The Role of Sp1 and Sp3 Transcription Factors in Hematopoiesis

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Cover: B and T cells in the spleen.

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The Role of Sp1 and Sp3 Transcription Factors in Hematopoiesis

De rol van Specificiteits transcriptie factoren Sp1 en Sp3 in hematopoiese

Thesis

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Prof.dr. H.G. Schmidt

and in accordance with the decision of the Doctorate Board

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<th>Full Form</th>
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<tbody>
<tr>
<td>AGM</td>
<td>Aorta-Gonad-Mesonephros</td>
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<tr>
<td>Btd</td>
<td>Buttonhead</td>
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<tr>
<td>BCR</td>
<td>B-cell receptor</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<tr>
<td>Bp</td>
<td>Base pair (s)</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>(c)DNA</td>
<td>(complementary) Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Cre recombinase</td>
<td>Causes recombination</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>ChIP-Seq</td>
<td>Chromatin Immuno Precipitation coupled to high-throughput Sequencing</td>
</tr>
<tr>
<td>(c)KO</td>
<td>(conditional) Knockout Out</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
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<tr>
<td>FL</td>
<td>Fetal Liver</td>
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<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorting</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocytes Myeloid Progenitor</td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic Stem Cells</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyl Transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>LacZ</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>LoxP</td>
<td>Locus of X-over (crossing over) of P1</td>
</tr>
<tr>
<td>(m)RNA</td>
<td>(Messenger) Ribonucleic Acid</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte Erythroid Progenitor</td>
</tr>
<tr>
<td>MK</td>
<td>Megakaryocyte</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic Activated Cell Sorting</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>PC</td>
<td>Peritoneal Cavity</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PLT</td>
<td>Platelet</td>
</tr>
<tr>
<td>RT-(PCR)</td>
<td>Reverse Transcriptase-(Polymerase Chain Reaction)</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>Sp/KLF</td>
<td>Specificity protein / Krüppel like factor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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<tr>
<td>YS</td>
<td>Yolk Sac</td>
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Chapter 1

General Introduction
Hematopoiesis is the generation of all mature blood cells from a rare pool of hematopoietic stem cells (HSCs). These blood cells serve a variety of essential functions in the vertebrate body, including gas transport, defense against pathogens, blood clotting and removal of apoptotic cells. Due to their limited life span, they need to be generated continuously throughout life in the stem cell niches of the bone marrow and several discrete anatomical niches that change rapidly, accompanying the highly dynamic processes characteristic of embryonic development [1-3]. Multi-potent hematopoietic stem cells give rise to a succession of progenitors from which mature blood cells are derived. The hematopoietic system is made up of all mature blood cell types including erythrocytes, platelets, and other cells of the myeloid and lymphoid lineages.

Multiple waves of hematopoiesis occur during embryonic development. The initial wave of blood production, termed primitive hematopoiesis, occurs in the mammalian yolk sac followed by definitive hematopoiesis originating from long term-hematopoietic stem cells that arise from the aorta-gonad-mesonephros (AGM) origin [2].

**Primitive hematopoiesis**

The first mesodermal cells emerge at the primitive streak stage and this gives rise to embryonic and extra-embryonic mesoderm. HSCs have been proposed to arise from mesodermal progenitors with both endothelial and hematopoietic potential called hemangioblasts. These hemangioblasts are characterized by expressing proteins such as Brachyury (Bry) and Fetal Liver Kinase 1 (Flk1). In the mouse, primitive hematopoietic cells first appear in the yolk sac (YS) from the hemanglioblast cells in the blood island (Figure 1) at 7.5 days postcoitum (dpc) [4-6]. Bry⁺/Flk⁺ hemangioblasts are first detected at the mid-streak stage of gastrulation and peak in number during the neural plate stage[7]. They function in producing red blood cells that facilitate tissue oxygenation as the embryo undergoes rapid growth. Primitive erythroid cells are transiently produced from Embryonic day 7 (E7) to E9. This is confined to the yolk sac blood islands and followed by production of definite hematopoietic progenitors[8]. Primitive erythrocytes are nucleated, larger than definitive erythrocytes and express the embryonic globin genes, ζ, εy and βh1[9, 10].
General Introduction

Figure 1. Mechanism of yolk sac (YS) blood island formation. a) Schematic view of the progressive evolution of blood island mesodermal cells to a functional vascular network and primitive erythroid cells. Two alternative mechanisms of the YS blood island formation are shown. b) The hemangioblast can directly give rise to both the endothelial and hematopoietic cell or these cells can arise via the intermediate production of hemogenic endothelial cells. c) Specification into blood cell and endothelial cells might even have occurred at earlier stages of differentiation. Numbers 1 and 2 indicates the biological processes occurring at the respective stages of development. Drawing has been adapted and modified from [11]

Definitive hematopoiesis

In the developing mouse embryo, the first definitive hematopoietic stem cells capable of giving rise to complete hematopoietic engraftment of adult recipients emerge in the dorsal aorta of the aorta-gonad-mesonephros (AGM) region at embryonic day (E) 10.5. They are thought to arise as clusters from the hemogenic endothelium [2, 12-14]. \textit{In vivo} cell lineage tracing and conditional deletion of transcription factors such as \textit{Runx1} and \textit{Gata 1} have provided strong evidence for a hemogenic endothelial origin of blood cells. Previous opposite views on the origin of blood cells either from the hemangioblast or the hemogenic endothelium has been linked together in a linear model in which the hemangioblast generates hematopoietic cells through a hemogenic endothelium intermediate
stage (figure 1) [15-17]. The yolk sac has also been mentioned as a possible site for multipotent hematopoietic stem cells that initiates definitive hematopoiesis [18, 19]. HSCs have also been detected in the placenta starting at E10.5- E11. Placental HSC activity parallels that of the AGM but expands significantly more than the AGM (>15fold more HSCs). This suggests that they are either generated there or originate from the AGM [20-23].

Later on in gestation, the fetal liver becomes the main organ harboring HSCs from the yolk sac, AGM and the placenta. De novo HSCs are not generated in the liver [2, 22, 24]. HSCs from the fetal liver migrate to the bone marrow before birth. They are maintained in a hematopoietic niche, which creates a microenvironment protective against apoptotic and differentiation stimuli and maintains quiescence of the pool of HSCs [1, 25].

Definitive HSCs can also differentiate into all cells of the hematopoietic lineage and are able to self-renew. These potentials are maintained throughout life [26].

Model for the hematopoietic system hierarchy

The adult hematopoietic system consists of at least eleven distinct lineages, representing mature cells with highly specialized functions (Figure 2). Despite this diversity, all of these lineages are descendants of the same hematopoietic stem cells (HSCs). During the initial step of differentiation, HSCs lose their self-renewing potential but retain the multilineage differentiation potential effectively generating the multipotent (multilineage) progenitor cell (MPP) [27]. Lympho-myeloid stem cells expressing Flt3 lack erythro-megakaryocytic potential [28]. MPPs differentiate into pluripotent progenitors (common lymphoid and common myeloid progenitors) which are more restricted in lineage commitment [29, 30].

Common lymphoid progenitors will give rise to adult cells that includes B-lymphocytes (B-cells), T-lymphocyte (T-cells), natural killer (NK) cells and dendritic cells (DCs) which form the adaptive (acquired) arm of the vertebrate immune system. B cells originate from HSCs in the bone marrow where they migrate to secondary lymphoid organs like the spleen and lymph node. Very early progenitors and/or HSCs seed the thymus, where they develop into T cells. During their differentiation in the thymus (T cells) and in the bone marrow (B cells), they are selected for tolerance (when they are still maturing). In addition, B cells undergo peripheral selection so that they only recognize foreign antigens.

The maturation and functions of B and T cells are closely linked to that of mononuclear phagocytes macrophages and DCs. When B cells recognize foreign antigens, they become activated and endocytose the antigens, process it and in the context of major histocompatibility complex (MHC) proteins, present this processed antigen to helper T cells (Th cells). B cell
responding to antigens may either differentiate to memory B cells or into plasma cells. Plasma cells produce large quantities of antibodies with the same specificity as the original B cell receptor but due to differential splicing, the immunoglobulins are no longer membrane-bound but secreted which fights the invaders. Memory B cells can mount a faster and stronger response to the same invading pathogens that they recognized.

**Figure 2. Hematopoietic Lineage specific Cre lines.** The figure shows the classical hierarchical model of hematopoiesis and the lineages and cell types where various Cre recombinase are expressed. The hematopoietic stem cell (HSC) gives rise to multilineage progenitors (MLP), which differentiate into all the hematopoietic lineages. MLPs become lineage-restricted to the lymphoid and myeloid lineage in the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) respectively. CLPs give rise to B and T-cells while CMPs can give rise to megakaryocyte/erythrocyte progenitors (MEP) and granulocyte/monocyte progenitor (GMP).

Dendritic cells are the most important antigen presenting cell (APC). They are very potent in internalizing antigens either by phagocytosis or receptor-mediated endocytosis, process it and in the context of MHC II molecule, display a fragment...
of the antigen on their membrane surface. T cell then recognizes the antigen-MHC II molecule complex for destruction. Helper T cells (CD4+) respond to foreign antigens by producing cytokines while cytotoxic T cells (CD8+) produce toxic granules which destroy infected cells. NK cells recognize virus infected cells in the absence of antibodies and MHC proteins and induce cell lysis or apoptosis.

Common myeloid progenitors (CMP) give rise to other hematopoietic cells including granulocytes, macrophages and erythroid cells. Neutrophils, eosinophils and basophils are called granulocytes because of the presence of granules in their cytoplasm. They are produced in the bone marrow and circulate in the blood stream. Macrophages are generally regarded as professional phagocytes as they specialized in removing dead or dying cells and cellular debris. Neutrophils constitute 50-60% of total circulating white blood cells. They rapidly engulf pathogens coated with antibodies and damage cells or cellular debris. They also attack invaders by releasing soluble anti-microbials as well as neutrophil extracellular traps (NETs) that trap the microbes. Eosinophils have limited ability to participate in phagocytosis. Their main role is in the killing of parasites by producing toxic granules that destroy the invading organism. The granules released by basophils and mast cells contain mediator agents such as histamines which contribute to the inflammatory response thereby contributing to the elimination of the pathogens. An unbalanced immune response for example due to excessive histamine production, can lead to an allergic disease.

