (Class II mutation) that blocks differentiation (Steffen et al., 2005).

Our experiments demonstrate for the first time that constitutive C-KIT signalling and downstream MAPkinase signalling has a direct influence on gene expression in addition to its classical role in survival and growth signal of leukemic cells. Our results suggest that in t(8;21) AML activated MAPkinase signalling deregulates polycomb repressive complexes and affects its association/dissociation to target loci. Since leukemic blasts are arrested at an early stage of differentiation they are likely to have a large number of genes under polycomb regulation. Therefore, signalling mediated deregulation of polycomb is likely to de-regulate a large number of genes.

siRNA mediated depletion of C-KIT in $Sca1^{+}Lin^{-}$ mouse HSCs transduced with RUNX1/ETO does not reduce the re-plating ability of these cells. However, the loss of C-KIT leads to the colony forming unit-GM of RUNX1/ETO positive HSCs to appear more spread out indicating an effect of C-KIT signalling on the differentiation status of these cells (Zheng et al., 2009). Furthermore, recently a clinical study has described that the treatment of a 52 year old male patient suffering from AML with t(8;21) translocation and activating N822K C-KIT mutation, with dasatinib resulted in robust in vivo differentiation of myeloblasts into neutrophilic granulocytes in the peripheral blood of the patient (Chevalier et al., 2010) pointing to a role of C-KIT in the differentiation block in this type of leukaemia. The mechanism elucidated by our study i.e. deregulation of key genes (in this case *PAX5*) via abrogation of polycomb mediated repression by aberrant signalling downstream of activating C-KIT mutation, may represent the main mechanism by which C-KIT affects differentiation of blasts in t(8;21) AML.

4.9 Summary

The mechanism by which *PAX5* is deregulated in t(8;21) AML is illustrated in figure 4.2. In B cells *PAX5* exhibits an active chromatin conformation at the promoters and enhancer, carries high levels of active histone makers (H3K4me3 and K3K9ac), recruits high level of RNA Polymerase II complex and is robustly expressed. In myeloid precursors without t(8;21) translocation the *PAX5* promoters show an active chromatin conformation and recruits RNA polymerase II. However, the gene is repressed by polycomb repressive complexes and the RNA polymerase II is held paused. In t(8;21) AML this polycomb repression is relieved due to aberrant MAPkinase signalling which is an effect of an activating mutation in receptor tyrosine kinases (C-KIT). This leads to the up-regulation of *PAX5* (see figure 4.2).

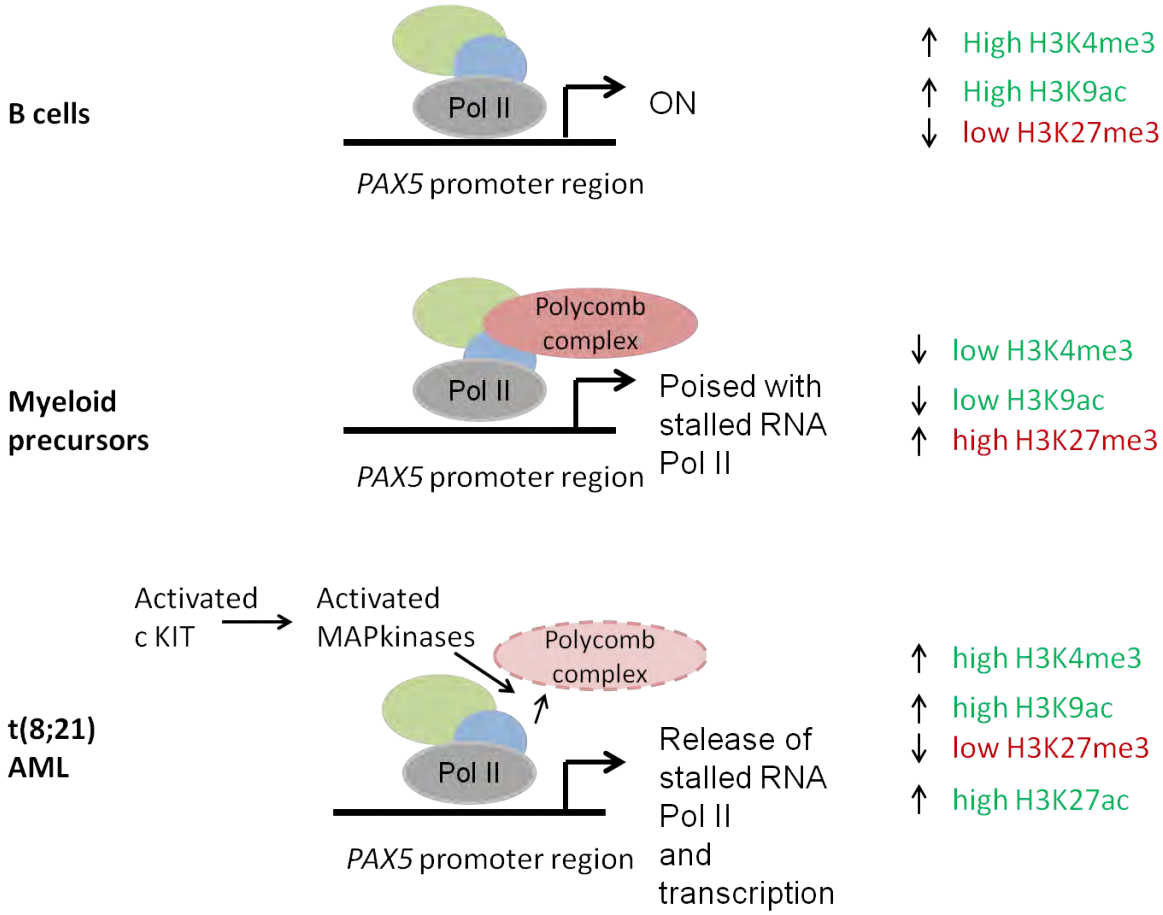


Figure 4.2: Aberrant activation of *PAX5* in t(8;21) AML involves abrogation of polycomb repression via constitutively activated MAPKinase pathways due to activating receptor tyrosine kinase mutations. In B cells the *PAX5* promoter remains active and robust transcription occurs. In myeloid precursors the gene is not expressed, but remains poised with a stalled RNA polymerase II and repressed by polycomb repressive complexes. In t(8;21) AML this polycomb repression is relieved via activated MAPkinase signalling leading to effective transcriptional elongation and aberrant expression of *PAX5.* The green and blue shapes indicate the basal transcription machinery associated with the RNA polymerase II complex.

4.10 Open questions and future work

Although our study sheds light on the general mechanism of *PAX5* deregulation in t(8;21) AML, there are a number of open questions. C-KIT mutations are most common in core binding factor leukaemia but receptor tyrosine kinase mutations are detected in a large variety of AML types irrespective of the karyotype. However *PAX5* expression is highly correlated to t(8;21) AML. It is therefore unlikely that activated receptor tyrosine kinase mediated aberrant signalling is the sole reason for up-regulation of *PAX5* in t(8;21) AML. It is therefore likely that RUNX1/ETO plays a role in the activation of *PAX5* in conjunction with aberrant signalling. As discussed in section 4.4 Dunne *et al* has suggested POU4F/BRN3A as a potential factor that co-operates with RUNX1/ETO to up-regulate *PAX5* (see section 4.4). It is therefore important to investigate whether BRN3A or any other RUNX1/ETO regulated factor co-operates with MAPkinase signalling in the de-regulation of *PAX5*. Transcription factors may be directly influenced by MAPkinase signalling to co-operate with RUNX1/ETO to mediate *PAX5* deregulation. Signalling mediated modification of transcription factors have been shown to affect transcription factor activity (Kowenz-Leutz et al., 2010, Yasuda et al., 2012).

Furthermore, since our experiments show that inhibition of signalling recruits polycomb on to the *PAX5* promoter hypersensitive sites, it is likely that aberrant MAPKinase signalling mediated loss of polycomb will affect a large number of polycomb targets in this type of leukaemia. If these genes are otherwise poised for transcription like *PAX5*, then these genes will also get de-repressed. Therefore this might be a mechanism of deregulation (up-regulated) of genes in

t(8;21) AML that are not directs targets of RUNX1/ETO. It is therefore important to perform microarray gene expression analysis before and after inhibitor treatment to examine the nature and number of deregulated genes. Genome-wide H3K27me3 and EZH2 ChIP-sequencing experiments to complement the microarray analysis will shed light on the genes that recruit polycomb after MAPkinase inhibition. These experiments are currently on-going in the lab and will help to unravel a novel mechanism of gene de-regulation via de-regulated polycomb in t(8;21) AML.

References

ADAMS, B., DORFLER, P., AGUZZI, A., KOZMIK, Z., URBANEK, P., MAURER-FOGY, I. & BUSSLINGER, M. 1992. Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev,* 6**,** 1589-607.

ADOLFSSON, J., MANSSON, R., BUZA-VIDAS, N., HULTQUIST, A., LIUBA, K., JENSEN, C. T., BRYDER, D., YANG, L., BORGE, O. J., THOREN, L. A., ANDERSON, K., SITNICKA, E., SASAKI, Y., SIGVARDSSON, M. & JACOBSEN, S. E. 2005. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell,* 121**,** 295-306.

AGGER, K., CLOOS, P. A., RUDKJAER, L., WILLIAMS, K., ANDERSEN, G., CHRISTENSEN, J. & HELIN, K. 2009. The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. *Genes Dev,* 23**,** 1171-6.

AKASAKA, T., TAKAHASHI, N., SUZUKI, M., KOSEKI, H., BODMER, R. & KOGA, H. 2002. MBLR, a new RING finger protein resembling mammalian Polycomb gene products, is regulated by cell cycle-dependent phosphorylation. *Genes Cells,* 7**,** 835-50.

AKASHI, K., TRAVER, D., MIYAMOTO, T. & WEISSMAN, I. L. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature,* 404**,** 193-7.

AKOULITCHEV, S., MAKELA, T. P., WEINBERG, R. A. & REINBERG, D. 1995. Requirement for TFIIH kinase activity in transcription by RNA polymerase II. *Nature,* 377**,** 557-60.

ALBRIGHT, S. C., WISEMAN, J. M., LANGE, R. A. & GARRARD, W. T. 1980. Subunit structures of different electrophoretic forms of nucleosomes. *J Biol Chem,* 255**,** 3673-84.

ALLAN, J., HARBORNE, N., RAU, D. C. & GOULD, H. 1982. Participation of core histone "tails" in the stabilization of the chromatin solenoid. *J Cell Biol,* 93**,** 285-97.

ALLAN, R. S., ZUEVA, E., CAMMAS, F., SCHREIBER, H. A., MASSON, V., BELZ, G. T., ROCHE, D., MAISON, C., QUIVY, J. P., ALMOUZNI, G. & AMIGORENA, S. 2012. An epigenetic silencing pathway controlling T helper 2 cell lineage commitment. *Nature,* 487**,** 249-53.

ALLMAN, D., SAMBANDAM, A., KIM, S., MILLER, J. P., PAGAN, A., WELL, D., MERAZ, A. & BHANDOOLA, A. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol,* 4**,** 168-74.

ANDERSON, K., RUSTERHOLZ, C., MANSSON, R., JENSEN, C. T., BACOS, K., ZANDI, S., SASAKI, Y., NERLOV, C., SIGVARDSSON, M. & JACOBSEN, S. E. 2007. Ectopic expression of PAX5 promotes maintenance of biphenotypic myeloid progenitors coexpressing myeloid and B-cell lineage-associated genes. *Blood,* 109**,** 3697-705.

ARAI, F., HIRAO, A., OHMURA, M., SATO, H., MATSUOKA, S., TAKUBO, K., ITO, K., KOH, G. Y. & SUDA, T. 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell,* 118**,** 149-61.

ASHMAN, L. K. 1999. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol,* 31**,** 1037-51.

ASOU, H., TASHIRO, S., HAMAMOTO, K., OTSUJI, A., KITA, K. & KAMADA, N. 1991. Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. *Blood,* 77**,** 2031-6.

ATCHISON, L., GHIAS, A., WILKINSON, F., BONINI, N. & ATCHISON, M. L. 2003. Transcription factor YY1 functions as a PcG protein in vivo. *EMBO J,* 22**,** 1347-58.

AWAD, S. & HASSAN, A. H. 2008. The Swi2/Snf2 bromodomain is important for the full binding and remodeling activity of the SWI/SNF complex on H3- and H4-acetylated nucleosomes. *Ann N Y Acad Sci,* 1138**,** 366-75.

BAER, B. W. & RHODES, D. 1983. Eukaryotic RNA polymerase II binds to nucleosome cores from transcribed genes. *Nature,* 301**,** 482-8.

BAI, L. & MOROZOV, A. V. 2010. Gene regulation by nucleosome positioning. *Trends Genet,* 26**,** 476-83.

BAIN, G., MAANDAG, E. C., IZON, D. J., AMSEN, D., KRUISBEEK, A. M., WEINTRAUB, B. C., KROP, I., SCHLISSEL, M. S., FEENEY, A. J., VAN ROON, M. & ET AL. 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell,* 79**,** 885-92.

BAKAYEV, V. V., BAKAYEVA, T. G. & VARSHAVSKY, A. J. 1977. Nucleosomes and subnucleosomes: heterogeneity and composition. *Cell,* 11**,** 619-29.

BAKER, S. J., KERPPOLA, T. K., LUK, D., VANDENBERG, M. T., MARSHAK, D. R., CURRAN, T. & ABATE, C. 1992. Jun is phosphorylated by several protein kinases at the same sites that are modified in serum-stimulated fibroblasts. *Mol Cell Biol,* 12**,** 4694-705.

BAKER, S. J., RANE, S. G. & REDDY, E. P. 2007. Hematopoietic cytokine receptor signaling. *Oncogene,* 26**,** 6724-37.

BANIAHMAD, A., STEINER, C., KOHNE, A. C. & RENKAWITZ, R. 1990. Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell,* 61**,** 505-14.

BANNISTER, A. J., ZEGERMAN, P., PARTRIDGE, J. F., MISKA, E. A., THOMAS, J. O., ALLSHIRE, R. C. & KOUZARIDES, T. 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature,* 410**,** 120-4.

BARRADAS, M., ANDERTON, E., ACOSTA, J. C., LI, S., BANITO, A., RODRIGUEZ-NIEDENFUHR, M., MAERTENS, G., BANCK, M., ZHOU, M. M., WALSH, M. J., PETERS, G. & GIL, J. 2009. Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev,* 23**,** 1177-82.

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**,** 823-37.

BECKER, H., PFEIFER, D., AFONSO, J. D., NIMER, S. D., VEELKEN, H., SCHWABE, M. & LUBBERT, M. 2008. Two cell lines of t(8;21) acute myeloid leukemia with activating KIT exon 17 mutation: models for the 'second hit' hypothesis. *Leukemia,* 22**,** 1792-4.

BEGHINI, A., BELLINI, M., MAGNANI, I., COLAPIETRO, P., CAIROLI, R., MORRA, E. & LARIZZA, L. 2005. STI 571 inhibition effect on KITAsn822Lys-mediated signal transduction cascade. *Exp Hematol,* 33**,** 682-8.

BEGHINI, A., MAGNANI, I., RIPAMONTI, C. B. & LARIZZA, L. 2002. Amplification of a novel c-Kit activating mutation Asn(822)-Lys in the Kasumi-1 cell line: a t(8;21)-Kit mutant model for acute myeloid leukemia. *Hematol J,* 3**,** 157-63.

BEISEL, C. & PARO, R. 2011. Silencing chromatin: comparing modes and mechanisms. *Nat Rev Genet,* 12**,** 123-35.

BENDER, M. A., BULGER, M., CLOSE, J. & GROUDINE, M. 2000. Beta-globin gene switching and DNase I sensitivity of the endogenous beta-globin locus in mice do not require the locus control region. *Mol Cell,* 5**,** 387-93.

BENDER, M. A., BYRON, R., RAGOCZY, T., TELLING, A., BULGER, M. & GROUDINE, M. 2006. Flanking HS-62.5 and 3' HS1, and regions upstream of the LCR, are not required for beta-globin transcription. *Blood,* 108**,** 1395-401.

BENEKLI, M., XIA, Z., DONOHUE, K. A., FORD, L. A., PIXLEY, L. A., BAER, M. R., BAUMANN, H. & WETZLER, M. 2002. Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival. *Blood,* 99**,** 252-7.

BENZ, C., COPLEY, M. R., KENT, D. G., WOHRER, S., CORTES, A., AGHAEEPOUR, N., MA, E., MADER, H., ROWE, K., DAY, C., TRELOAR, D., BRINKMAN, R. R. & EAVES, C. J. 2012. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell,* 10**,** 273-83.

BERNSTEIN, B. E., KAMAL, M., LINDBLAD-TOH, K., BEKIRANOV, S., BAILEY, D. K., HUEBERT, D. J., MCMAHON, S., KARLSSON, E. K., KULBOKAS, E. J., 3RD, GINGERAS, T. R., SCHREIBER, S. L. & LANDER, E. S. 2005. Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell,* 120**,** 169-81.

BERNSTEIN, B. E., MIKKELSEN, T. S., XIE, X., KAMAL, M., HUEBERT, D. J., CUFF, J., FRY, B., MEISSNER, A., WERNIG, M., PLATH, K., JAENISCH, R., WAGSCHAL, A., FEIL, R., SCHREIBER, S. L. & LANDER, E. S. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell,* 125**,** 315-26.

BHATIA, M., WANG, J. C., KAPP, U., BONNET, D. & DICK, J. E. 1997. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A,* 94**,** 5320-5.

BLACKWOOD, E. M. & KADONAGA, J. T. 1998. Going the distance: a current view of enhancer action. *Science,* 281**,** 60-3.

BLANK, U. & KARLSSON, S. 2011. The role of Smad signaling in hematopoiesis and translational hematology. *Leukemia,* 25**,** 1379-88.

BOISSET, J. C., VAN CAPPELLEN, W., ANDRIEU-SOLER, C., GALJART, N., DZIERZAK, E. & ROBIN, C. 2010. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature,* 464**,** 116-20.

BONIFER, C. & BOWEN, D. T. 2010. Epigenetic mechanisms regulating normal and malignant haematopoiesis: new therapeutic targets for clinical medicine. *Expert Rev Mol Med,* 12**,** e6.

BONIFER, C. & COCKERILL, P. N. 2011. Chromatin mechanisms regulating gene expression in health and disease. *Adv Exp Med Biol,* 711**,** 12-25.

BOYER, L. A., PLATH, K., ZEITLINGER, J., BRAMBRINK, T., MEDEIROS, L. A., LEE, T. I., LEVINE, S. S., WERNIG, M., TAJONAR, A., RAY, M. K., BELL, G. W., OTTE, A. P., VIDAL, M., GIFFORD, D. K., YOUNG, R. A. & JAENISCH, R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature,* 441**,** 349-53.

