Runx1 is Required for Erythroid Development

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Runx1 is benodigd voor ertroïde ontwikkeling

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Voor mijn ouders,
Lobke en Naud

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Chapter 1

Introduction

Basal transcription

The blueprint for life is encoded in the DNA (deoxyribonucleic acid) of all animals. DNA is a polymer strain of four different bases of two different types; the purines adenosine (A) and guanine (G) and the pyrimidines thymine (T) and cytosine (C). Every cell in our body, except germline cells, immune cells and erythrocytes, has the same set of DNA. The DNA contains genes that can be transcribed to messenger ribonucleic acid (mRNA) which serves as the template to be translated to protein. Proteins (and RNAs) carry out the biochemical processes in the cell like metabolism, signal transduction and DNA transcription. DNA in the mammalian cell is about two meter long and has to be compressed in a small nucleus that is about 5 micrometre wide. In order to fit all the DNA into this tiny space it is wrapped around proteins called histones. Around 200 base-pairs are wrapped around eight histones which are organised in a structure called a nucleosome creating a “beads on a string” conformation. Although the wrapping of DNA around nucleosomes condenses the DNA about seven times this is not sufficient to explain the compact structure of the genome. Multiple nucleosomes create chromatin that further compacts the DNA and forms the chromatid.

Chromatin can be in a closed or an open conformation and switch between these conformations. This is called chromatin remodelling. Genes in the closed conformation (called heterochromatin) are not transcribed, and genes that are active are in open conformation (called euchromatin). Heterochromatin is also associated with gene poor regions like telomeres. Also is the DNA of heterochromatin often methylated and are the histone modifications different than euchromatin.

The specific function of a cell is determined by set of genes that are transcribed; a large number of these are expressed in all cells to carry out general functions and a much smaller number that are cell type specific and code for the specific function of the cell. There are over 25000 genes in the mammalian genome and they are either transcribed or repressed.

The transcription of DNA to RNA is facilitated by RNA polymerases. Mammals have three types called RNApol I, II and III. RNApol II is responsible for the transcription of almost all protein coding genes. RNApol I and III transcribe ribosomal RNA and small nuclear RNA. RNA pol II is part of a large protein complex called TFII (transcription factor for RNA pol II) which consists of several subunits indicated from A to H. These transcription factors are called “general” transcription factors because they are important for the transcription of almost all protein coding genes. A large part of the TFII transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) are part of the transcription initiation complex that positions RNA pol II over the transcription start site (TSS). TFIIA binds to TFIID and aids this domain in binding to the TATA box present in many promoters. TFIIB serves as a bridge between TFIID and RNA pol II. Later TFIIE comes in to the complex and recruits TFIIH that is a DNA helicase and breaks the hydrogen bonds between the two DNA strands. TFIIH also catalyzes phosphorylation of the RNA pol II C-terminal tail that is needed for transcription

elongation. TFIIF is needed to stabilise the binding of RNA pol II to the whole complex. When the transcription initiation complex together with RNA pol II is loaded at the TSS the DNA present is partly melted and this is called transcription initiation. After this first initiation the gene can be re-initiated without the whole complex to be

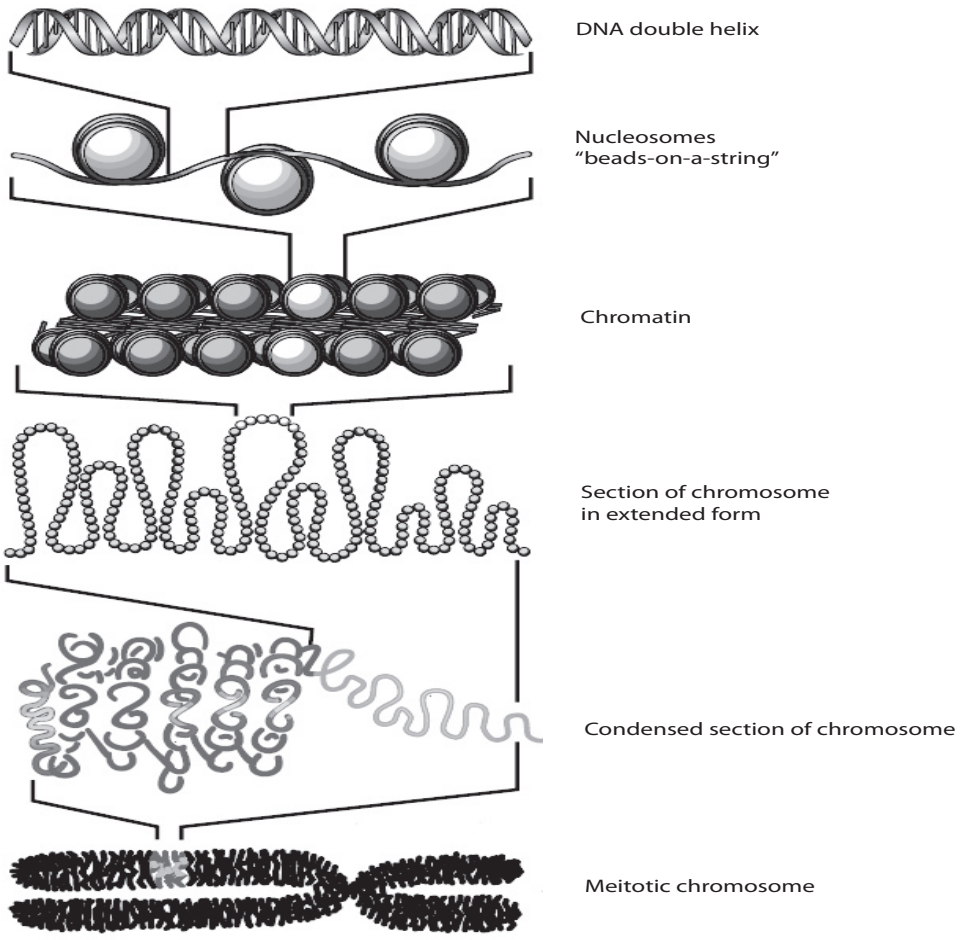

Figure 1: **Folding of DNA in eukaryotic cell.**
The double helix of DNA is wrapped around histones and form nucleosomes. Nucleosomes form chromatin and is condensed further in the nucleus forming chromosomes. Adapted from Gary Felsenfeld and Mark Groudine

assembled again[1]. When initiation has taken place the RNA pol II will move along the DNA in a process called elongation. Elongation consists of two steps with the first step phosphorylated RNA pol II that moves along the DNA strand and gets a stable grip. Then this movement is paused until RNA pol II is hyperphosphorylated and

elongation continues through the whole gene[2]. When a gene is fully transcribed RNA pol II disassociates from the DNA and the transcription is terminated. RNA synthesis by RNA pol I and III is terminated by recognition of a specific sequence called the terminator. The process of termination is poorly understood in RNA pol II transcription, but recent studies show a link to the initiation complex via TFIIB[3]. Another poorly understood process in transcription by RNA pol II is stalling. In this process RNA pol II stalls after having transcribed a fragment of the RNA and then falls of the DNA. The reason why stalling exists in the eukaryotic cell is unclear but research suggests that it is partly a mechanism for regulating transcription of developmental genes[4-5].

It is thought that this general TFII complex is recruited to genes by tissue specific and non-specific transcription factors. The basal transcription process alone is not sufficient for the process of transcription *in vivo*, but is dependent on other proteins called transcription factors (TF). TFs can bind promoters of genes that are located upstream of a gene and can be of variable size. Promoters are DNA elements of approximately 200 bp directly upstream of the transcription initiation site but can also extend into the gene. TFs can also bind enhancers/silencers and locus control regions; these are DNA elements that can act over very large distances, sometimes even more that 1Mb[6-8], on the transcription of genes. It is thought that these elements interact with the transcriptional start site via DNA looping and bring in factors to the transcriptional start transcription. These factors can influence transcription by e.g. modifying histones or start elongation.

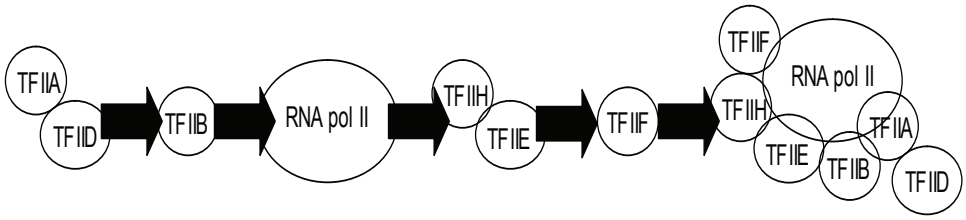

Figure 2: **Complex forming RNA pol II initiation complex.**
See text for explanation.

Development and differentiation

Animal life starts out with one omnipotent cell that is created by fertilization of an egg cell. For this cell to develop into an organism this single cell needs to proliferate to create more cells and these new cells need to differentiate into specialised cells and migrate to the proper location in the embryo. For a cell to differentiate from an embryonic stem (ES) cell to a specific tissue several intermediate cell stages are necessary. During this development the TF programmes change to proceed in the differentiation. An example is the TF Oct-4 that is expressed in ES cells and binds to the DNA sequence ATGCAAAT. Loss or altering the expression levels of Oct-4 in stem cells results is a loss of pluripotency and triggers differentiation[9-11]. When the

ES-cell for example starts to differentiate into the haematopoietic direction the TFs SCL and Gata2 start to be expressed. These TF are important for the generation of the definitive haematopoietic stem cell (HSC)[12-13]. The TF Gata2 is repressed when the HSC differentiates into the direction of erythrocytes and simultaneously Gata1 starts to express (reviewed in [14]).
The cell “knows” which transcription factors are necessary by activation of there genes by other transcription factors and extracellular signalling. An important group of proteins that regulate the specific pattern of gene expression in different cell types are the “tissue specific” TFs. These TFs bind directly or indirectly to the DNA and activate or repress transcription of specific genes.

Histone modifications

The nucleosome consists of five types of histones called H1, H2A, H2B, H3 en H4[15-16]. Histones can be modified at there N-terminal tails by various biochemical modifications like acetylation, methylation, phosphorylation, ubiquitination and sumolation (reviewed in[17]). In addition to these modifications also specialized histones exist like H2AZ and H2AX. These histones have a variation in there sequences and for example have a role in DNA repair and transcription[18]. Modifications of histones can be of a repressive or activative nature. The most frequent modifications are acetylation and methylation. Acetylation of lysine residues in the histone N-terminal tails is performed by Histone Acetyl Transferases (HAT) and is largely associated with active chromatin and mostly located at the transcriptional startsite of a gene[19-21]. The acetylation of histones is reversible and is removed by Histone Deacetylases (HDACs). Acetylation marks on the histone tails are recognised by proteins with a bromodomain. This domain is present in a wide range of transcription factors such as acetyltransferases (e.g. p300), chromatin remodelling proteins (e.g.SWI/SNF) and general transcription factors (e.g.TAFII250)[22-23].
The methylation of histones is more complex because the lysine residues in the tails can be mono, di or tri-methylated. All but one of the known histone methyltransferases (HMT) contain a SET domain that catalyzes the methylation activity. Histone methylation is associated with both gene activation and repression. Enhancers, the promoter and the body of a gene are methylated in a specific way contributing to the regulation of transcription[24]. Lysine methylation marks are recognised by the chromodomain that is associated with chromatin remodelling proteins[25].
Recently a histone demethylase was discovered called Lysine Specific demethylase1 (LSD1)[26]. It was shown that this enzyme removes histone 3 lysine 4 (H3K4) mono- and di- methyl marks and possibly also histone 3 lysine 9 (H3K9) mono- and di- methylation[27]. H3K4 methylation is associated with active genes and this suggests that LSD1 functions as a repressor[24, 28]. H3K9 di- and tri-methylation is associated with repressed genes thus LSD1 could possibly also have an activation role, although H3K9 mono-methylation is associated with active genes[29-31]. Other histone demethylases were discovered later e.g. LSD2 and some members of the Jumonji family of proteins[32-33], which have been shown to demethylate lysine trimethyl marks[34]. LSD1 has been shown to be important for

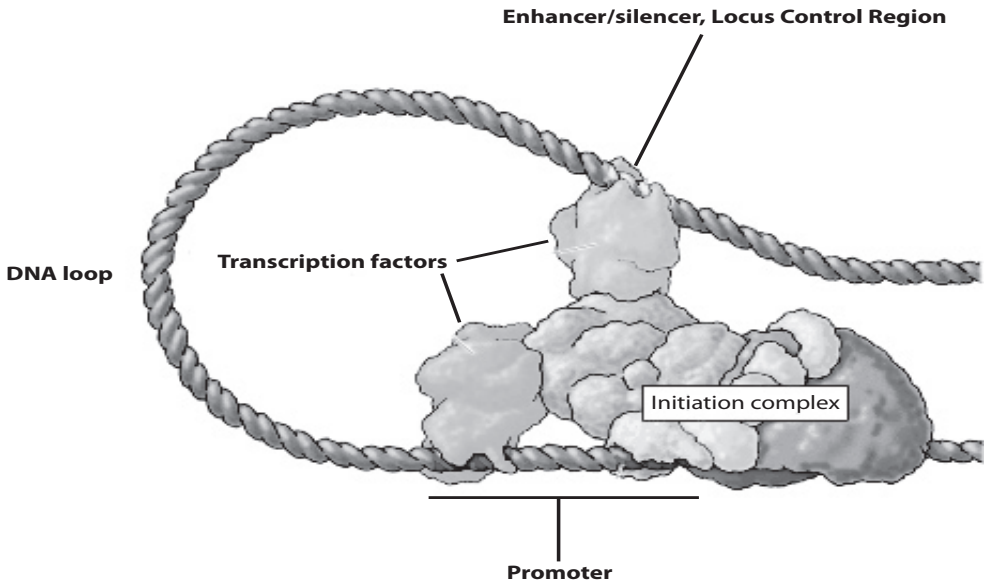

Figure 3: **Representation of DNA looping of enhancer to promoter of an RNA pol II transcribed gene.** Adapted from book: The cell; a molecular approach.

definitive erythroid development in mouse. It forms complexes in erythroid cells with important erythroid transcription factors like Gfi1b, TAL1 and Gata1[35-37]. Hence the methylation status of several erythropoietic genes has been suggested to be regulated by LSD1 but a specific role or function has not been assigned to LSD1 in erythropoiesis yet[37-38]. This is particularly relevant in the context of this thesis on definitive erythropoiesis.

Arginine residues in proteins can also be methylated by a different family of methyl transferases, the Protein Arginine Methyl Transferases (PRMT). This type of methylation is different from lysine methylation. Arginine methylation can occur in three fashions: 1) in a symmetrical dimethylated fashion with one methyl group placed on each of the terminal nitrogen atoms. 2) In an asymmetrical dimethylated fashion with two methyl groups placed on one of the two terminal nitrogen atoms. 3) In a monomethylated fashion with one of the terminal nitrogen atoms methylated. The arginine methylation has also been linked to histones and their modification. For example the histone 3 tail is methylated on a number of different arginine residues[39-40] and an arginine histone demethylase has been reported[41].

Chromatin remodelling complexes

The eukaryotic DNA is composed of chromatin that in turn makes the DNA compact. This creates a problem for transcription, DNA repair and replication. Nucleosomes need to be relocated or removed from the DNA to expose TF binding sites or start repair/replication process. For the chromatin to be remodelled between open and

closed conformation the cell uses chromatin remodelling complexes. Examples of these complexes in mouse are the BAF complex (SWI/SNF in yeast), INO80, NURD and ISWI, which are important for execution of a number of different developmental programs(reviewed in [42]). These complexes utilise ATP to remodel chromatin and do this with a specific subunit like BRG1 for BAF and SNF2L for ISWI. All these complexes bind nucleosomes and have domain that bind specific transcription factors, e.g. Oct-4 or epigenetic marks[43]. Every chromatin remodelling complex contains Actin Related Proteins (ARP) that are homologues to Actin, a spatial organizer in cells, and are thought to be needed to create 3D DNA structures. Mutations in these subunits result in a wide range of phenotypes[44-46]

Haematopoiesis

The production of all haematopoietic cells such as T-cells, B-cells megakaryocytes and erythrocytes is called haematopoiesis. All these cell types are derived from a common stem cell called the haematopoietic stem cell (HSC). The HSC is believed to be derived from a common mesodermal precursor for endothelial cells and haematopoietic cells called the hemangioblast. These hemangioblast cells express the mesodermal markers Brachyury and Fetal Liver Kinase 1 (FLK1)[47-48]. The hemangioblast gives rise to hemogenic endothelium regulated in part by SCL/TAL1[48-49]. In mammals there are two waves of haematopoiesis derived from this endothelium called the primitive haematopoiesis regulated in part by the Wnt signalling pathway, and the definitive haematopoiesis regulated in part by the Notch signalling pathway. These processes include a number of TFs some of which are essential for one stage, e.g. Runx1 is essential for definitive haematopoiesis[50-52]. The haematopoietic system is widely used as a model system for cellular and molecular mechanisms of differentiation and has provided many basic insights into gene regulatory processes that also apply to other cell systems. In addition it is an important system to study in the context of disease, such as leukaemias, anaemias and the hemoglobinopathies and provide the molecular basis for the development of novel therapies to treat these disorders.

Primitive Haematopoiesis

Primitive haematopoiesis or primitive erythropoiesis occurs in the yolk sac in blood islands at day 7.5 days post coitum (dpc) from hemangioblast cells that migrated there from the primitive streak[53-54]. These primitive HSC do not persist through the development of the embryo and are absent in the adult mouse. The primitive HSC's do not have the ability to selfrenew and colonize bone marrow. They give rise to primitive erythrocytes and disappear around day 9dpc, but primitive erythrocytes persist longer in the bloodstream[55]. They co-exist for a short while with erythrocytes from definitive HSC that will replace them fully later in development. Primitive erythrocytes are larger than there definitive counterparts and express the embryonic globin genes ζ, εy and βh1[56-57].

Definitive haematopoiesis

Transplantation studies have shown that there are multiple sites of definitive HSC generation. The first site is the para-aortic-splanchnopleura that's develops in the aorta-gonad-mesonephros (AGM) region. Definitive HSC derive on the ventral side of the dorsal aorta where they are generated de novo at day 10dpc from the endothelium as clusters[58]. Definitive HSCs can differentiate into every haematopoietic linage, they keep this potential throughout adult life and have the capacity to self-renew. The suggestion was made that there are secondary sites of definitive HSC generation based on the quantification of the number of HSCs generated in the AGM region and studies done with Runx1 and GATA1 haploinsufficient mice[59-61]. This resulted in the discovery that the mouse and human placenta also are a site of HSC generation or amplification[62-64]. The yolk sac has also been mentioned as a potential site for definitive HSC generation[65], however the evidence to support this flawed because when these cells were injected directly into adult mice they were incapable of engraftment.
Several TFs have been shown to be essential for the development of HSC including the Gata transcription factor family and the Core Binding Factor family, which are the focus of the work described in this thesis.

Erythropoiesis

Erythropoiesis is the generation of red blood cells or erythrocytes via several different cell stages from the HSC. They transport of oxygen throughout the body and contain haemoglobin, the protein that binds oxygen in the lungs and releases it in the tissues. Erythrocytes have a lifespan of 120 days in humans and make up about 45% of the blood volume. They are generated in large numbers during the primitive and definitive stages of erythropoiesis and are often described as EryP (P for primitive) and EryD (D for definitive). The differentiation to the mature erythrocyte starts with the self renewing HSC which gives rise to a multipotent progenitor (MPP). This cell is thought to have lost the ability to selfrenew but retain the same differentiation potential. In the direction of erythroid development this cell develops into the common myeloid progenitor (CMP) splitting of from the common lymphoid progenitor (CLP) that gives rise to B- and T-cells. The CMP gives rise to the megakaryocyte-erythroid progenitor (MEP), splitting off from all other myeloid cell lineages. The MEP is committed to either the megakaryocyte lineage or the erythrocyte linage. The next cell type in erythrocyte differentiation is the burst forming units-erythroid cell (BFU-E) followed by the colony forming unit-erythroid (CFU-E). With the differentiation into the proerythroblast "terminal erythroid differentiation" is initiated, i.e, the immature precursors become erythrocytes. From the proerythroblast the basophilic erythroblast is formed and then the polychromatic erythroblast. The next stage is the orthochromatic erythroblast that enucleates forming the reticulocyte. The shape of the cell changes into a biconcave and the mature erythrocyte is formed.