Platelets and erythrocytes are other hematopoietic cell types derived from CMPs. Platelets bud from megakaryocytes which are produced in the bone marrow. Their primary function is to participate in the formation of clot clots and wound healing thereby maintaining a normal homeostasis. Erythrocytes are generated in the bone marrow from erythroid precursor cells expressing CD71 and Ter119 [31-33]. They expel their nucleus and other organelles before entering the general circulation. Expelling their nucleus allows for maximum surface area for gas transportation [34].

Perturbations in hematopoiesis are the cause of a plethora of medical conditions, including leukemias, hereditary anemias and autoimmune disorders. The hematopoietic system is a self-renewal system and serves as important system to study these disorders and provide the molecular basis for the development of new therapies to combat many diseases. Transcription factors play an important role in regulating this system. This thesis focuses on the crucial role of Specificity (Sp) transcription factors in hematopoiesis. Transcription factors that regulate B cell and megakaryocyte (platelets) development will be discussed briefly below.
Transcriptional control of B cell lymphopoiesis

The development of antibody producing B lymphocytes from hematopoietic stem cells (HSC) occurs in the bone marrow (BM) in a step-wise process, involving a controlled lineage and locus-specific rearrangement of the immunoglobulin heavy chain (Igh, IgHC) and light chain loci, κ (Igk) or λ (Igl), mediated by the recombination activating genes (RAG-1 and RAG-2) [35-38] (Figure 3). The earliest committed B cell precursors are pre-pro B cells which can be identified by expression of the B cell-associated marker B220 (CD45R) and the activation of many B cell-lineage specific genes [39-41]. B lineage committed cells also express CD19 which is a target of the lineage committed factor Pax5 and have their immunoglobulin locus in germline configuration. Ig heavy-chain (Igh) V(D)J recombination is initiated at the next stage of development, the Pro B cell stage. Upon successful V_H to DJ_H rearrangement, the precursor B cell receptor (Pre-BCR) is expressed on the cell surface comprising of Igμ H chain, non-rearranged surrogate light chain (SLC) constituents, λ5 and VpreB in association with Ig-α and Ig-β signal transduction subunits. The expression of the pre-BCR serves as a key checkpoint regulator in B cell development which monitors the assembly of functional IgHC. The proper pre-BCR and interleukin-7 receptor (IL-7R) signaling induces a burst of pre B cell proliferation and survival [42-44]. Igh rearrangement is terminated and the large cycling pre-B cells further differentiate into the small resting pre-B cells at which point the Ig κ or λ light chain (Igl) V_L-to-J_L recombination is initiated along with changes in cell surface markers. IL7-R expression and CD43 are both terminated and the small pre-B cells start up-regulating the adhesion molecule CD2, CD25 (the IL-2 receptor) and major histocompatibility complex (MHC) class II [42, 43]. Successful Igl gene rearrangement leads to the expression of the BCR on the cell surface and the transit to immature B cells where they are checked for auto-reactivity before leaving the BM to the periphery.

The process of B lineage specification and commitment depends on the coordinated actions of various signaling cascades and transcriptional networks, which involves the initiation of B cell-specific gene expression and repression of alternative lineage-specific genes [45]. Fms-like tyrosine kinase 3 (Flt3) receptor and IL7 receptors are both crucial for CLP to develop into mature B cells as deficient mice have impaired lymphocyte expansion[46-51]. Transcription factors including PU.1 (Sfpi1), Ikaros (ikzf1), E2A (Tcfe2a), Early B cell factor, (Ebf1), Pax5, Aiolos (ikzf3), Sox4 and Bcl11a form networks that controls early B cell lymphopoiesis. PU.1, Ikaros and E2A are required for CLP (LMPP) to become lymphoid restricted by up-regulating the expression of IL-7R and Flt3 [49, 52-58]. The zinc finger transcription factor Bcl11A has also been implicated in early B and T lymphopoiesis [59-61]. B cell specification involves the expression of E2A and Ebf1 as well as up-regulation of II7-R signaling at the pre-pro B cell stage [51, 62].
They work separately and in concert to regulate the expression of most of the B cell-specific genes such as λ5, VpreB, Igα (Mb-1 or CD79a) and Igβ (B29 or CD97b) [63-67] (Figure 4). B cell lineage commitment (pro-B cells) is finally achieved by the induction of Pax5 by E2A and Ebf1 [67, 68]. Pax5 is a member of the paired box (PAX) family of transcription factors which activates the expression of B lineage genes and represses genes associated with other lineages [69-71].

Figure 3. The development of B cells begins in the bone marrow (BM) from the hematopoietic stem cells (HSC). Committed B cells at the pro-B cell stage move to the large pre-B cell stage by successful expression of Igμ and the surrogate light chain (SLC) on the cell surface. After proliferating, these cells internalize their SLC and become the small pre-B cell. IgL rearrangement is then initiated. Successful expression of IgL on the surface marks the transition from the small pre-B cell stage to the immature B cell stage. Non-autoreactive immature B cells expressing a fully recombined BCR migrate into the periphery as mature B cells.

Pax5 repressed genes include receptors involved in the differentiation of progenitors to myeloid, DC or T lineages for example, M-CSF receptor and Flt3 [71, 72]. Pax5 blocks T cell development and promotes B cell lymphopoiesis by blocking Notch1. It has also been shown to silence the myeloid-specific genes by binding to the myeloid specific colony-stimulating-factor-receptor (Csf1R) gene and reduce the frequency of binding of the basal transcription machinery to the promoter and by activating antisense RNA expression [73]. Pax5-deficient B-cell progenitors were able to reconstitute T cell development in vivo further strengthening the importance of Pax5 in B cell commitment. [74, 75]. It also regulates multiple components of the pre-B and the BCR and activates genes
which include CD19, Slp-65, and transcription factors implicated in B cell differentiation such as Id3, Ief1, SpiB, Irf4, Irf8, and Aiolos while down-regulating Flt3[76]. One of the components of the pre B cell receptor complex, Igα (encoded by the Mb-1 gene), is regulated by combinatorial action of E2A, EBF1, Pax5, Ets1, Sp1, and Runx1[62]. It is worth noting that in addition to Pax5, Ebf1 can also restrict alternative lineages options and promote B cell fate commitment independently of Pax5 [77, 78].

Figure 4: Transcription factors critical for B cell development. Transcription factors involved in various stages of B cell development, growth factor receptors and cell- surface markers are shown. From left to right the successive stages include LMPP (lymphoid-primed multipotent progenitor), ELP (early lymphoid progenitor), pre-pro-B cell, and committed pro- and pre-B cell [45].

Transcriptional control of Megakaryopoiesis

Megakaryopoiesis is the process by which platelets or thrombocytes are formed from megakaryocytes (MKs) originating from HSCs in the bone marrow. During MKs maturation, they undergo endomitotic cell cycles during which they replicate DNA but do not undergo cytokinesis and as a result acquire a DNA content of up to 128N (where 2N is the DNA content of a diploid somatic cell). MKs are large cells (~150μM in diameter) having a single, large, lobulated, polyploid nucleus[79]. Platelets bud off from the cytoplasm of MKs as proplatelet filaments. They lose their filaments thereby becoming platelets. Platelets do not have a nucleus but contain organelles such as mitochondria, and also microtubules and granules. They function in maintaining normal homeostasis by participating in the formation of platelet-rich clot when the continuity of the vasculature is interrupted and in healing wounds. In the immune system, they promote inflammation which could lead to the development of diseases such as atherosclerosis [80].

Transcription factors regulating the process of megakaryopoiesis include GATA1/2, FOG-1, Fli-1, Runx1, NF-E2 and Tal1 which can either act individually or in tandem. The Gata1 transcription factor is essential for maturation of erythroid and megakaryocytic cells as Gata1 knockout embryos do not survive due to severe
anemia while inactivation of Gata1 in megakaryocytes leads to thrombocytopenia and accumulation of immature MKs [81-84]. Gata1 and Gata2 have overlapping functions during MKs development [85]. The function of Gata1 is modulated by its interaction with other proteins including Sp1, EKLF, Fli1 and FOG-1 [86-88]. GATA1 and its co-factor FOGER (Friend of Gata1) are critical in promoting megakaryocyte-erythroid differentiation while at the same time inhibiting the expression of PU.1 and myeloid differentiation [89, 90]. FOGER binds to GATA protein and has

**Figure 5: Megakaryopoiesis.** Under the influence of TPO, HSC proliferate and differentiate into megakaryocytes [91].

similar expression pattern in erythroid cells and in MKs. Mice deficient of FOGER lack MKs and the erythroid defect is a phenocopy of that of GATA1 null mice [92, 93]. Fli-1 activates many MK-specific genes such as GATA1, GP11B, GPVI and GPIB and represses the activity of erythroid factors at erythroid promoters [94-97]. It is required for maturation of MKs as Fli-1 knockout MKs display immature morphology and a significantly reduced expression of the late MK gene GPIX [98, 99]. In addition to its role in the formation of HSCs during embryogenesis, Runx1 has an important role in the development of MKs as conditional deletion of Runx1 in mice leads to thrombocytopenia and impaired megakaryopoiesis [100-103]. Proplatelet formation requires microtubules (β1-tubulin) which is regulated by NF-E2 [104, 105] NF-E2 knockouts are embryonically lethal due to hemorrhage and the absence of circulatory platelets (thrombocytopenia). Targeted disruption of NF-E2 showed defective thrombopoiesis as a result of impaired proplatelet production suggesting its role in later stages of megakaryocyte maturation [106, 107]. Conditional inactivation of TAL1 (SCL) in the hematopoietic lineage resulted in a specific decrease in red cell and MK production suggesting its role in the MEP [108, 109]

Thrombopoietin (TPO) is by far the most potent regulator of megakaryopoiesis. Together with its receptor c-Mp1 it is critical for Mk growth and development. Knockout mice have reduced number of MKs and platelets [91, 110, 111]. HSCs also express TPO and c-Mp1. This is important for the maintenance of the hematopoietic pool (self-renewal) and expansion as TPO and c-Mp1 mutant
mice have reduced numbers of HSCs and progenitors of all lineages including MEPs\cite{111-113}. In addition to TPO, other thrombopoietic cytokines include stem cell factor (SCF), erythropoietin, IL-3, IL-6 and IL-11\cite{114-117}.