BRACKEN, A. P., DIETRICH, N., PASINI, D., HANSEN, K. H. & HELIN, K. 2006. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev,* 20**,** 1123-36.

BRANTJES, H., ROOSE, J., VAN DE WETERING, M. & CLEVERS, H. 2001. All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res,* 29**,** 1410-9.

BRAY, S. J. 2006. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol,* 7**,** 678-89.

BREILING, A., TURNER, B. M., BIANCHI, M. E. & ORLANDO, V. 2001. General transcription factors bind promoters repressed by Polycomb group proteins. *Nature,* 412**,** 651-5.

BROMBERG, J. F., WRZESZCZYNSKA, M. H., DEVGAN, G., ZHAO, Y., PESTELL, R. G., ALBANESE, C. & DARNELL, J. E., JR. 1999. Stat3 as an oncogene. *Cell,* 98**,** 295-303.

BROWN, C. J., HENDRICH, B. D., RUPERT, J. L., LAFRENIERE, R. G., XING, Y., LAWRENCE, J. & WILLARD, H. F. 1992. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell,* 71**,** 527-42.

BUCHHOLZ, F., REFAELI, Y., TRUMPP, A. & BISHOP, J. M. 2000. Inducible chromosomal translocation of AML1 and ETO genes through Cre/loxP-mediated recombination in the mouse. *EMBO Rep,* 1**,** 133-9.

BULGER, M. & GROUDINE, M. 1999. Looping versus linking: toward a model for long-distance gene activation. *Genes Dev,* 13**,** 2465-77.

BULLINGER, L., DOHNER, K., BAIR, E., FROHLING, S., SCHLENK, R. F., TIBSHIRANI, R., DOHNER, H. & POLLACK, J. R. 2004. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med,* 350**,** 1605-16.

BURGESS-BEUSSE, B., FARRELL, C., GASZNER, M., LITT, M., MUTSKOV, V., RECILLAS-TARGA, F., SIMPSON, M., WEST, A. & FELSENFELD, G. 2002. The insulation of genes from external enhancers and silencing chromatin. *Proc Natl Acad Sci U S A,* 99 Suppl 4**,** 16433-7.

BUSSLINGER, M. 2004. Transcriptional control of early B cell development. *Annu Rev Immunol,* 22**,** 55-79.

BUSSLINGER, M., KLIX, N., PFEFFER, P., GRANINGER, P. G. & KOZMIK, Z. 1996. Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. *Proc Natl Acad Sci U S A,* 93**,** 6129-34.

CAIRNS, B. R. 2009. The logic of chromatin architecture and remodelling at promoters. *Nature,* 461**,** 193-8.

CAIROLI, R., BEGHINI, A., GRILLO, G., NADALI, G., ELICE, F., RIPAMONTI, C. B., COLAPIETRO, P., NICHELATTI, M., PEZZETTI, L., LUNGHI, M., CUNEO, A., VIOLA, A., FERRARA, F., LAZZARINO, M., RODEGHIERO,

F., PIZZOLO, G., LARIZZA, L. & MORRA, E. 2006. Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study. *Blood,* 107**,** 3463-8.

CAIROLI, R., GRILLO, G., BEGHINI, A., TEDESCHI, A., RIPAMONTI, C. B., LARIZZA, L. & MORRA, E. 2003. C-Kit point mutations in core binding factor leukemias: correlation with white blood cell count and the white blood cell index. *Leukemia,* 17**,** 471-2.

CALES, C., ROMAN-TRUFERO, M., PAVON, L., SERRANO, I., MELGAR, T., ENDOH, M., PEREZ, C., KOSEKI, H. & VIDAL, M. 2008. Inactivation of the polycomb group protein Ring1B unveils an antiproliferative role in hematopoietic cell expansion and cooperation with tumorigenesis associated with Ink4a deletion. *Mol Cell Biol,* 28**,** 1018-28.

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., MILNER, L. A., KRONENBERG, H. M. & SCADDEN, D. T. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature,* 425**,** 841-6.

CAO, R., TSUKADA, Y. & ZHANG, Y. 2005. Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol Cell,* 20**,** 845-54.

CAPRON, C., LACOUT, C., LECLUSE, Y., JALBERT, V., CHAGRAOUI, H., CHARRIER, S., GALY, A., BENNACEUR-GRISCELLI, A., CRAMER-BORDE, E. & VAINCHENKER, W. 2010. A major role of TGF-beta1 in the homing capacities of murine hematopoietic stem cell/progenitors. *Blood,* 116**,** 1244-53.

CARE, R. S., VALK, P. J., GOODEVE, A. C., ABU-DUHIER, F. M., GEERTSMA-KLEINEKOORT, W. M., WILSON, G. A., GARI, M. A., PEAKE, I. R., LOWENBERG, B. & REILLY, J. T. 2003. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol,* 121**,** 775-7.

CARETTI, G., PALACIOS, D., SARTORELLI, V. & PURI, P. L. 2011. Phosphoryl-EZH-ion. *Cell Stem Cell,* 8**,** 262-5.

CARGNELLO, M. & ROUX, P. P. 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev,* 75**,** 50-83.

CAROW, C. E., HANGOC, G., COOPER, S. H., WILLIAMS, D. E. & BROXMEYER, H. E. 1991. Mast cell growth factor (c-kit ligand) supports the growth of human multipotential progenitor cells with a high replating potential. *Blood,* 78**,** 2216-21.

CHA, T. L., ZHOU, B. P., XIA, W., WU, Y., YANG, C. C., CHEN, C. T., PING, B., OTTE, A. P. & HUNG, M. C. 2005. Akt-mediated phosphorylation of

EZH2 suppresses methylation of lysine 27 in histone H3. *Science,* 310**,** 306-10.

CHALLEN, G. A., BOLES, N. C., CHAMBERS, S. M. & GOODELL, M. A. 2010. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell Stem Cell,* 6**,** 265-78.

CHEN, G., FERNANDEZ, J., MISCHE, S. & COUREY, A. J. 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev,* 13**,** 2218-30.

CHEN, M. J., YOKOMIZO, T., ZEIGLER, B. M., DZIERZAK, E. & SPECK, N. A. 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature,* 457**,** 887-91.

CHEN, S., BOHRER, L. R., RAI, A. N., PAN, Y., GAN, L., ZHOU, X., BAGCHI, A., SIMON, J. A. & HUANG, H. 2010. Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2. *Nat Cell Biol,* 12**,** 1108-14.

CHEN, X., LU, C., PRADO, J. R., EUN, S. H. & FULLER, M. T. 2011. Sequential changes at differentiation gene promoters as they become active in a stem cell lineage. *Development,* 138**,** 2441-50.

CHEN, Z., GIBSON, T. B., ROBINSON, F., SILVESTRO, L., PEARSON, G., XU, B., WRIGHT, A., VANDERBILT, C. & COBB, M. H. 2001. MAP kinases. *Chem Rev,* 101**,** 2449-76.

CHEN, Z., ZANG, J., WHETSTINE, J., HONG, X., DAVRAZOU, F., KUTATELADZE, T. G., SIMPSON, M., MAO, Q., PAN, C. H., DAI, S., HAGMAN, J., HANSEN, K., SHI, Y. & ZHANG, G. 2006. Structural insights into histone demethylation by JMJD2 family members. *Cell,* 125**,** 691-702.

CHEVALIER, N., SOLARI, M. L., BECKER, H., PANTIC, M., GARTNER, F., MAUL-PAVICIC, A., HUBNER, J., WASCH, R., SCHMITT-GRAFF, A. & LUBBERT, M. 2010. Robust in vivo differentiation of t(8;21)-positive acute myeloid leukemia blasts to neutrophilic granulocytes induced by treatment with dasatinib. *Leukemia,* 24**,** 1779-81.

CHIAN, R., YOUNG, S., DANILKOVITCH-MIAGKOVA, A., RONNSTRAND, L., LEONARD, E., FERRAO, P., ASHMAN, L. & LINNEKIN, D. 2001. Phosphatidylinositol 3 kinase contributes to the transformation of hematopoietic cells by the D816V c-Kit mutant. *Blood,* 98**,** 1365-73.

CHOI, C. Y., KIM, Y. H., KWON, H. J. & KIM, Y. 1999. The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J Biol Chem,* 274**,** 33194-7.

CHOI, K., KENNEDY, M., KAZAROV, A., PAPADIMITRIOU, J. C. & KELLER, G. 1998. A common precursor for hematopoietic and endothelial cells. *Development,* 125**,** 725-32.

CHOW, C. M., GEORGIOU, A., SZUTORISZ, H., MAIA E SILVA, A., POMBO, A., BARAHONA, I., DARGELOS, E., CANZONETTA, C. & DILLON, N. 2005. Variant histone H3.3 marks promoters of transcriptionally active genes during mammalian cell division. *EMBO Rep,* 6**,** 354-60.

CHRISTENSEN, J. L. & WEISSMAN, I. L. 2001. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci U S A,* 98**,** 14541-6.

CHUNG, Y. J., PARK, B. B., KANG, Y. J., KIM, T. M., EAVES, C. J. & OH, I. H. 2006. Unique effects of Stat3 on the early phase of hematopoietic stem cell regeneration. *Blood,* 108**,** 1208-15.

CIVIN, C. I., STRAUSS, L. C., BROVALL, C., FACKLER, M. J., SCHWARTZ, J. F. & SHAPER, J. H. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol,* 133**,** 157-65.

CLEMENTS, A., POUX, A. N., LO, W. S., PILLUS, L., BERGER, S. L. & MARMORSTEIN, R. 2003. Structural basis for histone and phosphohistone binding by the GCN5 histone acetyltransferase. *Mol Cell,* 12**,** 461-73.

CLOOS, P. A., CHRISTENSEN, J., AGGER, K., MAIOLICA, A., RAPPSILBER, J., ANTAL, T., HANSEN, K. H. & HELIN, K. 2006. The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature,* 442**,** 307-11.

COBALEDA, C., SCHEBESTA, A., DELOGU, A. & BUSSLINGER, M. 2007. Pax5: the guardian of B cell identity and function. *Nat Immunol,* 8**,** 463-70.

COBAS, M., WILSON, A., ERNST, B., MANCINI, S. J., MACDONALD, H. R., KEMLER, R. & RADTKE, F. 2004. Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. *J Exp Med,* 199**,** 221-9.

COCKERILL, P. N. 2000. Identification of DNaseI hypersensitive sites within nuclei. *Methods Mol Biol,* 130**,** 29-46.

COCKERILL, P. N. 2011. Structure and function of active chromatin and DNase I hypersensitive sites. *FEBS J,* 278**,** 2182-210.

COLUCCI, F., SAMSON, S. I., DEKOTER, R. P., LANTZ, O., SINGH, H. & DI SANTO, J. P. 2001. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood,* 97**,** 2625-32.

CONNEALLY, E., CASHMAN, J., PETZER, A. & EAVES, C. 1997. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc Natl Acad Sci U S A,* 94**,** 9836-41.

COPLEY, M. R., BEER, P. A. & EAVES, C. J. 2012. Hematopoietic stem cell heterogeneity takes center stage. *Cell Stem Cell,* 10**,** 690-7.

CORDEN, J. L. 1990. Tails of RNA polymerase II. *Trends Biochem Sci,* 15**,** 383-7.

CORONA, D. F., CLAPIER, C. R., BECKER, P. B. & TAMKUN, J. W. 2002. Modulation of ISWI function by site-specific histone acetylation. *EMBO Rep,* 3**,** 242-7.

COUREY, A. J. & JIA, S. 2001. Transcriptional repression: the long and the short of it. *Genes Dev,* 15**,** 2786-96.

COYAUD, E., STRUSKI, S., PRADE, N., FAMILIADES, J., EICHNER, R., QUELEN, C., BOUSQUET, M., MUGNERET, F., TALMANT, P., PAGES, M. P., LEFEBVRE, C., PENTHER, D., LIPPERT, E., NADAL, N., TAVIAUX, S., POPPE, B., LUQUET, I., BARANGER, L., ECLACHE, V., RADFORD, I., BARIN, C., MOZZICONACCI, M. J., LAFAGE-POCHITALOFF, M., ANTOINE-POIREL, H., CHARRIN, C., PEROT, C., TERRE, C., BROUSSET, P., DASTUGUE, N. & BROCCARDO, C. 2010. Wide diversity of PAX5 alterations in B-ALL: a Groupe Francophone de Cytogenetique Hematologique study. *Blood,* 115**,** 3089-97.

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., BOYER, L. A., YOUNG, R. A. & JAENISCH, R. 2010. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A,* 107**,** 21931-6.

CUI, K., ZANG, C., ROH, T. Y., SCHONES, D. E., CHILDS, R. W., PENG, W. & ZHAO, K. 2009. Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell Stem Cell,* 4**,** 80-93.

CUTHBERT, G. L., DAUJAT, S., SNOWDEN, A. W., ERDJUMENT-BROMAGE, H., HAGIWARA, T., YAMADA, M., SCHNEIDER, R., GREGORY, P. D., TEMPST, P., BANNISTER, A. J. & KOUZARIDES, T. 2004. Histone deimination antagonizes arginine methylation. *Cell,* 118**,** 545-53.

CZERNY, T., SCHAFFNER, G. & BUSSLINGER, M. 1993. DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev,* 7**,** 2048-61.

DAHL, R., WALSH, J. C., LANCKI, D., LASLO, P., IYER, S. R., SINGH, H. & SIMON, M. C. 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol,* 4**,** 1029-36.

DAHLE, O., KUMAR, A. & KUEHN, M. R. 2010. Nodal signaling recruits the histone demethylase Jmjd3 to counteract polycomb-mediated repression at target genes. *Sci Signal,* 3**,** ra48.

DAI, X. M., RYAN, G. R., HAPEL, A. J., DOMINGUEZ, M. G., RUSSELL, R. G., KAPP, S., SYLVESTRE, V. & STANLEY, E. R. 2002. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood,* 99**,** 111-20.

DAKIC, A., METCALF, D., DI RAGO, L., MIFSUD, S., WU, L. & NUTT, S. L. 2005. PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. *J Exp Med,* 201**,** 1487-502.

DALTON, W. T., JR., AHEARN, M. J., MCCREDIE, K. B., FREIREICH, E. J., STASS, S. A. & TRUJILLO, J. M. 1988. HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood,* 71**,** 242-7.

DE BRUIJN, M. F., SPECK, N. A., PEETERS, M. C. & DZIERZAK, E. 2000. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J,* 19**,** 2465-74.

DECKER, T., PASCA DI MAGLIANO, M., MCMANUS, S., SUN, Q., BONIFER, C., TAGOH, H. & BUSSLINGER, M. 2009. Stepwise activation of enhancer and promoter regions of the B cell commitment gene Pax5 in early lymphopoiesis. *Immunity,* 30**,** 508-20.

DEFTOS, M. L. & BEVAN, M. J. 2000. Notch signaling in T cell development. *Curr Opin Immunol,* 12**,** 166-72.

DEKOTER, R. P., LEE, H. J. & SINGH, H. 2002. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity,* 16**,** 297-309.

DEKOTER, R. P. & SINGH, H. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science,* 288**,** 1439-41.

DEKOTER, R. P., WALSH, J. C. & SINGH, H. 1998. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *EMBO J,* 17**,** 4456-68.

DELOGU, A., SCHEBESTA, A., SUN, Q., ASCHENBRENNER, K., PERLOT, T. & BUSSLINGER, M. 2006. Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity,* 24**,** 269-81.

DHALLUIN, C., CARLSON, J. E., ZENG, L., HE, C., AGGARWAL, A. K. & ZHOU, M. M. 1999. Structure and ligand of a histone acetyltransferase bromodomain. *Nature,* 399**,** 491-6.

DIAS, S., SILVA, H., JR., CUMANO, A. & VIEIRA, P. 2005. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. *J Exp Med,* 201**,** 971-9.

DOULATOV, S., NOTTA, F., LAURENTI, E. & DICK, J. E. 2012. Hematopoiesis: a human perspective. *Cell Stem Cell,* 10**,** 120-36.

DRUKER, B., OKUDA, K., MATULONIS, U., SALGIA, R., ROBERTS, T. & GRIFFIN, J. D. 1992. Tyrosine phosphorylation of rasGAP and associated proteins in chronic myelogenous leukemia cell lines. *Blood,* 79**,** 2215-20.

DUNNE, J., MANNARI, D., FARZANEH, T., GESSNER, A., VAN DELFT, F. W., HEIDENREICH, O., YOUNG, B. D. & GASCOYNE, D. M. 2012. AML1/ETO and POU4F1 synergy drives B-lymphoid gene expression typical of t(8;21) acute myeloid leukemia. *Leukemia,* 26**,** 1131-5.

DVIR, A., CONAWAY, R. C. & CONAWAY, J. W. 1997. A role for TFIIH in controlling the activity of early RNA polymerase II elongation complexes. *Proc Natl Acad Sci U S A,* 94**,** 9006-10.

DYKSTRA, B., KENT, D., BOWIE, M., MCCAFFREY, L., HAMILTON, M., LYONS, K., LEE, S. J., BRINKMAN, R. & EAVES, C. 2007. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell,* 1**,** 218-29.

DZIERZAK, E. & SPECK, N. A. 2008. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol,* 9**,** 129-36.

EBERHARD, D. & BUSSLINGER, M. 1999. The partial homeodomain of the transcription factor Pax-5 (BSAP) is an interaction motif for the retinoblastoma and TATA-binding proteins. *Cancer Res,* 59**,** 1716s-1724s; discussion 1724s-1725s.

ECKERT, R. L., ADHIKARY, G., RORKE, E. A., CHEW, Y. C. & BALASUBRAMANIAN, S. 2011. Polycomb group proteins are key regulators of keratinocyte function. *J Invest Dermatol,* 131**,** 295-301.

EILKEN, H. M., NISHIKAWA, S. & SCHROEDER, T. 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature,* 457**,** 896-900.

ELIASSON, P. & JONSSON, J. I. 2010. The hematopoietic stem cell niche: low in oxygen but a nice place to be. *J Cell Physiol,* 222**,** 17-22.

ENDOH, M., ENDO, T. A., ENDOH, T., ISONO, K., SHARIF, J., OHARA, O., TOYODA, T., ITO, T., ESKELAND, R., BICKMORE, W. A., VIDAL, M.,

BERNSTEIN, B. E. & KOSEKI, H. 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.

ENVER, T. & GREAVES, M. 1998. Loops, lineage, and leukemia. *Cell,* 94**,** 9-12.

ERICKSON, P., GAO, J., CHANG, K. S., LOOK, T., WHISENANT, E., RAIMONDI, S., LASHER, R., TRUJILLO, J., ROWLEY, J. & DRABKIN, H. 1992. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. *Blood,* 80**,** 1825-31.