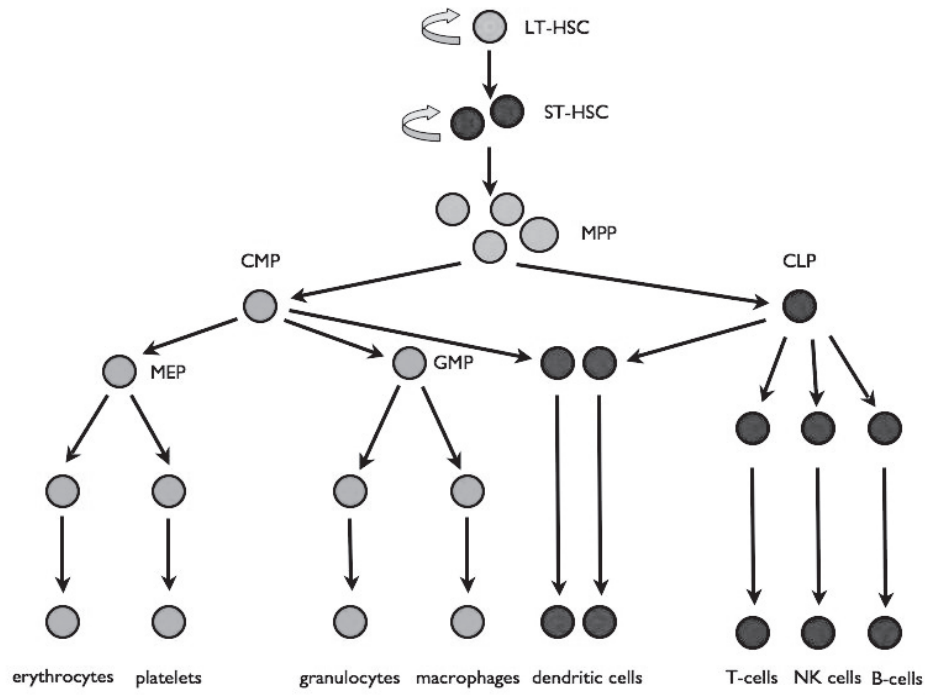

Figure 4: **Definitive mouse haematopoiesis.**
LT-HSC (long term haematopoietic stem cell), ST-HSC (short term haematopoietic stem cell), MPP (multipotent progenitor), CMP (common myeloid progenitor), MEP (megakaryocyte erythrocyte progenitor), GMP (granulocyte macrophage progenitor), CLP (common lymphoid progenitor). Adapted from Robert Margolin and Yusra Abidi

Examples of transcription factors known to be involved in erythropoiesis are Gata1, EKLF or KLF1, LDB1, LMO2, LMO4, p45/NF-E2 and TAL1/SCL [13, 66-71]. Gata1, TAL1/SCL and EKLF have been shown to be important in primitive and definitive erythropoiesis[70, 72-73]. Next to these essential transcription factors there are a number co-regulators essential for erythropoiesis. For example FOG1 which binds to the Gata proteins help to recruit important transcriptional complexes[74]. Similarly p300 a HAT which binds Gata1, TAL1/SCL and EKLF has been implicated in haematopoietic development[75-78] and BRG1 which is part of the BAF complex has been shown to bind to important erythroid genes. BRG1 hypomorphic mice also have a block in erythroid development [76-77, 79].

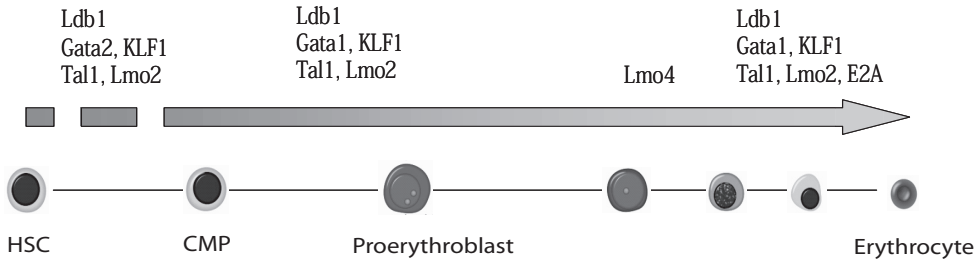

Figure 5: **Definitive mouse erythropoiesis.**
Differentiation of erythrocytes from the HSC (haematopoietic stem cells) via the CMP (common myeloid progenitor) to the proerythroblast. On top examples of transcription factors important in erythropoiesis.

Transcription factors involved in haematopoiesis

The generation of HSC and the process of differentiation to all the blood cells in the body is dependent on a large number of transcription factors and the process of unravelling the regulatory network of TFs involved in haematopoiesis is still in an early stage. With new technologies like very sensitive mass spectrometry and ”next generation” sequencing this process is speeding up rapidly to enhance our understanding of haematopoiesis.
Here I will focus on a small number of factors, namely SCL/Tal1, Gata2 and Runx1 known to be essential for the emergence of the early haematopoietic cells and Gata1, Pu.1 and GFI1b known to be essential for the differentiation towards myeloid/ erythroid blood cells.

Core binding factors

The core binding factors were first discovered as proteins binding to the core sequence of the Moloney virus enhancer[80]. The mammalian Core Binding Factor (CBF) family consists of the α subunits Runx1, 2 and 3 and their common partner the β subunit CBFβ. The α subunits are DNA binding proteins via there Runt domain to the consensus [TC]G[TC]GGT[TC][81-82]. This binding to the DNA is enhanced by binding of CBFβ[83]. The Runt domain is homologous to the drosophila *runt* gene involved in segmentation. Other names for the core binding factor family are papyloma enhancer binding protein (PEBP)[84] and AML due to the discovery that Runx1 is often a target for translocation in acute myeloid leukemia (AML). The CBF family is conserved in many animal species from human to sea urchin. Drosophila has four α subunit genes called *runt, lozenge, CG34145* and *CG42267,* and two β subunit genes called *brother* and *big brother*. C.elegans contains one α subunit called *rnt-1* and one β subunit called *bro-1*. Human and mouse have three α subunits called *RUNX1*, *RUNX2* and *RUNX3* and one β subunit called *CBF*β.
The CBFs were discovered as frequent targets of chromosomal translocations in leukaemias[85-86]. Examples of core binding factor translocations fusion proteins

are CBFβ-SMMHC, AML1-EVI1, TAL1-AML1 and AML1-MTG16. The most frequently occurring translocation is the t8:21 that generates the chimaeric protein AML1-ETO. This is a fusion of almost the entire ETO protein with the DNA binding domain of Runx1 and creates a protein that binds to Runx1 target genes but can only repress these genes[87]. In human acute myeloid leukaemias (AML) this translocation is present in 10% of all cases. Runx1 and CBFβ are often targets of translocations in AML[88-90].
Patients with an AML1-ETO translocation often show a hypoplasia, a disruption of the erythroid and megakaryocytic differentiation[91]. This observation was also seen in mouse models expressing AML1-ETO fusion protein[92]. Contradictory to these results is that a different AML-ETO mouse model does not show the hypoplasia phenotype[93]. A zebrafish model with inducible expression of AML1-ETO showed a downregulation of Gata1 and SCL, two factors important for haematopoiesis and erythropoiesis[94]. In *in vitro* cell cultures of erythroid cell lines it has also been shown that AML1-ETO inhibits the differentiation of K562 cells, MEL cells and human primary erythroid cells[95-97].
A knock-out (KO) model of Runx2 resulted in a bone formation phenotype and Runx2 was shown to be essential for the maturation of osteoblasts[98-99]. It also targets the Indian Hedgehog pathway that is important for chondrocyte development[100]. Runx2 has not been linked to human cancers like its family member Runx1. Runx3 is expressed in many cell types e.g. epithelial cells, blood cells and neural cells. A knock-out (KO) model of Runx3 gave a wide variety of phenotypes[101]. The most dominant phenotype was seen in the gastric system. Runx3 was later shown to function as a tumour suppressor in gastric mucosa. It is also involved in T-cell development[102-103]. This thesis will focus on Runx1 and its function.

Runt related factor 1

Runx1 was first cloned as a t(8;21) translocation that occurs frequently in Acute Myeloid Leukemia (AML)[85, 104]. In addition to the runt domain Runx1 also contains a transactivation domain and multiple repressive domains. It can therefore act as an activator and a repressor. Runx1 and CBFβ have been shown to be important for haematopoiesis in several studies. Runx1 deficient mice do not develop definitive HSC and closer inspection showed a complete absence of haematopoietic clusters in the AGM region[105-106]. The embryos die between 12.5dpc and 13.5dpc and have haemorrhages in the central nervous system. The CBFβ knock-out mouse shows a similar phenotype as the Runx1 knock-out mouse which illustrates that the complex of these two TFs is important for proper gene regulation[107-108]. Nevertheless some definitive haematopoietic progenitors were found in the fetal liver, but these appear to be defective as they are not capable of engrafting irradiated mice by transplantation.
People suffering from Down syndrome have trisomy 21. The *runx1* gene in humans is located of chromosome 21 and they can have an overexpression of Runx1. These people have a 50% higher incidence of leukaemias and a possible role in this could be linked to the *runx1* gene[109]. Also the generation of the hemogenic endothelium

is affected in trisomy 21 ES cells[110].
After the emergence of the HSC Runx1 is important for megakaryocytic, B-cell and T-cell development. Upon Runx1 deletion in adult mice a megakaryocytic phenotype was seen[111]. The maturation of megakaryocytes was arrested and this phenotype was also seen in adult mice that were CBFβ hypermorphic[112]. In several other studies it has been shown that Runx1 has a role in the development of T-cells and B-cells of the adult mouse[113-114].

Runx1 post-translational modifications

Like histone proteins Runx1 can also be modified by phosphorylation, methylation and acetylation. These modifications can have an important role in the regulation of Runx1 function in the cell.
Phosphorylation of Runx1 occurs on serine and threonine residues which changes its biochemical properties. Phosphorylation of serine 246 and 266 by ERK disrupts the binding of Runx1 with the repressor protein mSIN3a[115]. Arginine methylation by PRMT1 at positions 206 and 210 also disrupts the binding of Runx1 with mSIN3a[116]. This methylation is tissue specific and occurs for example in the erythroid linage but not in the megakaryocytic lineage[117]. Acytelation of lysine residues 24 and 43 by p300 increases the ability of Runx1 to bind DNA[118].

Runx1 gene locus

All the three *runx* genes contain two promoters, a proximal and distal promoter[119-120]. The proximal promoter resides in the first intron of the genes. The *runx1* first intron is about 130kb and contains at least one enhancer needed for proper Runx1 expression[121-122]. There are many conserved sites in the first intron, which are thought to be regulatory regions but the intron probably contains even more regulatory sites[49, 122]. During development the proximal promoter of the *runx1* gene is mainly used during the onset of haematopoietic development. Transcription via the distal promoter increases during embryo development and is almost the only promoter transcribed in the adult mouse[123]. The *runx1* gene in the mouse embryo is activated by Notch, SCL/TAL1, LMO2 and LDB1[49, 52, 124-126][Mylona et al unpublished]. Later in development the expression is regulated by Runx1 itself, Gata1 and SCL/TAL1[122-126]

Runx1 binding partners

Runx1 binds both co-repressors and co-activators. Therefore it can act as a transcriptional repressor or activator. The role of Runx1 in the development and differentiation of the haematopoietic system can be split into two: one for the emergence of the HSC for which Runx1 is absolutely essential and one after the emergence of the HSC. Mouse knock-in studies where the repressor fusion protein AML1-ETO replaces Runx1 show that Runx1 mostly acts as an activator in the development of definitive HSC. After the HSC emerge its function seem to switch to

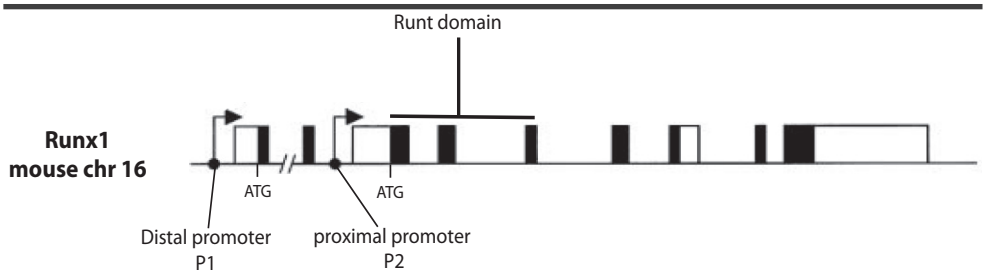

Figure 6: **Schematic representation of the structure of the Runx1 gene locus.** Promoters, transcription start sites (ATG) and exons (black blocks) shown. Runx1 gene locus contains two promotors. P1 or distal promoter and P2 or proximal promoter. Exons 3,4 and 5 encode runt domain of Runx1 protein.

being a repressor [93, 127-128]. How Runx1 alters its transcriptional role depends on the protein partners that cooperate with Runx1 in the different tissues.

Lim Domain Binding protein 1

Lim Domain Binding protein 1 (LDB1) is a homologue of the Drosophila CHIP protein that is thought to be involved in long range DNA-DNA interactions[129]. The mouse LDB1 knock-out model dies at 10.5dpc and displays a haematopoietic phenotype[130]. In the erythroid development LDB1 is suggested to be important for proper β-globin expression by organising the 3D chromatin structure of the locus[131-132]. Runx1 was found in a screen to identify protein partners of LDB1 in Mouse Erythroid Leukemic (MEL) cells [66], but its role in erythropoiesis is largely unknown. LDB1 binds DNA via GATA1 and TAL1 and mostly binds genes that are activated in the last steps of erythroid differentiation[133].

Groucho/TLE

All the Runx proteins contain a VWRPY amino acid sequence at the C-terminus. This domain recruits the repressor TLE, homologue of the *Drosophila* Groucho, to the Runx1 protein[134]. Deletion of this domain in mouse gives a T-cell phenotype[135-136]. Runx1 binds to the *cd4* silencer[103] in agreement with the observation that more CD4 positive T-cells are observed when Runx1 lacks the VWRPY domain. Runx3 also has a role in T-cell development, it regulates the *cd4* and *cd8* genes and binds to the *cd8* enhancer in mature T-cells[102, 137]. However it is unclear whether the VWRPY domain is obligatory for this function.

The TGFβ pathway

The TGFβ signalling pathway comprises a family of extracellular molecules (TGFβ) and receptors that regulate together with the Bone Morphogenetic proteins (BMP) the SMAD family of TFs. The Runx proteins have been shown to interact with some SMAD factors[138] and similar roles of the TGFβ pathway and Runx proteins

have been observed in bone formation and induction of IgA. These functions are performed by Runx2 and Runx3 respectively [138-140]. However even tough all Runx proteins bind to SMAD's no functional overlap has been found for TGFβ and Runx1.

The GATA transcription factor family

The GATA transcription factor family is essential for development of different tissues. The family consists of six members that are expressed in different tissues at different time points (reviewed in [141]) The proteins are zinc-finger proteins that bind DNA directly via the sequence (A/T)GATA(A/G) that also gave them their name.
Gata1 was the first of the family to be discovered as an specific binder to the beta-globin 3' enhancer[142]. Later Gata1 was shown to be essential for erythrocyte development but is also expressed in dendritic cells, megakaryocyte and eosinophil cell lineages[143-144]. Gata1 null erythrocytes arrest in the proerythroblast stage and fail to develop in mature erythrocytes. Runx1 has been shown to be a partner of Gata1 via direct binding in megakaryocytes and both Runx1 and Gata1 loss in adult mice results in a megakaryocytic phenotype[111, 114, 145-147]. Runx and Gata have also been linked to each other in transcriptional control in other cell types[148-149]. To date (excluding the study presented in this thesis) only the *cd41* gene has been linked to being regulated by both Gata1 and Runx1[112, 150-151]. In Drosophila the Runx1 and Gata1 orthologues, respectively lozenge and serpent, have also been shown to cooperate in the development of the haematopoietic system[152-153].

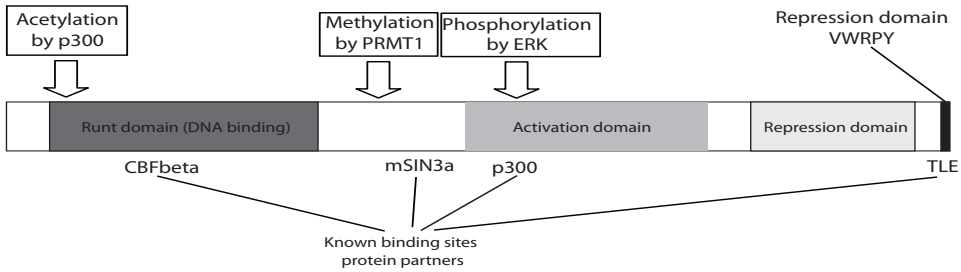

Figure 7: **Schematic representation of Runx1 protein.** The Runx1 protein is post translational modified on shown sites. Known protein partner binding sites show.

Cell cycle

Runx1 has also been linked to controlling the cell cycle. The first evidence for this was the observation that the fusion proteins CBFβ-SMMC and AML1-ETO slow down cell cycle progression in the G1 to S progression[154-155]. In several experimental systems it was shown that Runx1 stimulates progression of G1 to S phase[155-157]. These features seemed to be related to the observation that the cell cycle genes *p21*, *cdk4* and *cyclinD3* are target genes of Runx1[155, 158-159]. The level of Runx1 protein is regulated during the cell cycle via phosphorylation of serine 276 and 303 by CDK's and this mark is recognised by the Anaphase-Promoting Complex leading to the degradation of Runx1[160].

Aim of the project

Runx1 is known to be essential in the formation of the definitive haematopoietic stem cell, but it is not required for its maintenance[161]. Several studies have shown that Runx1 plays a role in the differentiation of B-cells, T-cells and megakaryocytes[111, 113-114]. Erythrocytes are generated together with megakaryocytes from the MEP and it is thought that Runx1 is a molecular switch between these two cell types (reviewed in [162]). This theory is supported by Runx1 conditional KO mice which have a more severe megakaryocytic phenotype in comparison to their erythroid phenotype. In addition the Runx1 protein levels drop when the MEP starts to differentiate towards the erythroid lineage[145, 163]. Evidence against this theory is the result that mice expressing the AML1-ETO fusion protein have a severe erythroid phenotype[92]. This is also seen in human suffering from acute myeloid leukaemia (AML) with this translocation[91]. In our lab Runx1 was identified to be part of a protein complex important for erythropoiesis called the LDB1 complex[66]. This complex contains proteins important for erythropoiesis like Gata1, LDB1, Tal1, ETO2 and CDK9.

This thesis describes a biochemical approach to understand the function of Runx1 in erythropoiesis. Chapter 2 describes how N-terminally tagged Runx1 cDNA with a Bio-V5-double tag is stably expressed in Mouse Erythroid Leukemic (MEL) cells. MEL cells represent the proerythroblast stage of erythroid differentiation and they can be induced to differentiate towards erythrocytes. Immunoprecipitations were performed and Runx1 protein partners were identified using mass spectrometry. This showed a number of protein partners that were known to be part of the LDB1 complex, but also two new partners LSD1 and Myef2. The new partner Myef2 mainly binds to Runx1 in the proerythroblast stage while LSD1 binds mostly in differentiating cells. The analysis of Runx1 genome wide binding shows that Runx1 binds erythroid specific genes in both non-differentiated and differentiated MEL cells. This chapter also shows that the genome wide binding pattern does not change dramatically during differentiation. By decreasing the expression of Runx1 in MEL cells using shRNA it was found that Runx1 functions as an activator and a repressor in MEL cells but that its function is diminished when MEL cell differentiate.

Myef2 was not known to have a role in erythropoiesis or haematopoiesis, but Chapter 2 describes that Myef2 represses important erythroid genes. The role of Myef2 in haematopoiesis is further confirmed using knock-down experiments in zebrafish (Chapter 3). Repression of erythroid genes by Myef2 is likely mediated via Runx1 in the proerythroblast stage (Chapter 2). During erythroid differentiation Runx1 regulates a significant smaller number of genes and in Chapter 4 evidence is shown that repression of part of these genes is likely to be mediated by LSD1. Enhanced binding of LSD1 to the DNA at Runx1 binding sites is observed after differentiation. This enhanced binding correlates with changes in the histone methylation of Runx1 repressed genes.

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Chapter 2

The role of the transcription factor Runx1 in erythropoiesis

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Abstract

It is known that Runx1 is an essential regulator to generate haematopoietic stem cells, but much less is known about its role in the downstream process of haematopoietic differentiation. In erythroid cells Runx1 was shown to be part of a large transcription factor complex together with LDB1, Gata1, Tal1 and Eto2[1]. By tagging Runx1 in erythroid cells we show here that Runx1 binds two repressor proteins LSD1 and Myef2. ChIP/seq analysis and microarray expression analysis were used to show that Runx1 binds approximately 18 thousand targets in erythroid cells. Functional analysis shows that Runx1 regulates these genes in part with the newly identified partner Myef2.