**The Sp/KLF family of transcription factors**

The Sp/KLF (Specificity Protein/ Krüppel-Like Factor) family of transcription factors is composed of a large number of different proteins. At the moment, 26 members have been identified comprising of nine different Sp proteins, designated Sp1 to Sp9 and seventeen KLF proteins also designated KLF1 to KLF 17 \cite{118-122}. The hallmark characteristic of the family members is the presence of a highly conserved DNA-binding domain composed of three zinc fingers of the Cys2His2 type, located at the C-terminus of the proteins (Fig. 6) \cite{119, 123}. These krüppel-like zinc fingers were first identified in the *Drosophila melanogaster* segmentation Krüppel (Kr) protein \cite{124}. Each zinc finger contains a motif of three amino acids which contributes to the DNA binding properties of the family members. In human, the krüppel-like three zinc fingers was first identified in the family founder Sp1, and is believed to contact DNA with amino acids KHA in the first, RER in the second and RHK in the third zinc finger \cite{119}. Zinc finger 1 has been shown to contribute at most moderately to total binding affinity and overall sequence specificity by the three zinc fingers of Sp1 \cite{125, 126}. Due to amino acid substitutions in the zinc fingers, Sp/KLF members recognize GC- (GGGGCGGG) and GT- (GGTGTGGGG) boxes with different affinities. GC and GT boxes are cis-acting elements found in the promoters and enhancers of many ubiquitous, tissue specific, viral and cell cycle specific genes and are important for their expression. Additionally, GC/GT motifs have been shown to be involved in the maintenance of methylation-free status of CpG islands for the adenine phosphoribosyltransferase (APRT) gene \cite{127, 128}.

Since the experiments presented in this thesis are based on the Sp transcription factors, a more detail description of the Sp family members will follow below.

**Structural characterization of the Sp family members**

Sp factors differ from their KLF counterparts by the presence of a Buttonhead (BTD) domain on the N-terminal side of the DNA binding domain (Fig. 6). BTD was first described in *Drosophila* as the mammalian homologue of Sp1 because it encodes a zinc finger transcription factor similar in sequence and function to mammalian Sp1 \cite{129}. The BTD of Sp1 and Sp3 are important for the synergistic activation of proteins such as the sterol regulatory element binding protein (SREBP) at promoters with a single SREBP binding site \cite{130-132}. The
BTD-box regulates transcription activities of Sp factors and deletion of domain C in Sp1 (figure 6) containing the BTD-box resulted in reduced Sp1-mediated transcriptional activation [133]. A stretch of amino acid (SPLALLAATCSK/RIG/E) of unknown function called the Sp-box (figure 6) is located at the N-termini of the Sp proteins [134]. In the past, Sp was the abbreviation of the sephacryl- and phosphocellulose column used to isolate and purify the Sp1 protein. Today it refers to specificity protein.

A very close evolutionary relationship exist between human Sp genes and the homeobox (HOX) gene clusters as they co-localize on the following chromosomes, Sp1 and Sp7 (osterix) co-localize near HOX C genes on 12q13.13 [135, 136], Sp2 and Sp6 near HOX B on 17q12.31/32 [137, 138], Sp3, Sp5 and Sp9 near HOX D on 2q31.1 [139, 140] and Sp4 and Sp8 near HOX A on 7q21.2 [118, 141].

Within the Sp family, Sp1, Sp2, Sp3 and Sp4 form a subgroup further characterized by a N-terminal glutamine-rich activation domain whereas Sp5 to Sp9 contain proline, alanine or serine/threonine- rich domains and are remarkably shorter in length [122]. Sp1, Sp3 and Sp4 each have two glutamine- rich activation domains (domains A and B) which are essential for transcriptional activation while Sp2 has only one (Figure 6). Serine and threonine-rich sequences are located close to the A and B domain which may be a target for post- translational modifications.

Sp1 and Sp3 are ubiquitously expressed while Sp4 is predominantly expressed in neuronal tissues [121]. Sp2 is expressed in several cells lines. Sp1, Sp3 and Sp4 recognize the same promoter elements with similar specificity and affinity [142]. Sp2 was thought to have a different consensus-binding site because of substitution of a critical histidine residue by a leucine in its zinc finger 1 but recently, Sp2 was shown to also binds to the GC-box motifs similar to Sp1 and Sp3[137].

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**Figure 6.** Structural features of Sp-proteins. Schematic representation of the four human Sp-family members Sp1, Sp2, Sp3 and Sp4. Amino acid length is indicated on the right. Colors for Sp box, buttonhead (BTD) box, zinc-finger domain, highly charged region, serine/threonine (S/T)-rich, and
Transactivational functions of Sp factors

Despite the high degree of similarity in the DNA-binding activities of the Sp proteins, family members differ broadly in the ability to regulate transcription. Traditional studies on the trans-activation potential of Sp factors were performed in *Drosophila* SL2 cells since Sp1-like proteins were thought not to be present in *Drosophila* making them ideal cells for functional analysis of Sp-like proteins. However, this view was challenged with the cloning of the *Drosophila* Sp1 homologue Buttonhead [129] and D-Sp1 [143]. Nonetheless, Sp1-like activities are reportedly absent in SL2 cell extracts [133, 144].

**Sp1**

Sp1 is the founding member of the Sp subfamily. It was the first to be cloned and identified as a transactivator of the SV40 (Simian Virus 40) early promoter [123, 145-147]. Human Sp1 can stimulate transcription both from proximal promoter elements and distal enhancers [148]. Their three zinc fingers recognize the GC- and GT-boxes [149]. Glutamine-rich activation domains (A and B) are essential for super-activation of Sp1-dependent transcription. This was shown using a DNA binding-deficient mutant that retains glutamine-rich activation domains interacting with proximally bound Sp1 to super-activate transcription [142, 148]. For synergistic activation through binding to multiple sites, domains A, B and the carboxyl-terminal domain D (figure 6) are all required [150]. Domain C has a weak activation potential when compared to Domains A and B [148]. Interacting with co-repressors like SMRT, NcoR and BCoR, Sp1 can repress transcription via interaction of its inhibitory domain located in the N terminus and zinc fingers when targeted to the proximal promoter [151].

**Sp2**

Sp2 was identified by screening human HUT78 (αβ T cells) cDNA libraries for GT box binding proteins homologous to Sp1 in its DNA-binding domain (zinc fingers) under low-stringency conditions [137]. It binds to the GT-box in the T-cell antigen receptor α (TCRα) and differs from Sp1 and Sp3 in its inability to activate promoters containing the classical GC- and GT- boxes [152, 153]. The differences in DNA binding affinity between Sp2 and Sp1 can be readily explained by the difference in amino acid composition in zinc finger 1, with a leucine residue in Sp1 being substituted by a histidine residue in Sp2. Furthermore, Sp2 has only one glutamine-rich activation domain and no domain D (Figure 6) in the C-terminus of the protein. The presence of one activation domain in Sp2 implies that it might
function as a transcriptional activator in a manner distinct from Sp1, Sp3 and Sp4, which all have two activation domains. In Sp1, these two activation domains are required for super-activation and synergistic activation[150]. Sp2 has also been shown to activate the expression of the Soxs1 gene mediated by interferon-γ [154]. It can also repress transcription in a cell type specific manner. It activates Sp1 and Sp3 driven transcription of the murine CTP:phosphocholine cytidylyltransferase promoter in insect cells but represses the same construct in mammalian cells [155]. It can repress the transcription of the tumor suppressor gene carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in certain tumors [156].

Sp3
Among all the Sp family members, Sp3 and Sp1 are structurally and evolutionary most closely related to each other [149]. Each has two activation domains necessary for transcriptional activation (figure 6). Sp3 recognizes the GC- and GT-boxes with similar affinity as Sp1. However, depending on the cellular context and the promoter activity in question, functional studies have revealed that unlike Sp1, Sp3 functions as transcriptional activator or repressor. Sp3 can activate transcription in many cell lines including Drosophila Schneider cells and mammalian cell lines [157, 158]. Sp1 and Sp3 do exhibit additive and synergistic effects on promoter activity [157-162].

However, under certain conditions, Sp3 is at best weakly active and in the case of a promoter containing multiple adjacent binding sites, Sp3 has been shown to repress transcription driven by Sp1 or other transcription factors [163, 164]. For example, Sp3 represses the Sp1-mediated trans-activation of human COL2A1 gene in chondrocytes [165]. The inhibitory domain (ID) located between the second glutamine-rich activation domain and the first zinc finger (figure 6) has been associated with the repressive function of Sp3 [166]. The amino acid triplicate KEE located in the ID has been shown to be absolutely crucial for the repressive function of Sp3 as substituting the triplicate amino acid residues with alanine turn the transcriptional inactive Sp3 into a strong activator [166]. KEE is a target for post-translational modification with SUMO [167]. Post-translational modifications of Sp factors will be discussed below.