ERILL, I. & O'NEILL, M. C. 2009. A reexamination of information theory-based methods for DNA-binding site identification. *BMC Bioinformatics,* 10**,** 57.

ESKELAND, R., LEEB, M., GRIMES, G. R., KRESS, C., BOYLE, S., SPROUL, D., GILBERT, N., FAN, Y., SKOULTCHI, A. I., WUTZ, A. & BICKMORE, W. A. 2010. Ring1B compacts chromatin structure and represses gene expression independent of histone ubiquitination. *Mol Cell,* 38**,** 452-64.

FAIRBAIRN, L. J., COWLING, G. J., REIPERT, B. M. & DEXTER, T. M. 1993. Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. *Cell,* 74**,** 823-32.

FANG, X., SUN, J., XIANG, P., YU, M., NAVAS, P. A., PETERSON, K. R., STAMATOYANNOPOULOS, G. & LI, Q. 2005. Synergistic and additive properties of the beta-globin locus control region (LCR) revealed by 5'HS3 deletion mutations: implication for LCR chromatin architecture. *Mol Cell Biol,* 25**,** 7033-41.

FARRELL, C. M., WEST, A. G. & FELSENFELD, G. 2002. Conserved CTCF insulator elements flank the mouse and human beta-globin loci. *Mol Cell Biol,* 22**,** 3820-31.

FEHLING, H. J., LACAUD, G., KUBO, A., KENNEDY, M., ROBERTSON, S., KELLER, G. & KOUSKOFF, V. 2003. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development,* 130**,** 4217-27.

FENSKE, T. S., PENGUE, G., MATHEWS, V., HANSON, P. T., HAMM, S. E., RIAZ, N. & GRAUBERT, T. A. 2004. Stem cell expression of the AML1/ETO fusion protein induces a myeloproliferative disorder in mice. *Proc Natl Acad Sci U S A,* 101**,** 15184-9.

FILION, G. J., VAN BEMMEL, J. G., BRAUNSCHWEIG, U., TALHOUT, W., KIND, J., WARD, L. D., BRUGMAN, W., DE CASTRO, I. J., KERKHOVEN, R. M., BUSSEMAKER, H. J. & VAN STEENSEL, B. 2010.

Systematic protein location mapping reveals five principal chromatin types in Drosophila cells. *Cell,* 143**,** 212-24.

FINCH, C. A., HARKER, L. A. & COOK, J. D. 1977. Kinetics of the formed elements of human blood. *Blood,* 50**,** 699-707.

FISCHLE, W., TSENG, B. S., DORMANN, H. L., UEBERHEIDE, B. M., GARCIA, B. A., SHABANOWITZ, J., HUNT, D. F., FUNABIKI, H. & ALLIS, C. D. 2005. Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature,* 438**,** 1116-22.

FITZSIMMONS, D., HODSDON, W., WHEAT, W., MAIRA, S. M., WASYLYK, B. & HAGMAN, J. 1996. Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev,* 10**,** 2198-211.

FLEMING, H. E., JANZEN, V., LO CELSO, C., GUO, J., LEAHY, K. M., KRONENBERG, H. M. & SCADDEN, D. T. 2008. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell,* 2**,** 274-83.

FODOR, B. D., KUBICEK, S., YONEZAWA, M., O'SULLIVAN, R. J., SENGUPTA, R., PEREZ-BURGOS, L., OPRAVIL, S., MECHTLER, K., SCHOTTA, G. & JENUWEIN, T. 2006. Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. *Genes Dev,* 20**,** 1557-62.

FORSBERG, E. C., PROHASKA, S. S., KATZMAN, S., HEFFNER, G. C., STUART, J. M. & WEISSMAN, I. L. 2005. Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genet,* 1**,** e28.

FORTUNEL, N., BATARD, P., HATZFELD, A., MONIER, M. N., PANTERNE, B., LEBKOWSKI, J. & HATZFELD, J. 1998. High proliferative potential-quiescent cells: a working model to study primitive quiescent hematopoietic cells. *J Cell Sci,* 111 (Pt 13)**,** 1867-75.

FRIEDMAN, A. D. 2002. Transcriptional regulation of granulocyte and monocyte development. *Oncogene,* 21**,** 3377-90.

FUDA, N. J., ARDEHALI, M. B. & LIS, J. T. 2009. Defining mechanisms that regulate RNA polymerase II transcription in vivo. *Nature,* 461**,** 186-92.

GALLOWAY, J. L., WINGERT, R. A., THISSE, C., THISSE, B. & ZON, L. I. 2005. Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. *Dev Cell,* 8**,** 109-16.

GARCIA, R., YU, C. L., HUDNALL, A., CATLETT, R., NELSON, K. L., SMITHGALL, T., FUJITA, D. J., ETHIER, S. P. & JOVE, R. 1997. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ,* 8**,** 1267-76.

GARRARD, W. T. 1991. Histone H1 and the conformation of transcriptionally active chromatin. *Bioessays,* 13**,** 87-8.

GEHANI, S. S., AGRAWAL-SINGH, S., DIETRICH, N., CHRISTOPHERSEN, N. S., HELIN, K. & HANSEN, K. 2010. Polycomb group protein displacement and gene activation through MSK-dependent H3K27me3S28 phosphorylation. *Mol Cell,* 39**,** 886-900.

GELMETTI, V., ZHANG, J., FANELLI, M., MINUCCI, S., PELICCI, P. G. & LAZAR, M. A. 1998. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. *Mol Cell Biol,* 18**,** 7185-91.

GEORGES, A. B., BENAYOUN, B. A., CABURET, S. & VEITIA, R. A. 2010. Generic binding sites, generic DNA-binding domains: where does specific promoter recognition come from? *FASEB J,* 24**,** 346-56.

GEORGOPOULOS, K., BIGBY, M., WANG, J. H., MOLNAR, A., WU, P., WINANDY, S. & SHARPE, A. 1994. The Ikaros gene is required for the development of all lymphoid lineages. *Cell,* 79**,** 143-56.

GEORGOPOULOS, K., MOORE, D. D. & DERFLER, B. 1992. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science,* 258**,** 808-12.

GIBSON, S. E., DONG, H. Y., ADVANI, A. S. & HSI, E. D. 2006. Expression of the B cell-associated transcription factors PAX5, OCT-2, and BOB.1 in acute myeloid leukemia: associations with B-cell antigen expression and myelomonocytic maturation. *Am J Clin Pathol,* 126**,** 916-24.

GOLDBERG, A. D., BANASZYNSKI, L. A., NOH, K. M., LEWIS, P. W., ELSAESSER, S. J., STADLER, S., DEWELL, S., LAW, M., GUO, X., LI, X., WEN, D., CHAPGIER, A., DEKELVER, R. C., MILLER, J. C., LEE, Y. L., BOYDSTON, E. A., HOLMES, M. C., GREGORY, P. D., GREALLY, J. M., RAFII, S., YANG, C., SCAMBLER, P. J., GARRICK, D., GIBBONS, R. J., HIGGS, D. R., CRISTEA, I. M., URNOV, F. D., ZHENG, D. & ALLIS, C. D. 2010. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell,* 140**,** 678-91.

GOLDSMITH, M. A., MIKAMI, A., YOU, Y., LIU, K. D., THOMAS, L., PHARR, P. & LONGMORE, G. D. 1998. Absence of cytokine receptor-dependent specificity in red blood cell differentiation in vivo. *Proc Natl Acad Sci U S A,* 95**,** 7006-11.

GONDOR, A. & OHLSSON, R. 2009. Chromosome crosstalk in three dimensions. *Nature,* 461**,** 212-7.

GRAF, T., MCNAGNY, K., BRADY, G. & FRAMPTON, J. 1992. Chicken "erythroid" cells transformed by the Gag-Myb-Ets-encoding E26 leukemia virus are multipotent. *Cell,* 70**,** 201-13.

GRANDIS, J. R., DRENNING, S. D., CHAKRABORTY, A., ZHOU, M. Y., ZENG, Q., PITT, A. S. & TWEARDY, D. J. 1998. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro. *J Clin Invest,* 102**,** 1385-92.

GRANGE, T., BERTRAND, E., ESPINAS, M. L., FROMONT-RACINE, M., RIGAUD, G., ROUX, J. & PICTET, R. 1997. In vivo footprinting of the interaction of proteins with DNA and RNA. *Methods,* 11**,** 151-63.

GRIFFITH, F. 1928. The significance of Pneumococcal types.

GRIGORYAN, T., WEND, P., KLAUS, A. & BIRCHMEIER, W. 2008. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev,* 22**,** 2308-41.

GROSVELD, F., VAN ASSENDELFT, G. B., GREAVES, D. R. & KOLLIAS, G. 1987. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell,* 51**,** 975-85.

GRUNSTEIN, M. 1998. Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell,* 93**,** 325-8.

GUILLEMETTE, B., BATAILLE, A. R., GEVRY, N., ADAM, M., BLANCHETTE, M., ROBERT, F. & GAUDREAU, L. 2005. Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. *PLoS Biol,* 3**,** e384.

HADLAND, B. K., HUPPERT, S. S., KANUNGO, J., XUE, Y., JIANG, R., GRIDLEY, T., CONLON, R. A., CHENG, A. M., KOPAN, R. & LONGMORE, G. D. 2004. A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood,* 104**,** 3097-105.

HAN, H., TANIGAKI, K., YAMAMOTO, N., KURODA, K., YOSHIMOTO, M., NAKAHATA, T., IKUTA, K. & HONJO, T. 2002. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol,* 14**,** 637-45.

HASSA, P. O., HAENNI, S. S., ELSER, M. & HOTTIGER, M. O. 2006. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol Mol Biol Rev,* 70**,** 789-829.

HATZFELD, J., LI, M. L., BROWN, E. L., SOOKDEO, H., LEVESQUE, J. P., O'TOOLE, T., GURNEY, C., CLARK, S. C. & HATZFELD, A. 1991. Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor beta 1 or Rb oligonucleotides. *J Exp Med,* 174**,** 925-9.

HAYAKAWA, F., TOWATARI, M., KIYOI, H., TANIMOTO, M., KITAMURA, T., SAITO, H. & NAOE, T. 2000. Tandem-duplicated Flt3 constitutively

activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. *Oncogene,* 19**,** 624-31.

HE, W., DORN, D. C., ERDJUMENT-BROMAGE, H., TEMPST, P., MOORE, M. A. & MASSAGUE, J. 2006. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. *Cell,* 125**,** 929-41.

HE, Z., MA, W. Y., LIU, G., ZHANG, Y., BODE, A. M. & DONG, Z. 2003. Arsenite-induced phosphorylation of histone H3 at serine 10 is mediated by Akt1, extracellular signal-regulated kinase 2, and p90 ribosomal S6 kinase 2 but not mitogen- and stress-activated protein kinase 1. *J Biol Chem,* 278**,** 10588-93.

HEIDENREICH, O. 2009. Targeting oncogenes with siRNAs. *Methods Mol Biol,* 487**,** 221-42.

HEINTZMAN, N. D., HON, G. C., HAWKINS, R. D., KHERADPOUR, P., STARK, A., HARP, L. F., YE, Z., LEE, L. K., STUART, R. K., CHING, C. W., CHING, K. A., ANTOSIEWICZ-BOURGET, J. E., LIU, H., ZHANG, X., GREEN, R. D., LOBANENKOV, V. V., STEWART, R., THOMSON, J. A., CRAWFORD, G. E., KELLIS, M. & REN, B. 2009. Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature,* 459**,** 108-12.

HEINTZMAN, N. D. & REN, B. 2009. Finding distal regulatory elements in the human genome. *Curr Opin Genet Dev,* 19**,** 541-9.

HEINTZMAN, N. D., STUART, R. K., HON, G., FU, Y., CHING, C. W., HAWKINS, R. D., BARRERA, L. O., VAN CALCAR, S., QU, C., CHING, K. A., WANG, W., WENG, Z., GREEN, R. D., CRAWFORD, G. E. & REN, B. 2007. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet,* 39**,** 311-8.

HELTEMES-HARRIS, L. M., WILLETTE, M. J., RAMSEY, L. B., QIU, Y. H., NEELEY, E. S., ZHANG, N., THOMAS, D. A., KOEUTH, T., BAECHLER, E. C., KORNBLAU, S. M. & FARRAR, M. A. 2011. Ebf1 or Pax5 haploinsufficiency synergizes with STAT5 activation to initiate acute lymphoblastic leukemia. *J Exp Med,* 208**,** 1135-49.

HENIKOFF, S. 2008. Nucleosome destabilization in the epigenetic regulation of gene expression. *Nat Rev Genet,* 9**,** 15-26.

HERSHEY, A. D. A. C., M. 1952. Independent functions of viral protein and nucleic acid in growth of bacteriophages.

HERZ, H. M., NAKANISHI, S. & SHILATIFARD, A. 2009. The curious case of bivalent marks. *Dev Cell,* 17**,** 301-3.

HEYWORTH, C., PEARSON, S., MAY, G. & ENVER, T. 2002. Transcription factor-mediated lineage switching reveals plasticity in primary committed progenitor cells. *EMBO J,* 21**,** 3770-81.

HIGUCHI, M., O'BRIEN, D., KUMARAVELU, P., LENNY, N., YEOH, E. J. & DOWNING, J. R. 2002. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell,* 1**,** 63-74.

HOCK, H., HAMBLEN, M. J., ROOKE, H. M., SCHINDLER, J. W., SALEQUE, S., FUJIWARA, Y. & ORKIN, S. H. 2004. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature,* 431**,** 1002-7.

HOFLINGER, S., KESAVAN, K., FUXA, M., HUTTER, C., HEAVEY, B., RADTKE, F. & BUSSLINGER, M. 2004. Analysis of Notch1 function by in vitro T cell differentiation of Pax5 mutant lymphoid progenitors. *J Immunol,* 173**,** 3935-44.

HOLMES, M. L., PRIDANS, C. & NUTT, S. L. 2008. The regulation of the B-cell gene expression programme by Pax5. *Immunol Cell Biol,* 86**,** 47-53.

HON, G., WANG, W. & REN, B. 2009a. Discovery and annotation of functional chromatin signatures in the human genome. *PLoS Comput Biol,* 5**,** e1000566.

HON, G. C., HAWKINS, R. D. & REN, B. 2009b. Predictive chromatin signatures in the mammalian genome. *Hum Mol Genet,* 18**,** R195-201.

HORCHER, M., SOUABNI, A. & BUSSLINGER, M. 2001. Pax5/BSAP maintains the identity of B cells in late B lymphopoiesis. *Immunity,* 14**,** 779-90.

IGARASHI, H., GREGORY, S. C., YOKOTA, T., SAKAGUCHI, N. & KINCADE, P. W. 2002. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity,* 17**,** 117-30.

IIDA, S., RAO, P. H., NALLASIVAM, P., HIBSHOOSH, H., BUTLER, M., LOUIE, D. C., DYOMIN, V., OHNO, H., CHAGANTI, R. S. & DALLA-FAVERA, R. 1996. The t(9;14)(p13;q32) chromosomal translocation associated with lymphoplasmacytoid lymphoma involves the PAX-5 gene. *Blood,* 88**,** 4110-7.

IKAWA, T., FUJIMOTO, S., KAWAMOTO, H., KATSURA, Y. & YOKOTA, Y. 2001. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. *Proc Natl Acad Sci U S A,* 98**,** 5164-9.

IWAMA, A., OGURO, H., NEGISHI, M., KATO, Y., MORITA, Y., TSUKUI, H., EMA, H., KAMIJO, T., KATOH-FUKUI, Y., KOSEKI, H., VAN LOHUIZEN, M. & NAKAUCHI, H. 2004. Enhanced self-renewal of hematopoietic stem

cells mediated by the polycomb gene product Bmi-1. *Immunity,* 21**,** 843-51.

IWASAKI-ARAI, J., IWASAKI, H., MIYAMOTO, T., WATANABE, S. & AKASHI, K. 2003. Enforced granulocyte/macrophage colony-stimulating factor signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. *J Exp Med,* 197**,** 1311-22.

IWASAKI, H., MIZUNO, S., ARINOBU, Y., OZAWA, H., MORI, Y., SHIGEMATSU, H., TAKATSU, K., TENEN, D. G. & AKASHI, K. 2006. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev,* 20**,** 3010-21.

IWASAKI, H., MIZUNO, S., WELLS, R. A., CANTOR, A. B., WATANABE, S. & AKASHI, K. 2003. GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity,* 19**,** 451-62.

IWASAKI, H., SOMOZA, C., SHIGEMATSU, H., DUPREZ, E. A., IWASAKI-ARAI, J., MIZUNO, S., ARINOBU, Y., GEARY, K., ZHANG, P., DAYARAM, T., FENYUS, M. L., ELF, S., CHAN, S., KASTNER, P., HUETTNER, C. S., MURRAY, R., TENEN, D. G. & AKASHI, K. 2005. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood,* 106**,** 1590-600.

JACOBS, J. J., KIEBOOM, K., MARINO, S., DEPINHO, R. A. & VAN LOHUIZEN, M. 1999. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature,* 397**,** 164-8.

JAFFREDO, T., BOLLEROT, K., SUGIYAMA, D., GAUTIER, R. & DREVON, C. 2005a. Tracing the hemangioblast during embryogenesis: developmental relationships between endothelial and hematopoietic cells. *Int J Dev Biol,* 49**,** 269-77.

JAFFREDO, T., GAUTIER, R., BRAJEUL, V. & DIETERLEN-LIEVRE, F. 2000. Tracing the progeny of the aortic hemangioblast in the avian embryo. *Dev Biol,* 224**,** 204-14.

JAFFREDO, T., NOTTINGHAM, W., LIDDIARD, K., BOLLEROT, K., POUGET, C. & DE BRUIJN, M. 2005b. From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp Hematol,* 33**,** 1029-40.

JALAL HOSSEINIMEHR, S., INANAMI, O., HAMASU, T., TAKAHASHI, M., KASHIWAKURA, I., ASANUMA, T. & KUWABARA, M. 2004. Activation of c-kit by stem cell factor induces radioresistance to apoptosis through ERK-dependent expression of survivin in HL60 cells. *J Radiat Res,* 45**,** 557-61.

JEANNET, G., SCHELLER, M., SCARPELLINO, L., DUBOUX, S., GARDIOL, N., BACK, J., KUTTLER, F., MALANCHI, I., BIRCHMEIER, W., LEUTZ,

A., HUELSKEN, J. & HELD, W. 2008. Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. *Blood,* 111**,** 142-9.

JENUWEIN, T. & ALLIS, C. D. 2001. Translating the histone code. *Science,* 293**,** 1074-80.