Introduction

The transcription factor Runx1 (AML1, CBFα2) is known to be important for the development of the haematopoietic system in mammals. It is part of a small family of core binding transcription factors that also consists of Runx2 (AML3, CBFα1) and Runx3 (AML2, CBFα3) and CBFβ. Runx1 was first discovered as a homologue of the Drosophila segmentation gene runt together with Runx2 and Runx3. The Runx1 protein binds directly to DNA via the consensus [TC]G[TC]GGT[TC] [2-3] and several studies have shown that Runx1 is important for the emergence of the haematopoietic stem cell (HSC). The Runx1 knock-out (KO) mouse does not develop the definitive haematopoietic system, and has minor defects in the primitive haematopoietic system[4-6]. The Runx proteins form a heterodimer with CBFβ that enhances the binding to DNA. This dimerization is important for the function of Runx1, which is confirmed by the CBFβ KO mouse also lacking definitive hematopoietic development[7-8].

How Runx1 functions after the emergence of the definitive HSC, and in particular in the erythroid compartment, is not well understood, although a conditional knock-out shows some defects in the differentiation of erythrocytes. In one model erythrocytes show a significantly higher number of Howell-Jolly bodies probably due to the hyposplenia. Another model showed an increase in the ratio of maturing myeloid to erythroid cells when compared to the controls[9-10]. A recent study has shown that Runx1 is also important in primitive erythropoiesis[11]. Defects were found in the morphology and Ter119 expression of the primitive erythrocytes. Finally the Runx1 homologues are also required for definitive erythropoiesis in non-mammalian vertebrates[12-13]. However none of these studies sheds much light on the molecular function of Runx1.

From studies using transgenic mice replacing the endogenous *runx1* gene in adults by a conditional Runx1-ETO fusion gene, a less severe phenotype was observed when compared to the mouse adult conditional knock-out of Runx1[9-10, 14]. The AML1-ETO fusion gene is a fusion of the Runx1 binding domain to almost the entire *eto* gene. This creates a protein that binds to Runx1 target genes but can only repress these genes. From this observation it appears that Runx1 functions mainly as a repressor in adult mice. From previous studies it is known that Runx1 forms a repressive complex with mSIN3a in hematopoietic stem and progenitor cells (HSPS)[15], but nothing is known about this complex at later stages.

We are interested in the function of Runx1 in adult erythropoiesis, because it was found to be present in a complex containing essential regulators of erythropoiesis like LDB1, GATA1 and TAL1[16-17]. Its protein partners and target genes were identified using mass spectrometry and ChIP-sequencing. A number of these Runx1 target genes are important for erythropoiesis and we show that Runx1 regulates these genes via Myef2, a previously unknown repressor important for erythropoiesis.

Materials and methods

Tagging Runx1 construct

A Nhe1 restriction site was cloned into the cDNA of the Runx1 distal promoter to replace the translation start site. The Bio-V5 double tag was ligated into the Nhe1 site to create N-terminally tagged Runx1 cDNA[18-20]. The tagged Runx1 cDNA was cloned into the Not1 site of the GATA1 minimal promoter[21-23].

Cell culture

Mouse Erythro-Leukaemia (MEL) cells were cultured in DMEM containing 10% fetal calf serum and penicillin/streptomycin. Addition of 2% dimethylsulfoxide (DMSO), was used to induce differentiation towards terminal erythrocytes. Cells were harvested after 4 days of differentiation.

Immunoprecipitations

N-terminally tagged Runx1 cDNA was stably expressed in MEL cells containing the bacterial biotin ligase BirA[24]. Nuclear extracts were made of MEL cells as described[24]. Immunoprecipitations (IP) were done as described [25]. Bio-V5-Runx1 Immunoprecipitations (IP) from nuclear extracts were preformed using V5 affinity agarose beads from Sigma[16]. Antibody IP's are described in the supplementary data S1. Washes were preformed using HENG150 0.3%NP40. IP's were preformed in the presence of benzonase endonuclease to exclude the identification of complex formed via DNA binding.

ChIP and ChIP-sequencing sample preparation

Chromatin Immunoprecipitations (ChIP) were performed using a sonication buffer as described[20]. Per ChIP $2x10^7$ and per ChIP-seq $10x10^7$ MEL cells were used. Antibodies used for ChIP are described in supplementary data S1.

shRNA in MEL cells

The TRC Mission human and mouse library from Sigma was used for shRNA mediated knock-down of proteins of interest. They were delivered to MEL cells via lentiviral infection. Virus was added to $0.5x10^6$ MEL cells and cultured for 48 hours. Puromycin was added and nuclear extracts and/or total RNA was harvested 48 hours later. For induced MEL cells DMSO was added to the medium together with the puromycin and cells were harvested 4 days later.

Bio-informatical analysis

The bio-informatical analysis of the microarray and ChIP-sequencing data were carried out as described in[19].

Results

Tagging Runx1 and generating stable MEL cell lines

The Runx1 protein was tagged with a Bio-V5-tag at the N-terminus by inserting the sequence coding for this tag at the 5'-end of the *runx1* cDNA starting from the initiation of translation site of the distal promoter (Fig 1A) and the cDNA was stably expressed in BirA MEL cells using a GATA1 expression vector[24]. Several clones were tested to avoid potential overexpression artefacts and clone 7 was chosen because it gave low Bio-V5-Runx1 expression close to the endogenous levels (Fig 1B). Clone 7 also did not show a difference in normal or induced tissue culture compared to non transfected MEL cells. The immunoprecipitation (IP) of Bio-V5-Runx1 is efficient because it is almost absent in the supernatant of the IP (Fig 1C).

Identifying Runx1 protein partners via V5 immunoprecipitations and mass spectrometry

Single-step purifications of Bio-V5-Runx1 complexes were performed using V5-agarose beads (Sigma) and analyzed by mass-spectrometry (LC-MSMS). Control V5-Immunoprecipitations were performed in BirA-MEL cells not containing the Bio-V5-Runx1 vector. The mass spectrometry (MS) data (Table 1) show the proteins that that were pulled down with the Bio-V5-Runx1. These were not found in the control V5-IP. A number of proteins were pulled down that were previously found to interact with Runx1, e.g. CBFβ and GATA1[26-29], which shows that the tagged Runx1 forms the appropriate complexes. As expected Eto2 and Tal1 were also found in the mass spec data confirming that Runx1 is part of the LDB1 complex in erythroid cells, although LDB1 itself was not found in our data. This suggests that Runx1 binds to this complex via Eto2 and/or Tal1.

The LSD1 complex was also found, albeit only in differentiated cells. It contains LSD1, Gfi1b and Co-rest and has been shown to be important in haematopoiesis[30-31]. LSD1, the first demethylase identified in mammals, demethylates H3K4 to enable gene repression[32-33]. However recent evidence showed that LSD1 can also function as an activator by demethylating H3K9 via an as yet unknown mechanism [34-36]. The Runx1-LSD1 interaction was validated by immunoprecipitations using antibodies against endogenous Runx1 (Fig 2) and LSD1 (supplementary data S6). Also here the proteins were mainly found to interact in cells induced to differentiate.

Another potential repressor that was identified in the V5-IP data was Myelin expression factor 2 (Myef2) (Table1), which has not been shown previously to be expressed or form complexes in hematopoietic cells. Myef2 has been identified as a repressor of the mouse myelin basic protein gene and binds DNA directly[37]. It contains two RNA recognition motifs (RRM) that have been shown to be responsible for binding to DNA[38]. The binding of Myef2 to endogenous Runx1 was confirmed with antibody IP although only a weak band was visible, due to the quality of the antibody used, which results in an inefficient Runx1 endogenous IP (Fig 2). Supplementary figure S7 shows Myef2 binding to Bio-V5-Runx1 via the more

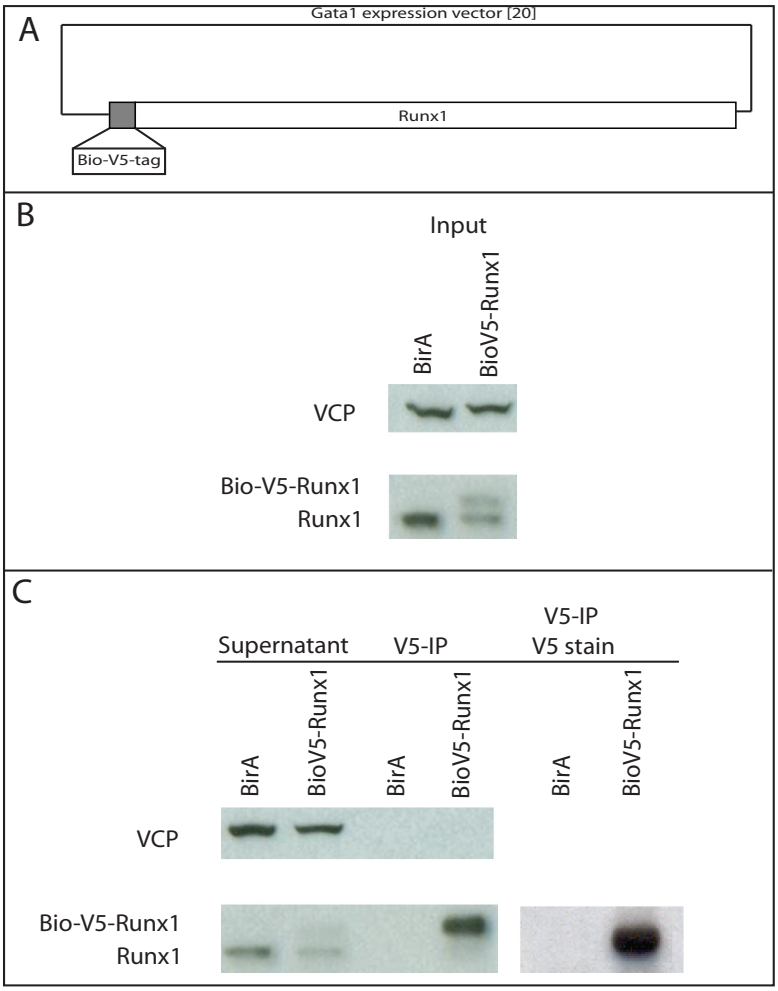


Figure 1. **Bio-V5-Runx1 and V5 immunoprecipitation**. A) Schematic view of Bio-V5-Runx1. A Bio-V5 double tag was placed on the N-terminus of the Runx1 cDNA starting from the distal promoter. B) Expression of Bio-V5-Runx1 in MEL cells clone 7 and compared to endogenous levels of Runx1 in BirA control cells. C) The V5-IP was analyzed on western blots using anti-Runx1 or anti- V5 staining. The VCP protein was used as loading control.

efficient V5 IP. This shows that the Runx1-Myef2 complex is much more prominent in non-induced MEL cells. The interaction can be seen in induced cells but only with the more efficient V5-Runx1 IP and a longer exposure time (supplementary data S7). It was not possible to carry out the reverse IP for Myef2 with the presently available antibodies. Also a tagging approach at the N or C terminus of the Myef2 was also not successful.

Proteins	C88/BirA	Bio-V5-Runx1 non-induced	Bio-V5-Runx1 induced
Core binding Factors			
CBF-beta	-	+	+
ETO-2	-	-	+
Haematopoietic proteins			
GATA-1	-	+	+
TAL1	-	+	+
LSD1complex			
LSD1	-	-	+
Co-Rest1	-	-	+
GFI1b	-	-	+
genetic suppressor element 1	-	-	+
Repressors			
Myef2	-	+	+/-

Table1. **Mass spectrometry results Bio-V5-Runx1 V5 immunoprecipitation.** (-) no binding found (+) strong binding found (+/-) medium binding found.

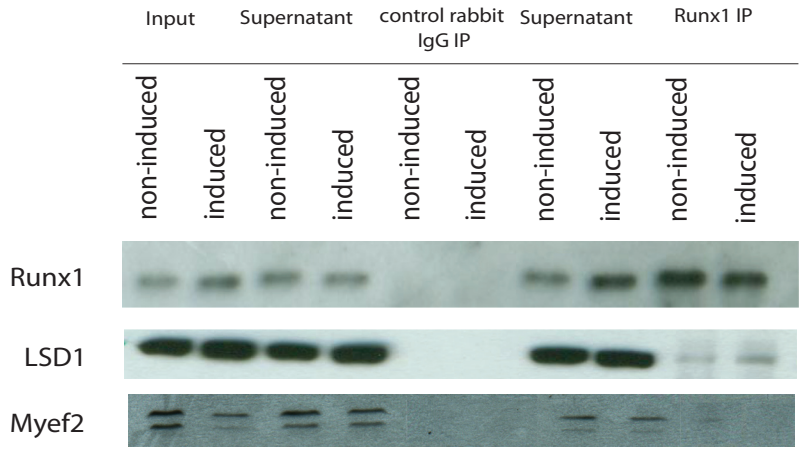


Figure 2. **Confirmation of the mass spec data using endogenous Runx1 immunoprecipitations.** Rabbit anti-Runx1 and Rabbit control IgG IP of nuclear extracts form non-induced and induced MEL cells and analysed by western blots stained for Runx1, LSD1 and Myef2.

Genome wide Runx1 DNA binding sites

The Bio-V5-tagged version of Runx1 was then used to identify the genome wide DNA binding sites of Runx1 by chromatin immunoprecipitations followed by sequence analysis of the bound DNA (ChIP-seq). The Runx1 +23.5 enhancer was used as a positive control for the ChIP[39] to show that the Bio-V5 tagged Runx1 and endogenous Runx1 were bound to this enhancer (supplementary data S8). This site is also a prime binding site in the ChIP-seq analysis. In total 13 million reads were mapped back to the mouse genome. These genome wide binding data were combined with microarray data of differentially expressed genes in differentiating MEL cells[17] and visualised in Figure3a with on the x-axis the position of Runx1 binding to the transcriptional startsite (TSS) of the differential expressed gene and the y-axis the fold expression change during differentiation. The bubbles in the plots are Runx1 binding sites and the size represents the peak height in the ChIP-seq data. The result shows that Runx1 binds to a significant number of genes that are up or down regulated upon differentiation in non-induced and induced MEL cells, many of which are involved in erythroid differentiation. Induced cells show less binding of Runx1 to upregulated genes, although the overall binding pattern does not change.
A motif discovery analysis in 200bp around Runx1 binding peaks shows a Gata1 binding motif to be present at 74% of these peaks (Fig3b). Two other motifs were also seen to be overrepresented around Runx1 binding peaks, of course the E box binding Tal1 and an ets/elk/elf binding site.

Runx1 binding to erythroid specific genes

To verify the binding to regulatory elements of important or typical haematopoietic genes we checked binding to the genes *gata1*, *eto2*, and *epb4.2*. Gata1 is an important regulator of erythropoiesis and essential for the terminal differentiation (reviewed in [40]). Eto-2 was shown to be part of the Gata1/Ldb1/Tal1 complex in erythroid cells and its absence causes an erythroid phenotype in mice[16, 41]. Both the *gata1* and *eto2* gene were top hits in the Runx1 ChIP-seq data and are transcription factors. Gata1 binds DNA while Eto2 does not. Epb4.2 is a structural membrane protein of erythrocytes that is highly upregulated in differentiating erythroid cells (reviewed in [42]). It is not a transcription factor like Eto2 and Gata1 and is an example, of Runx1 binding to a totally different type of gene in erythroid cells.

	Overlap Runx1 and Gata1 binding			
	non-induced		induced	
	Upregulated genes	Down regulated genes	Upregulated genes	Down regulated genes
Number of genes	745	553	130	123
Percentage of overlap	70.7%	57.4%	75.1%	38.8%

Table 2. Overlap binding of Runx1/Gata1 shown on Runx1 target genes. Number of genes and percentage of overlap total target genes Runx1 shown.

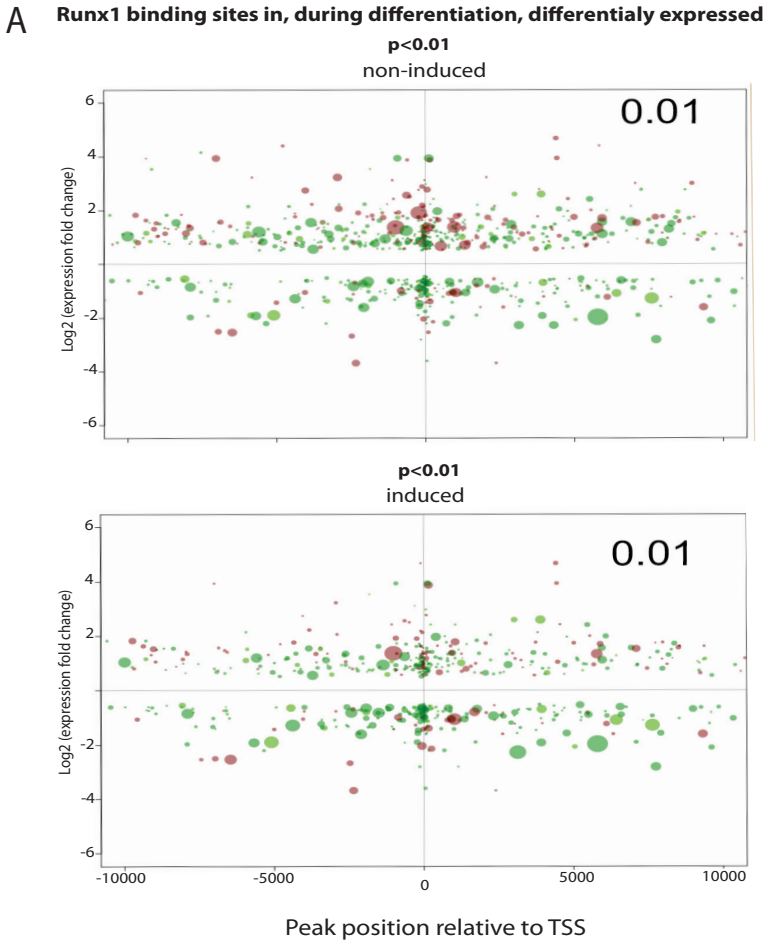


Figure 3. **Runx1 genome wide binding patterns** A) Non-induced and induced MEL cells. With on the x-axis the position of Runx1 binding to the transcriptional startsite (TSS) of the differential expressed gene and the y-axis the fold expression change during differentiation. The bubbles in the plots are Runx1 binding sites and the size represents the peak height in the ChIP-seq data. Brown bubbles: TSS in non CpG region. Green bubbles: TSS in CpG region B) Motif discovery analysis 200bp around Runx1 binding sites. Gata motif discovered around 72% of Runx1 binding site. Also the E-box sequence and ets/elk/elf binding motief were discovered around Runx1 binding sites.

Runx1 binds to the promoter of *eto2* and *epb4.2* and to the promoter and upstream erythroid HS-3.5 enhancer of *gata1* (Fig4 a,b,c). This binding was observed in both non-induced and induced MEL cells. No binding was observed in the negative controls, the 3'UTR of the *gata1* and *epb4.2* gene and 2kb downstream of the *eto2* promoter.

Knock-down of Runx1 shows a function as a transcriptional repressor

Five Runx1 shRNA's from the TCR library were tested by lentiviral infection followed by puromycin selection. Western blots of nuclear extracts showed that shRunx1#1 and shRunx1#2 transduction resulted in a knock-down (KD) of Runx1

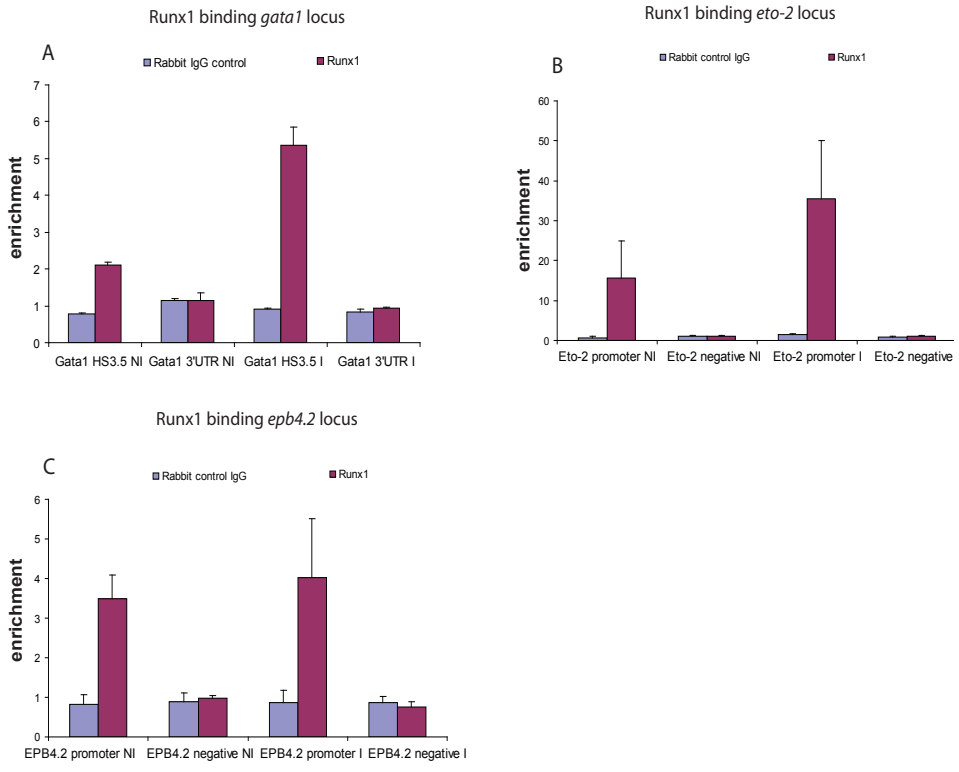


Figure 4. **Confirmation ChIP-sequencing results via endogenous Runx1 ChIP.** ChIP preformed in MEL cells non-induced (NI) and MEL cells induced (I). A) Rabbit anti-Runx1 and Rabbit control IgG ChIP. RT-PCR done with primers against gata1 HS3.5 enhancer and negative region at 3'UTR gata1 gene. B) Rabbit anti-Runx1 and Rabbit control IgG ChIP, RT-PCR done with primers against eto-2 promoter and negative region 2kb downstream of startsite C) Rabbit anti-Runx1 and Rabbit control IgG ChIP, primers against epb4.2 promoter and negative region at 3'UTR epb4.2 gene.

in MEL cells versus the control shTCR (Fig5a). Microarray data of the non-induced and induced KD MEL cells (supplementary table S3 and S4) were correlated to the Runx1 ChIP-seq data to identify direct target genes and these are shown in Figure 5b. Different genes are up- or down regulated before differentiation after a Runx1 KD, which suggests that Runx1 can function both as an activator and repressor in erythroid cells. Runx1 appears to activate its own gene, but is a repressor of a number of others. Most shows a moderate increase of expression after the Runx1 KD, but others such as Ebp4.2 show a dramatic increase in expression suggesting that Runx1 is the major repressor of this gene in undifferentiated cells. When the cells are differentiated much fewer genes are affected and to a lower extent which correlates with a decrease of bound Runx1. This suggests that its role in late erythroid differentiation is much less important.