Sp4
Sp4 is expressed predominantly in neuronal tissues and was first cloned along with Sp3 by recognition site screening using GT-box of the uteroglobin gene promoter [149]. The GT box is also found in the SV40 enhancer. Sp4 is similar in structure to Sp1 and Sp3 and via its three highly conserved zinc fingers, binds the GC- and GT-boxes with similar affinity and specificity as Sp1. Having two glutamine-rich activation domains (Figure 6), Sp4 is also able to activate transcription. Co-transfection experiments into insect SL2 cells revealed that Sp4 is an activator like Sp1 and that the N terminus of Sp1 is able to super-activate Sp4 mediated
transcriptional activity suggesting that the glutamine rich activation domain of Sp1 and Sp4 are functional related. However, it differs from Sp1 in that it is not able to synergistically activate promoters due to the lack of a functionally active domain D which is crucial for the synergistic activation observed in Sp1 [142]. Several promoters can be regulated by Sp4 and other Sp family members in mammalian cells as well as in Drosophila [168-170]

**Post-translational modifications of Sp factors**

Trans-activation of target genes by Sp proteins is dependent on post-translational modifications as well as physical interaction of the Sp proteins with each other, with additional sequence-specific DNA-binding proteins, and with components of the basal transcription complex. The modifications include phosphorylation, glycosylation, sumoylation and acetylation.

**Phosphorylation**

Protein phosphorylation is a post-translational modification in which a serine, threonine or tyrosine residue is phosphorylated by a kinase by adding a covalently bound phosphate group. Phosphorylation of Sp1 proteins has been shown to affect its DNA binding ability and its stability. Different kinases phosphorylate the three amino acid residues listed above in different parts of Sp1. Sp1 phosphorylation can influence transcription by either resulting in an increase or decrease in its DNA binding activity and this has an effect on the expression of genes that control cell growth, differentiation, survival and cell cycle progression. Examples of phosphorylation sites in Sp1 and their related kinases are discussed below.

Protein kinase C (PKC), which regulates cell cycle and cell growth can mediate the phosphorylation of Sp1 at Thre739 of Sp1 [171]. This phosphorylation is important for the transcription of myelin basic protein during oligodendrocyte differentiation [172]. PKC-ζ selectively interacts with and phosphorylates the zinc finger domain of Sp1 [173-175]. Angiotensin II which activates PKC-ζ phosphorylation, stimulates Sp1 phosphorylation in the zinc finger domain (Thre668, Ser670 and Thre681) and increase Sp1 binding to platelet-derived growth factor-D promoter [176]. Other PKC isotypes (-α and –δ) have also been shown to partake in the signaling pathways that induce phosphorylation [177]. PKA and PKG are other kinases that regulate cell growth and cell cycle which can phosphorylate Sp1 and enhance its DNA binding [178]. The stability of Sp1 can be regulated by its phosphorylation by JNK at Thr278/Thr739 during mitosis [179, 180]. Sp1 phosphorylation in HAE cells has also been reported [179]. DNA-dependent protein kinase (DNA-PK) was the first kinase to be shown to phosphorylate Sp1 at the serine/threonine residues [181]. MAP kinase/Erk kinase modulates the interaction between Sp1 zinc finger binding domain or Sp1ID (figure
with co-repressors such as SMRT, NcoR and BCoR leading to transcriptional repression under certain circumstances [151]. p38 MAP kinase phosphorylates Sp1 at Thr453 in a rat pancreatic β cell line [182, 183].

Cyclin A dependent activation of cyclin-dependent kinases (CDKs) is essential for many stages in the cell cycle. Cyclin A-CDK can interact and phosphorylate Sp1 at Ser59 and enhance its DNA binding ability thereby increasing the expression of target genes [184, 185].

Phosphorylation of Sp3 has also been reported to have functional consequences. Erk1/2 phosphorylates Sp3 and induces gene expression in Caco-cells. Unlike Sp1, phosphorylation of Sp3 doesn’t appear to influence its DNA binding ability or its stability but instead enhances its transcriptional activity on VEGF gene expression [186].

Glycosylation

Many cytoplasmic and nuclear proteins are modified post-translationally at the hydroxyl groups of specific serine and threonine residues by a single monosaccharide, acetyl-D-glucosamine (GlcNAc). The covalent o-glycosidic bond formation is catalyzed by O-GlcNac transferase (OGT). Glycosylation has been shown to modulate the activity of proteins via influencing protein-protein interactions, DNA binding affinity, subcellular localization, half-life and proteolytic processing. Sp1 has several O-linked GlcNAc-residues on serines and threonines located mostly at the C-terminal half of the protein. The O-GlcNAcylated form of Sp1 was more active than non-O-GlcNAcylated proteins [187, 188]. Insulin-mediated O-GlcNaclylation of Sp1 facilitates its migration to the nucleus where it is sequentially deglycosylated and then phosphorylated [189, 190]. O-GlcNacylation of Sp1 has also been shown to be required for the expression of a number of target genes including the Ogt gene which encodes the enzyme OGT. Inactivating Ogt resulted in a decrease in O-GlcNAc-modified Sp1 and an increased accumulation of phosphorylated Sp1. This suggest a balance of O-glycosylation and phosphorylated is needed to regulate the activity of Sp1 and other proteins [191, 192].

In vitro O-glycosylation of a Sp1-derived peptide containing glutamine-rich transactivation domain B-C prevents it from untimely ectopic interacting with itself or full length Sp1 protein or TAF110 [193, 194].

SUMOylation

Small Ubiquitin-like Modifier (SUMO) proteins are attached to and detached from other proteins in cells to alter their functions. They play a role in various cellular processes including nuclear-cytosolic transport, apoptosis, protein stability, cell cycle progression and transcriptional regulation. SUMO modification of Sp3 transcription factor is linked to repression of transcription [195]. The inhibitory domain (ID) of Sp3 located between the second glutamine-rich activation domain
and the DNA binding domain (figure 6), has been shown to negatively regulate the activity of Sp3. The repressive activity is mediated by the attachment of SUMO to a key lysine (K551) which lies within the SUMO consensus motif IKEE in the ID [196]. PIAS1 (protein inhibitor of activated STAT1) can silence Sp3 by mediating the conjugation of SUMO to K551 without altering its DNA binding ability [195]. SUMOylated Sp3 then initiates a local heterochromatic gene silencing [167, 197]. Covalent attachment of SUMO-1 to Sp3 changes its location to the nuclear periphery and nuclear dots [195]. SUMOylation of Sp3 regulates its ability to activate or repress transcription in a context-dependent manner [198]. Another post-translational modification, known as acetylation, also targets the same key lysine residue in the IKEE triplicate leading to transcriptional repression by Sp3 [199]. Acetylation of Sp1 has been shown to inhibit PTEN expression by binding to PTEN promoter and recruiting histone modifies such as HDAC [200].

**In vivo functional role of Sp factors and their expression patterns.**

Even though Sp proteins share similar structural features, gene knockout studies have revealed that they have different biological functions as knockout embryos exhibit distinct phenotypes.

**Sp1**

Sp1 is expressed in many if not all mammalian cell types and has been implicated in regulating the expression of many genes including housekeeping genes, tissue specific genes, developmental specific genes as well as genes involved in the cell cycle [201-207]. The broad expression pattern of Sp1 suggests its significance in all cell types, but surprisingly, mouse ES cells deficient of Sp1 are viable, grow normally and can be differentiated *in vitro* into embryoid bodies [208]. However, in the developing embryo, Sp1 is crucial as Sp1 knockout embryos are severely retarded in development and die around embryonic day 11 (E11) showing a wide range of developmental abnormalities. There is a huge heterogeneity in the phenotype of Sp1 knockout embryos as some show differentiated structures such as developing heart, otic vesicles, somites, erythroid cells and extra-embryonic tissues while others resemble just a mass of undifferentiated cells. Sp1KO causes a cell autonomous defect as knockout mouse ES cells injected into wild-type blastocyst contribute to chimeric embryonic tissue only during early development. Contribution declines rapidly around E10 and is not detectable in the newborn animals. This suggest that that Sp1 deficiency causes a cellular defect and cell death only after commitment and/or differentiation have occurred and is not essential for a particular cell type or lineage [208].
**Sp2**

Sp2 is the least studied member among the Sp factor sub-group containing the glutamine-rich activation domain. Unlike Sp1 which is ubiquitously expressed, the expression of Sp2 is restricted to several cell lines [137, 152]. In mouse embryonic fibroblast (MEF) cells, Sp2 is essential for cell autonomous proliferation and Sp2 knockout embryos are delayed in development and do not survive beyond E9.5. The knockout embryos have an open cranial neural tube [134]. Also, Sp2 has been shown to play a crucial role in the proliferation of cultured HEK293 cells as siRNA mediated knockdown of Sp2 in these cells led to severe reduction in proliferation compared to the control [153]. In COS-1 cells, Sp2 is associated with the nuclear matrix and localizes predominantly within sub-nuclear foci different from Sp1 and Sp3. These foci may play an important role in regulating the function of Sp2 and could in part explain the differences in its function when compared to Sp1 and Sp3 [209].

**Sp3**

Sp3 is ubiquitously expressed and is closely related to Sp1. However, classical gene knockout studies have revealed that they have different and redundant roles during embryogenesis.

Sp3 knockout embryos are growth retarded and survive throughout gestation but die immediately after birth due to respiratory failure as well as various other developmental abnormalities [210]. Lung-specific genes are not affected suggesting that the inability for Sp3KO mice to breath was not due to Sp3 regulating those lung specific genes. E14.5 Sp3 null mutants have severe cardiac malformations which could be the cause of the prenatal lethality [211]. Sp3-deficient mice also showed impaired tooth development [210]. There was decreased ossification or bone formation in the skull of E18.5 Sp3 knockout embryos due to a decrease in the expression of osteocalcin by mature osteoblasts. Osteoblasts (bone forming cells) exclusively express Osteocalcin and other genes that are important for mineralization [212]. The hematopoietic system of Sp3 null mice was also affected. E18.5 embryos displayed an impaired B/T cell development as well as a cell autonomous defect of the erythroid and myeloid lineages which could explain the delay observed in the formation of definitive erythrocytes [213].