JIN, C. & FELSENFELD, G. 2007. Nucleosome stability mediated by histone variants H3.3 and H2A.Z. *Genes Dev,* 21**,** 1519-29.

JIN, Q., YU, L. R., WANG, L., ZHANG, Z., KASPER, L. H., LEE, J. E., WANG, C., BRINDLE, P. K., DENT, S. Y. & GE, K. 2011. Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation. *EMBO J,* 30**,** 249-62.

JUNG, D., GIALLOURAKIS, C., MOSTOSLAVSKY, R. & ALT, F. W. 2006. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu Rev Immunol,* 24**,** 541-70.

KAGALWALA, M. N., GLAUS, B. J., DANG, W., ZOFALL, M. & BARTHOLOMEW, B. 2004. Topography of the ISW2-nucleosome complex: insights into nucleosome spacing and chromatin remodeling. *EMBO J,* 23**,** 2092-104.

KAMMINGA, L. M., BYSTRYKH, L. V., DE BOER, A., HOUWER, S., DOUMA, J., WEERSING, E., DONTJE, B. & DE HAAN, G. 2006. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood,* 107**,** 2170-9.

KANEKO, S., LI, G., SON, J., XU, C. F., MARGUERON, R., NEUBERT, T. A. & REINBERG, D. 2010. Phosphorylation of the PRC2 component Ezh2 is cell cycle-regulated and up-regulates its binding to ncRNA. *Genes Dev,* 24**,** 2615-20.

KANG, S. H., JEONG, S. J., KIM, S. H., KIM, J. H., JUNG, J. H., KOH, W., KIM, D. K. & CHEN, C. Y. 2012. Icariside II induces apoptosis in U937 acute myeloid leukemia cells: role of inactivation of STAT3-related signaling. *PLoS One,* 7**,** e28706.

KANG, Y., CHAO, N. J. & AVERSA, F. 2008. Unmanipulated or CD34 selected haplotype mismatched transplants. *Curr Opin Hematol,* 15**,** 561-7.

KANHERE, A., VIIRI, K., ARAUJO, C. C., RASAIYAAH, J., BOUWMAN, R. D., WHYTE, W. A., PEREIRA, C. F., BROOKES, E., WALKER, K., BELL, G. W., POMBO, A., FISHER, A. G., YOUNG, R. A. & JENNER, R. G. 2010. Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2. *Mol Cell,* 38**,** 675-88.

KARANU, F. N., MURDOCH, B., GALLACHER, L., WU, D. M., KOREMOTO, M., SAKANO, S. & BHATIA, M. 2000. The notch ligand jagged-1

represents a novel growth factor of human hematopoietic stem cells. *J Exp Med,* 192**,** 1365-72.

KAUSTOV, L., OUYANG, H., AMAYA, M., LEMAK, A., NADY, N., DUAN, S., WASNEY, G. A., LI, Z., VEDADI, M., SCHAPIRA, M., MIN, J. & ARROWSMITH, C. H. 2011. Recognition and specificity determinants of the human cbx chromodomains. *J Biol Chem,* 286**,** 521-9.

KIKUCHI, J., KINOSHITA, I., SHIMIZU, Y., KIKUCHI, E., KONISHI, J., OIZUMI, S., KAGA, K., MATSUNO, Y., NISHIMURA, M. & DOSAKA-AKITA, H. 2010. Distinctive expression of the polycomb group proteins Bmi1 polycomb ring finger oncogene and enhancer of zeste homolog 2 in nonsmall cell lung cancers and their clinical and clinicopathologic significance. *Cancer,* 116**,** 3015-24.

KIKUCHI, K., LAI, A. Y., HSU, C. L. & KONDO, M. 2005. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. *J Exp Med,* 201**,** 1197-203.

KIM, J., SIF, S., JONES, B., JACKSON, A., KOIPALLY, J., HELLER, E., WINANDY, S., VIEL, A., SAWYER, A., IKEDA, T., KINGSTON, R. & GEORGOPOULOS, K. 1999. Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. *Immunity,* 10**,** 345-55.

KIM, T. H., BARRERA, L. O., ZHENG, M., QU, C., SINGER, M. A., RICHMOND, T. A., WU, Y., GREEN, R. D. & REN, B. 2005. A high-resolution map of active promoters in the human genome. *Nature,* 436**,** 876-80.

KIRSTETTER, P., ANDERSON, K., PORSE, B. T., JACOBSEN, S. E. & NERLOV, C. 2006. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol,* 7**,** 1048-56.

KNOBLICH, J. A. 2008. Mechanisms of asymmetric stem cell division. *Cell,* 132**,** 583-97.

KNOEPFLER, P. S. & EISENMAN, R. N. 1999. Sin meets NuRD and other tails of repression. *Cell,* 99**,** 447-50.

KOCH, U., WILSON, A., COBAS, M., KEMLER, R., MACDONALD, H. R. & RADTKE, F. 2008. Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood,* 111**,** 160-4.

KONDO, M., SCHERER, D. C., MIYAMOTO, T., KING, A. G., AKASHI, K., SUGAMURA, K. & WEISSMAN, I. L. 2000. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature,* 407**,** 383-6.

KONDO, M., WEISSMAN, I. L. & AKASHI, K. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell,* 91**,** 661-72.

KONUMA, T., OGURO, H. & IWAMA, A. 2010. Role of the polycomb group proteins in hematopoietic stem cells. *Dev Growth Differ,* 52**,** 505-16.

KOPAN, R. & ILAGAN, M. X. 2009. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell,* 137**,** 216-33.

KORZUS, E., TORCHIA, J., ROSE, D. W., XU, L., KUROKAWA, R., MCINERNEY, E. M., MULLEN, T. M., GLASS, C. K. & ROSENFELD, M. G. 1998. Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science,* 279**,** 703-7.

KOUZARIDES, T. 2007. Chromatin modifications and their function. *Cell,* 128**,** 693-705.

KOWENZ-LEUTZ, E., PLESS, O., DITTMAR, G., KNOBLICH, M. & LEUTZ, A. 2010. Crosstalk between C/EBPbeta phosphorylation, arginine methylation, and SWI/SNF/Mediator implies an indexing transcription factor code. *EMBO J,* 29**,** 1105-15.

KOZMIK, Z., WANG, S., DORFLER, P., ADAMS, B. & BUSSLINGER, M. 1992. The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol Cell Biol,* 12**,** 2662-72.

KRYSINSKA, H., HOOGENKAMP, M., INGRAM, R., WILSON, N., TAGOH, H., LASLO, P., SINGH, H. & BONIFER, C. 2007. A two-step, PU.1-dependent mechanism for developmentally regulated chromatin remodeling and transcription of the c-fms gene. *Mol Cell Biol,* 27**,** 878-87.

KU, M., KOCHE, R. P., RHEINBAY, E., MENDENHALL, E. M., ENDOH, M., MIKKELSEN, T. S., PRESSER, A., NUSBAUM, C., XIE, X., CHI, A. S., ADLI, M., KASIF, S., PTASZEK, L. M., COWAN, C. A., LANDER, E. S., KOSEKI, H. & BERNSTEIN, B. E. 2008. Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS Genet,* 4**,** e1000242.

KULESSA, H., FRAMPTON, J. & GRAF, T. 1995. GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. *Genes Dev,* 9**,** 1250-62.

KUMANO, K., CHIBA, S., KUNISATO, A., SATA, M., SAITO, T., NAKAGAMI-YAMAGUCHI, E., YAMAGUCHI, T., MASUDA, S., SHIMIZU, K., TAKAHASHI, T., OGAWA, S., HAMADA, Y. & HIRAI, H. 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity,* 18**,** 699-711.

LACORAZZA, H. D., YAMADA, T., LIU, Y., MIYATA, Y., SIVINA, M., NUNES, J. & NIMER, S. D. 2006. The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. *Cancer Cell,* 9**,** 175-87.

LAI, A. Y. & KONDO, M. 2006. Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *J Exp Med,* 203**,** 1867-73.

LAIOSA, C. V., STADTFELD, M. & GRAF, T. 2006a. Determinants of lymphoid-myeloid lineage diversification. *Annu Rev Immunol,* 24**,** 705-38.

LAIOSA, C. V., STADTFELD, M., XIE, H., DE ANDRES-AGUAYO, L. & GRAF, T. 2006b. Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. *Immunity,* 25**,** 731-44.

LAM, K. & ZHANG, D. E. 2012. RUNX1 and RUNX1-ETO: roles in hematopoiesis and leukemogenesis. *Front Biosci,* 17**,** 1120-39.

LANCRIN, C., MAZAN, M., STEFANSKA, M., PATEL, R., LICHTINGER, M., COSTA, G., VARGEL, O., WILSON, N. K., MOROY, T., BONIFER, C., GOTTGENS, B., KOUSKOFF, V. & LACAUD, G. 2012. GFI1 and GFI1B control the loss of endothelial identity of hemogenic endothelium during hematopoietic commitment. *Blood,* 120**,** 314-22.

LANDEIRA, D., SAUER, S., POOT, R., DVORKINA, M., MAZZARELLA, L., JORGENSEN, H. F., PEREIRA, C. F., LELEU, M., PICCOLO, F. M., SPIVAKOV, M., BROOKES, E., POMBO, A., FISHER, C., SKARNES, W. C., SNOEK, T., BEZSTAROSTI, K., DEMMERS, J., KLOSE, R. J., CASANOVA, M., TAVARES, L., BROCKDORFF, N., MERKENSCHLAGER, M. & FISHER, A. G. 2010. Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. *Nat Cell Biol,* 12**,** 618-24.

LANSDORP, P. M., SUTHERLAND, H. J. & EAVES, C. J. 1990. Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. *J Exp Med,* 172**,** 363-6.

LARIZZA, L., MAGNANI, I. & BEGHINI, A. 2005. The Kasumi-1 cell line: a t(8;21)-kit mutant model for acute myeloid leukemia. *Leuk Lymphoma,* 46**,** 247-55.

LARSSON, J., BLANK, U., HELGADOTTIR, H., BJORNSSON, J. M., EHINGER, M., GOUMANS, M. J., FAN, X., LEVEEN, P. & KARLSSON, S. 2003. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. *Blood,* 102**,** 3129-35.

LARSSON, J., BLANK, U., KLINTMAN, J., MAGNUSSON, M. & KARLSSON, S. 2005. Quiescence of hematopoietic stem cells and maintenance of the stem cell pool is not dependent on TGF-beta signaling in vivo. *Exp Hematol,* 33**,** 592-6.

LASLO, P., PONGUBALA, J. M., LANCKI, D. W. & SINGH, H. 2008. Gene regulatory networks directing myeloid and lymphoid cell fates within the immune system. *Semin Immunol,* 20**,** 228-35.

LASLO, P., SPOONER, C. J., WARMFLASH, A., LANCKI, D. W., LEE, H. J., SCIAMMAS, R., GANTNER, B. N., DINNER, A. R. & SINGH, H. 2006. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell,* 126**,** 755-66.

LAU, P. N. & CHEUNG, P. 2011a. Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. *Proc Natl Acad Sci U S A,* 108**,** 2801-6.

LAU, P. N. & CHEUNG, P. 2011b. Unlocking polycomb silencing through histone H3 phosphorylation. *Cell Cycle,* 10**,** 1514-5.

LAWSON, G. M., TSAI, M. J. & O'MALLEY, B. W. 1980. Deoxyribonuclease I sensitivity of the nontranscribed sequences flanking the 5' and 3' ends of the ovomucoid gene and the ovalbumin and its related X and Y genes in hen oviduct nuclei. *Biochemistry,* 19**,** 4403-41.

LEBIEN, T. W. 2000. Fates of human B-cell precursors. *Blood,* 96**,** 9-23.

LEDDIN, M., PERROD, C., HOOGENKAMP, M., GHANI, S., ASSI, S., HEINZ, S., WILSON, N. K., FOLLOWS, G., SCHONHEIT, J., VOCKENTANZ, L., MOSAMMAM, A. M., CHEN, W., TENEN, D. G., WESTHEAD, D. R., GOTTGENS, B., BONIFER, C. & ROSENBAUER, F. 2011. Two distinct auto-regulatory loops operate at the PU.1 locus in B cells and myeloid cells. *Blood,* 117**,** 2827-38.

LEE, M. G., VILLA, R., TROJER, P., NORMAN, J., YAN, K. P., REINBERG, D., DI CROCE, L. & SHIEKHATTAR, R. 2007. Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. *Science,* 318**,** 447-50.

LEE, N., MAURANGE, C., RINGROSE, L. & PARO, R. 2005. Suppression of Polycomb group proteins by JNK signalling induces transdetermination in Drosophila imaginal discs. *Nature,* 438**,** 234-7.

LEE, T. I., JENNER, R. G., BOYER, L. A., GUENTHER, M. G., LEVINE, S. S., KUMAR, R. M., CHEVALIER, B., JOHNSTONE, S. E., COLE, M. F., ISONO, K., KOSEKI, H., FUCHIKAMI, T., ABE, K., MURRAY, H. L., ZUCKER, J. P., YUAN, B., BELL, G. W., HERBOLSHEIMER, E., HANNETT, N. M., SUN, K., ODOM, D. T., OTTE, A. P., VOLKERT, T. L., BARTEL, D. P., MELTON, D. A., GIFFORD, D. K., JAENISCH, R. & YOUNG, R. A. 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell,* 125**,** 301-13.

LEE, T. I. & YOUNG, R. A. 2000. Transcription of eukaryotic protein-coding genes. *Annu Rev Genet,* 34**,** 77-137.

LEHMANN, L., FERRARI, R., VASHISHT, A. A., WOHLSCHLEGEL, J. A., KURDISTANI, S. K. & CAREY, M. 2012. Polycomb Repressive Complex 1 (PRC1) Disassembles RNA Polymerase II Preinitiation Complexes. *J Biol Chem*.

LESSARD, J., SCHUMACHER, A., THORSTEINSDOTTIR, U., VAN LOHUIZEN, M., MAGNUSON, T. & SAUVAGEAU, G. 1999. Functional antagonism of the Polycomb-Group genes eed and Bmi1 in hemopoietic cell proliferation. *Genes Dev,* 13**,** 2691-703.

LETTERIO, J. J., GEISER, A. G., KULKARNI, A. B., DANG, H., KONG, L., NAKABAYASHI, T., MACKALL, C. L., GRESS, R. E. & ROBERTS, A. B. 1996. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest,* 98**,** 2109-19.

LEWIS, E. B. 1978. A gene complex controlling segmentation in Drosophila. *Nature,* 276**,** 565-70.

LI, B., CAREY, M. & WORKMAN, J. L. 2007. The role of chromatin during transcription. *Cell,* 128**,** 707-19.

LI, G., MARGUERON, R., KU, M., CHAMBON, P., BERNSTEIN, B. E. & REINBERG, D. 2010. Jarid2 and PRC2, partners in regulating gene expression. *Genes Dev,* 24**,** 368-80.

LI, L., JOTHI, R., CUI, K., LEE, J. Y., COHEN, T., GORIVODSKY, M., TZCHORI, I., ZHAO, Y., HAYES, S. M., BRESNICK, E. H., ZHAO, K., WESTPHAL, H. & LOVE, P. E. 2011. Nuclear adaptor Ldb1 regulates a transcriptional program essential for the maintenance of hematopoietic stem cells. *Nat Immunol,* 12**,** 129-36.

LI, Q., HARJU, S. & PETERSON, K. R. 1999. Locus control regions: coming of age at a decade plus. *Trends Genet,* 15**,** 403-8.

LI, X. Y., MACARTHUR, S., BOURGON, R., NIX, D., POLLARD, D. A., IYER, V. N., HECHMER, A., SIMIRENKO, L., STAPLETON, M., LUENGO HENDRIKS, C. L., CHU, H. C., OGAWA, N., INWOOD, W., SEMENTCHENKO, V., BEATON, A., WEISZMANN, R., CELNIKER, S. E., KNOWLES, D. W., GINGERAS, T., SPEED, T. P., EISEN, M. B. & BIGGIN, M. D. 2008. Transcription factors bind thousands of active and inactive regions in the Drosophila blastoderm. *PLoS Biol,* 6**,** e27.

LICHTINGER, M., HOOGENKAMP, M., KRYSINSKA, H., INGRAM, R. & BONIFER, C. 2010. Chromatin regulation by RUNX1. *Blood Cells Mol Dis,* 44**,** 287-90.

LIEFKE, R., OSWALD, F., ALVARADO, C., FERRES-MARCO, D., MITTLER, G., RODRIGUEZ, P., DOMINGUEZ, M. & BORGGREFE, T. 2010. Histone demethylase KDM5A is an integral part of the core Notch-RBP-J repressor complex. *Genes Dev,* 24**,** 590-601.

LIESCHKE, G. J., GRAIL, D., HODGSON, G., METCALF, D., STANLEY, E., CHEERS, C., FOWLER, K. J., BASU, S., ZHAN, Y. F. & DUNN, A. R. 1994a. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood,* 84**,** 1737-46.

LIESCHKE, G. J., STANLEY, E., GRAIL, D., HODGSON, G., SINICKAS, V., GALL, J. A., SINCLAIR, R. A. & DUNN, A. R. 1994b. Mice lacking both macrophage- and granulocyte-macrophage colony-stimulating factor have macrophages and coexistent osteopetrosis and severe lung disease. *Blood,* 84**,** 27-35.

LIN, H. & GROSSCHEDL, R. 1995. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature,* 376**,** 263-7.

LIN, Y. C., JHUNJHUNWALA, S., BENNER, C., HEINZ, S., WELINDER, E., MANSSON, R., SIGVARDSSON, M., HAGMAN, J., ESPINOZA, C. A., DUTKOWSKI, J., IDEKER, T., GLASS, C. K. & MURRE, C. 2010. A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat Immunol,* 11**,** 635-43.

LINNEKIN, D. 1999. Early signaling pathways activated by c-Kit in hematopoietic cells. *Int J Biochem Cell Biol,* 31**,** 1053-74.

LIS, J. T., MASON, P., PENG, J., PRICE, D. H. & WERNER, J. 2000. P-TEFb kinase recruitment and function at heat shock loci. *Genes Dev,* 14**,** 792-803.

LO, M. C., PETERSON, L. F., YAN, M., CONG, X., JIN, F., SHIA, W. J., MATSUURA, S., AHN, E. Y., KOMENO, Y., LY, M., OMMEN, H. B., CHEN, I. M., HOKLAND, P., WILLMAN, C. L., REN, B. & ZHANG, D. E. 2012. Combined gene expression and DNA occupancy profiling identifies potential therapeutic targets of t(8;21) AML. *Blood,* 120**,** 1473-84.

LOMBERK, G., WALLRATH, L. & URRUTIA, R. 2006. The Heterochromatin Protein 1 family. *Genome Biol,* 7**,** 228.