Previous results from our lab show that the full Gata1/Tal1/Ldb1 complex acts mainly as an activator in MEL cells. Recent data (unpublished) show that this activation during differentiation is primarily achieved through the release of repression in the undifferentiated cells. Runx1 binds to this complex of activators and may activate genes in MEL cells via this complex. The ChIP/seq profiles in Table2 show that the overlap in binding of Runx1 with Gata1 binding is highest in genes repressed by Runx1 consistent with the role of the Gata1/Tal1/Ldb1 complex.

Because the Runx1 protein levels were difficult to detect, the RNA expression levels were also measured using RT-PCR. The result (Fig6a) confirms that Runx1 expression is indeed reduced in the MEL cells transduced with shRunx1#1 and shRunx1#2. *Eto2*, *epb4.2* and *gata1* expression was clearly increased in the Runx1 KD samples before induction of differentiation.

The mass spec and IP data show that only Myef2 is bound to Runx1 in non-induced cells and that this binding is lost after induction. This suggests that Myef2 is the factor that represses transcription together with Runx1.

Knock-down of Myef2 shows similar gene regulation as Runx1

The KD data above suggest Myef2 is the repressive co-factor of Runx1 before differentiation. To test this possibility shRNA vectors Myef2 were tested to determine if Myef2 is important in repression.

Fig 6b shows that the two shRNA vectors against Myef2 transcripts result in a KD of 50% or more in the undifferentiated MEL cells. This results in a doubling of the transcripts of the *runx1*, *eto2*, and *gata1* genes, which corresponds with the repression that was also seen in the Runx1 KD and strongly suggests that Runx1 represses these genes via Myef2. The transcripts of *epb4.2* were much less dramatically increased compared to the Runx1 KD and will be elaborated on in the discussion. It is unfortunately not possible to determine whether Myef2 binds to the same site as Runx1 due to the quality of the Myef2 antibody. Nevertheless we conclude that Runx1 has a suppressive role only before induction and that this role is mediated via Myef2.

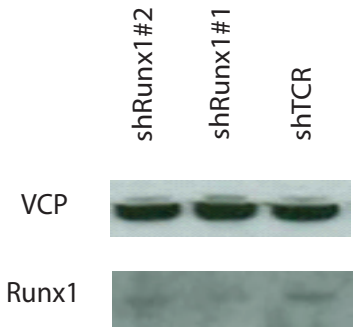
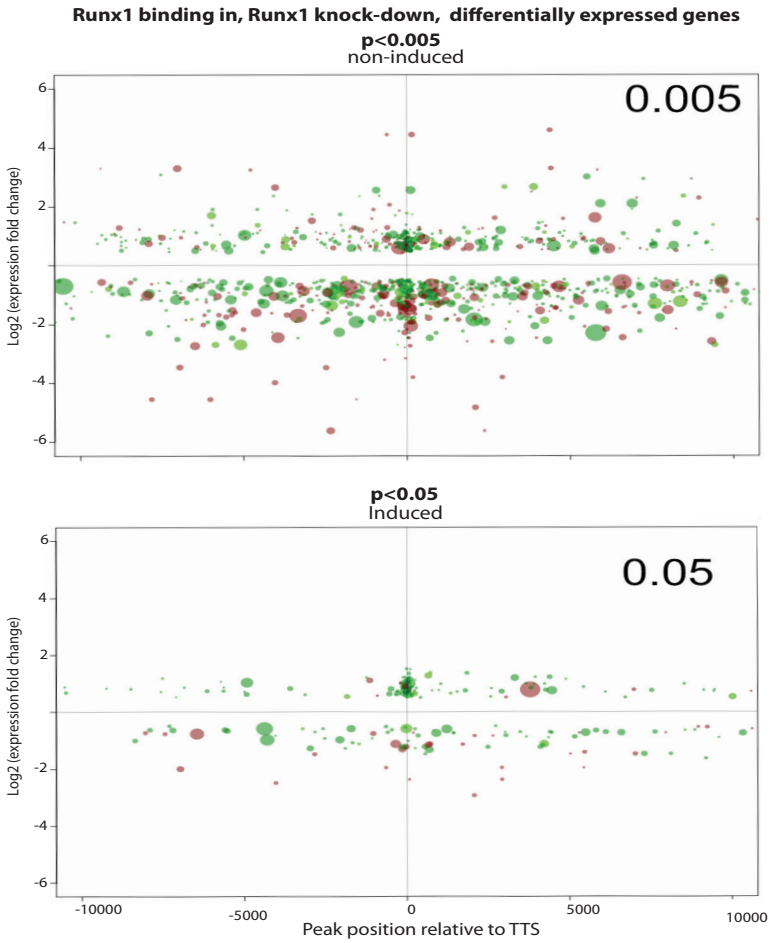


Figure 5. **Genome wide identification of Runx1 target genes** A) Western blot shRunx1#1 and shRunx1#2 versus shTCR control. Runx1 stain and VCP as loading control. B) Bubble plot representation of Runx1 ChIP-sequencing binding data to differentially expressed genes after Runx1 knock-down. With on the x-axis the position of Runx1 binding to the transcriptional startsite (TSS) of the differential expressed gene and the y-axis the fold expression change during differentiation. The bubbles in the plots are Runx1 binding sites and the size represents the peak height in the ChIP-seq data. Brown bubbles: TSS in non CpG region. Green bubbles: TSS in CpG region



The binding site of Myef2 is TGTCCT and it is expected that this site is more represented in Runx1 repressed genes when compared to the activated genes. Table 3 shows that indeed the Myef2 binding site is represented more in genes repressed by Runx1, confirming the observation that Runx1 represses erythroid genes via Myef2.

Discussion

One of the essential regulators in the emergence of the haematopoietic stem cell is the transcription factor Runx1. However the role of Runx1 beyond haematopoietic stem cell formation and maintenance in erythroid cells is poorly understood. In this study a previously unknown role of Runx1 in erythropoiesis is uncovered by showing that it acts as a repressor of a number of erythroid genes via the repressor protein Myef2. This function of Runx1 is very different from that described previously in primitive erythropoiesis in zebrafish, xenopus and mouse and makes use of the newly discovered partner protein Myef2

Runx1 was known to have a function in the development of megakaryocytes, which develop from the same progenitor cell as erythroid cells and where Runx1 also functions as an repressor (reviewed in [43]). The complex of Runx1 and Gata1 has been described in megakaryocytes[28-29], but it is not clear what function it has during megakaryocytic development and whether a complex is formed with LSD1 or Myef2.

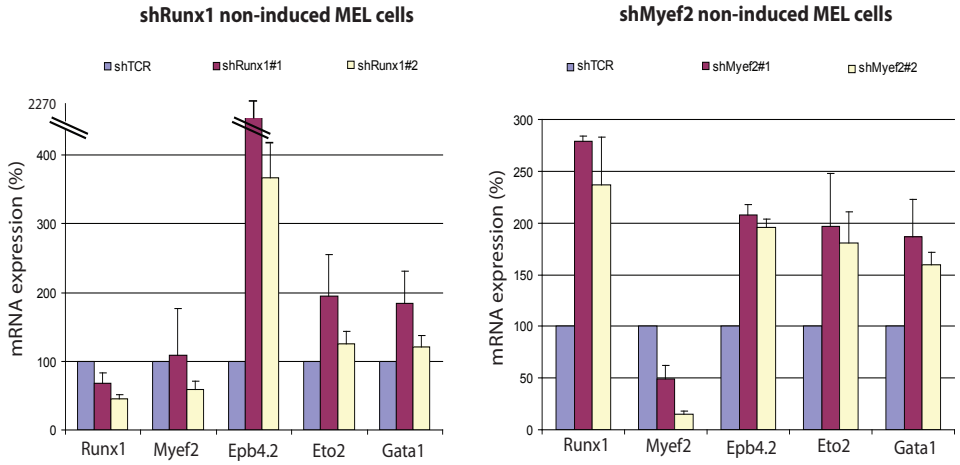


Figure 6. **Knock-down experiments Runx1 and Myef2.** Expression levels of mRNA were measured via quantitative PCR of Runx1, Myef2, Epb4.2, Eto-2 and Gata1 A) mRNA shRunx1 RT-PCR measured versus shTCR control in non induced MEL cells. B) mRNA shMyef2 RT-PCR measured versus shTCR control in non induced MEL cells.

The interaction of Runx1 with LSD1 was confirmed in MEL cells via co-IP with antibodies against Runx1 and LSD1 in both non-induced and induced cells. However when the IP is performed with an antibody against LSD1 the interaction is seen solely in induced MEL cells. This suggests that the interaction between Runx1 and LSD1 is stronger in induced MEL cells and possibly only needed in these cells. However the present data do not provide any direct evidence that this particular interaction has a function in the induced cells.

	Overlap Runx1 and TGTCCT occurrence 200bp +/- of peak position			
	non-induced		induced	
	Upregulated genes	Down regulated genes	Upregulated genes	Down regulated genes
Number of genes	451	339	81	101
Percentage of overlap	42.8%	35.2%	46.8%	31.9%

Table 3. Overlap Runx1 binding to target genes and Myef2 binding sequence "TGTCCT". Number of genes and percentage of overlap total target genes Runx1 shown.

The interaction of Runx1 with Myef2 is seen in the mass spectrometry data from non-induced and induced cells with similar mascot scores. However an interaction was confirmed by an anti-Runx1 IP in non-induced cells only. This suggests that the interaction between Runx1 and Myef2 is much weaker or even lost after induction which may explain why the suppressive role of Runx1 on the target genes that were analysed is lost. The trigger of this disassociation is not known but a possible explanation could be the recruitment of LSD1 to Runx1.

It has been shown that Runx1 has a role in primitive erythropoiesis. In the Runx1$^{-/-}$ mouse the morphology of primitive erythrocytes is affected and Gata1 is down regulated, although it is not clear whether this downregulation is a primary effect or a secondary effect[11]. Here we show Runx1 binding to the *gata1* locus and that Runx1 regulates Gata1 expression. If Runx1 would also bind the *gata1* locus and regulate the gene directly in primitive erythropoiesis it would do so as an activator and not as a repressor via Myef2.

Our genome wide correlation of Runx1 ChIP-seq and KD suggests that Runx1 acts as a repressor and an activator in MEL cells. The complex that would be in part responsible for gene repression would be Runx1-Myef2. Most Runx1 target genes show a moderate increase of expression after the Runx1 KD, like *gata1* and *eto2*. Others such as *ebp4.2* show a dramatic increase in expression. This observation suggests that Runx1 is the major repressor of certain genes in undifferentiated cells and a less important repressor of other genes.

Gata1 acts as an activator in MEL cells and also interact with Runx1. This complex could be responsible in part for Runx1 gene activation. We show however that these complexes are more present at genes repressed by Runx1 which corresponds with the recent observation that activation (by the Gata1-Tal1-LDB1 complex) during differentiation is primarily achieved through the release of repression in the undifferentiated cells. How Runx1 activates genes in MEL cells remains unclear from our data.

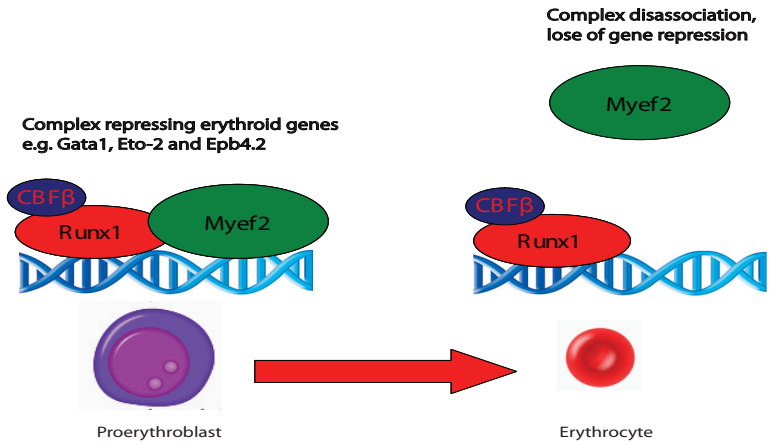


Figure 7. **Model of Runx1-Myef2 complex function in erythroid differentiation.** See text for explanation.

Deletion of Runx1 in adult mice does gives extramedullary hematopoiesis composed of maturing myeloid and erythroid cells[9-10], suggesting an increase in activity at the precursor stages (as represented undifferentiated MEL cells). Runx1 may be even more essential for stress erythropoiesis. For example Eto2 which is part of the same complex is important for erythropoiesis *in vitro* and stress erythropoiesis *in vivo*[16-17, 41].

In summary we report a novel function of the Runx1 protein in erythrocyte development. It acts as a repressor of important erythroid genes like *eto2*, *gata1* and *epb4.2*. The repression of these genes is mediated via Myef2 which we show to be a binding partner of Runx1. The repressive function of Runx1 and the complex binding with Myef2 are lost after induction of differentiation. This suggests that Runx1 keeps erythroid specific genes repressed before terminal differentiation

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Chapter 3

Myef2 is essential for haematopoietic development in zebrafish

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Manuscript in preperation

Abstract

Runx1 is essential for the emergence of the definitive haematopoietic stem cell and plays a role in haematopoiesis thereafter. Runx1 binds and regulates a number of important erythroid genes in combination with Myef2. In order to further investigate the role of Myef2 in haematopoiesis morpholino knock-down studies were performed in zebrafish embryos. These experiments show that Myef2 has an important role in zebrafish erythropoiesis and T-cell development presumably mediated via Runx1.

Introduction

Runx1 is important in the development of the definitive haematopoietic stem cell (HSC) in e.g. mouse and zebrafish[1-3]. Later in development Runx1 is important in the differentiation of the HSC into T-cells and erythrocytes[4-5][van Riel et al]. How Runx1 functions after the emergence of the definitive HSC, and in particular in the erythroid compartment, is largely unknown. It is known to function as a transcriptional repressor and activator on different genes. Runx1 activates for example the *pu.1* gene early in definitive haematopoietic development and later in development the *CSFR* gene[6-7]. Runx1 can form activating complexes with p300, a histone acetyl transferase[8]. It has been shown that Runx1 represses the *cd4* gene[9-10] in the development of T-cells probably via a repressive complex formed with Groucho/TLE[10]. From previous studies it is also known that Runx1 forms a repressive complex with mSIN3a in hematopoietic stem and progenitor cells (HSPS)[11].
Myelin expression factor 2 (Myef2) has previously been identified as a repressor of the mouse *myelin basic protein* gene and it was shown to bind DNA directly[12]. Myef2 contains two RNA recognition motifs (RRM) that have been shown to be responsible for binding to DNA[13]. It was recently identified by us as a partner of Runx1 in erythroid cells and shown to repress important erythroid genes[vanRiel et al], which was surprising as it had not been shown to be expressed or form complexes in haematopoietic cells previously.
In order to further study its role in haematopoiesis we carried out an oligonucleotide morpholino knock-down of Myef2 in zebrafish. This results in an erythroid and T-cell phenotype in the definitive haematopoiesis, which shows that Myef2 indeed has an important role in zebrafish haematopoiesis which is presumably mediated via Runx1.

Materials and methods

Zebrafish maintenance and morpholino injections

Fish were bred and maintained as described[14] and staged as described[15]. Morpholino oligonucleotides (MO) were designed to target splice junctions in the un-spliced myef2 messenger RNA: Myef2 MO 5'-CTCACCAACTACATGAGACATA CAA-3', targeting the intron2-exon3 junction. Typically, 1nl of MO were injected in 1-2 cell stage embryos (Myef2 MO – 6.5ng/nl) and their efficiency verified by PCR with the following primers: myef2 F- CAGAACCAAGACGACACGAA and myef2 R-CGATGGATGGAGGAATGTTT.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out as described [16]. DIG-labelled antisense RNA probes were transcribed from linearized templates using T3, T7 or Sp6 RNA polymerases (Roche, Burgess Hill, United Kingdom). After hybridization,

embryos were bleached in 5% formamide, 0.5% SSC, 10% H2O2 for 10-30 minutes, washed in PBST (PBS, 0.1% Tween-20) and transferred to 80% glycerol for imaging.

Results

Myef2 morpholino injected zebrafish show low Runx1 and Gata1 mRNA levels

Myef2 zebrafish knock-down experiments were carried out to verify a potential role of Myef2 in haematopoiesis. A splice MO was injected into the zebrafish zygote. Figure 1a shows that it targets Myef2 mRNA because an extra band appears in the PCR analysis of Myef2 mRNA when compared to the control shown by the yellow arrow. This extra band is alternatively spliced mRNA that cannot be translated into functional protein. The mRNA of the housekeeping gene *ef1*α is unchanged.
mRNA levels of a number of haematopoietic genes expressed in developing zebrafish were visualized by in situ hybridization. Runx1 and Gata1 mRNA were stained 20 hours post fertilizations (hpf) which is the period of time that the primitive hematopoietic system develops. Figure 1b shows that the levels of Gata1 and Runx1 mRNA are unchanged in the MO injected zebrafish when compared to wildtype. Figure 1c shows that the Runx1 levels are much lower when compared to wildtype after 26hpf which represents the onset of the definitive haematopoietic system. Four days post fertilization (dpf) also shows a drop in Gata1 expression when compared to wildtype resulting in a defective erythropoiesis.