Remarkably, Sp1 and Sp3 compound heterozygous embryos are not viable. They displayed various developmental abnormalities which resemble a combination of Sp1 and Sp3 knockout embryos. These include growth retardation, impaired lung development, placenta defects, impaired ossification and anemia. The development of anemia in Sp1/Sp3 compound heterozygous mutant embryos is linked to a delay in the maturation of erythrocytes. This study revealed that a
threshold activity of both Sp1 and Sp3 is essential for normal embryogenesis and that Sp1 and Sp3 do have redundant functions in regulating target genes [214].

**Sp4**
The expression pattern of Sp4 is more restricted to neuronal tissues [149, 215]. Even though it structurally resembles Sp1 and Sp3, co-transfection experiments have revealed that Sp4 exhibits specific functional properties distinct from Sp1 and Sp3 [142]. Disruption of mouse Sp4 revealed its importance in growth, viability and male fertility [141]. Few days after birth, two third of Sp4 knockout embryos die and the surviving ones are significantly smaller than the wild-type littermates. Interestingly, Sp4 mutant males do not breed despite having normal testes containing mature sperm. The fertility of female mutants appeared normal. This indicated that Sp4 is required for a normal male reproductive behavior [141]. In the brain, Sp4 regulates patterning of dendritic cells during cerebellar maturation [169, 216] and in the hippocampus, impaired postnatal development of the dentate gyrus are observed [217].
Scope of the thesis

Hematopoiesis is a self-renewal system and therefore an attractive target for therapy, since healthy hematopoietic stem cells can in principle replace faulty cells. In order to unravel this therapeutic potential, it is necessary to understand how hematopoiesis is regulated at the molecular level particularly by transcription factors. Hematopoietic lineage commitment and maturation involves a progressive restriction of differentiation potential and the establishment of lineage-specific gene expression profiles, which depend on the concerted action of lineage specific and ubiquitously expressed transcription factors. In this thesis, we focused on the role of Sp transcription factors in regulating adult hematopoiesis.

Sp1 and Sp3 are critical for the normal embryonic development as Sp1 and Sp3 knockouts are embryonic lethal with various developmental abnormalities including the hematopoietic system. We intended to study the function of Sp1 and Sp3 in the adult hematopoietic system. Therefore we generated Sp1 and Sp3 conditional knockout mice and used the Cre-loxP system to delete Sp1/Sp3 from the entire adult hematopoietic system (Chapter 2). We demonstrated a severe disruption of the adult hematopoietic system upon Sp1 and Sp3 deletion (Chapter 2). To get a comprehensive analysis of the role Sp1/Sp3 in the B lineage which was affected upon pan-hematopoietic deletion of Sp1/Sp3 deletion, we used B lineage-specific Cre line. This allowed us to investigate the in vivo function of Sp1/Sp3 at different stages of B cell development (Chapter 3). We demonstrated developmental arrest upon Sp1/Sp3 deletion and used an Ig transgene to particularly rescue the differentiation defect (Chapter 3).

Pan-hematopoietic deletion of Sp1/Sp3 also revealed an impeded megakaryocyte development. Therefore, we used megakaryocyte specific Cre line to simultaneously inactivate Sp1 and Sp3 in the megakaryocyte lineage (Chapter 4). We unravel the molecular mechanisms underlying the redundant role of Sp1 and Sp3 during megakaryocyte development (Chapter 4). In Chapter 5, we discuss the significance and implications of the main studies described in Chapter 2-4 and proposed directions for future research.
References


138. Scohy, S., et al., Assignment1 of Sp genes to rat chromosome bands 7q36 (Sp1), 10q31-->q32.1 (Sp2), 3q24-->q31 (Sp3) and 6q33 (Sp4) and of the


Chapter 2

Sp1 and Sp3 regulate adult hematopoiesis
Sp1/Sp3 regulate hematopoiesis

Severe hematopoietic defects after Mx1-Cre-mediated depletion of Sp1 and Sp3 transcription factors in mice

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Abstract

Sp1 and Sp3 belong to the family of Specificity Protein (Sp) transcription factors which play crucial roles in mouse embryonic development. Sp1 knockout embryos die around embryonic day 10.5. Sp3 knockout embryos survive gestation but die immediately after birth. Sp3 knockout embryos display a multitude of development abnormalities including placental, cardiac, bone and hematopoietic defects. Analysis of $Sp1^{wt/ko}$::$Sp3^{wt/ko}$ compound heterozygous embryos revealed a series of developmental abnormalities resulting in late prenatal mortality. Although these classical gene knockout studies have demonstrated the importance of Sp factors in developmental processes including hematopoiesis, prenatal lethality of $Sp$ mutants precludes analysis of their function in adult animals. To examine the role of Sp1 and Sp3 beyond embryogenesis, we generated conditional knockout mice for $Sp1$ and $Sp3$ which we crossed with the well-characterized inducible pan-hematopoietic Cre deleter line $Mx1$-Cre in order to inactivate the conditional knockout alleles in the hematopoietic system. We achieved efficient $Mx1$-Cre mediated deletion of $Sp1$ and $Sp3$ in hematopoietic tissues. Surprisingly, depletion of either Sp1 or Sp3 had very little impact on adult hematopoiesis. In contrast, simultaneous inactivation of the $Sp1$ and $Sp3$ genes resulted in severe hematopoietic defects affecting both the lymphoid and myeloid lineages. It appears that, unlike during embryonic development, Sp1 and Sp3 have largely overlapping roles in adult hematopoiesis.
Sp1/Sp3 regulate hematopoiesis

Introduction

The proliferation and differentiation of hematopoietic stem cells resulting in all mature blood cell types involves a progressive restriction of differentiation potential and the establishment of lineage-specific gene expression profiles. These expression profiles depend on the concerted action of lineage-specific and ubiquitously expressed transcription factors [1]. Members of the Specificity protein/Krüppel-Like Factor (Sp/KLF) family play a crucial role in regulating the expression of many genes, including developmental specific, tissue specific, and cell cycle regulated genes [2-7]. They are united by their highly conserved 81 amino acid DNA binding domain, which consists of three classical Cys2-His2 zinc fingers related to those found in the *Drosophila melanogaster* regulator protein Krüppel [8-10]. Together, these three zinc fingers form the sequence-specific DNA binding domain recognizing the widely distributed G-rich elements such as the GC (GGGGCGGGG)-box and the related GT (GGGGTGTGG)-box. These sequences are recurring motifs in promoters and more distal regulatory elements of many mammalian genes.

Based on the conservation of their non-DNA binding N-terminal domains Sp1-4 form a sub-branch of the family. Of these four factors, Sp1 and Sp3 display the highest similarity. Sp2 has a defining amino acid substitution in the DNA binding domain which sets it apart from all other family members, while Sp4 has a restricted expression pattern including neuronal tissues [8, 11, 12].

The overall structural similarity and ubiquitous expression patterns shared by Sp1 and Sp3 suggest that these two proteins are functionally equivalent. However, classical gene knock studies have revealed that they have different functions as they exhibit distinct phenotypes. Sp1 knockout embryos are severely retarded in development and do not survive beyond embryonic day (E) 10.5 [4]. Sp3 null embryos develop throughout gestation but die shortly after birth due to a series of complications which include delay in lung, tooth and heart development and defects in hematopoiesis [2, 13, 14]. *Sp1*<sup>wt/ko</sup>::*Sp3*<sup>wt/ko</sup> compound heterozygous mice are not viable and are retarded in development, displaying placental and hematopoietic defects. These observations suggest that a critical threshold of Sp1 and Sp3 activity is required for normal embryonic development and that these two proteins have additive effects in regulating downstream target genes [3].

To start exploring the role of Sp1 and Sp3 in the adult hematopoietic system, we generated mice with conditional knockout alleles for Sp1 and Sp3 which we crossed with a well characterized pan-hematopoietic Cre line, *Mx1-Cre* [15]. In *Mx1-Cre* mice, Cre recombinase is under the control of a type-I interferon-inducible promoter (*Mx1*) which is silent in healthy mice and can be induced to high levels of transcription in interferon-responsive cells by administering interferon alpha/beta (IFN-α/β) or, alternatively, synthetic double stranded RNA such as
polyriboinosinic::polyribocytidylic acid [Poly (I:C)]. The inducible Mx1-Cre/poly (I:C) system has been widely used for pan-hematopoietic inducible deletion of “floxed” target genes [5-7]. Our results reveal efficient Mx1-Cre-mediated deletion of Sp1 and Sp3 in hematopoietic tissues. Surprisingly, depletion of either Sp1 or Sp3 had very little impact on adult hematopoiesis. In contrast, simultaneous inactivation of the Sp1 and Sp3 genes resulted in severe hematopoietic defects affecting both the lymphoid and myeloid lineages. We conclude that, as opposed to their functions in embryonic development, Sp1 and Sp3 have largely overlapping roles in adult hematopoiesis. Since these deletions affect the lymphoid and myeloid lineages, these two transcription factors appear to be critical regulators of adult hematopoiesis.

Materials and Methods

Generation of Sp1 and Sp3 conditional knockout mice targeting constructs
The Sp1 and Sp3 targeting constructs were linearized with Not1 and transfected into E14 embryonic stem (ES) cells. After Gancyclovir counter selection and G418 resistance selection, clones were picked and analyzed for homologous recombination events. Positive clones which had retained both loxP sites were karyotyped, and clones with the correct karyotype were used to generate chimeric mice.