LUDWIG, S., ENGEL, K., HOFFMEYER, A., SITHANANDAM, G., NEUFELD, B., PALM, D., GAESTEL, M. & RAPP, U. R. 1996. 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. *Mol Cell Biol,* 16**,** 6687-97.

LUIS, T. C., ICHII, M., BRUGMAN, M. H., KINCADE, P. & STAAL, F. J. 2012a. Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. *Leukemia,* 26**,** 414-21.

LUIS, T. C., KILLMANN, N. M. & STAAL, F. J. 2012b. Signal transduction pathways regulating hematopoietic stem cell biology: introduction to a series of Spotlight Reviews. *Leukemia,* 26**,** 86-90.

LUIS, T. C., NABER, B. A., FIBBE, W. E., VAN DONGEN, J. J. & STAAL, F. J. 2010. Wnt3a nonredundantly controls hematopoietic stem cell function and its deficiency results in complete absence of canonical Wnt signaling. *Blood,* 116**,** 496-7.

LUIS, T. C., NABER, B. A., ROOZEN, P. P., BRUGMAN, M. H., DE HAAS, E. F., GHAZVINI, M., FIBBE, W. E., VAN DONGEN, J. J., FODDE, R. & STAAL, F. J. 2011. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell Stem Cell,* 9**,** 345-56.

LUSTIG, A. J. 1998. Mechanisms of silencing in Saccharomyces cerevisiae. *Curr Opin Genet Dev,* 8**,** 233-9.

LUTTERBACH, B., WESTENDORF, J. J., LINGGI, B., PATTEN, A., MONIWA, M., DAVIE, J. R., HUYNH, K. D., BARDWELL, V. J., LAVINSKY, R. M., ROSENFELD, M. G., GLASS, C., SETO, E. & HIEBERT, S. W. 1998. ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Mol Cell Biol,* 18**,** 7176-84.

LYAKHOV, I. G., KRISHNAMACHARI, A. & SCHNEIDER, T. D. 2008. Discovery of novel tumor suppressor p53 response elements using information theory. *Nucleic Acids Res,* 36**,** 3828-33.

MACDONALD, N., WELBURN, J. P., NOBLE, M. E., NGUYEN, A., YAFFE, M. B., CLYNES, D., MOGGS, J. G., ORPHANIDES, G., THOMSON, S., EDMUNDS, J. W., CLAYTON, A. L., ENDICOTT, J. A. & MAHADEVAN, L. C. 2005. Molecular basis for the recognition of phosphorylated and phosphoacetylated histone h3 by 14-3-3. *Mol Cell,* 20**,** 199-211.

MAEDA, T., MERGHOUB, T., HOBBS, R. M., DONG, L., MAEDA, M., ZAKRZEWSKI, J., VAN DEN BRINK, M. R., ZELENT, A., SHIGEMATSU, H., AKASHI, K., TERUYA-FELDSTEIN, J., CATTORETTI, G. & PANDOLFI, P. P. 2007. Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF. *Science,* 316**,** 860-6.

MAENNER, S., BLAUD, M., FOUILLEN, L., SAVOYE, A., MARCHAND, V., DUBOIS, A., SANGLIER-CIANFERANI, S., VAN DORSSELAER, A., CLERC, P., AVNER, P., VISVIKIS, A. & BRANLANT, C. 2010. 2-D structure of the A region of Xist RNA and its implication for PRC2 association. *PLoS Biol,* 8**,** e1000276.

MAHADEVAN, L. C., WILLIS, A. C. & BARRATT, M. J. 1991. Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell,* 65**,** 775-83.

MAHONY, S., AURON, P. E. & BENOS, P. V. 2007. DNA familial binding profiles made easy: comparison of various motif alignment and clustering strategies. *PLoS Comput Biol,* 3**,** e61.

MAHONY, S. & BENOS, P. V. 2007. STAMP: a web tool for exploring DNA-binding motif similarities. *Nucleic Acids Res,* 35**,** W253-8.

MAILLARD, I., KOCH, U., DUMORTIER, A., SHESTOVA, O., XU, L., SAI, H., PROSS, S. E., ASTER, J. C., BHANDOOLA, A., RADTKE, F. & PEAR, W. S. 2008. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. *Cell Stem Cell,* 2**,** 356-66.

MAKELA, T. P., PARVIN, J. D., KIM, J., HUBER, L. J., SHARP, P. A. & WEINBERG, R. A. 1995. A kinase-deficient transcription factor TFIIH is functional in basal and activated transcription. *Proc Natl Acad Sci U S A,* 92**,** 5174-8.

MALHOTRA, S. & KINCADE, P. W. 2009. Canonical Wnt pathway signaling suppresses VCAM-1 expression by marrow stromal and hematopoietic cells. *Exp Hematol,* 37**,** 19-30.

MANCINI, S. J., MANTEI, N., DUMORTIER, A., SUTER, U., MACDONALD, H. R. & RADTKE, F. 2005. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood,* 105**,** 2340-2.

MARGUERON, R. & REINBERG, D. 2011. The Polycomb complex PRC2 and its mark in life. *Nature,* 469**,** 343-9.

MARSDEN, M. P. & LAEMMLI, U. K. 1979. Metaphase chromosome structure: evidence for a radial loop model. *Cell,* 17**,** 849-58.

MARSON, A., FOREMAN, R., CHEVALIER, B., BILODEAU, S., KAHN, M., YOUNG, R. A. & JAENISCH, R. 2008. Wnt signaling promotes reprogramming of somatic cells to pluripotency. *Cell Stem Cell,* 3**,** 132-5.

MARTINEZ-AGOSTO, J. A., MIKKOLA, H. K., HARTENSTEIN, V. & BANERJEE, U. 2007. The hematopoietic stem cell and its niche: a comparative view. *Genes Dev,* 21**,** 3044-60.

MASSAGUE, J., SEOANE, J. & WOTTON, D. 2005. Smad transcription factors. *Genes Dev,* 19**,** 2783-810.

MATOZAKI, S., NAKAGAWA, T., KAWAGUCHI, R., AOZAKI, R., TSUTSUMI, M., MURAYAMA, T., KOIZUMI, T., NISHIMURA, R., ISOBE, T. & CHIHARA, K. 1995. Establishment of a myeloid leukaemic cell line (SKNO-1) from a patient with t(8;21) who acquired monosomy 17 during disease progression. *Br J Haematol,* 89**,** 805-11.

MCARTHUR, G. A., ROHRSCHNEIDER, L. R. & JOHNSON, G. R. 1994. Induced expression of c-fms in normal hematopoietic cells shows evidence for both conservation and lineage restriction of signal transduction in response to macrophage colony-stimulating factor. *Blood,* 83**,** 972-81.

MCKENZIE, J. L., TAKENAKA, K., GAN, O. I., DOEDENS, M. & DICK, J. E. 2007. Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin-CD34+CD38- population. *Blood,* 109**,** 543-5.

MCMANUS, S., EBERT, A., SALVAGIOTTO, G., MEDVEDOVIC, J., SUN, Q., TAMIR, I., JARITZ, M., TAGOH, H. & BUSSLINGER, M. 2011. The transcription factor Pax5 regulates its target genes by recruiting chromatin-modifying proteins in committed B cells. *EMBO J,* 30**,** 2388-404.

MEDINA, K. L., PONGUBALA, J. M., REDDY, K. L., LANCKI, D. W., DEKOTER, R., KIESLINGER, M., GROSSCHEDL, R. & SINGH, H. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev Cell,* 7**,** 607-17.

MEDVEDOVIC, J., EBERT, A., TAGOH, H. & BUSSLINGER, M. 2011. Pax5: a master regulator of B cell development and leukemogenesis. *Adv Immunol,* 111**,** 179-206.

MEDVINSKY, A. & DZIERZAK, E. 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell,* 86**,** 897-906.

METCALF, D. & NICOLA, N. A. 1984. The regulatory factors controlling murine erythropoiesis in vitro. *Prog Clin Biol Res,* 148**,** 93-105.

MIKKELSEN, T. S., HANNA, J., ZHANG, X., KU, M., WERNIG, M., SCHORDERET, P., BERNSTEIN, B. E., JAENISCH, R., LANDER, E. S. & MEISSNER, A. 2008. Dissecting direct reprogramming through integrative genomic analysis. *Nature,* 454**,** 49-55.

MIKKELSEN, T. S., KU, M., JAFFE, D. B., ISSAC, B., LIEBERMAN, E., GIANNOUKOS, G., ALVAREZ, P., BROCKMAN, W., KIM, T. K., KOCHE, R. P., LEE, W., MENDENHALL, E., O'DONOVAN, A., PRESSER, A., RUSS, C., XIE, X., MEISSNER, A., WERNIG, M., JAENISCH, R., NUSBAUM, C., LANDER, E. S. & BERNSTEIN, B. E. 2007. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature,* 448**,** 553-60.

MILLER, J. P., IZON, D., DEMUTH, W., GERSTEIN, R., BHANDOOLA, A. & ALLMAN, D. 2002. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. *J Exp Med,* 196**,** 705-11.

MIN, J., ZHANG, Y. & XU, R. M. 2003. Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. *Genes Dev,* 17**,** 1823-8.

MIRANDA, M. B., MCGUIRE, T. F. & JOHNSON, D. E. 2002. Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. *Leukemia,* 16**,** 683-92.

MIYOSHI, H., KOZU, T., SHIMIZU, K., ENOMOTO, K., MASEKI, N., KANEKO, Y., KAMADA, N. & OHKI, M. 1993. The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. *EMBO J,* 12**,** 2715-21.

MIYOSHI, H., SHIMIZU, K., KOZU, T., MASEKI, N., KANEKO, Y. & OHKI, M. 1991. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. *Proc Natl Acad Sci U S A,* 88**,** 10431-4.

MIZUGUCHI, G., SHEN, X., LANDRY, J., WU, W. H., SEN, S. & WU, C. 2004. ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science,* 303**,** 343-8.

MOCHIZUKI-KASHIO, M., WENDT, G. R. & IWAMA, A. 2012. Tumor suppressor function of the polycomb group genes. *Cell Cycle,* 11**,** 2043-4.

MOHAMMAD, H. P. & BAYLIN, S. B. 2010. Linking cell signaling and the epigenetic machinery. *Nat Biotechnol,* 28**,** 1033-8.

MOHN, F., WEBER, M., REBHAN, M., ROLOFF, T. C., RICHTER, J., STADLER, M. B., BIBEL, M. & SCHUBELER, D. 2008. Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. *Mol Cell,* 30**,** 755-66.

MONTEIRO, R., POUGET, C. & PATIENT, R. 2011. The gata1/pu.1 lineage fate paradigm varies between blood populations and is modulated by tif1gamma. *EMBO J,* 30**,** 1093-103.

MORRISON, A. M., JAGER, U., CHOTT, A., SCHEBESTA, M., HAAS, O. A. & BUSSLINGER, M. 1998. Deregulated PAX-5 transcription from a translocated IgH promoter in marginal zone lymphoma. *Blood,* 92**,** 3865-78.

MORRISON, S. J., HEMMATI, H. D., WANDYCZ, A. M. & WEISSMAN, I. L. 1995. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A,* 92**,** 10302-6.

MORRISON, S. J. & WEISSMAN, I. L. 1994. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity,* 1**,** 661-73.

MUJTABA, S., MANZUR, K. L., GURNON, J. R., KANG, M., VAN ETTEN, J. L. & ZHOU, M. M. 2008. Epigenetic transcriptional repression of cellular genes by a viral SET protein. *Nat Cell Biol,* 10**,** 1114-22.

MULLER-SIEBURG, C. E., CHO, R. H., KARLSSON, L., HUANG, J. F. & SIEBURG, H. B. 2004. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. *Blood,* 103**,** 4111-8.

MULLER-SIEBURG, C. E., SIEBURG, H. B., BERNITZ, J. M. & CATTAROSSI, G. 2012. Stem cell heterogeneity: implications for aging and regenerative medicine. *Blood,* 119**,** 3900-7.

MULLER, A. M., MEDVINSKY, A., STROUBOULIS, J., GROSVELD, F. & DZIERZAK, E. 1994. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity,* 1**,** 291-301.

MULLER, J. & KASSIS, J. A. 2006. Polycomb response elements and targeting of Polycomb group proteins in Drosophila. *Curr Opin Genet Dev,* 16**,** 476-84.

MULLIGHAN, C. G., GOORHA, S., RADTKE, I., MILLER, C. B., COUSTAN-SMITH, E., DALTON, J. D., GIRTMAN, K., MATHEW, S., MA, J., POUNDS, S. B., SU, X., PUI, C. H., RELLING, M. V., EVANS, W. E., SHURTLEFF, S. A. & DOWNING, J. R. 2007. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature,* 446**,** 758-64.

MULLIGHAN, C. G., MILLER, C. B., RADTKE, I., PHILLIPS, L. A., DALTON, J., MA, J., WHITE, D., HUGHES, T. P., LE BEAU, M. M., PUI, C. H., RELLING, M. V., SHURTLEFF, S. A. & DOWNING, J. R. 2008. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature,* 453**,** 110-4.

MUNARI, F., SOEROES, S., ZENN, H. M., SCHOMBURG, A., KOST, N., SCHROEDER, S., KLINGBERG, R., REZAEI-GHALEH, N., STUETZER, A., GELATO, K. A., WALLA, P. J., BECKER, S., SCHWARZER, D., ZIMMERMANN, B., FISCHLE, W. & ZWECKSTETTER, M. 2012. Methylation of K9 in histone H3 directs alternative modes of highly dynamic interaction of heterochromatin protein hHP1beta with the nucleosome. *J Biol Chem*.

MURRAY, P. 1932. The development in vitro of the blood of the early chick embryo. *Proc Roy Soc London,* 11**,** 497-521.

NARLIKAR, G. J., FAN, H. Y. & KINGSTON, R. E. 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell,* 108**,** 475-87.

NEBRAL, K., DENK, D., ATTARBASCHI, A., KONIG, M., MANN, G., HAAS, O. A. & STREHL, S. 2009. Incidence and diversity of PAX5 fusion genes in childhood acute lymphoblastic leukemia. *Leukemia,* 23**,** 134-43.

NELSON, C. J., SANTOS-ROSA, H. & KOUZARIDES, T. 2006. Proline isomerization of histone H3 regulates lysine methylation and gene expression. *Cell,* 126**,** 905-16.

NEPH, S., VIERSTRA, J., STERGACHIS, A. B., REYNOLDS, A. P., HAUGEN, E., VERNOT, B., THURMAN, R. E., JOHN, S., SANDSTROM, R., JOHNSON, A. K., MAURANO, M. T., HUMBERT, R., RYNES, E.,

WANG, H., VONG, S., LEE, K., BATES, D., DIEGEL, M., ROACH, V., DUNN, D., NERI, J., SCHAFER, A., HANSEN, R. S., KUTYAVIN, T., GISTE, E., WEAVER, M., CANFIELD, T., SABO, P., ZHANG, M., BALASUNDARAM, G., BYRON, R., MACCOSS, M. J., AKEY, J. M., BENDER, M. A., GROUDINE, M., KAUL, R. & STAMATOYANNOPOULOS, J. A. 2012. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature,* 489**,** 83-90.

NERLOV, C. & GRAF, T. 1998. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev,* 12**,** 2403-12.

NERLOV, C., QUERFURTH, E., KULESSA, H. & GRAF, T. 2000. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood,* 95**,** 2543-51.

NGUYEN, S., LEBLANC, T., FENAUX, P., WITZ, F., BLAISE, D., PIGNEUX, A., THOMAS, X., RIGAL-HUGUET, F., LIOURE, B., AUVRIGNON, A., FIERE, D., REIFFERS, J., CASTAIGNE, S., LEVERGER, G., HAROUSSEAU, J. L., SOCIE, G. & DOMBRET, H. 2002. A white blood cell index as the main prognostic factor in t(8;21) acute myeloid leukemia (AML): a survey of 161 cases from the French AML Intergroup. *Blood,* 99**,** 3517-23.

NICHOGIANNOPOULOU, A., TREVISAN, M., NEBEN, S., FRIEDRICH, C. & GEORGOPOULOS, K. 1999. Defects in hemopoietic stem cell activity in Ikaros mutant mice. *J Exp Med,* 190**,** 1201-14.

NIESSEN, H. E., DEMMERS, J. A. & VONCKEN, J. W. 2009. Talking to chromatin: post-translational modulation of polycomb group function. *Epigenetics Chromatin,* 2**,** 10.

NIMER, S. D. & MOORE, M. A. 2004. Effects of the leukemia-associated AML1-ETO protein on hematopoietic stem and progenitor cells. *Oncogene,* 23**,** 4249-54.

NING, Z. Q., LI, J. & ARCECI, R. J. 2001. Signal transducer and activator of transcription 3 activation is required for Asp(816) mutant c-Kit-mediated cytokine-independent survival and proliferation in human leukemia cells. *Blood,* 97**,** 3559-67.

NIWA, H., OGAWA, K., SHIMOSATO, D. & ADACHI, K. 2009. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature,* 460**,** 118-22.

NOTTA, F., DOULATOV, S., LAURENTI, E., POEPPL, A., JURISICA, I. & DICK, J. E. 2011. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science,* 333**,** 218-21.

NOVINA, C. D. & ROY, A. L. 1996. Core promoters and transcriptional control. *Trends Genet,* 12**,** 351-5.

NOWAK, S. J. & CORCES, V. G. 2004. Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet,* 20**,** 214-20.

NUSSLEIN-VOLHARD, C., KLUDING, H. & JURGENS, G. 1985. Genes affecting the segmental subdivision of the Drosophila embryo. *Cold Spring Harb Symp Quant Biol,* 50**,** 145-54.

NUTT, S. L., HEAVEY, B., ROLINK, A. G. & BUSSLINGER, M. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature,* 401**,** 556-62.

NUTT, S. L., MORRISON, A. M., DORFLER, P., ROLINK, A. & BUSSLINGER, M. 1998. Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO J,* 17**,** 2319-33.

NUTT, S. L., URBANEK, P., ROLINK, A. & BUSSLINGER, M. 1997. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev,* 11**,** 476-91.

O'NEILL, D. W., SCHOETZ, S. S., LOPEZ, R. A., CASTLE, M., RABINOWITZ, L., SHOR, E., KRAWCHUK, D., GOLL, M. G., RENZ, M., SEELIG, H. P., HAN, S., SEONG, R. H., PARK, S. D., AGALIOTI, T., MUNSHI, N., THANOS, D., ERDJUMENT-BROMAGE, H., TEMPST, P. & BANK, A. 2000. An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells. *Mol Cell Biol,* 20**,** 7572-82.