Myef2 phenotype is limited to the haematopoietic system

Closer examination of the expression patterns in the early development of zebrafish haematopoietic system shows that the expression levels of cMYB, Fli1, Ikaros, Pax2.1 and deltaC seem to be normal at the onset (28hpf) of the definitive hematopoietic system (Figure 2a). cMYB is essential for mouse definitive haematopoiesis but it is also expressed during primitive haematopoiesis[17-18]. In zebrafish cMYB is expressed in the ventral wall of the dorsal aorta in Runx1 positive cells[19]. Ikaros is also detected in primitive and definitive haematopoietic precursors in both mice and zebrafish[20-21] and is essential for development of all B- and T-cell lineages[22]. Fli1 is expressed in the primitive and definitive stage of haematopoiesis is zebrafish[18] and is, together with deltaC, essential for normal vascularisation needed for haematopoietic development[23]. Figure 2a show that this vascularisation is unaffected in the MO injected zebrafish. In addition the development of the pronephric duct is unaffected as shown by normal expression

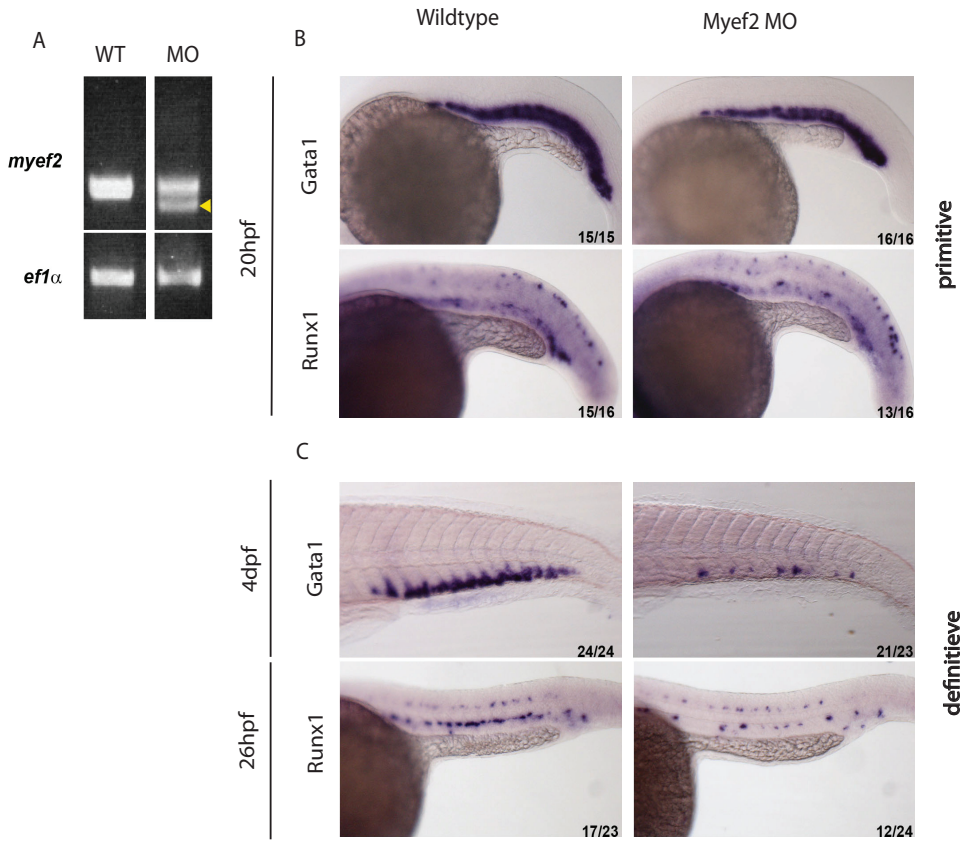

Figure 1 **Zebrafish splice morpholino injections against zebrafish Myef2**.
A) PCR on Myef2 and housekeeping gene ef1α mRNA. In MO injected zebrafish an extra PCR band was seen, that represents spliced mRNA under the influence of the MO (yellow arrow), compared to the control. B) Staining of Gata1 and Runx1 at 20hpf, which represents primitive haematopoiesis. C) Staining of Runx1 26dpf and Gata1 4dpf which represent definitive haematopoiesis

of PAX2.1[24]. By 4dpf the definitive HSC migrate to the kidney marrow and remain there throughout the zebrafish lifespan. Malformations in its development could explain haematopoietic phenotypes. Thus the results show no haematopoietic phenotype in the Myef2 MO injected zebrafish in the early stages of definitive haematopoiesis apart from the low levels of Runx1 (Fig1c).
Figure 2b shows the expression levels of Rag1 and Ikaros (T-cell markers) at 4dpf in the zebrafish thymus[22, 25]. No or very few T-cells are present in the thymus of MO injected zebrafish when compared to the controls, which show a clear T-cell staining. Furthermore we see less staining of cMYB in the kidney marrow of MO-treated zebrafish that represents lower amounts of HSC.

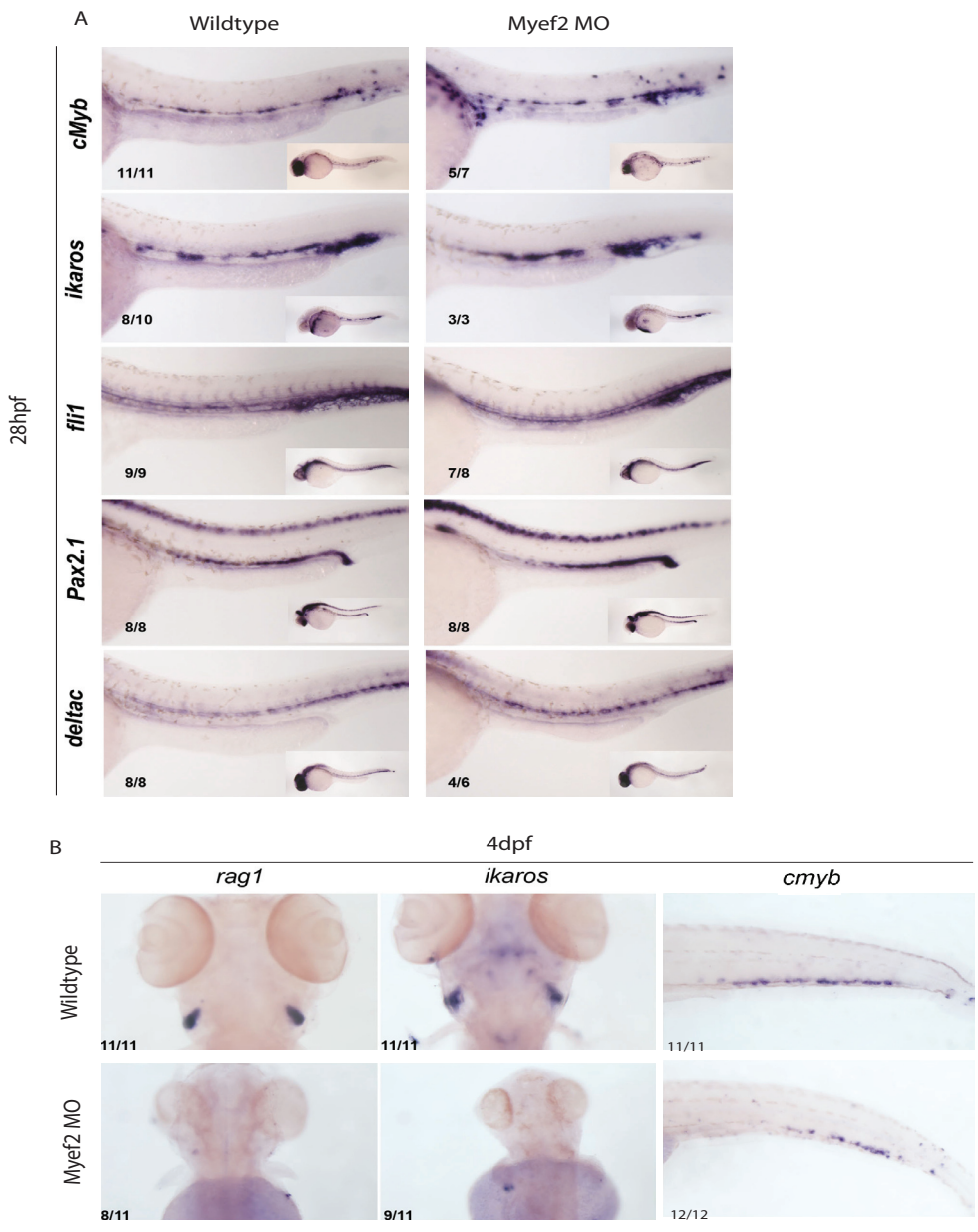

Figure 2 **Zebrafish splice morpholino injections against zebrafish Myef2.**
A) Staining of cMYB, Ikaros and Fli1 to show HSC development. Staining of Fli1, Pax2 and deltaC to show morphology of haematopoietic tissue. Staining at 28hpf represents the onset of definitive haematopoiesis. B) Staining of rag1 and Ikaros (T-cell markers) in the thymus and cMYB (HSC marker) in the kidney marrow at 4dpc.

Discussion

One of the essential regulators in the emergence of the haematopoietic stem cell is the transcription factor Runx1. However the role of Runx1 beyond haematopoietic stem cell formation and maintenance in erythroid cells is poorly understood. In this study we show that a previously unknown partner of Runx1, named Myef2, has an important role in haematopoietic development in zebrafish.
A haematopoietic phenotype was observed in the zebrafish injected with MO against Myef2 mRNA. A striking observation was that no effect could be seen in the development of the primitive haematopoietic system similar to what is observed in Runx1 knock-out mice and zebrafish knock-down studies[1-3]. The levels of Gata1 and Runx1 were both comparable to the control. When the levels of Runx1 and Gata1 were measured in definitive haematopoiesis the levels were lower in the zebrafish injected with the MO against Myef2 compared to the control resulting in less definitive HSC seen at 4dpc by cMYB staining. This phenotype is probably caused due to the lower levels of Runx1 caused by the lower levels of its partner protein Myef2. The lower Gata1 levels seen in 4dpc in the kidney marrow indicates a erythroid phenotype that corresponds with the role of Runx1 and Myef2 seen in mouse erythropoiesis [van Riel et al]
When the phenotype was investigated further the levels of haematopoietic markers like cMYB, FLI1 and Ikaros were indeed normal during the early stages of definitive haematopoiesis. This suggests that the development of the haemogenic endothelium is unaffected by the Myef2 KD, but that becomes important later. The morphology of the vascular system and the pronephric duct were also normal as shown by Fli1, Pax2 and DeltaC staining and we therefore conclude that the phenotype seen in the MO injected zebrafish is not due to morphological defects in kidney development. At 4dpf the levels of T-cell markers Rag1 and Ikaros are almost undetectable in the thymus. Runx1 is known to have an important role in T-cell development [5, 10, 26] and we therefore hypothesize that the T cell phenotype seen with Myef2 deficiency is the same as seen with a loss of Runx1.

We conclude that number of definitive HSC is lower due to improper Runx1 function via Myef2. Similarly the development of erythrocytes and T-cells is probably also aberrant due to improper Runx1 function.

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Chapter 4

Runx1 recruits LSD1 to target genes during terminal erythroid differentiation and appears to be involved in Runx1 mediated transcriptional repression.

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Work in progress

Abstract

The haematopoietic transcription factor Runx1 is important for the differentiation of T-cells, B-cells, megakaryocytes and erythrocytes. Most of these observations were made using mouse knock-out studies and give little information about the molecular mechanisms. Here we show that Runx1 forms a complex with the histone modification enzyme LSD1 in erythroid cells. LSD1 is recruited via Runx1 to genes in erythroid cells and appears to be involved in the repression of these genes.

Introduction

Runx1 is an important transcription factor in the differentiation of the haematopoietic stem cell towards T-cells, megakaryocytic and erythrocytes[1-3][vanRiel et al], but the molecular mechanism how and which genes are regulated by Runx1 during differentiation is largely unknown. Using Mouse Erythro-Leukaemia (MEL) cells it has been shown that Runx1 represses a number of erythroid genes like *epb4.2, gata1* and *eto2* in the proerythroblast state of terminal differentiation[vanRiel et al]. Very little is known how Runx1 activates genes in the proerythroblast stage and during the downstream erythroid differentiation.

Histone tails can be modified in several different ways, like methylation, phosphorylation and acetylation, and have been associated with transcription regulation[4-8]. The methylation of histones lysine residues in the tails can be mono-, di- or tri-methylated. Histone methylation is associated with both gene activation and repression and is recognised by the chromodomain that is associated with chromatin remodelling proteins[9]. Histone methylation was thought to be a permanent epigenetic mark until a histone demethylase was discovered called Lysine Specific Demethylase1 (LSD1)[10-11]. LSD1 demethylates H3K4 mono and di-methylation that results in gene repression. Recently evidence has been found that LSD1 may also function as an activator by demethylating the repressive mono and di-methylation mark on H3K9[12-14]. LSD1 was shown to be important in erythropoiesis and LSD1 depleted MEL cells do not differentiate properly towards terminal erythrocytes[15-16]. These studies also showed Tal1 and Gfi1b to regulate erythroid genes via LSD1. Our lab showed that Runx1 is in the same complex with LSD1, Tal1 and Gfi1b in erythroid cells[17][vanRiel et al].

Here we investigate whether the Runx1-LSD1 complex functions as a histone demethylating complex after differentiation of MEL cells i.e. during terminal erythroid differentiation after the proerythroblast stage. We used RNAi knock-down and ChIP-sequencing studies to identify genes regulated by Runx1. We show that the binding of LSD1 to Runx1 repressed genes is upregulated during terminal erythroid differentiation and appears to be involved in their repression.

Materials and methods

Cell culture

Mouse Erythro-Leukaemia (MEL) cells were cultured in DMEM containing 10% fetal calf serum and penicillin/streptomycin. These cells represent the proerythroblast stage in erythropoiesis. Addition of 2% dimethylsulfoxide (DMSO), was used to induce differentiation towards terminal erythrocytes. Cells were harvested after 4 days of differentiation.

gene symbol	chromosome	logFoldChange	adjusted P value
Ppbp	5	3.13289	0.016281
Acp5	9	2.47846	0.012924
Aqp8	7	2.26321	0.016281
Csf2rb2	15	2.00402	0.034224
Mybpc3	2	1.99324	0.012924
Pkhd1l1	15	1.95249	0.057030
Speer4d	5	1.67309	0.022900
Rph3al	11	1.61124	0.048133
Cd59a	2	1.58084	0.026256
Mt2	8	1.55024	0.020874
Pfkp	13	1.54721	0.055885
Aldoc	11	1.48925	0.043525
Rogdi	16	1.46784	0.016281
Hba-x	11	1.38273	0.016281
Csf2rb	15	1.37419	0.052173
Prokr1	6	1.35853	0.023114
Cntn3	6	1.35306	0.016281
Ube2l6	2	1.34797	0.016281
Tcp11l2	10	1.31306	0.030923
Sly	Y	1.28335	0.044876
Art4	6	1.28204	0.048186
Ccng2	5	1.26220	0.042861
Man2b1	8	1.25007	0.016281

Table 1: **Selection of upregulated genes Runx1 KD induced MEL cells**

ChIP and ChIP-sequencing sample preparation

Chromatin Immunoprecipitations (ChIP) were performed using a sonication buffer as described[18]. Per ChIP $2x10^7$ and per ChIP-seq $10x10^7$ MEL cells were used. Antibodies used for ChIP are described in supplementary data S1.

shRNA in MEL cells

The TRC Mission human and mouse library from Sigma was used for shRNA mediated knock-down of proteins of interest. They were delivered to MEL cells via lentiviral infection. Virus was added to $0.5x10^6$ MEL cells and cultured for 48 hours. For induced MEL cells DMSO was added to the medium together with the puromycin and cells were harvested 4 days later.

Bio-informatical analysis

The bio-informatical analysis of the microarray and ChIP-sequencing data were carried out as described in[19]. ChIP-sequencing data is shown in the UCSC genome browser with on top the gene of interests. Transcription factor and modified histones localization on chromosome are shown via peaks. Height of the peak represents number of sequences enriched in ChIP. Scores before and during differentiation are normalized to each other with the total amount of sequences found in the ChIP-sequencing.

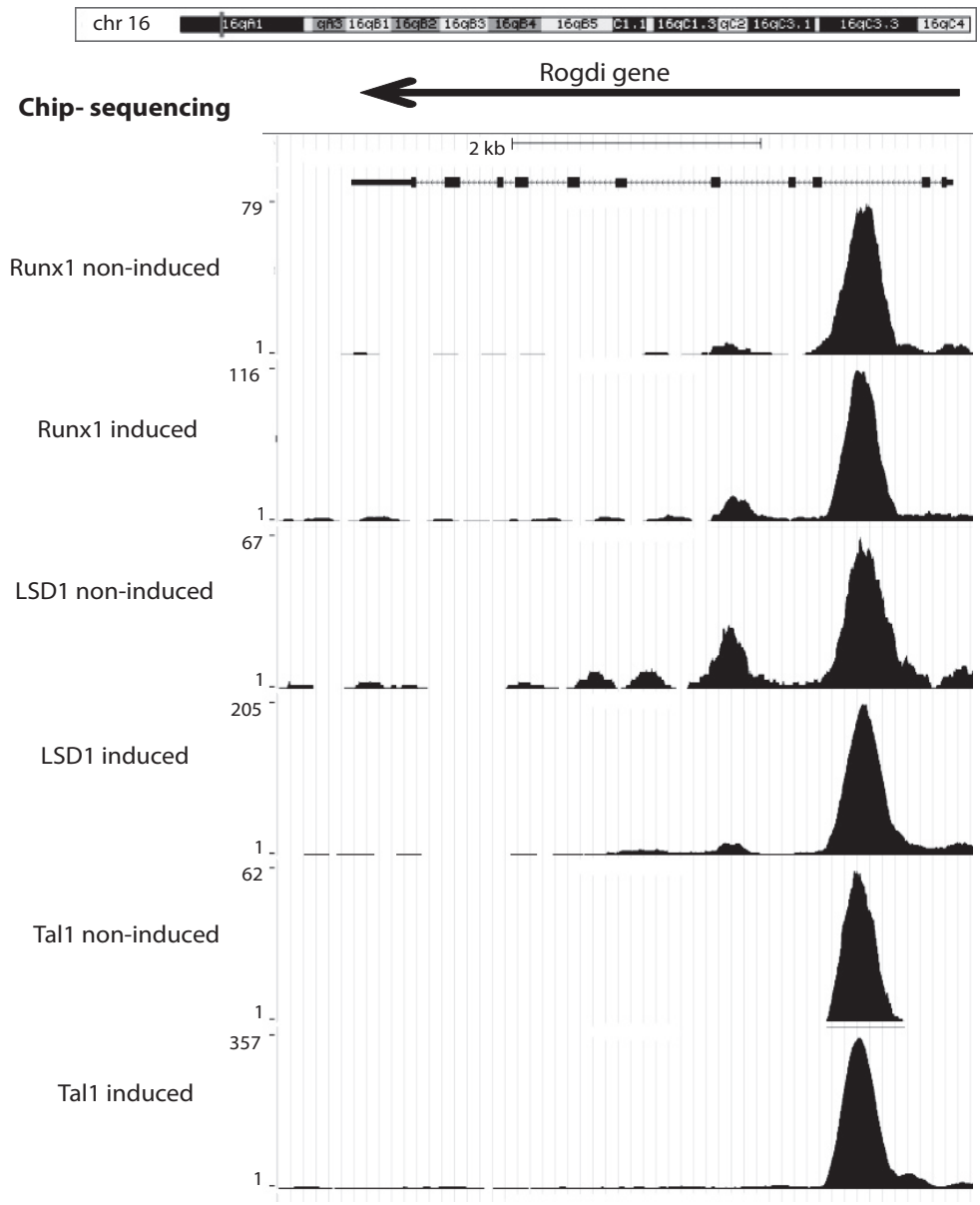

Figure 1: **Runx1, LSD1 and Tal1 binding to Runx1 repressed gene *rogdi* in induced MEL cells:** ChIP-sequencing data is shown in the UCSC genome browser with on top the gene of interests. Transcription factor binding in the region of the relevant gene are shown by the peaks. The height of the peak represents the number of sequences enriched in ChIP-sequence and hence is representative of the relative amount of Runx1 or LSD1 bound.

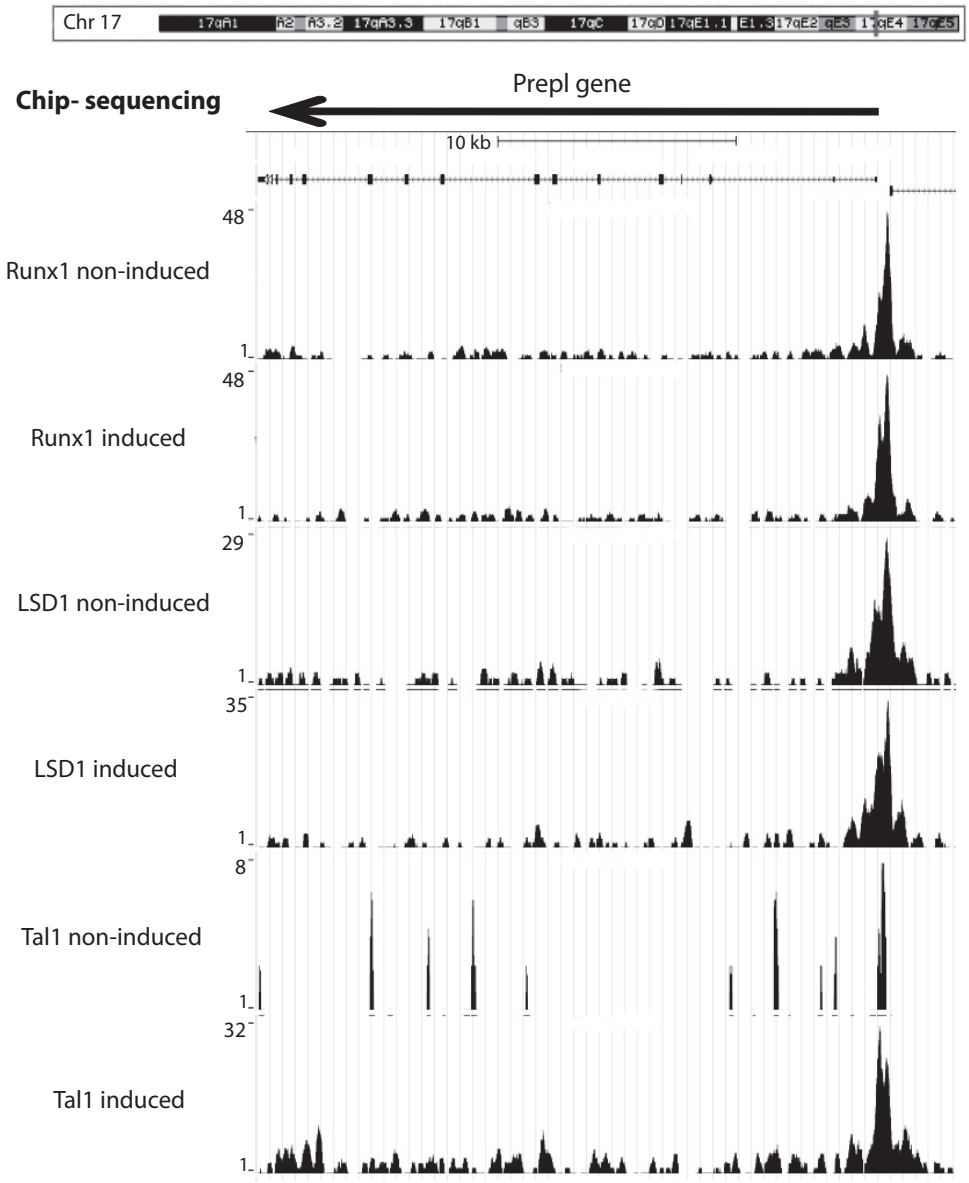

Figure 2: **Runx1, LSD1 and Tal1 binding to Runx1 activated gene *prepl* in induced MEL cells:** ChIP-sequencing data is shown in the UCSC genome browser with on top the gene of interests. Transcription factor binding are shown as described in figure 1.