Mice
Mice were generated and maintained in the Erasmus MC animal care facility (EDC) under specific pathogen-free conditions. All the animal experiments were approved by the Erasmus MC Animal Ethics Committee. Correctly targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimeric animals. Chimeric males, as judged by coat color, were mated to C57BL/6 females and germline transmission was scored by coat color. F1 animals were genotyped for the presence of the targeted Sp1 or Sp3 allele and positive animals were selected for further breeding. To delete the IRES-lacZ-Neo cassette from the targeted Sp3 locus, animals were bred to the Rosa26<sup>F<sub>loxP</sub></sup> line [16]. Animals carrying floxed Sp1 or Sp3 alleles were bred to homozygosity and crossed with the Mx1-Cre line [15]. Additional rounds of breeding resulted in compound mice homozygous for the floxed Sp1 and Sp3 alleles, with or without the Mx1-Cre transgene.
Genotyping and Southern blotting

PCR genotyping of Sp1 alleles. A sense primer (5´-GAGGCCTTGTGCAAAAGTAAG-3´) and antisense primer 2 (5´-ACACCACCAGATTCAAGACTCT-3´) flanked the loxP-sequence inserted in intron 5. This yields PCR products of 339 bp for the wildtype allele, and 447 bp for the floxed allele. For the detection of the recombed allele, an additional antisense primer (5´-TTGGACCCATGCTACCTTG-C-3´) was used, flanking the downstream loxP site. In combination with the sense primer, this yields a PCR fragment of 313 bp upon Cre-mediated deletion. The annealing temperature for PCR was 55 °C.

Southern blotting of Sp1 alleles. Genomic DNA was isolated from bone marrow or liver, and digested with BamH1 and PstI. After fractionation on 0.7% agarose gels, the DNA was transferred to nylon membranes and probed with a ~1 kb fragment generated by PCR amplification of genomic DNA, using the primer pair 5´-GCATCCCACCAGAGTAAGCA-3´ and 5´-AAGTCTCAGTGCCAACGC-3´. This yields a 4.2 kb band for the floxed Sp1 allele, and a 3.7 band for the recombed Sp1 allele.

PCR Genotyping of Sp3 alleles. To distinguish the wildtype and floxed alleles, we used a forward primer in exon 4 (5´-TGTTAGACACTCAGCTTG-3´) and a reverse primer in intron 4 (5´-GTCTACAGCAAGTTCCAG-3´). This yields a product size of 530 bp for the Sp3 wildtype allele, and 599 bp for the Sp3 floxed allele. To detect the recombed allele, we used a three primer PCR strategy with forward primers located in intron 3 (5´-GGAGGGCTTTAATATTACC-3´) and exon 4 (5´-TGTTAGACACTCAGCTTG-3´), and a common reverse primer in intron 4 (5´-GTCTAGCTACAGCTCCAG-3´). This yields a product size of 592 bp for the Sp3 floxed allele, and 629 bp for the Sp3 recombed allele. The annealing temperature for PCR was 59 °C.

Southern blotting of Sp3 alleles. Genomic DNA was isolated from bone marrow or liver, and digested with EcoRV. After fractionation on 0.7% agarose gels, the DNA was transferred to nylon membranes and probed with a ~1.7 kb fragment generated by PCR amplification of genomic DNA, using the primer pair 5´-AGGCCTGAGATTTAGTGGGT-3´ and 5´-CTGAGCAAGAAATGCTGATCC-3´. This yields a 6.6 kb band for the floxed Sp3 allele, and a 5.2 kb band for the recombed allele.

Induction of Mx1-Cre by plpC treatment

Animals were injected intraperitoneally with poly (I:C) (Sigma) dissolved in PBS (plpC/PBS; 25 µg plpC per g body weight) every other day for a total of 5 days (3 treatment course). The mice were allowed to recover from the induced interferon response for an additional 7 or 14 days before being analyzed. In some cases, the
plpC treatment was started with 7-day old pups receiving a total of 7 poly (I:C)/PBS injections over a 6-week period (7 treatment course).

**Western Blot analysis**

Nuclear extracts from bone marrow cells and splenocytes were prepared as follows: cells in lysis buffer (10 mM/HEPES-KOH [pH = 7.9], 1.5 mM MgCl₂, 10 mM KCl, supplemented with complete protease inhibitor cocktail (Roche Diagnostics) and Pefablock (Merck)) were incubated on ice for 15 minutes to extract cytoplasmic proteins. After a short spin, the pellets were re-suspended in a high salt buffer (20 mM/HEPES-KOH [pH = 7.9], 1.5 mM MgCl₂, 10 mM KCl, 25% glycerol, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA) to extract the nuclear proteins followed by high speed centrifugation to remove cellular debris. Proteins were size-fractionated by 7.5% SDS-PAGE. The gels were transferred to nitrocellulose membranes which were blocked with 1% (w/v) bovine serum albumin (BSA), 0.05% Tween 20 (v/v) (Roche Diagnostics) in PBS, followed by probing with the appropriate primary antibodies. Secondary stainings were performed using goat-anti-mouse/rabbit IR-Dye 680 or 800 antibodies in PBS containing 5% (w/v) blotting grade non-fat dry milk powder (Bio-Rad Laboratories, Hercules, USA) and 0.05% (v/v) Tween 20. The blots were scanned with an Odyssey Infrared Imaging System (Li-Cor Biosciences). Western blots were probed with the following primary antibodies: Sp1 rabbit polyclonal (home-made), Sp3 rabbit polyclonal (D-20): sc-644, Nucleophosmin (NPM1) mouse monoclonal: sc-32256 (all from Santa Cruz Biotechnology).

**Hematological analysis**

Peripheral blood was collected from mice (6-12 weeks old) through the mandibular vein. An automated counter (Scil Vet ABC, Viernheim, Germany) was used to measure the standard blood parameters.

**Flow cytometry**

Femurs and tibiae were crushed and subsequently filtered through a 100 µm cell strainer to obtain bone marrow single-cell suspensions. Splenic single-cell suspensions were obtained by mincing through 40 µm cell strainers. Cells were washed with FACS buffer (PBS, 0.25% (w/v) BSA, 0.5 mM EDTA, 0.05% (w/v) NaN₃) and incubated with the respective monoclonal antibodies (mAbs) at room temperature for 10 minutes. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and the data analyzed with Tree Star FlowJo software. Primary antibodies used is listed is supplemental table 1.
Statistical Analysis
To analyze statistical significance of the hematological parameters and flow cytometry data, we used Mann-Whitney tests. \( P < 0.05 \) was considered significant.

Results

Generation of mice with Sp1 and Sp3 conditional knockout alleles

To generate mice with a floxed \( Sp1 \) allele (\( Sp1^{fl} \)), we employed a strategy based on that used for the knockout allele. Deleting exon 5 and 6 of the \( Sp1 \) gene removes the C-terminal part of the protein including the entire DNA binding domain, resulting in an \( Sp1 \) null mutation for DNA binding [3, 4]. We constructed an \( Sp1 \) conditional targeting vector by introducing two loxP sites flanking exons 5 and 6 of the \( Sp1 \) gene, and inserted a \( LacZ-Neo \) fusion gene under the control of the \( PGK \) promoter downstream of the last exon, in the antisense direction. Cre-induced recombination between the two loxP sites would result in the deletion of exon 5 and 6 thereby creating the \( Sp1 \) conditional knockout allele (Fig. 1A).

Clones which had the \( PGK-LacZ-Neo \) gene at the 3’ end of the \( Sp1 \) gene and the loxP site 5’ to exon 6 were karyotyped, and a clone with the correct karyotype was injected into C57BL/6 blastocysts. Male chimeras were bred to C57BL/6 females to obtain germline transmission of the \( Sp1^{fl} \) allele. Mating between \( Sp1^{wt/fl} \) mice resulted in \( Sp1^{fl/fl} \) offspring at the expected Mendelian ratio. These animals appeared normal and were fertile. To test the functionality of the \( Sp1^{fl} \) allele, \( Sp1^{fl/fl} \) mice were crossed with the \( CAG-Cre \) line which expresses Cre protein ubiquitously under the control of the chicken \( \beta-actin \) promoter [17]. As expected, \( CAG-Cre::Sp1^{fl/fl} \) embryos died around E9–E10 (data not shown) which is similar to the developmental stage at which \( Sp1 \) knockout embryos die [4].

The strategy for the generation of \( Sp3^{fl} \) allele is outlined in (Fig. 1B). A targeting vector was constructed with loxP sites flanking exon 4. The adjacent selection marker (\( IRES-lacZ-Neo \)) was flanked by FRT sites, rendering expression of the \( LacZ-Neo \) fusion gene under control of the endogenous \( Sp3 \) promoter. Verification of the orientation and functionality of the loxP and FRT sites in the targeting vector was performed in \( E. coli \) strains expressing Cre- or Flp recombinase [18]. The linearized targeting vector was transfected into E14 ES cells and G418-resistant colonies were analyzed by Southing blotting and PCR. After karyotyping, ES clones were injected into C57BL/6 blastocysts.

Germline transmission was obtained and the mice were crossed with the \( Rosa26^{Flp} \) line to remove the \( IRES-lacZ-Neo \) cassette [19]. F1 animals were propagated to breed out the \( Rosa26^{Flp} \) allele and to obtain \( Sp3^{fl/fl} \) animals. Further matings of
Sp1<sup>fl/fl</sup> with Sp3<sup>fl/fl</sup> animals resulted in mice with homozygous conditional knockout alleles for both Sp1 and Sp3 (Sp1<sup>fl/fl</sup>::Sp3<sup>fl/fl</sup>). Mice of all three genotypes (Sp1<sup>fl/fl</sup>, Sp3<sup>fl/fl</sup> and Sp1<sup>fl/fl</sup>::Sp3<sup>fl/fl</sup>) were bred with Mx1-Cre mice [15] to obtain animals with pIpC-inducible Cre expression.

**Mx1-Cre** mediated deletion of the Sp1 and Sp3 genes is efficient in hematopoietic tissues and leads to the loss of Sp1 and Sp3 proteins.