OGAWA, M., MATSUZAKI, Y., NISHIKAWA, S., HAYASHI, S., KUNISADA, T., SUDO, T., KINA, T. & NAKAUCHI, H. 1991. Expression and function of c-kit in hemopoietic progenitor cells. *J Exp Med,* 174**,** 63-71.

OGURO, H. & IWAMA, A. 2007. Life and death in hematopoietic stem cells. *Curr Opin Immunol,* 19**,** 503-9.

OGURO, H., IWAMA, A., MORITA, Y., KAMIJO, T., VAN LOHUIZEN, M. & NAKAUCHI, H. 2006. Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. *J Exp Med,* 203**,** 2247-53.

OGURO, H., YUAN, J., ICHIKAWA, H., IKAWA, T., YAMAZAKI, S., KAWAMOTO, H., NAKAUCHI, H. & IWAMA, A. 2010. Poised lineage specification in multipotential hematopoietic stem and progenitor cells by the polycomb protein Bmi1. *Cell Stem Cell,* 6**,** 279-86.

OKUDA, K., SANGHERA, J. S., PELECH, S. L., KANAKURA, Y., HALLEK, M., GRIFFIN, J. D. & DRUKER, B. J. 1992. Granulocyte-macrophage colony-stimulating factor, interleukin-3, and steel factor induce rapid tyrosine phosphorylation of p42 and p44 MAP kinase. *Blood,* 79**,** 2880-7.

OKUDA, T., CAI, Z., YANG, S., LENNY, N., LYU, C. J., VAN DEURSEN, J. M., HARADA, H. & DOWNING, J. R. 1998. Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. *Blood,* 91**,** 3134-43.

OKUMURA, A. J., PETERSON, L. F., OKUMURA, F., BOYAPATI, A. & ZHANG, D. E. 2008. t(8;21)(q22;q22) Fusion proteins preferentially bind to duplicated AML1/RUNX1 DNA-binding sequences to differentially regulate gene expression. *Blood,* 112**,** 1392-401.

ORFORD, K., KHARCHENKO, P., LAI, W., DAO, M. C., WORHUNSKY, D. J., FERRO, A., JANZEN, V., PARK, P. J. & SCADDEN, D. T. 2008. Differential H3K4 methylation identifies developmentally poised hematopoietic genes. *Dev Cell,* 14**,** 798-809.

ORKIN, S. H., SHIVDASANI, R. A., FUJIWARA, Y. & MCDEVITT, M. A. 1998. Transcription factor GATA-1 in megakaryocyte development. *Stem Cells,* 16 Suppl 2**,** 79-83.

ORKIN, S. H. & ZON, L. I. 2008. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell,* 132**,** 631-44.

OSAWA, M., HANADA, K., HAMADA, H. & NAKAUCHI, H. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science,* 273**,** 242-5.

PABST, T., MUELLER, B. U., HARAKAWA, N., SCHOCH, C., HAFERLACH, T., BEHRE, G., HIDDEMANN, W., ZHANG, D. E. & TENEN, D. G. 2001. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. *Nat Med,* 7**,** 444-51.

PAJCINI, K. V., SPECK, N. A. & PEAR, W. S. 2011. Notch signaling in mammalian hematopoietic stem cells. *Leukemia,* 25**,** 1525-32.

PALACIOS, D., MOZZETTA, C., CONSALVI, S., CARETTI, G., SACCONE, V., PROSERPIO, V., MARQUEZ, V. E., VALENTE, S., MAI, A., FORCALES, S. V., SARTORELLI, V. & PURI, P. L. 2010. TNF/p38alpha/polycomb signaling to Pax7 locus in satellite cells links inflammation to the epigenetic control of muscle regeneration. *Cell Stem Cell,* 7**,** 455-69.

PAN, G., TIAN, S., NIE, J., YANG, C., RUOTTI, V., WEI, H., JONSDOTTIR, G. A., STEWART, R. & THOMSON, J. A. 2007. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell,* 1**,** 299-312.

PAN, Y., TSAI, C. J., MA, B. & NUSSINOV, R. 2010. Mechanisms of transcription factor selectivity. *Trends Genet,* 26**,** 75-83.

PANDEY, R. R., MONDAL, T., MOHAMMAD, F., ENROTH, S., REDRUP, L., KOMOROWSKI, J., NAGANO, T., MANCINI-DINARDO, D. & KANDURI,

C. 2008. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell,* 32**,** 232-46.

PARK, S. H., CHI, H. S., MIN, S. K., PARK, B. G., JANG, S. & PARK, C. J. 2011. Prognostic impact of c-KIT mutations in core binding factor acute myeloid leukemia. *Leuk Res,* 35**,** 1376-83.

PASINI, D., BRACKEN, A. P., HANSEN, J. B., CAPILLO, M. & HELIN, K. 2007. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Mol Cell Biol,* 27**,** 3769-79.

PASINI, D., CLOOS, P. A., WALFRIDSSON, J., OLSSON, L., BUKOWSKI, J. P., JOHANSEN, J. V., BAK, M., TOMMERUP, N., RAPPSILBER, J. & HELIN, K. 2010. JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. *Nature,* 464**,** 306-10.

PENG, J. C., VALOUEV, A., SWIGUT, T., ZHANG, J., ZHAO, Y., SIDOW, A. & WYSOCKA, J. 2009. Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. *Cell,* 139**,** 1290-302.

PETERSON, L. F., BOYAPATI, A., AHN, E. Y., BIGGS, J. R., OKUMURA, A. J., LO, M. C., YAN, M. & ZHANG, D. E. 2007a. Acute myeloid leukemia with the 8q22;21q22 translocation: secondary mutational events and alternative t(8;21) transcripts. *Blood,* 110**,** 799-805.

PETERSON, L. F., LO, M. C., OKUMURA, A. J. & ZHANG, D. E. 2007b. Inability of RUNX1/AML1 to breach AML1-ETO block of embryonic stem cell definitive hematopoiesis. *Blood Cells Mol Dis,* 39**,** 321-8.

PETRUK, S., SEDKOV, Y., RILEY, K. M., HODGSON, J., SCHWEISGUTH, F., HIROSE, S., JAYNES, J. B., BROCK, H. W. & MAZO, A. 2006. Transcription of bxd noncoding RNAs promoted by trithorax represses Ubx in cis by transcriptional interference. *Cell,* 127**,** 1209-21.

PHARR, P. N., OGAWA, M., HOFBAUER, A. & LONGMORE, G. D. 1994. Expression of an activated erythropoietin or a colony-stimulating factor 1 receptor by pluripotent progenitors enhances colony formation but does not induce differentiation. *Proc Natl Acad Sci U S A,* 91**,** 7482-6.

PHILLIPS, J. E. & CORCES, V. G. 2009. CTCF: master weaver of the genome. *Cell,* 137**,** 1194-211.

PINHEIRO, I., MARGUERON, R., SHUKEIR, N., EISOLD, M., FRITZSCH, C., RICHTER, F. M., MITTLER, G., GENOUD, C., GOYAMA, S., KUROKAWA, M., SON, J., REINBERG, D., LACHNER, M. & JENUWEIN, T. 2012. Prdm3 and Prdm16 are H3K9me1 Methyltransferases Required for Mammalian Heterochromatin Integrity. *Cell,* 150**,** 948-60.

PRICE, D. H. 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol Cell Biol,* 20**,** 2629-34.

PRIDANS, C., HOLMES, M. L., POLLI, M., WETTENHALL, J. M., DAKIC, A., CORCORAN, L. M., SMYTH, G. K. & NUTT, S. L. 2008. Identification of Pax5 target genes in early B cell differentiation. *J Immunol,* 180**,** 1719-28.

PRLIC, M. & BEVAN, M. J. 2011. Cutting edge: beta-catenin is dispensable for T cell effector differentiation, memory formation, and recall responses. *J Immunol,* 187**,** 1542-6.

PTASINSKA, A., ASSI, S. A., MANNARI, D., JAMES, S. R., WILLIAMSON, D., DUNNE, J., HOOGENKAMP, M., WU, M., CARE, M., MCNEILL, H., CAUCHY, P., CULLEN, M., TOOZE, R. M., TENEN, D. G., YOUNG, B. D., COCKERILL, P. N., WESTHEAD, D. R., HEIDENREICH, O. & BONIFER, C. 2012a. Depletion of RUNX1/ETO in t(8;21) AML cells leads to genome-wide changes in chromatin structure and transcription factor binding. *Leukemia*.

PTASINSKA, A., ASSI, S. A., MANNARI, D., JAMES, S. R., WILLIAMSON, D., DUNNE, J., HOOGENKAMP, M., WU, M., CARE, M., MCNEILL, H., CAUCHY, P., CULLEN, M., TOOZE, R. M., TENEN, D. G., YOUNG, B. D., COCKERILL, P. N., WESTHEAD, D. R., HEIDENREICH, O. & BONIFER, C. 2012b. Depletion of RUNX1/ETO in t(8;21) AML cells leads to genome-wide changes in chromatin structure and transcription factor binding. *Leukemia,* 26**,** 1829-41.

RAAPHORST, F. M. 2005. Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. *Hum Mol Genet,* 14 Spec No 1**,** R93-R100.

RADA-IGLESIAS, A., BAJPAI, R., SWIGUT, T., BRUGMANN, S. A., FLYNN, R. A. & WYSOCKA, J. 2011. A unique chromatin signature uncovers early developmental enhancers in humans. *Nature,* 470**,** 279-83.

RADTKE, F., WILSON, A., STARK, G., BAUER, M., VAN MEERWIJK, J., MACDONALD, H. R. & AGUET, M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity,* 10**,** 547-58.

RAISNER, R. M., HARTLEY, P. D., MENEGHINI, M. D., BAO, M. Z., LIU, C. L., SCHREIBER, S. L., RANDO, O. J. & MADHANI, H. D. 2005. Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. *Cell,* 123**,** 233-48.

RAMPALLI, S., LI, L., MAK, E., GE, K., BRAND, M., TAPSCOTT, S. J. & DILWORTH, F. J. 2007. p38 MAPK signaling regulates recruitment of Ash2L-containing methyltransferase complexes to specific genes during differentiation. *Nat Struct Mol Biol,* 14**,** 1150-6.

REDELL, M. S., RUIZ, M. J., ALONZO, T. A., GERBING, R. B. & TWEARDY, D. J. 2011. Stat3 signaling in acute myeloid leukemia: ligand-dependent and -independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor. *Blood,* 117**,** 5701-9.

REINBERG, D. & SIMS, R. J., 3RD 2006. de FACTo nucleosome dynamics. *J Biol Chem,* 281**,** 23297-301.

REKHTMAN, N., RADPARVAR, F., EVANS, T. & SKOULTCHI, A. I. 1999. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev,* 13**,** 1398-411.

REVILLA, I. D. R., BILIC, I., VILAGOS, B., TAGOH, H., EBERT, A., TAMIR, I. M., SMEENK, L., TRUPKE, J., SOMMER, A., JARITZ, M. & BUSSLINGER, M. 2012. The B-cell identity factor Pax5 regulates distinct transcriptional programmes in early and late B lymphopoiesis. *EMBO J,* 31**,** 3130-46.

REYA, T., DUNCAN, A. W., AILLES, L., DOMEN, J., SCHERER, D. C., WILLERT, K., HINTZ, L., NUSSE, R. & WEISSMAN, I. L. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature,* 423**,** 409-14.

RHOADES, K. L., HETHERINGTON, C. J., HARAKAWA, N., YERGEAU, D. A., ZHOU, L., LIU, L. Q., LITTLE, M. T., TENEN, D. G. & ZHANG, D. E. 2000. Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. *Blood,* 96**,** 2108-15.

RHODES, J., HAGEN, A., HSU, K., DENG, M., LIU, T. X., LOOK, A. T. & KANKI, J. P. 2005. Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell,* 8**,** 97-108.

RICHLY, H., LANGE, M., SIMBOECK, E. & DI CROCE, L. 2010. Setting and resetting of epigenetic marks in malignant transformation and development. *Bioessays,* 32**,** 669-79.

RICHMOND, T. J., FINCH, J. T., RUSHTON, B., RHODES, D. & KLUG, A. 1984. Structure of the nucleosome core particle at 7 A resolution. *Nature,* 311**,** 532-7.

RIEGER, M. A., HOPPE, P. S., SMEJKAL, B. M., EITELHUBER, A. C. & SCHROEDER, T. 2009. Hematopoietic cytokines can instruct lineage choice. *Science,* 325**,** 217-8.

RINGROSE, L. & PARO, R. 2007. Polycomb/Trithorax response elements and epigenetic memory of cell identity. *Development,* 134**,** 223-32.

ROBB, L. 2007. Cytokine receptors and hematopoietic differentiation. *Oncogene,* 26**,** 6715-23.

ROBERT-MORENO, A., ESPINOSA, L., DE LA POMPA, J. L. & BIGAS, A. 2005. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development,* 132**,** 1117-26.

ROBERT-MORENO, A., ESPINOSA, L., SANCHEZ, M. J., DE LA POMPA, J. L. & BIGAS, A. 2007. The notch pathway positively regulates programmed cell death during erythroid differentiation. *Leukemia,* 21**,** 1496-503.

ROBINSON, P. J., AN, W., ROUTH, A., MARTINO, F., CHAPMAN, L., ROEDER, R. G. & RHODES, D. 2008. 30 nm chromatin fibre decompaction requires both H4-K16 acetylation and linker histone eviction. *J Mol Biol,* 381**,** 816-25.

ROESSLER, S., GYORY, I., IMHOF, S., SPIVAKOV, M., WILLIAMS, R. R., BUSSLINGER, M., FISHER, A. G. & GROSSCHEDL, R. 2007. Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol,* 27**,** 579-94.

ROLINK, A. G., NUTT, S. L., MELCHERS, F. & BUSSLINGER, M. 1999. Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. *Nature,* 401**,** 603-6.

ROLINK, A. G., SCHANIEL, C., BRUNO, L. & MELCHERS, F. 2002. In vitro and in vivo plasticity of Pax5-deficient pre-B I cells. *Immunol Lett,* 82**,** 35-40.

RONNSTRAND, L. 2004. Signal transduction via the stem cell factor receptor/c-Kit. *Cell Mol Life Sci,* 61**,** 2535-48.

ROVIDA, E., GOZZINI, A., BARBETTI, V., GIUNTOLI, S., SANTINI, V. & DELLO SBARBA, P. 2006. The c-Jun-N-terminal-Kinase inhibitor SP600125 enhances the butyrate derivative D1-induced apoptosis via caspase 8 activation in Kasumi 1 t(8;21) acute myeloid leukaemia cells. *Br J Haematol,* 135**,** 653-9.

SABIN, F. 1920. Studies on the origin of blood vessels and red blood corpuscles as seen in living blastoderm of chicks during second day of incubation. *Carnegie Inst Wash Pub,* 272.

SAEED, S., LOGIE, C., FRANCOIJS, K. J., FRIGE, G., ROMANENGHI, M., NIELSEN, F. G., RAATS, L., SHAHHOSEINI, M., HUYNEN, M., ALTUCCI, L., MINUCCI, S., MARTENS, J. H. & STUNNENBERG, H. G. 2012. Chromatin accessibility, p300 and histone acetylation define PML-RARalpha and AML1-ETO binding sites in acute myeloid leukemia. *Blood*.

SAHA, A., WITTMEYER, J. & CAIRNS, B. R. 2006. Chromatin remodelling: the industrial revolution of DNA around histones. *Nat Rev Mol Cell Biol,* 7**,** 437-47.

SAURIN, A. J., SHAO, Z., ERDJUMENT-BROMAGE, H., TEMPST, P. & KINGSTON, R. E. 2001. A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. *Nature,* 412**,** 655-60.

SAWARKAR, R. & PARO, R. 2010. Interpretation of developmental signaling at chromatin: the Polycomb perspective. *Dev Cell,* 19**,** 651-61.

SCHANIEL, C., BRUNO, L., MELCHERS, F. & ROLINK, A. G. 2002a. Multiple hematopoietic cell lineages develop in vivo from transplanted Pax5-deficient pre-B I-cell clones. *Blood,* 99**,** 472-8.

SCHANIEL, C., GOTTAR, M., ROOSNEK, E., MELCHERS, F. & ROLINK, A. G. 2002b. Extensive in vivo self-renewal, long-term reconstitution capacity, and hematopoietic multipotency of Pax5-deficient precursor B-cell clones. *Blood,* 99**,** 2760-6.

SCHELLER, M., HUELSKEN, J., ROSENBAUER, F., TAKETO, M. M., BIRCHMEIER, W., TENEN, D. G. & LEUTZ, A. 2006. Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat Immunol,* 7**,** 1037-47.

SCHESSL, C., RAWAT, V. P., CUSAN, M., DESHPANDE, A., KOHL, T. M., ROSTEN, P. M., SPIEKERMANN, K., HUMPHRIES, R. K., SCHNITTGER, S., KERN, W., HIDDEMANN, W., QUINTANILLA-MARTINEZ, L., BOHLANDER, S. K., FEURING-BUSKE, M. & BUSKE, C. 2005. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. *J Clin Invest,* 115**,** 2159-68.

SCHMITT, T. M., CIOFANI, M., PETRIE, H. T. & ZUNIGA-PFLUCKER, J. C. 2004a. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med,* 200**,** 469-79.

SCHMITT, T. M., DE POOTER, R. F., GRONSKI, M. A., CHO, S. K., OHASHI, P. S. & ZUNIGA-PFLUCKER, J. C. 2004b. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol,* 5**,** 410-7.

SCHOEFTNER, S., SENGUPTA, A. K., KUBICEK, S., MECHTLER, K., SPAHN, L., KOSEKI, H., JENUWEIN, T. & WUTZ, A. 2006. Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. *EMBO J,* 25**,** 3110-22.

SCHUBELER, D., MACALPINE, D. M., SCALZO, D., WIRBELAUER, C., KOOPERBERG, C., VAN LEEUWEN, F., GOTTSCHLING, D. E., O'NEILL, L. P., TURNER, B. M., DELROW, J., BELL, S. P. & GROUDINE, M. 2004. The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev,* 18**,** 1263-71.

SCHUTTE, J., MOIGNARD, V. & GOTTGENS, B. 2012. Establishing the stem cell state: insights from regulatory network analysis of blood stem cell development. *Wiley Interdiscip Rev Syst Biol Med,* 4**,** 285-95.

SCHWARTZ, B. E. & AHMAD, K. 2005. Transcriptional activation triggers deposition and removal of the histone variant H3.3. *Genes Dev,* 19**,** 804-14.

SCHWARTZ, Y. B., KAHN, T. G., NIX, D. A., LI, X. Y., BOURGON, R., BIGGIN, M. & PIRROTTA, V. 2006. Genome-wide analysis of Polycomb targets in Drosophila melanogaster. *Nat Genet,* 38**,** 700-5.