Results

Knock-down of Runx1 after the proerythroblast stage shows that a small group of genes is upregulated.

In order to investigate which genes are regulated by Runx1 in induced MEL cells RNA expression levels were analysed using microarray based experiments on induced MEL cells after Runx1 shRNA knock down (KD). The entire microarray data is shown in supplementary table S4. Table 1 shows that the expression level of 23 genes has increased by the knockdown when compared to control suggesting that these are repressed by Runx1 during the differentiation of MEL cells. Other genes show the opposite pattern and are downregulated by the Runx1 KD. Many of these genes are also bound by Runx1 on or near the transcriptional start site (TSS) (visualised in Chapter 2 Fig 5b). Because Runx1 forms a complex with LSD1 during differentiation suggests that LSD1 plays a role in these two sets of genes and that the patterns of histone methylation may change differently in the sets after differentiation of the MEL cells. We therefore analysed the binding of Runx1 and LSD1 to these genes and compared this to the distribution of H3K4 methylation marks at these genes.

LSD1 and Tal1 are recruited to Runx1 repressed genes in differentiating MEL cells

ChIP-sequencing was carried out in MEL cells before and after differentiation for LSD1, Tal1, Gfi1b and a number of histone H3K4 mono- and di-methylation marks using antibodies specific for these factors or epigenetic marks. Runx1 ChIP-sequencing was preformed using a V5 ChIP of an N-terminal Bio-V5 tagged Runx1. The data were analysed and displayed in the UCSC genome browser[20]. Figure 1 shows detailed examples of the binding of Runx1 and LSD1 to the gene *rogdi*. This gene is upregulated in induced MEL cells in the Runx1 KD (Table 1). The results show that Runx1 binds in the second intron of the gene in close proximity to the TSS together with LSD1 both in non-induced and induced MEL cells. However in contrast to Runx1, which shows only a small increase, the binding of LSD1 increases more that 3 fold during differentiation. Figure 1 also shows an increase of Tal1 binding of almost 6 fold in agreement with the observation that Tal1 binding increases in general (Andrieu/Soler *et al i*n prep). Tal1 and LSD1 have been shown to be partners in erythroid cells and that LSD1 is not recruited to genes by Tal1 during differentiation [16]. We propose that the elevated LSD1 level of binding is caused by the binding of LSD1 to Runx1. This corresponds with previously shown data where we show an increase in binding of LSD1 to Runx1 during erythroid differentiation[vanRiel et al]. This suggests that the recruitment of LSD1 by Runx1 to these genes may be responsible for their repression.

Top ten Runx1 repressed genes differentiating erythroid cells

Gene symbol	Chromosome	Log fold change Runx1 KD	Runx1		LSD1		Tal1		Gfi1b		H3K4 monoMethyl		H3K4 diMethyl		H3K4 triMethyl		Expression during differentiation	
			NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN
Ppbp	5	3.1329	105	56	16	44	0	70	0	0	12	14	52	20	4	8	57	0
Acp5	9	2.4785	96	51	70	102	132	127	57	64	34	21	104	56	10	9	73	299
Aqp8	7	2.2632	66	32	48	125	53	168	20	54	20	10	73	57	4	5	0	0
Csf2rb2	15	2.0040	19	18	64	101	25	52	17	35	19	15	67	46	6	14	1064	903
Mybpc3	2	1.9932	39	44	9	15	16	88	11	26	21	14	18	14	3	6	0	53
Pkhd1l1	15	1.9525	79	47	16	61	44	139	12	19	30	20	63	63	7	11	245	1368
Rph3al	11	1.6112	128	70	27	62	32	161	20	48	26	20	76	40	4	8	16	24
Cd59a	2	1.5808	81	50	30	37	125	79	9	19	39	24	79	43	4	6	75	82
Pfkp	13	1.5472	21	23	15	29	0	14	15	27	11	9	58	50	16	16	1817	1374
Rogdi	16	1.4678	90	101	56	209	109	203	19	34	26	9	43	36	4	4	92	36

Top ten Runx1 activated genes differentiating erythroid cells

Gene symbol	Chromosome	Log fold change Runx1 KD	Runx1		LSD1		Tal1		Gfi1b		H3K4 monoMethyl		H3K4 diMethyl		H3K4 triMethyl		Expression during differentiation	
			NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN
Eif3i	4	-1.8728	23	23	25	17	11	35	21	24	13	7	57	48	13	14	918	483
Prepl	17	-1.7712	55	42	28	36	11	18	45	70	15	16	54	51	17	20	621	405
Lypla1	1	-1.7709	5	27	73	105	23	31	0	6	10	7	5	4	3	4	694	315
Hrsp12	15	-1.6476	24	24	18	17	14	7	26	39	22	15	78	58	13	22	260	86
ccdc104	11	-1.6451	23	24	13	16	7	5	16	26	17	15	41	39	10	15	281	231
Tfb2m	1	-1.6343	35	42	21	13	7	7	24	38	17	16	58	48	11	17	282	223
Ranbp1	16	-1.5417	46	37	27	21	14	8	52	57	9	10	65	47	16	13	677	270
Ppia	11	-1.4763	16	18	23	19	9	4	33	26	13	14	65	37	10	15	1039	740
Thg1l	11	-1.4249	26	23	18	22	9	9	43	57	10	9	50	36	15	16	291	65
Nol8	13	-1.3874	19	15	19	27	12	9	23	24	21	11	46	36	17	19	782	287

Ten genes bound but not regulated by Runx1 in differentiating erythroid cells

Gene symbol	Chromosome	Log fold change Runx1 KD	Runx1		LSD1		Tal1		Gfi1b		H3K4 monoMethyl		H3K4 diMethyl		H3K4 triMethyl		Expression during differentiation	
			NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN	NI	IN
EpoR	9	0.0000	131	124	60	94	359	163	84	122	43	30	110	96	45	58	1068	1291
Arhgap17	7	0.0000	182	155	46	152	194	169	36	37	20	16	69	45	9	10	382	326
Rac2	15	0.0000	93	51	28	66	123	149	64	82	21	15	57	53	9	15	1014	713
Crk	11	0.0000	52	46	13	18	7	11	28	34	17	15	53	41	12	14	942	811
Fbxo3	2	0.0000	44	44	18	22	19	52	31	32	29	18	85	79	28	29	921	737
Pitrm1	13	0.0000	27	19	12	13	14	9	19	24	15	11	57	50	11	13	1387	513
TCFap4	16	0.0000	23	10	16	17	9	19	23	42	7	7	39	40	11	9	255	96
Ptprj	2	0.0000	135	109	36	58	62	127	21	39	35	26	80	56	10	18	1055	1389
Sdad1	5	0.0000	36	38	15	33	12	7	24	25	12	13	54	45	15	22	1290	421
Saps2	15	0.0000	99	90	31	46	25	31	31	40	26	20	68	50	11	14	429	658

Table 2: **Normalised ChIP-sequencing binding peaks of transcription factors Runx1, LSD1, Tal1, Gfi1b and the histone marks H3K4 mono- di- and tri-methyl**. Three sets of genes were analysed: Runx1 repressed genes during erythroid differentiating, Runx1 activated genes during erythroid differentiation and Runx1 bound but not regulated genes during erythroid differentiation. Green= significantly (at least 20%) downregulated. Red= significantly (at least 20%) upregulated.

If true it would be expected that the genes which are downregulated due to the Runx1 KD would not show a change (or even a decrease) of LSD1 binding during differentiation. An example (Figure 2, see also supplementary table S4 microarray data Runx1 KD differentiating erythroid cells) of such a gene is *prepl* which shows that Runx1 and LSD1 bind to these genes in non-induced and induced MEL cells at comparable levels. Tal1 binding is upregulated from no binding at all to a low level of binding.

LSD1 recruitment correlates with loss of histone methylation of Runx1 repressed genes in differentiating cells

Table 2 shows the binding scores of Runx1, LSD1, Tal1, Gfi1b and histone H3K4 mono-, di- and tri-methylation marks of the ten most up and down regulated genes in differentiation cells depleted of Runx1. All these genes have a Runx1 binding site within 10kb of the gene locus. The table also shows ten genes that bind Runx1 but are not regulated by Runx1. LSD1 is recruited to all ten genes, Tal1 in eight of the ten and Gfi1b in eight of the ten most upregulated genes. In the ten most downregulated genes we show that LSD is recruited to four genes, Tal1 to two genes and Gfi1b to six genes. Finally ten of the genes not regulated by Runx1 show that LSD1 is recruited to seven genes, Tal1 to four genes and Gfi1b to seven genes.

Almost all the genes that are repressed by Runx1 and show LSD1 binding show a decrease in H3K4 mono- and di-methylation, while tri-methylation remains unchanged, suggesting that LSD1 has a role in the Runx1 mediated repression during differentiation. The obvious next experiment would be to reduce the level of LSD1 during MEL cell differentiation, however LSD1 depleted MEL cells (supplementary figure S5) do not differentiate properly and hence it was not possible to obtain meaningful expression arrays of induced MEL cells with low levels of LSD1. It is therefore difficult to determine whether LSD1 and histone demethylation play a role in this group of genes because there is no correlation with the genes being up- or down-regulated during differentiation. There is however a reasonable correlation with LSD1 binding and a decrease of histone mono- and di-methylation of H3K4.

There is a clear correlation between the genes that are activated by Runx1 and downregulation during development. However there is only a weak correlation with decreased mono- and di-methylation of H3K4. There is no correlation with LSD1 binding in this group, except that the binding is very low before and after induction. Clearly this group is regulated by Runx1 but not LSD1.

In the group of genes that binds Runx1 but is not regulated by it there appears to be no correlation between histone methylation and the up- or down regulation of expression during differentiation. There is however some correlation between increased LSD1 binding and H3K4 mono- or di-demethylation.

Discussion

Runx1 is known to regulate genes important for erythropoiesis in the proerythroblast [vanRiel et al]. After differentiation of the proerythroblast stage it was shown that Runx1 still regulates a number of genes, although this number is much smaller [vanRiel et al]. We have previously shown that Runx1 forms a complex with LSD1 in induced MEL cells. Here we have investigated whether the Runx1-LSD1 complex regulates any genes during terminal erythropoiesis using ChIP-sequencing and knock-down studies.

As shown by the microarray data and the results in Chapter 2 it is clear that Runx1 activates and represses a number of genes in induced MEL cells. This number is much smaller when compared to the number of genes that are regulated by Runx1 in non-induced cells. Many of these genes, such as *rogdi*, have an unknown function, but others have a known role in haematopoiesis in general or in erythropoiesis specifically. For example the *ppbp* gene (Pro-Platelets basic protein) is repressed by Runx1 in induced MEL cells and encodes the chemokine Cxcl7 in platelets[21-22]. Also the *pfkp* gene (Phosphofructokinase platelets), which is highly expressed in megakaryocytes and platelets and important for glycolysis[23]. The *csf2rb2* gene (colony stimulating factor 2 receptor, beta 2) encodes a common subunit of the IL-3 (interleukin 3) receptor[24]. The *il-3* gene is regulated by Runx1[25-26], however we find no binding of Runx1 to the *il-3* gene in MEL cells (vanRiel et al unpublished data). IL-3 represses erythroid differentiation by inhibiting the erythroid regulatory factors produced via erythropoietin (EPO) (reviewed in [27]). The *csf2rb* gene (colony stimulating factor 2 receptor beta) which is also repressed by Runx1 (table 1), is part of the IL-3 receptor. These results show that Runx1 is involved in the repression of genes that are important for other haematopoietic lineages or genes that themselves inhibit erythroid differentiation.

The Runx1 DNA binding pattern does not change much during differentiation from proerythroblast towards erythrocytes, however the protein complexes formed with Runx1 do change during erythroid differentiation [vanRiel et al]. In particular the Runx1-LSD1 complex is mainly formed in induced MEL cells, suggesting that LSD1 may be the functional component of Runx1 in at least a subset of the genes repressed by Runx1. Indeed LSD1 binding increases at all of the genes repressed by Runx1 in induced MEL cells, however this was also seen in genes not regulated but bound by Runx1. The first group also shows an increase Tal1 binding to these genes in differentiating MEL cells. The possibility remains that both are needed by Runx1 to repress these genes.

We show in Table 2 that the H3K4 mono- and di- methylation mark is reduced in most genes repressed by Runx1 although the mark does not disappear. We also show that the di-methyl mark is reduced in the majority of Runx1 activated genes while the mono-methyl mark is reduced in most non Runx1 regulated genes. In the top ten Runx1 repressed genes the changes in methylation marks correlate strong with the increased binding of LSD1. However these changes do not correlate with the

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Chapter 5

General discussion

General discussion

Defects in erythropoiesis lead to reduced numbers or dysfunctional erythrocytes and causes diseases like anaemia, thalassemia and sickle cell anaemia. By understanding the molecular mechanisms involved in erythropoiesis novel therapies may be developed. In these studies the Mouse Erythro-Leukaemia (MEL) cell line was used as a model system of terminal erythroid differentiation in order to identify transcription factors involved in this process. MEL cells represent the proerythroblast stage and can be induced to differentiate towards terminal erythrocytes.

The transcription factor Runx1 is important for the development of the definitive haematopoietic stem cell (HSC), T-cells, B-cells and megakaryocytes[1-6]. Erythrocytes and megakaryocytes develop from a common precursor cell called the Megakaryocyte-Erythrocyte Progenitor (MEP) cell[7-9] and the hypothesis was that Runx1 functions as a molecular switch between these two cell types[10]. This idea is supported by the observation that Runx1 transcription is downregulated during erythropoiesis[11-12]. Expression of Runx1 would push the MEP towards the megakaryocyte direction while Runx1 downregulation would result in erythroid differentiation. However this hypothesis was never proven experimentally. Our lab identified Runx1 to be part of a large protein complex in MEL cells. This complex also contains proteins like Gata1, LDB1 and Tal1 that are important for erythroid development and this complex was shown to regulate erythroid genes[13-16].

Runx1 is also known as AML1 because its gene locus is often translocated in acute myeloid leukaemia (AML)[17-18]. The most common translocation is the t8;21 that creates a fusion protein of the DNA binding Runt-domain of Runx1 and almost the entire *eto* gene[19]. Eto contains four conserved nervy homology regions that recruit corepressors such as N-coR, mSIN3a and also HDAC's[20-22]. This AML1-ETO fusion protein binds to Runx1 target genes and represses these genes[23-24]. Patients with an AML1-ETO translocation often show a hypoplasia, resulting in a disruption of erythroid and megakaryocytic differentiation[25]. This observation is also seen in mouse models expressing the AML1-ETO fusion protein[26]. Contradictory to these results is that a different AML-ETO mouse model does not show the hypoplasia phenotype[27]. These two mouse models are generated differently what could account for the difference shown. Also is the erythroid phenotype shown in older mice than tested in the mouse model not showing a phenotype. Furthermore it has also been shown that AML1-ETO inhibits the differentiation of K562 cells, MEL cells and human primary erythroid cells *in vitro* [28-30]. These results and the observation that Runx1 is part of a protein complex important for erythropoiesis suggest it has a role in this process.

Chapter 2 describes the DNA binding sites in the proerythroblast and differentiated cells of a Bio-V5 tagged version of Runx1 using ChIP-sequencing. Analysis of the DNA sequence around these binding sites showed an overrepresentation of Gata1 and Tal1 binding sequences confirming that these transcription factors cooperate at the DNA level. The genome-wide comparison of Runx1 binding sites around or in the

differentially expressed genes in proerythroblast and differentiated cells shows that Runx1 binding to erythroid genes does not change much during terminal erythroid differentiation. The same analysis but with expression data from Runx1 depleted cells shows that Runx1 functions as an activator and a repressor in both stages but the number of affected genes is greatly reduced later in differentiated cells. A mass spectrometry screen of immunoprecipitated Bio-V5-Runx1 was used to identify the partners of Runx1 in the proerythroblast and differentiated MEL cells. This confirmed the presence of the proteins that are part of the LDB1 complex, like Gata1 and Tal1, but not LDB1 itself. This suggests that the binding of LDB1 and Runx1 is very indirect and probably mediated via another factor in the complex. This analysis also identified the new partners Myef2 and LSD1. Myef2 was originally shown to be a repressor of the mouse *myelin basic protein* gene and binds DNA directly via the sequence "TGTCCT"[31-32], while LSD1 was the first demethylase identified and functions as a repressor by removing the H3K4 mono- and di-methylation marks associated with gene activity[33]. Such lysine methylation marks are recognised by the chromodomain associated with chromatin remodelling proteins[34].

Suppression of Runx1 by RNAi shows that it represses important erythroid genes like *epb4.2, gata1* and *eto2* at the proerythroblast stage. This repression is lifted when MEL cells are induced to enter terminal erythroid differentiation. The binding data show that Runx1 is bound to these genes before and after differentiation but that the binding with Myef2 is lost when cells differentiate. Knock-down of Myef2 shows repression of the mentioned genes at the proerythroblast stage but not in the differentiated cells similar to the results obtained with the Runx1 knock-down. These results suggest that Runx1 needs to be bound to Myef2 in order to repress these genes.

The Runx1 RNAi induced knock down shows that the transcription of *epb4.2* is upregulated 20 times while the transcripts of *gata1* and *eto2* only increase two-fold. When Myef2 is knocked down all these three genes are upregulated only 1.5 to 2 times. This appears to be a contradictory result because if all three genes are regulated by the Runx1-Myef2 complex a higher upregulation of the *epb4.2* gene would be expected in the Myef2 knock-down. One possible explanation for this may be that another factor is involved in the suppression of *epb4.2* that itself is regulated by the Runx1 complex. Another possible explanation is that the levels of Runx1 are actually not lowered in the Myef2 KD (it shows higher transcript levels of Runx1), resulting in more Runx1 loaded onto the genes using the remaining Myef2 protein pool more efficiently which may be resulting in a mild effect on the *epb4.2* gene in the Myef2 KD. It was unfortunately not possible to perform immunoprecipitations and chromatin immunoprecipitations of Myef2 due to the poor quality of the available antibodies. To resolve this question new antibodies would have to be raised and tagging Myef2 would be the other option to help identify Myef2 partners and target genes.

Myef2 was unknown to have a function in erythropoiesis or haematopoiesis and its potential role in haematopoiesis was therefore tested by morpholino mediated knock down in zebrafish (Chapter 3) and coupled to in situ hybridizations to visualize

mRNA expression of important haematopoietic genes. The results show no effect in primitive haematopoiesis, similar to Runx1 knock-down in zebrafish and mouse[6, 35]. However with the onset of definitive haematopoiesis Runx1 mRNA levels are lower when compared to control probably as a result of a decreased number of cells rather than a decrease in Runx1 transcription. The levels of haematopoietic markers like cMYB, Ikaros and Fli1 are unchanged and also the morphology is unaffected shown by staining by deltaC and Pax2.1. These results suggest that the development of the haemogenic endothelium and the HSC is normal until Myef2 is needed. We suggest that this would occur late in HSC development because of the levels of haematopoietic markers are normal or even downstream of the HSC. It would be interesting to investigate whether the loss of Myef2 would have a similar phenotype in the mouse and when exactly Runx1 is needed in the development from the haemogenic endothelium to HSC.

At later stages in development a clear phenotype is seen. Less HSCs are present in the kidney marrow and the remaining cells appear to have a differentiation defect towards myeloid and lymphoid cells, since T-cells and erythroid cells are affected. Runx1 plays a role in the differentiation of both lineages in the mouse (Chapter 2 and [27, 36-37]) and it would therefore seem reasonable to assume that the Myef2 morpholino phenotypes seen in zebrafish are mediated via Runx1.