Mice age between 6-12 weeks were subjected to 3 poly (I:C) treatment course. At the end of treatment course and recovery period, the deletion of exon 5 and 6 (Sp1) and exon 4 (Sp3) in the bone marrow and spleen was verified by PCR. A 450 bp band represented the Sp1cko allele and the recombined allele was 280 bp in size. The Sp3cko PCR fragment had a size of 2851 bp while the recombinant allele was 630 bp (Fig. 2A). The efficiencies of Sp1 and Sp3 deletion in the bone marrow of Mx1-Cre::Sp1<sup>fl/fl</sup>::Sp3<sup>fl/fl</sup> (double KO, dKO) and Sp1<sup>fl/fl</sup>::Sp3<sup>fl/fl</sup> (WT) mice were analyzed by Southern blotting. This revealed that the efficiency of Sp1 and Sp3 deletion was >90% (Fig. 2B). To confirm that deletion of Sp1 exon 5/6 and Sp3 exon 4 resulted in the absence of Sp1 and Sp3 proteins, we used nuclear extracts from bone marrow and splenic cells for western blotting. The results revealed that Sp1 was almost completely absent in the bone marrow of Mx1-Cre::Sp1<sup>fl/fl</sup>::Sp3<sup>fl/fl</sup> animals. Sp3 was also not present in the spleen of the dKO animals (Fig. 2C). We noted that dKO animals displayed a larger spleen size than the WT animals which became even more severe by allowing the animals to recover for 14 days (Fig. 2D). These data demonstrate that Sp1 and Sp3 were efficiently deleted upon poly (I:C) treatment of Mx1-Cre::Sp1<sup>fl/fl</sup>::Sp3<sup>fl/fl</sup> animals.
Figure 1: Generation of Sp1 and Sp3 mutant alleles. Schematic drawings of the strategy to create the alleles used for the Mx1-Cre-mediated conditional knockout of the Sp1 and Sp3 genes. The PGK-HSV-TK cassette is deleted upon homologous recombination and was used for counter-selection to enrich for ES cell clones with homologous recombination events. (A) Sp1 mutant allele. Sp1 exons are numbered Ex5 and Ex6. (B) Sp3 floxed allele. Flp recombinase was used to remove the IRES-LacZ-Neo selection cassette, leaving exon 4 (Ex4) flanked by loxP sites. Glutamine-rich activation domains are indicated in red, the zinc fingers as green boxes, exons are represented by black boxes.
Figure 2: Mx1-Cre mediated deletion of floxed Sp1 and Sp3 alleles in hematopoietic tissues. 
(A) PCR analysis on bone marrow and spleen. (B) Southern blot analysis of genomic DNA isolated from the bone marrow after pIpC treatment course. (C) Western blot analysis of nuclear extracts from bone marrow (Sp1) and splenic (Sp3) cells after the pIpC treatment course. The three Sp3 isoforms can be seen. Nucleophosmin (Npm1) was used as a loading control. (D) Spleen sizes 14 days after 3 poly (I:C) treatment course.
Sp1/Sp3 regulate hematopoiesis

No hematopoietic defects in *Mx1-Cre::Sp1fl/fl* or *Mx1-Cre::Sp3fl/fl* animals

Poly I:C was administered to mice aged between 6-12 weeks old for Sp3 analysis (3 treatment course over a 5 day period) and 7-day old pup for a period of 6 weeks (7 treatment course). After the 7 pIpC treatment course single knockouts, *Mx1-Cre::Sp1fl/fl* (Sp1 sKO), *Mx1-Cre::Sp3fl/fl* (Sp3 sKO) and their littermate controls, *Sp1fl/fl* and *Sp3fl/fl* were allowed to recover for 7 days. At the end of the recovery period, peripheral blood was collected and complete blood cell count was performed using a Scil Vet ABC blood counter. The results revealed that *Mx1-Cre* mediated deletion of Sp1 from the hematopoietic system showed no obvious phenotype as the hematological parameters of Sp1 sKO animals were comparable to those observed in the controls (Fig. 3A). Similarly, Sp3 sKO mice also displayed no obvious hematological phenotype (Fig. 3B). Also, extensive flow cytometry analysis on precursor and mature blood cells in the bone marrow and spleen didn’t reveal any difference between the sKO and the WT (data not shown).
Figure 3. No obvious hematopoietic defects in Sp1 or Sp3 mutants. Peripheral blood analysis of Sp1KO (n=12), Sp3KO (n=16) and their corresponding littermates Sp1fl/fl (n=12), Sp3fl/fl (n=14). The values for the white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), mean cell volume (MCV), Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were all normalized to 100% in the Sp1fl/fl or Sp3fl/fl controls.

Hematopoietic defects in *Mx1-Cre::Sp1fl/fl::Sp3fl/fl* animals

After pIpC 7 treatment course and recovery, peripheral blood was collected from *Mx1-Cre::Sp1fl/fl::Sp3fl/fl* animals and littermate controls, Sp1fl/fl::Sp3fl/fl. Standard blood parameters were measured. DKO animals displayed mild microcytic hypochromic anemia as shown by the reduced RBCs number, mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). In addition, the WBC compartment was also reduced to 50% when compared to WT littermates. Strikingly, dKO mice were severely thrombocytopenic (Fig. 4). These data suggests that Sp1 and Sp3 play a critical overlapping role in the development of these hematopoietic cell lineages.

Figure 4. Hematopoietic defects in Sp1/Sp3 double mutant mice. Peripheral blood analysis of DKO mice (n=14) and littermate CT mice (n=12). Counts for white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), mean cell volume (MCV), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) are all normalized to 100% in CT animals. Error bars indicate standard deviation (SD), * p<0.05, ** P<0.01, *** p<0.001
Sp1 and Sp3 are required for normal myelopoiesis and lymphopoiesis

Peripheral blood analysis revealed significantly reduced total WBC, RBC and platelet counts in the dKO animals. We therefore analyzed by flow cytometry the myeloid and lymphoid compartments in different organs of dKO and WT animals. First we characterized the B-cell lineage cells in the bone marrow and spleen by analysing the expression of IgM and IgD on the CD19+B220+ B cell population. We categorized B-cell subsets as follows pre/pro (IgM⁻IgD⁻), immature (IgM⁺IgD⁻) and mature/re-circulating (IgM⁺IgD⁺) B cells (Fig. 5A). The absolute numbers of total B cells in the BM and spleen of dKO animals was reduced when compared to WT littermates. Subpopulation analysis also showed a significant reduction of pre/pro B cells and immature B cells while re-circulatory B cell numbers were not significantly affected in the BM of dKO animals when compared to WT littermates. In concordance, the numbers of transitional (IgM⁺IgDlow) and mature (IgM⁺IgDhigh) B cells were also reduced in the spleens of dKO animals (Fig. 5B).

Next, we characterized by flow cytometry T cells in the spleen and thymus of analyzed animals. We calculated the absolute numbers CD3, CD4 and CD8 T cell subsets from the proportion in live gate. The total number of T cells (including CD4⁺ and CD8⁺ subsets) and Natural Killer (NK) cells were reduced in the spleen of the dKO animals (Fig. 5C).

Flow cytometry analysis also revealed decrease numbers of CD19⁺ B cells, CD4⁺CD8⁻ T-cells and CD41⁺CD62P⁺ platelets in the peripheral blood of the dKO animals (Fig. 5D).

Analysis of the general myeloid marker CD11b (granulocytes, monocytes/macrophages, dendritic cell subsets) in Spleen and Ly6c (granulocyte-monocyte/macrophage precursors) in BM revealed no significant changes in absolute numbers in dKO mice compared to WT littermates, as shown in Fig. 5E. This suggests that the reduction in WBCs is the consequence of lymphocyte reduction, i.e. Sp1 and Sp3 are required in B and T cell development, but not in the monocyte/granulocyte lineages.

We next set out to analyze the erythroid compartment in the BM and spleen of dKO animals. The absolute numbers of erythrocyte precursors in the bone marrow of the dKO animals were comparable to those in the WT despite a lower level of erythrocytes in peripheral blood (Fig. 5E).
Figure 5. Impeded myelopoiesis and lymphopoiesis in Mx1-Cre::Sp1fl/fl::Sp3fl/fl mice. Hematopoietic tissues from Mx1-Cre::Sp1fl/fl::Sp3fl/fl (DKO) (n=16) and Sp1fl/fl::Sp3fl/fl (CT) (n=14) litters were analysed by flow cytometry after the plpC 3 treatment course. (A&B) B cell analysis in the bone marrow (BM) and spleen respectively. (C) T cell analysis in the spleen of the CT and DKO animals respectively. (D) Platelets, B- and T- cell analysis in peripheral blood. (E) General myeloid cells in the spleen (CD11b+), Erythrocyte (Ter119+) and granulocyte-monocyte (Ly6C+) precursors in the BM. Absolute cell numbers of the different cell types calculated from the number of cells in the live gates are indicated on the right of each FACS plot (mean and standard deviation (SD), * P<0.05).
Discussion

Since Sp1 and Sp3 knockouts are embryonic lethal [4, 13], this study aimed to generate mice with conditional knockout alleles for Sp1 and Sp3 and study the function of these genes in the adult hematopoietic system. Disrupting the Sp1 or Sp3 gene alone showed no obvious phenotype in the adult hematopoietic system. This suggests that a redundant function exists between Sp1 and Sp3 in the adult hematopoietic system similar to what was observed in Sp1/Sp3 compound heterozygous embryos [3]. We went further to delete both Sp1 and Sp3 in the entire adult hematopoietic system.

At the end of the plpC treatment course and recovery, blood parameters measured revealed that test animals were severely thrombocytopenic, anemic and leukopenic and did not recover even when the recovery period was extended to another 7 days, while the control animals displayed normal blood parameters. Next we set out to determine whether the defective blood parameters observed in the dKO were a result of defective blood precursors or Sp1/Sp3 are required for the maintenance of mature blood cell types.

Analysis of the lymphoid compartment revealed that dKO animals had defective B cell precursor populations in the bone marrow. Pre/pro B cells were present in similar numbers in the bone marrow of the WT and dKO animals but the numbers of immature, re-circulating and total B cells were reduced in the DKO. This suggests that during B cell development, Sp1/Sp3 are not required at stages prior to the pre/pro B stage but crucial for B cells to differentiate and progress to stages beyond the pre/pro B cell stage. This developmental blockage could explain why B cell numbers were also reduced in the spleen of the dKO animals. The number of T cells and NK cells were all reduced in the spleen of the DKO animals.