SCHWARTZ, Y. B., KAHN, T. G., STENBERG, P., OHNO, K., BOURGON, R. & PIRROTTA, V. 2010. Alternative epigenetic chromatin states of polycomb target genes. *PLoS Genet,* 6**,** e1000805.

SCHWARZ, B. A. & BHANDOOLA, A. 2004. Circulating hematopoietic progenitors with T lineage potential. *Nat Immunol,* 5**,** 953-60.

SCOTT, E. W., SIMON, M. C., ANASTASI, J. & SINGH, H. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science,* 265**,** 1573-7.

SEKINE, R., KITAMURA, T., TSUJI, T. & TOJO, A. 2008. Efficient retroviral transduction of human B-lymphoid and myeloid progenitors: marked inhibition of their growth by the Pax5 transgene. *Int J Hematol,* 87**,** 351-62.

SERVE, H., YEE, N. S., STELLA, G., SEPP-LORENZINO, L., TAN, J. C. & BESMER, P. 1995. Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. *EMBO J,* 14**,** 473-83.

SHARMA, S., GURUDUTTA, G. U., SATIJA, N. K., PATI, S., AFRIN, F., GUPTA, P., VERMA, Y. K., SINGH, V. K. & TRIPATHI, R. P. 2006. Stem cell c-KIT and HOXB4 genes: critical roles and mechanisms in self-renewal, proliferation, and differentiation. *Stem Cells Dev,* 15**,** 755-78.

SHEN, X., KIM, W., FUJIWARA, Y., SIMON, M. D., LIU, Y., MYSLIWIEC, M. R., YUAN, G. C., LEE, Y. & ORKIN, S. H. 2009. Jumonji modulates polycomb activity and self-renewal versus differentiation of stem cells. *Cell,* 139**,** 1303-14.

SHI, Y., LAN, F., MATSON, C., MULLIGAN, P., WHETSTINE, J. R., COLE, P. A. & CASERO, R. A. 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell,* 119**,** 941-53.

SHILATIFARD, A. 2006. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. *Annu Rev Biochem,* 75**,** 243-69.

SHIVDASANI, R. A., MAYER, E. L. & ORKIN, S. H. 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature,* 373**,** 432-4.

SHOGREN-KNAAK, M., ISHII, H., SUN, J. M., PAZIN, M. J., DAVIE, J. R. & PETERSON, C. L. 2006. Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science,* 311**,** 844-7.

SHOGREN-KNAAK, M. & PETERSON, C. L. 2006. Switching on chromatin: mechanistic role of histone H4-K16 acetylation. *Cell Cycle,* 5**,** 1361-5.

SIMON, J. A. & KINGSTON, R. E. 2009. Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol,* 10**,** 697-708.

SIMPSON, R. T. 1978. Structure of the chromatosome, a chromatin particle containing 160 base pairs of DNA and all the histones. *Biochemistry,* 17**,** 5524-31.

SIMPSON, R. T., WHITLOCK, J. P., JR., BINA-STEIN, M. & STEIN, A. 1978. Histone-DNA interactions in chromatin core particles. *Cold Spring Harb Symp Quant Biol,* 42 Pt 1**,** 127-36.

SIMS, R. J., 3RD, NISHIOKA, K. & REINBERG, D. 2003. Histone lysine methylation: a signature for chromatin function. *Trends Genet,* 19**,** 629-39.

SING, A., PANNELL, D., KARAISKAKIS, A., STURGEON, K., DJABALI, M., ELLIS, J., LIPSHITZ, H. D. & CORDES, S. P. 2009. A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. *Cell,* 138**,** 885-97.

SIPKINS, D. A., WEI, X., WU, J. W., RUNNELS, J. M., COTE, D., MEANS, T. K., LUSTER, A. D., SCADDEN, D. T. & LIN, C. P. 2005. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature,* 435**,** 969-73.

SITNICKA, E., BRAKEBUSCH, C., MARTENSSON, I. L., SVENSSON, M., AGACE, W. W., SIGVARDSSON, M., BUZA-VIDAS, N., BRYDER, D., CILIO, C. M., AHLENIUS, H., MARASKOVSKY, E., PESCHON, J. J. & JACOBSEN, S. E. 2003. Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. *J Exp Med,* 198**,** 1495-506.

SITNICKA, E., BRYDER, D., THEILGAARD-MONCH, K., BUZA-VIDAS, N., ADOLFSSON, J. & JACOBSEN, S. E. 2002. Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity,* 17**,** 463-72.

SMALE, S. T. 2010. Pioneer factors in embryonic stem cells and differentiation. *Curr Opin Genet Dev,* 20**,** 519-26.

SMALE, S. T., JAIN, A., KAUFMANN, J., EMAMI, K. H., LO, K. & GARRAWAY, I. P. 1998. The initiator element: a paradigm for core promoter heterogeneity within metazoan protein-coding genes. *Cold Spring Harb Symp Quant Biol,* 63**,** 21-31.

SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T cell activation. *Annu Rev Immunol,* 27**,** 591-619.

SMITH, R. D., YU, J., ANNUNZIATO, A. & SEALE, R. L. 1984. beta-Globin gene family in murine erythroleukemia cells resides within two chromatin domains differing in higher order structure. *Biochemistry,* 23**,** 2970-6.

SOCOLOVSKY, M., DUSANTER-FOURT, I. & LODISH, H. F. 1997. The prolactin receptor and severely truncated erythropoietin receptors support differentiation of erythroid progenitors. *J Biol Chem,* 272**,** 14009-12.

SOLOAGA, A., THOMSON, S., WIGGIN, G. R., RAMPERSAUD, N., DYSON, M. H., HAZZALIN, C. A., MAHADEVAN, L. C. & ARTHUR, J. S. 2003. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J,* 22**,** 2788-97.

SOLOMON, M. J., LARSEN, P. L. & VARSHAVSKY, A. 1988. Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. *Cell,* 53**,** 937-47.

SOMA, T., YU, J. M. & DUNBAR, C. E. 1996. Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of transforming growth factor-beta but not macrophage inflammatory protein-1 alpha activities. *Blood,* 87**,** 4561-7.

SOUABNI, A., COBALEDA, C., SCHEBESTA, M. & BUSSLINGER, M. 2002. Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity,* 17**,** 781-93.

SPANGRUDE, G. J., HEIMFELD, S. & WEISSMAN, I. L. 1988. Purification and characterization of mouse hematopoietic stem cells. *Science,* 241**,** 58-62.

SPIEKERMANN, K., BIETHAHN, S., WILDE, S., HIDDEMANN, W. & ALVES, F. 2001. Constitutive activation of STAT transcription factors in acute myelogenous leukemia. *Eur J Haematol,* 67**,** 63-71.

SRINIVASAN, S., DORIGHI, K. M. & TAMKUN, J. W. 2008. Drosophila Kismet regulates histone H3 lysine 27 methylation and early elongation by RNA polymerase II. *PLoS Genet,* 4**,** e1000217.

STALDER, J., LARSEN, A., ENGEL, J. D., DOLAN, M., GROUDINE, M. & WEINTRAUB, H. 1980. Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNAase I. *Cell,* 20**,** 451-60.

STANLEY, E., LIESCHKE, G. J., GRAIL, D., METCALF, D., HODGSON, G., GALL, J. A., MAHER, D. W., CEBON, J., SINICKAS, V. & DUNN, A. R. 1994. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A,* 91**,** 5592-6.

STARCK, J., COHET, N., GONNET, C., SARRAZIN, S., DOUBEIKOVSKAIA, Z., DOUBEIKOVSKI, A., VERGER, A., DUTERQUE-COQUILLAUD, M. & MORLE, F. 2003. Functional cross-antagonism between transcription factors FLI-1 and EKLF. *Mol Cell Biol,* 23**,** 1390-402.

STEENSMA, D. P., MCCLURE, R. F., KARP, J. E., TEFFERI, A., LASHO, T. L., POWELL, H. L., DEWALD, G. W. & KAUFMANN, S. H. 2006. JAK2 V617F is a rare finding in de novo acute myeloid leukemia, but STAT3 activation is common and remains unexplained. *Leukemia,* 20**,** 971-8.

STEFFEN, B., MULLER-TIDOW, C., SCHWABLE, J., BERDEL, W. E. & SERVE, H. 2005. The molecular pathogenesis of acute myeloid leukemia. *Crit Rev Oncol Hematol,* 56**,** 195-221.

STERNER, D. E. & BERGER, S. L. 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev,* 64**,** 435-59.

STOCK, J. K., GIADROSSI, S., CASANOVA, M., BROOKES, E., VIDAL, M., KOSEKI, H., BROCKDORFF, N., FISHER, A. G. & POMBO, A. 2007. Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol,* 9**,** 1428-35.

STOFFEL, R., ZIEGLER, S., GHILARDI, N., LEDERMANN, B., DE SAUVAGE, F. J. & SKODA, R. C. 1999. Permissive role of thrombopoietin and granulocyte colony-stimulating factor receptors in hematopoietic cell fate decisions in vivo. *Proc Natl Acad Sci U S A,* 96**,** 698-702.

STOPKA, T., AMANATULLAH, D. F., PAPETTI, M. & SKOULTCHI, A. I. 2005. PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *EMBO J,* 24**,** 3712-23.

STRUHL, G. 1981. A gene product required for correct initiation of segmental determination in Drosophila. *Nature,* 293**,** 36-41.

STRUHL, K. 1998. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev,* 12**,** 599-606.

SU, I. H., BASAVARAJ, A., KRUTCHINSKY, A. N., HOBERT, O., ULLRICH, A., CHAIT, B. T. & TARAKHOVSKY, A. 2003. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. *Nat Immunol,* 4**,** 124-31.

SU, I. H., DOBENECKER, M. W., DICKINSON, E., OSER, M., BASAVARAJ, A., MARQUERON, R., VIALE, A., REINBERG, D., WULFING, C. &

TARAKHOVSKY, A. 2005. Polycomb group protein ezh2 controls actin polymerization and cell signaling. *Cell,* 121**,** 425-36.

SU, W., PORTER, S., KUSTU, S. & ECHOLS, H. 1990. DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter. *Proc Natl Acad Sci U S A,* 87**,** 5504-8.

SUN, G., PORTER, W. & SAFE, S. 1998. Estrogen-induced retinoic acid receptor alpha 1 gene expression: role of estrogen receptor-Sp1 complex. *Mol Endocrinol,* 12**,** 882-90.

SWIGUT, T. & WYSOCKA, J. 2007. H3K27 demethylases, at long last. *Cell,* 131**,** 29-32.

TAGOH, H., COCKERILL, P. N. & BONIFER, C. 2006a. In vivo genomic footprinting using LM-PCR methods. *Methods Mol Biol,* 325**,** 285-314.

TAGOH, H., INGRAM, R., WILSON, N., SALVAGIOTTO, G., WARREN, A. J., CLARKE, D., BUSSLINGER, M. & BONIFER, C. 2006b. The mechanism of repression of the myeloid-specific c-fms gene by Pax5 during B lineage restriction. *EMBO J,* 25**,** 1070-80.

TANIGAKI, K., HAN, H., YAMAMOTO, N., TASHIRO, K., IKEGAWA, M., KURODA, K., SUZUKI, A., NAKANO, T. & HONJO, T. 2002. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat Immunol,* 3**,** 443-50.

TAVARES, L., DIMITROVA, E., OXLEY, D., WEBSTER, J., POOT, R., DEMMERS, J., BEZSTAROSTI, K., TAYLOR, S., URA, H., KOIDE, H., WUTZ, A., VIDAL, M., ELDERKIN, S. & BROCKDORFF, N. 2012. RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. *Cell,* 148**,** 664-78.

TENEN, D. G. 2003. Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer,* 3**,** 89-101.

THOMA, F., KOLLER, T. & KLUG, A. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J Cell Biol,* 83**,** 403-27.

THURMAN, R. E., RYNES, E., HUMBERT, R., VIERSTRA, J., MAURANO, M. T., HAUGEN, E., SHEFFIELD, N. C., STERGACHIS, A. B., WANG, H., VERNOT, B., GARG, K., JOHN, S., SANDSTROM, R., BATES, D., BOATMAN, L., CANFIELD, T. K., DIEGEL, M., DUNN, D., EBERSOL, A. K., FRUM, T., GISTE, E., JOHNSON, A. K., JOHNSON, E. M., KUTYAVIN, T., LAJOIE, B., LEE, B. K., LEE, K., LONDON, D., LOTAKIS, D., NEPH, S., NERI, F., NGUYEN, E. D., QU, H., REYNOLDS, A. P., ROACH, V., SAFI, A., SANCHEZ, M. E., SANYAL, A., SHAFER, A., SIMON, J. M., SONG, L., VONG, S., WEAVER, M., YAN, Y., ZHANG, Z., LENHARD, B., TEWARI, M., DORSCHNER, M. O.,

HANSEN, R. S., NAVAS, P. A., STAMATOYANNOPOULOS, G., IYER, V. R., LIEB, J. D., SUNYAEV, S. R., AKEY, J. M., SABO, P. J., KAUL, R., FUREY, T. S., DEKKER, J., CRAWFORD, G. E. & STAMATOYANNOPOULOS, J. A. 2012. The accessible chromatin landscape of the human genome. *Nature,* 489**,** 75-82.

TIACCI, E., PILERI, S., ORLETH, A., PACINI, R., TABARRINI, A., FRENGUELLI, F., LISO, A., DIVERIO, D., LO-COCO, F. & FALINI, B. 2004. PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. *Cancer Res,* 64**,** 7399-404.

TIAN, K., YANG, S., REN, Q., HAN, Z., LU, S., MA, F. & ZHANG, L. 2010. p38 MAPK contributes to the growth inhibition of leukemic tumor cells mediated by human umbilical cord mesenchymal stem cells. *Cell Physiol Biochem,* 26**,** 799-808.

TILL, J. E., MCCULLOCH, E. A. & SIMINOVITCH, L. 1964. A Stochastic Model of Stem Cell Proliferation, Based on the Growth of Spleen Colony-Forming Cells. *Proc Natl Acad Sci U S A,* 51**,** 29-36.

TIMOKHINA, I., KISSEL, H., STELLA, G. & BESMER, P. 1998. Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. *EMBO J,* 17**,** 6250-62.

TING, C. N., OLSON, M. C., BARTON, K. P. & LEIDEN, J. M. 1996. Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature,* 384**,** 474-8.

TIRODE, F., BUSSO, D., COIN, F. & EGLY, J. M. 1999. Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. *Mol Cell,* 3**,** 87-95.

TIWARI, V. K., STADLER, M. B., WIRBELAUER, C., PARO, R., SCHUBELER, D. & BEISEL, C. 2012. A chromatin-modifying function of JNK during stem cell differentiation. *Nat Genet,* 44**,** 94-100.

TONKS, A., PEARN, L., MUSSON, M., GILKES, A., MILLS, K. I., BURNETT, A. K. & DARLEY, R. L. 2007. Transcriptional dysregulation mediated by RUNX1-RUNX1T1 in normal human progenitor cells and in acute myeloid leukaemia. *Leukemia,* 21**,** 2495-505.

TREMBLAY, M., HERBLOT, S., LECUYER, E. & HOANG, T. 2003. Regulation of pT alpha gene expression by a dosage of E2A, HEB, and SCL. *J Biol Chem,* 278**,** 12680-7.

TSUJIMURA, T., KANAKURA, Y. & KITAMURA, Y. 1997. Mechanisms of constitutive activation of c-kit receptor tyrosine kinase. *Leukemia,* 11 Suppl 3**,** 396-8.

TSUKADA, Y., FANG, J., ERDJUMENT-BROMAGE, H., WARREN, M. E., BORCHERS, C. H., TEMPST, P. & ZHANG, Y. 2006. Histone demethylation by a family of JmjC domain-containing proteins. *Nature,* 439**,** 811-6.

TUAN, D. & LONDON, I. M. 1984. Mapping of DNase I-hypersensitive sites in the upstream DNA of human embryonic epsilon-globin gene in K562 leukemia cells. *Proc Natl Acad Sci U S A,* 81**,** 2718-22.

TURNER, B. M. 2012. The adjustable nucleosome: an epigenetic signaling module. *Trends Genet,* 28**,** 436-44.

URBANEK, P., WANG, Z. Q., FETKA, I., WAGNER, E. F. & BUSSLINGER, M. 1994. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell,* 79**,** 901-12.

VALBUENA, J. R., MEDEIROS, L. J., RASSIDAKIS, G. Z., HAO, S., WU, C. D., CHEN, L. & LIN, P. 2006. Expression of B cell-specific activator protein/PAX5 in acute myeloid leukemia with t(8;21)(q22;q22). *Am J Clin Pathol,* 126**,** 235-40.

VALK, P. J., VERHAAK, R. G., BEIJEN, M. A., ERPELINCK, C. A., BARJESTEH VAN WAALWIJK VAN DOORN-KHOSROVANI, S., BOER, J. M., BEVERLOO, H. B., MOORHOUSE, M. J., VAN DER SPEK, P. J., LOWENBERG, B. & DELWEL, R. 2004. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med,* 350**,** 1617-28.

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., VAN LOHUIZEN, M. & ET AL. 1994. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. *Genes Dev,* 8**,** 757-69.

VAN DER VLAG, J. & OTTE, A. P. 1999. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nat Genet,* 23**,** 474-8.

VAQUERO, A., SCHER, M. B., LEE, D. H., SUTTON, A., CHENG, H. L., ALT, F. W., SERRANO, L., STERNGLANZ, R. & REINBERG, D. 2006. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev,* 20**,** 1256-61.

VARDIMAN, J. W. 2010. The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. *Chem Biol Interact,* 184**,** 16-20.

VARDIMAN, J. W., HARRIS, N. L. & BRUNNING, R. D. 2002. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood,* 100**,** 2292-302.

VARDIMAN, J. W., THIELE, J., ARBER, D. A., BRUNNING, R. D., BOROWITZ, M. J., PORWIT, A., HARRIS, N. L., LE BEAU, M. M., HELLSTROM-LINDBERG, E., TEFFERI, A. & BLOOMFIELD, C. D. 2009. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood,* 114**,** 937-51.

VARNUM-FINNEY, B., BRASHEM-STEIN, C. & BERNSTEIN, I. D. 2003. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood,* 101**,** 1784-9.

VEPRINTSEV, D. B. & FERSHT, A. R. 2008. Algorithm for prediction of tumour suppressor p53 affinity for binding sites in DNA. *Nucleic Acids Res,* 36**,** 1589-98.

VICENT, G. P., NACHT, A. S., ZAURIN, R., BALLARE, C., CLAUSELL, J. & BEATO, M. 2010. Minireview: role of kinases and chromatin remodeling in progesterone signaling to chromatin. *Mol Endocrinol,* 24**,** 2088-98.