Runx1 and LSD1 occur in a complex during terminal erythropoiesis after the proerythroblast stage (Chapter 2). What triggers this complex formation is unknown but Runx1 is dephosphorylated in induced MEL cells (vanRiel et al, unpublished data). Eto2 is also part of the complex with Runx1 in induced MEL cells and this is a direct partner of LSD1 (Baymaz et al., unpublished data). Possibly LSD1 is recruited to Runx1 via Eto2. This could be proven by knock down of Eto2 or expressing a non LSD1 binding mutant and perform immunoprecipitations of Runx1 in these conditions to test if LSD1 is still a partner.

The ChIP-sequencing results show that Runx1 and LSD1 DNA binding sites overlap in genes repressed by Runx1 in induced MEL cells. Moreover the binding peaks of LSD1 increase in induced MEL cells when compared to non-induced MEL cells correlating with LSD1 forming a complex with Runx1. This suggests that LSD1 is recruited to the DNA by Runx1 binding and would agree with the observation that LSD1 and Runx1 are part of a complex that is dynamic during differentiation[14][S oler et al unpublished data]. However other explanations are possible, for example the increase in LSD1 bound sequences in induced MEL cells may simply be due to better availability of the epitope of the anti-LSD1 antibody. To control for this particular explanation, the ChIP-seq should also be repeated with a second antibody recognising a different epitope or with a tagged version of LSD1. However genes activated by Runx1 do not show an increase in LSD1 occupancy in induced MEL cells suggesting that the effect is not an experimental artefact and these genes are not regulated by LSD1.

The higher LSD1 occupancy correlates with a reduction in H3K4 mono- and di-methylation in Runx1 repressed genes in induced MEL cells. Such reduction would lower the recruitment of chromatin remodelling factors and result in the repression

of the target gene. The methylation change however does not correlate with the changes in expression during differentiation of these genes. It is therefore difficult to determine if methylation changes play a role in the repression in any of these genes. To confirm this mRNA genome-wide expression levels and an analysis of the epigenetic marks should be carried out after LSD1 depletion in induced MEL cells. Unfortunately such an analysis could not be carried out because LSD1 depleted MEL cells do not differentiate properly[38] (data not shown). This problem may be solved by generating a system to deplete MEL cells of LSD1 in a rapid and inducible fashion[39] (Jorna et al unpublished).

It would also be interesting to study how LSD1 is recruited to particular Runx1 binding sites and not to others. A possible answer may be found in the DNA sequence of the different sites. For example this may show that other specific and cooperating factors are bound in the different classes of genes at or near the Runx1-LSD1 binding sequence which may for example stabilize the binding of LSD1 to the complex.

Runx1 forms a complex with Myef2 and LSD1 (Chapter 2), but it is not clear whether the binding of these partners is direct or indirect and what domain(s) of the Runx1 protein is important to form these complexes. These questions could be answered by co-immunoprecipitations of bacterially expressed versions of normal and mutated Runx1 and one of the partners.

This work shows two modes of how Runx1 represses genes in terminal erythropoiesis but Runx1 also activates genes in the differentiation process (Figure 1), which would correlate with the observation Runx1 is associated with the Gata1/Tal1 complex of proteins, which mainly has an activating role in late erythroid differentiation [13,14]. However the comparison of Runx1 and Gata1 binding sites shows more overlap between binding sites in genes repressed by Runx1 (Chapter 2). This is in agreement with the finding that erythroid genes are activated by the LDB1/Gata1/Tal1 complex by a loss of repression rather than the recruitment of activators (Soler et al., unpublished data). However this still sheds no light on how Runx1 activates genes in erythroid cells. In other cell types Runx1 binds the acetyl transferase p300 that has been linked to transcription activation[40-41] but this protein was not found by us to bind Runx1 in erythroid cells. Since LSD1 has been reported to also demethylate H3K9 mono- and di-methylation marks[42], which correlates with gene activation it is tempting to speculate such a release of repression would be an obvious mechanism of how Runx1 mediates “activation” and would agree with the observation in Chapter 4 that LSD1 binds to genes activated by Runx1 in induced MEL cells. However in chapter 4 is also shown that LSD1 does not regulate Runx1 activated genes.

In summary Runx1 plays an important role in the regulation of transcription during “late” erythroid differentiation and shows at least three modes of action. Two of these, the transcriptional suppression of particular genes in proerythroblast cells and the suppression of a set of genes in differentiated cells, are (at least in part) mediated by the co-factor Myef2 and (likely) LSD1 respectively. It is presently not clear how Runx1 activates genes during this late differentiation process, but it may in fact use LSD1 also for this process by demethylating the transcription factor themselves rather than demethylating histone H3. The next question to be answered would be

how these different processes are carried out by Runx1 and this presents a clear challenge for the next stage of unravelling this part of the erythroid transcriptional network.

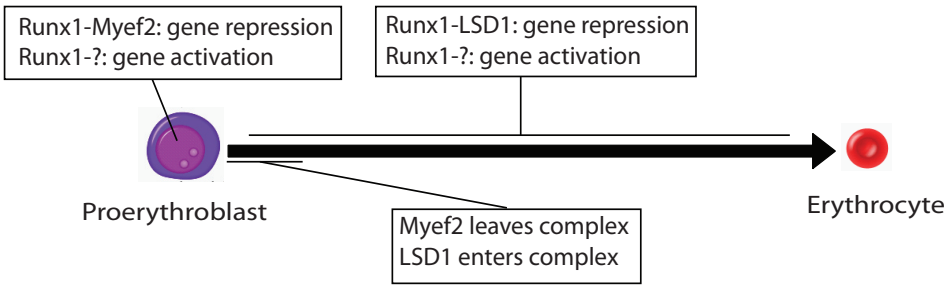

Figure 1 **Model of Runx1 function in terminal erythroid differentiation**

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Supplementary data

Antibody	application	company	catalogue number
Rabbit anti-Runx1	ChIP, IP	santa cruz	sc-28679
Goat anti-Runx1	WB	santa cruz	sc-8563
Rabbit anti-LSD1	WB, ChIP-seq	Abcam	ab17721
Rabbit anti-Myef2	WB	Avia	NP_057216
Mouse anti-VCP	WB	Abcam	ab11433
Goat anti-Gfi1b	ChIP-seq	santa cruz	sc-8559
Rabbit anti-Tal1	ChIP-seq	santa cruz	sc-22809
Normal Rabbit IgG	ChIP, IP	santa cruz	sc-2027
Normal goat IgG	ChIP, IP	santa cruz	sc-2028

Table S1. Antibodies used for experiments. Immunoprecipitation (IP), Western Blot (WB), Chromatin immunoprecipitation (ChIP)

ChIP Target gene	Sequence
Amylase	Reverse primer CTCCTTGTACGGGTTGGT
	Forward primer AATGATGTGCACAGCTGAA
Gata1 HS3.5	Reverse primer CCGGGTTGAAGCGTCTTCT
	Forward primer TCAGGGAAGGATCCAAGGAA
Gata1 3'UTR	Reverse primer GAATAGCCTTGACCTTGTGGC
	Forward primer GCAGGAGAATGGGAAATGTGG
Eto2 promoter	Reverse primer GAGGGCAGTTGGTGTTTG
	Forward primer CCACTCCTTCCTTATCTATCG
Eto2 intragenic	Reverse primer GGGACAGGAGAAAGAAAGG
	Forward primer TTCCACAGACACTCACTCTAT
Epb4.2 promoter	Reverse primer AAGGAGGAAGCAGAAGGAC
	Forward primer CCACGCTCTTTGGAGATGA
Epb4.2 3'UTR	Reverse primer AGAACCTGACCGGCTACAGA
	Forward primer AGACGGTTGAGGGTTGTTTG
Runx1+23enhancer	Reverse primer CGAAAAATAAACCGGCAGTTGA
	Forward primer CAAGCTGCCCACGTTATCAGT
Transcripts	**Sequence**
RI1	Reverse primer TGCAGGCACTGAAGCACCA
	Forward primer TCCAGTGTGAGCAGCTGAG
Gata1	Reverse primer TCCCAGTCCTTTCTTCTCTC
	Forward primer TCCACAGTTCACACACTCTC
Eto2	Reverse primer CTTCACACCTCACACACAT
	Forward primer CGTTCATCAAGAGACAGACC
Runx1	Reverse primer TAGCGAGATTCAACGACCT
	Forward primer CTTGTGGCGGATTTGTAAAG
Epb4.2	Reverse primer TCCCAAACAACCCTCAACCGTC
	Forward primer TGGTATGAAACATCTGAACACCCC
Myef2	Reverse primer CAGCGAACAGGAACATCA
	Forward primer ATTGTGGAACCAAGTCTACC

Table S2. Primer sequence RT PCR used

gene symbol	chromosome	logFoldChange	adjusted P value
Mcpt4	14	-4.54366	0.000103
Mcpt2	14	-4.29734	0.000057
Erv3	2	-3.43546	0.000112
Tcrg-V2	13	-3.32504	0.000643
Nyx	X	-3.29649	0.000241
Lcp1	14	-3.01143	0.000107
Plxnc1	10	-2.89529	0.000031
Phlda2	7	-2.79519	0.000181
Lgals1	15	-2.58265	0.000057
Gzmb	14	-2.53060	0.000247
Slc18a2	19	-2.52303	0.000477
Vim	2	-2.50461	0.000103
Plac8	5	-2.39611	0.000112
Gsta4	9	-2.36564	0.000255
Dpp4	2	-2.35290	0.000107
Lgmn	12	-2.31035	0.000064
Slc45a3	1	-2.29088	0.000210
Fam198b	3	-2.28806	0.000191
Dazl	17	-2.24230	0.000723
Psen2	1	-2.21252	0.000240
Ass1	2	-2.18963	0.000250
Ifih1	2	-2.16847	0.000142
P2rx4	5	-2.15900	0.000107
Ephx2	14	-2.13600	0.001017
Rangrf	11	-2.12511	0.017121
Klhl6	16	-2.12191	0.000171
Arhgap15	2	-2.10519	0.000209
Gpr128	16	-2.10182	0.000262
Muc13	16	-2.07240	0.000584
BC035947	1	-2.05250	0.000275
Ifngr1	10	-2.01583	0.000240
Utp14a	X	-1.99767	0.000220
Eif3i	4	-1.98140	0.000191
Zadh2	18	-1.97941	0.000477
Myb	10	-1.97807	0.000103
Cmtm7	9	-1.92546	0.000173
Myo1d	11	-1.91515	0.000171
Fam129a	1	-1.89427	0.000589
Ccnb1ip1	14	-1.83198	0.001208
Gtf2h1	7	-1.81373	0.000171
Nudt19	7	-1.78905	0.000114
Txndc12	4	-1.78108	0.000103
Aldh3a1	11	-1.77922	0.000616
BC028528	3	-1.77688	0.000261
Mns1	9	-1.75885	0.001067
Fam158a	14	-1.75263	0.000171
Zfp637	6	-1.75186	0.000233
Fdx1	9	-1.69495	0.000484
Mcpt9	14	-1.68909	0.000107

Lipa	19	-1.68625	0.000555
Hemt1	15	-1.68562	0.000854
Rpp40	13	-1.65247	0.001279
Tubb2b	13	-1.64779	0.000570
Ccbl2	3	-1.64663	0.000540
Jmjd1c	10	-1.61264	0.000672
Cpne3	4	-1.61186	0.000114
L2hgdh	12	-1.60232	0.000557
St6galnac3	3	-1.59210	0.000735
BC005685	4	-1.58498	0.002492
Mex3a	3	-1.57051	0.001354
Gm5662	12	-1.56312	0.012634
Acadsb	7	-1.55881	0.000503
4921507P07Rik	6	-1.55144	0.000301
Ivns1abp	1	-1.54356	0.000262
Ndst2	14	-1.54073	0.000388
Lhpp	7	-1.53947	0.000255
Xkr9	1	-1.53709	0.001341
Slc22a3	17	-1.53611	0.000181
Lypla1	1	-1.50653	0.000411
Ikzf2	1	-1.50374	0.000719
Hdc	2	-1.49928	0.002826
Intu	3	-1.49436	0.000240
Sep-06	X	-1.48612	0.000366
Far2	6	-1.48357	0.005634
Sh3kbp1	X	-1.48342	0.000727
Gm13023	4	-1.47913	0.000540
Ahcy	2	-1.47489	0.021071
St13	15	-1.47297	0.000255
Gna15	10	-1.47184	0.000240
Cd53	3	-1.47171	0.000327
Rnf125	18	-1.45930	0.000261
Gm8580	10	-1.44453	0.008802
Cdk6	5	-1.43467	0.000834
Dynlt3	X	-1.41799	0.000171
Scpep1	11	-1.41587	0.000262
Mum1l1	X	-1.41330	0.000668
Tgtp	11	-1.41018	0.003782
Fastkd1	2	-1.40967	0.001210
Ica1l	1	-1.39997	0.000672
Cpd	11	-1.38982	0.000247
Nfia	4	-1.38720	0.000638
H2afy	13	-1.38505	0.000240
Top1mt	15	-1.38209	0.000925
Slc4a11	2	-1.37950	0.001208
Tec	5	-1.37183	0.000698
Ptger4	15	-1.36960	0.000592
Aldh2	5	-1.36659	0.001445
Ogdhl	14	-1.36133	0.000756
Vps13c	9	-1.36108	0.000890
Rftn1	17	-1.36055	0.000857
Slc4a1	11	5.40952	0.000012

Ppbp	5	4.84237	0.000012
Hbb-y	7	4.26905	0.000107
Aqp8	7	3.87234	0.000103
Acp5	9	3.85063	0.000057
Alas2	X	3.33518	0.000057
Csf2rb2	15	3.24487	0.000240
Epb4.2	2	3.10671	0.000057
Mybpc3	2	2.93576	0.000103
Fmo1	1	2.73506	0.000171
Gypa	8	2.69372	0.000114
Smox	2	2.59000	0.000107
Fam55b	9	2.56653	0.000103
Ephb2	4	2.52975	0.000194
Mgst3	1	2.51785	0.000254
Alad	4	2.51551	0.000103
Hemgn	4	2.49664	0.000112
Ahsp		2.47451	0.000107
Apol10a	15	2.46745	0.000967
Butr1	11	2.42574	0.000103
Tfrc	16	2.38377	0.000178
Glipr2	4	2.37402	0.000639
Fam110c	12	2.35880	0.000103
Plek2	12	2.33631	0.000553
Aim1	10	2.33278	0.000171
Dhrs11	11	2.33122	0.000103
Rab3il1	19	2.31303	0.000107
Itgb7	15	2.26140	0.000107
Itga2b	11	2.21809	0.000171
Gabrr1	4	2.18572	0.000499
Btg2	1	2.17810	0.000103
Tmem8	17	2.17298	0.000112
Rhd	4	2.15210	0.000314
F830116E18Rik	11	2.11057	0.000103
Dnajb2	1	2.09911	0.000255
Epb4.9	14	2.09649	0.000240
Grap2	15	2.07242	0.000201
Vangl1	3	2.02441	0.000103
Unkl	17	2.02396	0.000181
Hba-a2	11	2.01894	0.000103
Slamf1	1	1.98781	0.000141
Rph3al	11	1.98210	0.000107
Abcb10	8	1.98116	0.000137
Ube2l6	2	1.97777	0.000240
Lrrc39	3	1.93516	0.000114
Gm16494	17	1.92995	0.000309
E2f2	4	1.91889	0.000220
Hist1h2bc	13	1.90628	0.001435
Trim10	17	1.88604	0.000112
Slc30a10	1	1.88538	0.000112
Gm5226	17	1.88325	0.000366
Tmod1	4	1.88165	0.000141
1190002A17Rik	2	1.88134	0.000171

Gpcpd1	2	1.87357	0.000107
Pdia2	17	1.86898	0.000854
Ank1	8	1.86609	0.000112
Arhgap23	11	1.85749	0.000103
Abcg2	6	1.85657	0.000114
Abcg4	9	1.85413	0.000107
Tmem86b	7	1.84906	0.000295
Arrdc3	13	1.83501	0.000327
Uros	7	1.83196	0.000255
Sema4b	7	1.82747	0.000347
Tbxas1	6	1.82627	0.000210
Slc6a9	4	1.82312	0.000191
2310046K01Rik	2	1.81926	0.000210
Pigq	17	1.81149	0.000114
Suv420h2	7	1.80867	0.000134
Josd2	7	1.80144	0.000262
Mylip	13	1.79264	0.000107
Mare	11	1.78912	0.000220
Slc43a1	2	1.77045	0.000112
Mageb16	X	1.74660	0.005780
4632428N05Rik	10	1.74261	0.000201
Gm6651	1	1.74190	0.001076
Fam132b	1	1.73687	0.000247
Hbb-b1	7	1.73282	0.002844
Micall2	5	1.72986	0.000692
Atp6v0a1	11	1.72186	0.000114
Ostb	9	1.71356	0.008197
Hmbs	9	1.70967	0.000211
Snca	6	1.70946	0.000361
1200009I06Rik	12	1.70152	0.001438
Appl2	10	1.68698	0.000114
Hmgn2	4	1.68331	0.000262
Il1r1	1	1.68097	0.000114
Cpeb4	11	1.67771	0.000215
Rrm2	12	1.67205	0.001089
Pkhd1l1	15	1.64859	0.001215
Ptdss2	7	1.64525	0.000181
Ccrl2	9	1.63923	0.001278
Capn5	7	1.63791	0.001292
Spns2	11	1.63635	0.001011
Hba-a1	11	1.63008	0.000171
Ampd3	7	1.62505	0.000262
Trp53inp1	4	1.61671	0.000112
Ptp4a3	15	1.61501	0.000233
Tlcd1	11	1.61429	0.000524
Cldn13	5	1.61357	0.000262
Slc6a3	13	1.61167	0.000112

Table S3 Top 100 up- and down regulated genes Runx1 KD non-induced MEL cells

gene symbol	chromosome	logFoldChange	adjusted P value
Gas5	1	-1.98301	0.016281
Rps25	9	-1.91058	0.033697
Eif3i	4	-1.87279	0.014645
Rps23	13	-1.83624	0.028973
Prepl	17	-1.77117	0.016281
Lypla1	1	-1.77093	0.016281
Hrsp12	15	-1.64764	0.022308
Ccdc104	11	-1.64512	0.020354
Tfb2m	1	-1.63433	0.023068
Acadsb	7	-1.55720	0.017310
Myb	10	-1.54417	0.027500
Snhg1	19	-1.54204	0.028973
Ranbp1	16	-1.54171	0.014645
Zdhhc2	8	-1.48375	0.016636
Ppia	11	-1.47632	0.017310
Lclat1	17	-1.46484	0.016636
Zfp120	2	-1.43627	0.016281
L2hgdh	12	-1.42852	0.016281
Thg1l	11	-1.42486	0.016281
Snora69	X	-1.41979	0.022504
Wdr35	12	-1.40076	0.016281
Dynlt3	X	-1.39749	0.017310
1700106N22Rik	17	-1.39601	0.029563
Eif2s2	2	-1.39017	0.017310
Nol8	13	-1.38737	0.043525
Gtf2h1	7	-1.38657	0.032245
Vkorc1	7	-1.38644	0.017310
Myc	15	-1.38418	0.022654
1110004F10Rik	7	-1.37133	0.016281
Nme2	11	-1.36668	0.017310
H2afy	13	-1.36426	0.016281
Tsr1	11	-1.35208	0.020354
Ccnb1ip1	14	-1.34342	0.028155
Slc38a1	15	-1.32545	0.024864
Snord58b	14	-1.32412	0.025108
Ktelc1	16	-1.32056	0.016281
Hspa13	16	-1.31962	0.037573
Uba6	5	-1.31856	0.016281
Echdc1	10	-1.31683	0.016281
Taf9b	X	-1.31594	0.024155
Tipin	9	-1.29866	0.016281
Rpp38	2	-1.29800	0.016688
Ttc26	6	-1.28315	0.017504
Etf1	18	-1.27638	0.016281
Mnd1	3	-1.27581	0.034089
2610524H06Rik	5	-1.27405	0.017310
Mtmr1	X	-1.27385	0.017310
Dnajc10	2	-1.27359	0.016636
Iars	13	-1.26995	0.033791

Tpr	1	-1.26458	0.018582
Dhx33	11	-1.26411	0.016281
Tmem48	4	-1.26291	0.016281
Lars	18	-1.25805	0.016281
Gpatch4	3	-1.25598	0.016281
Tmed5	5	-1.25536	0.023281
Rrp15	1	-1.25307	0.016281
Ppbp	5	3.13289	0.016281
Acp5	9	2.47846	0.012924
Aqp8	7	2.26321	0.016281
Csf2rb2	15	2.00402	0.034224
Mybpc3	2	1.99324	0.012924
Pkhd1l1	15	1.95249	0.057030
Speer4d	5	1.67309	0.022900
Rph3al	11	1.61124	0.048133
Cd59a	2	1.58084	0.026256
Mt2	8	1.55024	0.020874
Pfkp	13	1.54721	0.055885
Gm6560	5	1.51192	0.017310
Aldoc	11	1.48925	0.043525
Rogdi	16	1.46784	0.016281
Hba-x	11	1.38273	0.016281
Csf2rb	15	1.37419	0.052173
Prokr1	6	1.35853	0.023114
Cntn3	6	1.35306	0.016281
Ube2l6	2	1.34797	0.016281
Tcp11l2	10	1.31306	0.030923
Sly	Y	1.28335	0.044876
Art4	6	1.28204	0.048186
2010011I20Rik	2	1.26355	0.023068
Ccng2	5	1.26220	0.042861
Man2b1	8	1.25007	0.016281