VISNJIC, D., KALAJZIC, Z., ROWE, D. W., KATAVIC, V., LORENZO, J. & AGUILA, H. L. 2004. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood,* 103**,** 3258-64.

VONCKEN, J. W., NIESSEN, H., NEUFELD, B., RENNEFAHRT, U., DAHLMANS, V., KUBBEN, N., HOLZER, B., LUDWIG, S. & RAPP, U. R. 2005. MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the polycomb group protein Bmi1. *J Biol Chem,* 280**,** 5178-87.

VONCKEN, J. W., SCHWEIZER, D., AAGAARD, L., SATTLER, L., JANTSCH, M. F. & VAN LOHUIZEN, M. 1999. Chromatin-association of the Polycomb group protein BMI1 is cell cycle-regulated and correlates with its phosphorylation status. *J Cell Sci,* 112 (Pt 24)**,** 4627-39.

WAKITA, S., YAMAGUCHI, H., MIYAKE, K., MITAMURA, Y., KOSAKA, F., DAN, K. & INOKUCHI, K. 2011. Importance of c-kit mutation detection method sensitivity in prognostic analyses of t(8;21)(q22;q22) acute myeloid leukemia. *Leukemia,* 25**,** 1423-32.

WALTER, K., BONIFER, C. & TAGOH, H. 2008. Stem cell-specific epigenetic priming and B cell-specific transcriptional activation at the mouse Cd19 locus. *Blood,* 112**,** 1673-82.

WALTER, K., COCKERILL, P. N., BARLOW, R., CLARKE, D., HOOGENKAMP, M., FOLLOWS, G. A., RICHARDS, S. J., CULLEN, M. J., BONIFER, C. & TAGOH, H. 2010. Aberrant expression of CD19 in AML with t(8;21) involves a poised chromatin structure and PAX5. *Oncogene,* 29**,** 2927-37.

WANG, H., WANG, L., ERDJUMENT-BROMAGE, H., VIDAL, M., TEMPST, P., JONES, R. S. & ZHANG, Y. 2004a. Role of histone H2A ubiquitination in Polycomb silencing. *Nature,* 431**,** 873-8.

WANG, H., ZHAI, L., XU, J., JOO, H. Y., JACKSON, S., ERDJUMENT-BROMAGE, H., TEMPST, P., XIONG, Y. & ZHANG, Y. 2006. Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol Cell,* 22**,** 383-94.

WANG, J., SUN, Q., MORITA, Y., JIANG, H., GROSS, A., LECHEL, A., HILDNER, K., GUACHALLA, L. M., GOMPF, A., HARTMANN, D., SCHAMBACH, A., WUESTEFELD, T., DAUCH, D., SCHREZENMEIER, H., HOFMANN, W. K., NAKAUCHI, H., JU, Z., KESTLER, H. A., ZENDER, L. & RUDOLPH, K. L. 2012. A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage. *Cell,* 148**,** 1001-14.

WANG, J. H., NICHOGIANNOPOULOU, A., WU, L., SUN, L., SHARPE, A. H., BIGBY, M. & GEORGOPOULOS, K. 1996a. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity,* 5**,** 537-49.

WANG, L., GURAL, A., SUN, X. J., ZHAO, X., PERNA, F., HUANG, G., HATLEN, M. A., VU, L., LIU, F., XU, H., ASAI, T., DEBLASIO, T., MENENDEZ, S., VOZA, F., JIANG, Y., COLE, P. A., ZHANG, J., MELNICK, A., ROEDER, R. G. & NIMER, S. D. 2011a. The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation. *Science,* 333**,** 765-9.

WANG, Q., STACY, T., BINDER, M., MARIN-PADILLA, M., SHARPE, A. H. & SPECK, N. A. 1996b. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A,* 93**,** 3444-9.

WANG, Q., STACY, T., MILLER, J. D., LEWIS, A. F., GU, T. L., HUANG, X., BUSHWELLER, J. H., BORIES, J. C., ALT, F. W., RYAN, G., LIU, P. P., WYNSHAW-BORIS, A., BINDER, M., MARIN-PADILLA, M., SHARPE, A. H. & SPECK, N. A. 1996c. The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. *Cell,* 87**,** 697-708.

WANG, X., BAI, L., BRYANT, G. O. & PTASHNE, M. 2011b. Nucleosomes and the accessibility problem. *Trends Genet,* 27**,** 487-92.

WANG, Y., WYSOCKA, J., SAYEGH, J., LEE, Y. H., PERLIN, J. R., LEONELLI, L., SONBUCHNER, L. S., MCDONALD, C. H., COOK, R. G., DOU, Y., ROEDER, R. G., CLARKE, S., STALLCUP, M. R., ALLIS, C. D. & COONROD, S. A. 2004b. Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science,* 306**,** 279-83.

WANG, Y. Y., ZHAO, L. J., WU, C. F., LIU, P., SHI, L., LIANG, Y., XIONG, S. M., MI, J. Q., CHEN, Z., REN, R. & CHEN, S. J. 2011c. C-KIT mutation

cooperates with full-length AML1-ETO to induce acute myeloid leukemia in mice. *Proc Natl Acad Sci U S A,* 108**,** 2450-5.

WANG, Y. Y., ZHOU, G. B., YIN, T., CHEN, B., SHI, J. Y., LIANG, W. X., JIN, X. L., YOU, J. H., YANG, G., SHEN, Z. X., CHEN, J., XIONG, S. M., CHEN, G. Q., XU, F., LIU, Y. W., CHEN, Z. & CHEN, S. J. 2005. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. *Proc Natl Acad Sci U S A,* 102**,** 1104-9.

WANG, Z., ZANG, C., ROSENFELD, J. A., SCHONES, D. E., BARSKI, A., CUDDAPAH, S., CUI, K., ROH, T. Y., PENG, W., ZHANG, M. Q. & ZHAO, K. 2008. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet,* 40**,** 897-903.

WATSON, A. D., EDMONDSON, D. G., BONE, J. R., MUKAI, Y., YU, Y., DU, W., STILLMAN, D. J. & ROTH, S. Y. 2000. Ssn6-Tup1 interacts with class I histone deacetylases required for repression. *Genes Dev,* 14**,** 2737-44.

WEERKAMP, F., BAERT, M. R. M., NABER, B. A. E., KOSTER, E. E. L., DE HAAS, E. F. E., ATKURI, K. R., VAN DONGEN, J. J. M., HERZENBERG, L. A. & STAAL, F. J. T. 2006. Wnt signaling in the thymus is regulated by differential expression of intracellular signaling molecules. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 3322-3326.

WEI, L., VAHEDI, G., SUN, H. W., WATFORD, W. T., TAKATORI, H., RAMOS, H. L., TAKAHASHI, H., LIANG, J., GUTIERREZ-CRUZ, G., ZANG, C., PENG, W., O'SHEA, J. J. & KANNO, Y. 2010. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity,* 32**,** 840-51.

WEI, Y., CHEN, Y. H., LI, L. Y., LANG, J., YEH, S. P., SHI, B., YANG, C. C., YANG, J. Y., LIN, C. Y., LAI, C. C. & HUNG, M. C. 2011. CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. *Nat Cell Biol,* 13**,** 87-94.

WEISHAUPT, H., SIGVARDSSON, M. & ATTEMA, J. L. 2010. Epigenetic chromatin states uniquely define the developmental plasticity of murine hematopoietic stem cells. *Blood,* 115**,** 247-56.

WHITCOMB, S. J., FIERZ, B., MCGINTY, R. K., HOLT, M., ITO, T., MUIR, T. W. & ALLIS, C. D. 2012. Histone Monoubiquitylation Position Determines Specificity and Direction of Enzymatic Cross-talk with Histone Methyltransferases Dot1L and PRC2. *J Biol Chem,* 287**,** 23718-25.

WIEMELS, J. L., XIAO, Z., BUFFLER, P. A., MAIA, A. T., MA, X., DICKS, B. M., SMITH, M. T., ZHANG, L., FEUSNER, J., WIENCKE, J., PRITCHARD-JONES, K., KEMPSKI, H. & GREAVES, M. 2002. In utero origin of

t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. *Blood,* 99**,** 3801-5.

WILLIAMS, A. B., NGUYEN, B., LI, L., BROWN, P., LEVIS, M., LEAHY, D. & SMALL, D. 2012. Mutations of FLT3/ITD confer resistance to multiple tyrosine kinase inhibitors. *Leukemia*.

WILSON, A., MURPHY, M. J., OSKARSSON, T., KALOULIS, K., BETTESS, M. D., OSER, G. M., PASCHE, A. C., KNABENHANS, C., MACDONALD, H. R. & TRUMPP, A. 2004. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev,* 18**,** 2747-63.

WOO, C. J., KHARCHENKO, P. V., DAHERON, L., PARK, P. J. & KINGSTON, R. E. 2010. A region of the human HOXD cluster that confers polycomb-group responsiveness. *Cell,* 140**,** 99-110.

WOODCOCK, C. L., SKOULTCHI, A. I. & FAN, Y. 2006. Role of linker histone in chromatin structure and function: H1 stoichiometry and nucleosome repeat length. *Chromosome Res,* 14**,** 17-25.

WU, H., LIU, X., JAENISCH, R. & LODISH, H. F. 1995. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell,* 83**,** 59-67.

WU, J., SUKA, N., CARLSON, M. & GRUNSTEIN, M. 2001. TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. *Mol Cell,* 7**,** 117-26.

XU, R. H., SAMPSELL-BARRON, T. L., GU, F., ROOT, S., PECK, R. M., PAN, G., YU, J., ANTOSIEWICZ-BOURGET, J., TIAN, S., STEWART, R. & THOMSON, J. A. 2008. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell,* 3**,** 196-206.

XU, Y., BANERJEE, D., HUELSKEN, J., BIRCHMEIER, W. & SEN, J. M. 2003. Deletion of beta-catenin impairs T cell development. *Nat Immunol,* 4**,** 1177-82.

YAMANE, K., TOUMAZOU, C., TSUKADA, Y., ERDJUMENT-BROMAGE, H., TEMPST, P., WONG, J. & ZHANG, Y. 2006. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell,* 125**,** 483-95.

YANG, L., BRYDER, D., ADOLFSSON, J., NYGREN, J., MANSSON, R., SIGVARDSSON, M. & JACOBSEN, S. E. 2005. Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood,* 105**,** 2717-23.

YAP, K. L., LI, S., MUNOZ-CABELLO, A. M., RAGUZ, S., ZENG, L., MUJTABA, S., GIL, J., WALSH, M. J. & 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**,** 662-74.

YASUDA, T., HAYAKAWA, F., KURAHASHI, S., SUGIMOTO, K., MINAMI, Y., TOMITA, A. & NAOE, T. 2012. B cell receptor-ERK1/2 signal cancels PAX5-dependent repression of BLIMP1 through PAX5 phosphorylation: a mechanism of antigen-triggering plasma cell differentiation. *J Immunol,* 188**,** 6127-34.

YE, M., ERMAKOVA, O. & GRAF, T. 2005. PU.1 is not strictly required for B cell development and its absence induces a B-2 to B-1 cell switch. *J Exp Med,* 202**,** 1411-22.

YERGEAU, D. A., HETHERINGTON, C. J., WANG, Q., ZHANG, P., SHARPE, A. H., BINDER, M., MARIN-PADILLA, M., TENEN, D. G., SPECK, N. A. & ZHANG, D. E. 1997. Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nat Genet,* 15**,** 303-6.

YIE, J., SENGER, K. & THANOS, D. 1999. Mechanism by which the IFN-beta enhanceosome activates transcription. *Proc Natl Acad Sci U S A,* 96**,** 13108-13.

YOKOTA, Y., MANSOURI, A., MORI, S., SUGAWARA, S., ADACHI, S., NISHIKAWA, S. & GRUSS, P. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature,* 397**,** 702-6.

YOON, M. J., KOO, B. K., SONG, R., JEONG, H. W., SHIN, J., KIM, Y. W., KONG, Y. Y. & SUH, P. G. 2008. Mind bomb-1 is essential for intraembryonic hematopoiesis in the aortic endothelium and the subaortic patches. *Mol Cell Biol,* 28**,** 4794-804.

YOSHIDA, T., NG, S. Y., ZUNIGA-PFLUCKER, J. C. & GEORGOPOULOS, K. 2006. Early hematopoietic lineage restrictions directed by Ikaros. *Nat Immunol,* 7**,** 382-91.

YOUNG, R. A. 1991. RNA polymerase II. *Annu Rev Biochem,* 60**,** 689-715.

YU, M., MAZOR, T., HUANG, H., HUANG, H. T., KATHREIN, K. L., WOO, A. J., CHOUINARD, C. R., LABADORF, A., AKIE, T. E., MORAN, T. B., XIE, H., ZACHAREK, S., TANIUCHI, I., ROEDER, R. G., KIM, C. F., ZON, L. I., FRAENKEL, E. & CANTOR, A. B. 2012. Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. *Mol Cell,* 45**,** 330-43.

YU, X., LI, P., ROEDER, R. G. & WANG, Z. 2001. Inhibition of androgen receptor-mediated transcription by amino-terminal enhancer of split. *Mol Cell Biol,* 21**,** 4614-25.

YUAN, Y., ZHOU, L., MIYAMOTO, T., IWASAKI, H., HARAKAWA, N., HETHERINGTON, C. J., BUREL, S. A., LAGASSE, E., WEISSMAN, I. L., AKASHI, K. & ZHANG, D. E. 2001. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. *Proc Natl Acad Sci U S A,* 98**,** 10398-403.

YUN, M., WU, J., WORKMAN, J. L. & LI, B. 2011. Readers of histone modifications. *Cell Res,* 21**,** 564-78.

ZAMAN, Z., ANSARI, A. Z., KOH, S. S., YOUNG, R. & PTASHNE, M. 2001. Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression. *Proc Natl Acad Sci U S A,* 98**,** 2550-4.

ZARET, K. S. & CARROLL, J. S. 2011. Pioneer transcription factors: establishing competence for gene expression. *Genes Dev,* 25**,** 2227-41.

ZENG, L., ZHANG, Q., LI, S., PLOTNIKOV, A. N., WALSH, M. J. & ZHOU, M. M. 2010. Mechanism and regulation of acetylated histone binding by the tandem PHD finger of DPF3b. *Nature,* 466**,** 258-62.

ZHANG, D. E., ZHANG, P., WANG, N. D., HETHERINGTON, C. J., DARLINGTON, G. J. & TENEN, D. G. 1997. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A,* 94**,** 569-74.

ZHANG, H., ROBERTS, D. N. & CAIRNS, B. R. 2005. Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell,* 123**,** 219-31.

ZHANG, J., KALKUM, M., YAMAMURA, S., CHAIT, B. T. & ROEDER, R. G. 2004a. E protein silencing by the leukemogenic AML1-ETO fusion protein. *Science,* 305**,** 1286-9.

ZHANG, J., NIU, C., YE, L., HUANG, H., HE, X., TONG, W. G., ROSS, J., HAUG, J., JOHNSON, T., FENG, J. Q., HARRIS, S., WIEDEMANN, L. M., MISHINA, Y. & LI, L. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature,* 425**,** 836-41.

ZHANG, P., BEHRE, G., PAN, J., IWAMA, A., WARA-ASWAPATI, N., RADOMSKA, H. S., AURON, P. E., TENEN, D. G. & SUN, Z. 1999. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc Natl Acad Sci U S A,* 96**,** 8705-10.

ZHANG, P., IWASAKI-ARAI, J., IWASAKI, H., FENYUS, M. L., DAYARAM, T., OWENS, B. M., SHIGEMATSU, H., LEVANTINI, E., HUETTNER, C. S., LEKSTROM-HIMES, J. A., AKASHI, K. & TENEN, D. G. 2004b. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity,* 21**,** 853-63.

ZHANG, P., ZHANG, X., IWAMA, A., YU, C., SMITH, K. A., MUELLER, B. U., NARRAVULA, S., TORBETT, B. E., ORKIN, S. H. & TENEN, D. G. 2000. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood,* 96**,** 2641-8.

ZHANG, Y. 2003. Transcriptional regulation by histone ubiquitination and deubiquitination. *Genes Dev,* 17**,** 2733-40.

ZHANG, Y., AND REINBERG, D. 2006. Transcription regulation by histone methylation:interplay between differnt covalent modifications of the core histone tails. *genes dev,* 15**,** 2343-2360

ZHANG, Y. & REINBERG, D. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev,* 15**,** 2343-60.

ZHAO, J., SUN, B. K., ERWIN, J. A., SONG, J. J. & LEE, J. T. 2008. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science,* 322**,** 750-6.

ZHAO, J., XU, Y., ZONG, Y., ZHANG, S., SONG, Y., YU, K., LI, Z., JI, Y., QIU, Y. & CHEN, F. 2011. Inhibition of Stat3 expression induces apoptosis and suppresses proliferation in human leukemia HL-60 cells. *Hematology,* 16**,** 232-5.

ZHAO, X. D., HAN, X., CHEW, J. L., LIU, J., CHIU, K. P., CHOO, A., ORLOV, Y. L., SUNG, W. K., SHAHAB, A., KUZNETSOV, V. A., BOURQUE, G., OH, S., RUAN, Y., NG, H. H. & WEI, C. L. 2007. Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. *Cell Stem Cell,* 1**,** 286-98.

ZHENG, X., OANCEA, C., HENSCHLER, R. & RUTHARDT, M. 2009. Cooperation between constitutively activated c-Kit signaling and leukemogenic transcription factors in the determination of the leukemic phenotype in murine hematopoietic stem cells. *Int J Oncol,* 34**,** 1521-31.

ZHONG, S., JANSEN, C., SHE, Q. B., GOTO, H., INAGAKI, M., BODE, A. M., MA, W. Y. & DONG, Z. 2001a. Ultraviolet B-induced phosphorylation of histone H3 at serine 28 is mediated by MSK1. *J Biol Chem,* 276**,** 33213-9.

ZHONG, S., ZHANG, Y., JANSEN, C., GOTO, H., INAGAKI, M. & DONG, Z. 2001b. MAP kinases mediate UVB-induced phosphorylation of histone H3 at serine 28. *J Biol Chem,* 276**,** 12932-7.

ZHONG, S. P., MA, W. Y. & DONG, Z. 2000. ERKs and p38 kinases mediate ultraviolet B-induced phosphorylation of histone H3 at serine 10. *J Biol Chem,* 275**,** 20980-4.

ZHU, B., ZHENG, Y., PHAM, A. D., MANDAL, S. S., ERDJUMENT-BROMAGE, H., TEMPST, P. & REINBERG, D. 2005. Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation. *Mol Cell,* 20**,** 601-11.

ZINK, B. & PARO, R. 1989. In vivo binding pattern of a trans-regulator of homoeotic genes in Drosophila melanogaster. *Nature,* 337**,** 468-71.