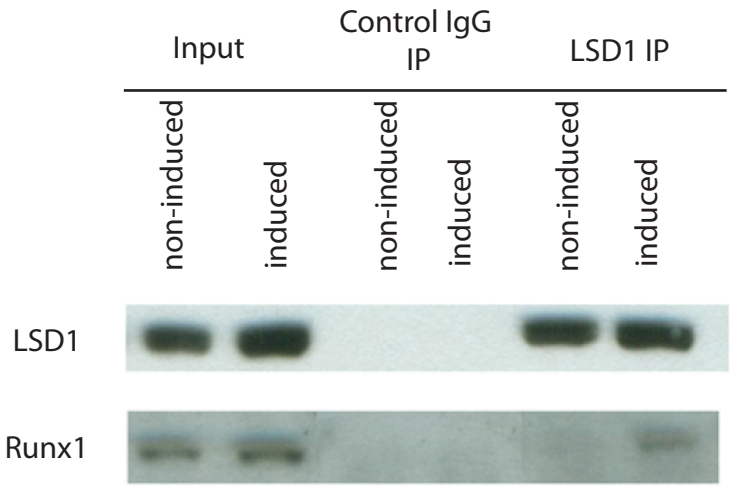
Table S4 All up and down regulated genes Runx1 KD induced MEL cells

gene symbols	chromosome	logFoldChange	adjusted P value
Slc4a1	11	-3,071385284	0,000320193
Apol10a	15	-2,76746958	0,001361583
Kdm1a	4	-2,598380651	0,000359823
Gm13646	13	-2,448156868	0,01543828
Epb4.2	2	-2,421292559	0,000337324
Cbx3	6	-2,340707079	0,006708892
Fam132b	1	-2,316888562	0,000654757
Hbb-b1	7	-2,260884131	0,001564986
Rhag	17	-2,174250988	0,001018278
Cldn13	5	-1,791683742	0,001490332
Atp11c	X	-1,740076499	0,002717412
Rhd	4	-1,738413174	0,001490332
Ptges3	10	-1,707186342	0,002591066
Mageb16	X	-1,706987419	0,02363356
Cstf3	2	-1,649006653	0,006942271
Grsf1	5	-1,646208427	0,001564986
Abcb4	5	-1,605701172	0,001490332
Slc25a37	14	-1,59396606	0,002608395
Gp5	16	-1,586127892	0,007754596
Butr1	11	-1,560957695	0,002717412
Tmem56	3	-1,558833963	0,002553892
Ppia	11	-1,551112346	0,01109665
Trim10	17	-1,486077347	0,002751119
Slc7a11	3	-1,479915882	0,002553892
Ndufa4l2	10	-1,471813907	0,002553892
Cbr1	16	-1,466193756	0,006942271
Kcnn4	7	-1,450955808	0,001534655
Mgst3	1	-1,449082746	0,00266996
Tmem59	4	-1,440820716	0,002553892
Gm16494	17	-1,427717905	0,00293519
Kel	6	-1,426307026	0,00266996
Rps23	13	-1,400163393	0,022951837
Aldh1a1	19	-1,378854133	0,001564986
Slc22a4	11	-1,372145205	0,004129658
Rpl15	14	-1,370357905	0,025123647
100043387	2	-1,362770259	0,024577727
Unkl	17	-1,358645396	0,001597451
Mif	10	-1,355021083	0,018113779
Hmgb1	5	-1,353180082	0,00629465
2610002M06Rik	X	-1,352038265	0,003169919
Trim2	3	-1,348298873	0,003488361
Hemgn	4	-1,317432641	0,001564986
Abcg2	6	-1,314953293	0,001564986
Taok1	11	-1,310373366	0,007639919
Hist1h2an	13	-1,307049468	0,006185187
Arhgdig	17	-1,30005765	0,003224985
Lrrc39	3	-1,293032002	0,004142438
Tfrc	16	-1,279955231	0,002591066
Gbp6	3	-1,259795254	0,002553892

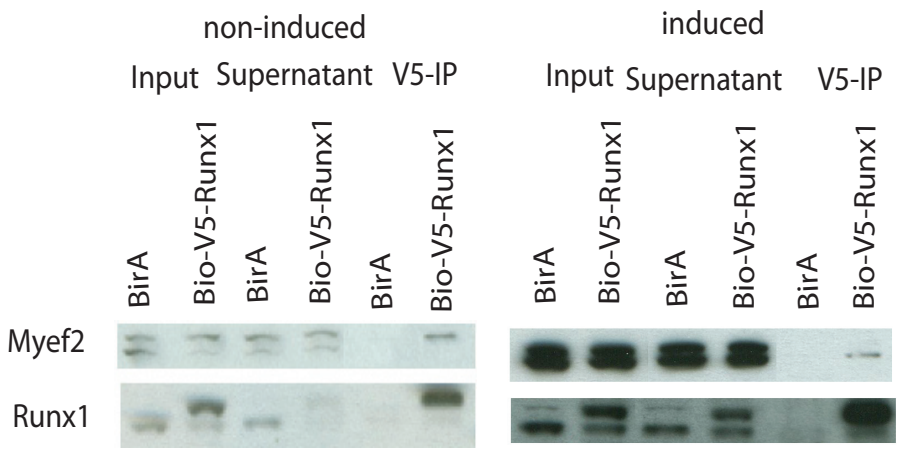
Ralgapa1	12	-1,258556619	0,001953449
Atf5	7	-1,252651069	0,002256776
Slc1a4	11	-1,25131079	0,003586649
Lgals1	15	3,122148997	0,000182282
Lgmn	12	2,775683482	0,000337324
Hemt1	15	2,651913263	0,002591066
Cd53	3	2,596621368	0,001534655
Rogdi	16	2,55575549	0,001564986
Tmem173	18	2,364107669	0,000337324
Spna2	2	2,293372372	0,000601245
Vim	2	2,151154018	0,000952682
Mcpt2	14	2,046652533	0,001271512
Mcpt4	14	1,985344267	0,000683854
Hmox1	8	1,89357069	0,001509079
5530401N12Rik	17	1,88679884	0,004110834
BC028528	3	1,867500409	0,000906269
Fmo1	1	1,861758213	0,000906269
Srsy	Y	1,840141266	0,034427865
F2r	13	1,794914899	0,001490332
Ahsg	16	1,773199588	0,002553892
Gm8995	7	1,727061829	0,004129658
Neurl3	1	1,69408237	0,00293519
Meis1	11	1,685913396	0,002553892
1700025G04Rik	1	1,656045354	0,002717412
Pld4	12	1,641867589	0,001057325
Rel	11	1,6332725	0,001490332
Lcp1	14	1,620450797	0,002553892
Snord116	7	1,599535673	0,008622841
Muc13	16	1,578501563	0,002553892
Arhgdib	6	1,563847073	0,002591066
Sgpl1	10	1,561484616	0,001351164
Scpep1	11	1,548985133	0,001490332
Syne2	12	1,541229637	0,001564986
Cep55	19	1,506144886	0,001351164
F2rl2	13	1,493165206	0,003380506
Lpin2	17	1,480049737	0,001564986
Map3k3	11	1,455578567	0,002717412
Appl2	10	1,43821225	0,003285437
Pls3	X	1,433348452	0,003255274
Plxna3	X	1,425773796	0,002591066
Ehd3	17	1,413546794	0,001490332
Nrgn	9	1,372240569	0,001564986
Tmem50b	16	1,369507309	0,001953449
Elmo1	13	1,351166108	0,00266996
Plp2	X	1,315625563	0,002928708
S100a6	3	1,307663168	0,002412225
Tnfaip8	18	1,301414621	0,002717412
Cd7	11	1,298444157	0,003009361
Vat1	11	1,289627766	0,011834943
1500035H01Rik	9	1,287397674	0,002735748
Lamb2	9	1,280845868	0,004142438
Tnfaip1	11	1,280452917	0,004653312

Plac8	5	1,278718234	0,002591066
Schip1	3	1,271496084	0,006837863
Egfl7	2	1,266266586	0,002591066
Klhl6	16	1,265073595	0,004142438
Ifngr2	16	1,253443738	0,002591066

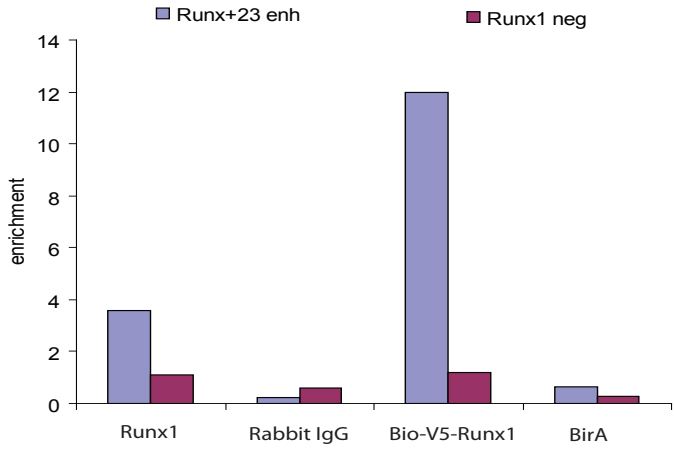
Table S5 All up and down regulated genes LSD1 KD induced MEL cells



S6. LSD1 immunoprecipitation. Antibody IP of endogenous LSD1 in non-induced and induced MEL cells. Control IP normal Rabbit serum. Western blot stained for LSD1 and Runx1.



S7. Bio-V5-Runx1 immunoprecipitation. V5 IP of Bio-V5-Runx1 in non-induced and induced MEL cells. V5 IP in MEL cells used as control. Western blot stained for Runx1 and Myef2



S8. Runx1 and Bio-V5-Runx1 binding to +23 enhancer runx1 locus. Runx1 antibody ChIP and V5 ChIP of Bio-V5-Runx1 in non-induced MEL cells. Control normal Rabbit IgG and V5 ChIP in MEL cells.

Summary

Summary

Erythropoiesis is the generation of erythrocytes from the haematopoietic stem cell (HSC). This process is regulated by transcription factor complexes that change as erythropoiesis progresses. Our lab uses the Mouse Erythro-Leukaemia (MEL) cell line as a model system for terminal erythroid differentiation to identify transcription factors involved in this process. MEL cells overexpress the transcription factor Pu.1, which is an antagonist of Gata1 and halts differentiation at the proerythroblast stage but they can be chemically induced to differentiate towards terminal erythrocytes.
A complex was identified in erythroid cells that is important for erythropoiesis and contains important erythroid transcription factors like Gata1, LDB1, Tal1 and Eto2. The transcription factor Runx1 was also identified as part of this complex while it was unknown to have a role in erythropoiesis. To investigate the potential role of Runx1 in erythropoiesis a Bio-V5 tagged version of Runx1 was expressed in MEL cells to first identify its protein partners using immunoprecipitations and mass spectrometry analysis. This yielded a number of proteins that were previously described as part of the complex but also new partners. Two novel factors were also identified to form a complex with Runx1, Myef2 in the proerythroblast stage of erythropoiesis and LSD1 later in erythropoiesis. Next the genome wide Runx1 DNA binding sites were determined in the proerythroblast and later stages of erythroid development using ChIP-sequencing. In a genome-wide analysis comparing Runx1 binding sites with differentially expressed genes from proerythroblast to erythrocyte differentiation showed that Runx1 binds erythroid specific genes during erythroid development. Binding to the important erythroid genes gata1, eto2 and epb4.2 was confirmed. Further more it was shown that the DNA binding sequences of Gata1 and Tal1 were overrepresented near Runx1 binding sites suggesting that cooperation of these transcription factors is important for erythroid development.
When a genome-wide expression analysis of Runx1 depleted MEL cells showed that Runx1 functions as an activator and repressor at the proerythroblast stage. When the cells differentiate towards erythrocytes much fewer genes are regulated by Runx1 although its genome-wide binding pattern does not change much. The expression levels of the genes gata1, eto2 and epb4.2 were measured independently after Runx1 knock-down which showed that these are indeed upregulated. Thus Runx1 functions as a repressor of these genes in the proerythroblast stage. A knock-down of Myef2 also showed an upregulation of the transcription of these three genes, suggesting that Runx1 forms a complex with Myef2 in the proerythroblast stage to represses a group of erythroid genes. After the proerythroblast stage this complex disassociates and repression is lifted although Runx1 stays bound to these genes. This role of Myef2 was unknown in erythropoiesis and we confirmed its function by zebrafish oligonucleotide morpholino injections against Myef2 mRNA. This confirmed a role for Myef2 in erythropoiesis but also showed an effect on T-cell development and HSC development probably mediated via Runx1.
After the proerythroblast stage Runx1 still regulates a number of genes while it forms

a complex with LSD1. LSD1 is and histone demethylase and represses genes by removing the activating mark on histone 3 lysine 4 (H3K4) mono and di-metylation. When LSD1 is recruited to genes repressed by Runx1 in differentiating cells the H3K4 mono- and di-methyl mark is partially removed. This suggests that Runx1 represses genes during terminal erythropoiesis by recruiting LSD1 to these genes after the proerythroblast stage. Runx1 also activates genes during erythropoiesis but it is unclear from our data how this function is mediated.

Samenvatting

Samenvatting

Erytropoiese is de differentiatie van de hematopoietische stamcel (HSC) naar rode bloedcel (erytrocyt). Dit proces wordt gereguleerd door complexen van transcriptiefactoren die van samenstelling veranderen tijdens de erytropoiese. Tijdens het onderzoek is gebruik gemaakt van de Mouse Erythro-Leukaemia (MEL) cellijn, die model staat voor terminale erytropoiese, om transcriptie factoren te identificeren die bij dit proces betrokken zijn. MEL cellen brengen de transcriptiefactor Pu.1 tot overexpressie die een antagonist is van Gata1 en stopt de differentiatie in de pro-erytroblast fase. Door toevoegingen van bepaalde chemische stoffen kan differentiatie worden geïnduceerd in de richting van terminale erythropoiese.
In erytroide cellen was een eiwit complex geïdentificeerd die belangrijk is voor de erytropoiese en bevat belangrijke erythroide transcriptiefactoren zoals Gata1, LDB1, Tal1 en Eto2. De transcriptiefactor Runx1 was ook geïdentificeerd als een deel van dit complex terwijl een rol in erytropoiese onbekend was. Om deze potentiële rol in erytropoiese te onderzoeken werd er een Bio-V5 gelabelde versie van Runx1 geëxpresseerd in MEL cellen om eerst zijn eiwit partners te identificeren via immunoprecipitatie en massa spectrometrie. In deze analyse werden eiwitten geïdentificeerd waarvan bekend was dat ze onderdeel uitmaakten van het complex. Ook werden twee nieuwe eiwitten ontdekt die in een complex zitten met Runx1. Dit waren Myef2 in de pro-erytroblast stadium en LSD1 in latere erytropoiese. Hierna werden alle genomische bindingsplaatsen van Runx1 bepaald in de pro-erytroblast en latere stadia van de erytroïde ontwikkeling via ChIP-sequencing. In een vergelijking tussen alle genomische Runx1 bindingsplaatsen en differentieel geëxpreseerde genen van de pro-erytroblast naar erytrocyten vinden we dat Runx1 aan erytroïde specifieke genen bindt. De binding aan belangrijke erytroïden genen zoals gata1, eto2 en epb4.2 werd bevestigd.
Wanneer dezelfde analyse werd gedaan maar nu met MEL cellen met een verminderd Runx1 expressie vinden we dat Runx1 zowel als een activator als een repressor functioneert tijdens de pro-erytroblast stadium. Na differentiatie vinden we dat er veel minder genen door Runx1 gereguleerd worden, maar de totale binding aan het genoom veranderd niet veel. De expressie van gata1, eto2 en epb4.2 is verhoogd in MEL cellen met een verminderde Runx1 expressie. Hieruit bleek dat Runx1 functioneert als een repressor van deze genen in de pro-erytroblast stadium. Ook in MEL cellen met een verminderde expressie van Myef2 zijn deze drie genen opgereguleerd, wat suggereert dat Runx1 een complex vormt met Myef2 in het pro-erytroblast stadium en een groep erytroïde genen represeert. Deze rol van Myef2 in erytropoiese was onbekend en werd bevestigd door zebravis oligonucleotide morpholino injecties tegen het mRNA van Myef2. Dit bevestigde de rol van Myef2 in erytropoiese. Verder werd een effect gezien in T-cel en HSC ontwikkeling waarschijnlijk gemedieerd via Runx1.
Na het pro-erytroblast stadium reguleert Runx1 nog steeds een aantal genen en vormt een complex met LSD1. LSD1 is een histon demethylase en represeert genen

door het verwijderen van de activerende markering op histon 3 lysine 4 (H3K4) mono- en di-methylatie. LSD1 wordt gerekruteerd naar genen door Runx1. Deze genen worden gerepreseerd tijdens differentiatie door Runx1. Waarschijnlijk word door de rekrutering van LSD1 de H3K4 mono en di-methylatie gedeeltelijk verwijderd. Dit suggereert dat Runx1 genen represeert tijdens terminale erythropoiese door het rekruteren van LSD1 naar deze genen na het pro-erytroblast stadium. Runx1 activeert ook genen tijdens de erytropoiese maar hoe dit werkt blijkt niet uit onze data.

Dankwoord

Dankwoord

Het is nu bijna 5 jaar geleden dat ik op de Erasmus begon met werken. In die tijd is er veel in mijn leven veranderd en ben ik ook als mens veel veranderd.

First of all I want to thank my promoter and supervisor Frank Grosveld for giving me the opportunity to work in his lab. During the years you have taught me great deal about performing research and interpreting results. Even though you have an immensely busy schedule you always had time for me to discuss results and help me to proceed in my promotion. I want to thank you for you kindness and patience during the years in your lab.

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Boet, 2011

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Education
1997-2001
Medical biology at laboratory sciences in Etten-Leur (HLO).
Education direction: biochemistry and immunology.
Education successfully finished in 2001.

2001-2005
Medical biology at the Radboud University Nijmegen.
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2006-2010
PhD student in the laboratory of Prof. Dr. Frank Grosveld.
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Title of thesis: Role of Runx1 in definitive erythropoiesis
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Research experience
2000-2001
Numico research B.V. in Wageningen.
Research title: Influence of DGLA on cytokine production PBMC's in vitro.
Supervisor: Dr Dooper MM
2004
Nijmegen centre for molecular life sciences (NCMLS) department Molecular Biology.
Research title: p63γ exhibits enhanced p21 promoter binding after DNA damage in vitro.
Supervisor: Dr M Lorhum and Prof.dr. HG Stunnenberg
2005
University medical centrum St. Radboud Department of Haematology.
Research title: Enhanced expression of chemokines in follicular lymphoma tumour B-cells and surrounding T-cells.
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Publication list

Dihomo-gamma-linolenic acid inhibits tumour necrosis factor-alpha production by human leucocytes independently of cyclooxygenase activity. Dooper MM, van Riel B, Graus YM, M'Rabet L. Immunology. 2003 Nov;110(3):348-57

The role of the transcription factor Runx1 in erythropoiesis
Boet van Riel, Tibor Pakozdi, Rutger Brouwer, Rui Monteiro, Roger Patient, Jan Christian Bryne, Erik-Jan Rijkers, Wilfred van Ijken, Charlotte Andrieu-Soler, Eric Soler, Jeroen Demmers, Boris Lenhard and Frank Grosveld (*in prep*)

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PhD training

Courses
2005
Course laboratory animal sciences, article 9.
2006
Reading and Discussing Literature course
Molecular and Cell Biology course
2007
BAC recombination techniques course
Radioactivity Dutch License 5B and completed the institute (in-house; Erasmus MC) course.
2009
English writing course (in-house; Erasmus MC)

Presentations
March 2007
Kleinwalsertal, Austria. 4th Winterschool of the International PhD program, Transcriptional Control of Developmental Processes. Presentation: 'Runx1 complexes formed in definitive erythroid cells'
June 2007
15th MGC Graduate Student Workshop Heidelberg. Presentation: 'Target gene identification of Runx1 via next generation sequencing'
August 2009
16th Runx workshop 'RUNX Transcription Factors in Development & Disease'. Presentation: 'Proteomics of hematopoietic transcription factors'

Extracurricular Activities
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Medical Genetics Centre (MGC) PhD students organizing committee: Organising 16th MGC Graduate Student Workshop Brugge.