Our findings clearly show the importance of Sp1 and Sp3 in hematopoiesis indicating that Sp1/Sp3 are essential for the proliferation or differentiation of lymphoid progenitors in the adult hematopoiesis. Defects in the myeloid compartment was manifested by reduced numbers of erythrocytes in the blood of the dKO animals despite a similar number of erythrocyte precursors in the BM. Sp1/Sp3 could be required for the maintenance of erythrocytes.

Low platelet count observed in peripheral blood was confirmed by flow cytometry which also revealed that dKO platelets were not able to up-regulate CD41 and CD62P. CD41 is expressed on platelets and megakaryocytes which together with CD61 form a receptor complex for several agonists including fibrinogen. CD41/CD61 (αIIb/β3 integrin heterodimer, also known as glycoprotein Ib/IIa) is an important molecule in the function of platelets contributing to expansion of clot
formation and platelet adhesion to the microvasculature [20, 21]. Its low expression in the dKO could lead to defective platelet coagulation. CD62P is expressed on the surface of activated platelets and indicates platelet activation. These results suggest that Sp1/Sp3 could have a role in the production and function of platelets. The larger spleen size indicated that extramedullary erythropoiesis occurred in the dKO mice to a higher extent due to their response to a more severe acute anemia when compared to the WT.

Collectively, our data show that Sp1 and Sp3 are required for normal hematopoiesis starting from the hematopoietic progenitors. Using lineage-specific Cre lines, further studies will delineate the specific roles of Sp1/Sp3 in the affected hematopoietic lineages in adult animals.
References


Supplemental Table 1

The following antibodies were used for flow cytometry

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Chapter 3

Sp1 and Sp3 co-operate to regulate B cell development
Sp1 and Sp3 co-operate to regulate B cell development

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Abstract

The ubiquitously expressed zinc finger transcription factors Sp1 and Sp3 belong to the family of Specificity protein (Sp) transcription factors that play crucial roles in mouse embryonic development. Sp1 knockout embryos die around embryonic day 10.5. Sp3 knockout embryos survive gestation but die immediately after birth. These knockout embryos display a multitude of developmental abnormalities including placental, cardiac, bone and hematopoietic defects. Analysis of Sp1ko/wt::Sp3ko/wt compound heterozygous embryos display a series of developmental abnormalities resulting in late prenatal mortality. Although these classical gene knockout studies have demonstrated the importance of Sp factors in developmental processes including lymphopoiesis, prenatal lethality of Sp mutants precludes analysis of their function in adult animals.

Here, we generated conditional knockout mice for Sp1 and Sp3 and crossed them with Mb1-Cre mice in order to inactivate the conditional knockout alleles in the B cell lineage.

Our findings revealed that deficiency for Sp1 or Sp3 had no apparent effect on the B cell lineage. However, simultaneous depletion of Sp1 and Sp3 led to an almost complete block in B cell development at the pre-B cell stage. The introduction a functionally pre-arranged Igµ, VH81X, partially rescued the phenotype. B cell receptor signaling appears to be normal in Sp1/Sp3 KO mice, but knockout cells do not proliferate. These studies demonstrate that redundant functions of Sp1 and Sp3 are critical for pre-B cell proliferation, differentiation and survival.
Chapter 3

Introduction

The development of B lymphocytes from hematopoietic stem cells (HSC) occurs in the bone marrow (BM) in a step-wise process, involving a controlled lineage and locus–specific rearrangement of the immunoglobulin heavy chain (Igh, IgHC) and light chain loci, κ (Igk) or λ (IgL), mediated by the recombination activating genes (RAG-1 and RAG-2)(1-3). The process of B lineage specification and commitment depends on the coordinated actions of various signaling cascades and transcriptional networks, which involves the initiation of B cell-specific gene expression profile and repression of alternative lineage-specific genes (4). PU.1, E2A, Ebf1 and Pax5/BSAP are among the transcription factors required for normal B cell development (5-8). These proteins initiate and control the sequential steps of V(D)J recombination necessary for formation of functional Ig genes (3, 9).

The earliest committed B cell precursors are pre-pro B cells and they have their immunoglobulin locus in germline configuration (10). Ig heavy-chain (Igh) V(D)J recombination is initiated at the next stage of development, the pro B cell stage. Upon successful V(H) to DJ(H) rearrangement, the precursor B cell receptor (Pre-BCR) is expressed on the cell surface comprising Igμ H chain, non-rearranged surrogate light chain (SLC) constituents, λ5 and VpreB, in association with Ig-α and Ig-β signal transduction subunits. The expression of the pre B cell receptor serves as a key checkpoint in B cell development which precedes the assembly of functional IgHC. Appropriate pre-BCR and interleukin-7 receptor (IL-7R) signaling induces a burst of pre B cell proliferation and is required for survival (11-13). Igh rearrangement is terminated and the large cycling pre-B cells further differentiate into the small resting pre B cells at which point the Ig κ or λ light chain (IgL) V_{L}-to-J_{L} recombination is initiated along with changes in cell surface markers. IL7-R and CD43 expression are both terminated and the small pre B cells start expressing CD2, CD25 and major histocompatibility complex (MHC) class II (11, 12). Successful IgL gene rearrangement leads to the expression of BCR on the cell surface and the transit to immature B cells where they are checked for auto-reactivity before leaving the BM to the periphery.

Sp1 and Sp3 are members of the Specificity protein/Krüppel-like Factor (SP/KLF) transcription factor family which are characterized by three highly conserved Cys2-His2 zinc fingers present at the C-termini of the proteins forming the sequence-specific DNA binding domain (14, 15). They regulate the expression of many housekeeping, cell cycle-specific and tissue-specific genes by binding to the GC- and GT-boxes in the regulatory regions of these genes with similar specificity and affinity. Sp1, Sp3 and Sp6 are expressed in all mammalian cells while Sp4 is expressed mainly in neuronal tissues (16). Although Sp1 and Sp3 have similar structures and high homology in their DNA binding domains, in vitro and in vivo studies reveal that these transcription factors have strikingly different
functions. *Sp1* knockout embryos are severely retarded in development and do not survive beyond embryonic day (E) 10.5 (17). *Sp3 null* embryos develop throughout gestation but die immediately after birth due to respiratory failure and cardiac malformations. There was also impaired B- and T cell development in Sp3-deficient embryos (18-20). Sp1/Sp3 compound heterozygous mice are not viable and are retarded in development, suggesting that a threshold of Sp1/Sp3 activity is required for normal embryonic development, and that these two proteins have redundant effects in regulating downstream target genes (18, 21).

To circumvent the problem of embryonic lethality, we generated mice with conditional knockout alleles for both *Sp1* and Sp3 and deleted these two genes from the B cell lineage using *Mb1-Cre* mice (22). Here, we show that Sp1 and Sp3 redundantly regulate differentiation and proliferation as the knockout B cells do not progress beyond the large pre-B cell stage. However, Sp1/Sp3 KO B cells can progress beyond the pre-B cell stage upon the introduction of pre-arranged Igµ, VH81X. Interestingly, Sp1/Sp3 knockout results in cell cycle arrest and apoptosis. Activated mature Sp1/Sp3 deficient B cells are significantly smaller than the control cells suggesting a metabolic defect. Therefore, we propose that co-operation between Sp1 and Sp3 is critical for the survival and growth of B cells.

**Material and Methods**

**Generation of Mice with conditional Sp1 and Sp3 knockout allele**

To study the functions of *Sp1* beyond embryogenesis, we generated mice with conditional knockout alleles for *Sp1* and Sp3 in C57BL/6 background based on the strategy followed for the knockout allele (17, 21). For detail description refer to chapter 2. Rag1-/- (23), VH81X (24) and *Mb1-Cre* (22) mice have been described previously. Genotyping was performed using standard PCR procedures. Primers are listed in Supplemental Table 1. Mice were bred and maintained in the Erasmus MC animal care facility (EDC) under specific pathogen free conditions and used at 6-12 weeks of age. All the animal studies were approved by the Erasmus MC Animal Ethics Committee.

**Cell Cycle analysis**

For cell cycle analysis, cells were kept in ice-cold ethanol for >2 hours followed by a 30 minutes room temperature incubation in PBS containing 0.02 mg/ml propidium iodide, 0.1% v/v Triton X-100 and 0.2 ml/ml RNase, left overnight at 4°C, and analyzed with a LSRII flow cytometer (BD Biosciences).

**Pre-B, B cell Culture and RNA sequencing**
Total BM B cells were cultured with 100 units/ml of IL-7 as described previously (25). To analyze the response of splenic B cells to BCR stimulation and lipopolysaccharide (LPS), B cells were MACS purified from total spleen cell suspensions and stimulated in vitro for two days with 10μg/ml [F(ab’)]2 α-IgM (Jackson ImmunoResearch Laboratories) or 5μg/ml LPS in IMDM (10%FCS, 50Ug/mL gentamycin and 0.05mM β-mercapto-ethanol). The procedure for magnetic-activated cell sorting (MACS) has been described earlier (26).

Total RNA was extracted from >1X10⁶ cells using the GeneElute mammalian total RNA miniprep system (Sigma-Aldrich) in accordance with the manufacturer’s instructions. For genome-wide expression analysis, at least 10ng of total RNA was used in Illumina Next Generation Sequencing (Erasmus Center for Biomics).

**Flow cytometry**

Femurs and tibiae were crushed and subsequently filtered through a 100 μm cell strainer to obtain BM single-cell suspensions. Splenic single-cell suspensions were obtained by mincing them through 40μm cell strainers. Cells were washed with FACS buffer (PBS, 0.25% BSA, 0.5mM EDTA, 0.05% NaN₃) and incubated with the appropriate monoclonal antibodies (mAbs) at room temperature for 10 minutes. They were washed and analyzed. Intracellular IgM (Igμ) was analyzed by first fixing and permeabilizing cells with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Bioscience) according to the manufacturer’s instructions. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed with Tree Star FlowJo software. Monoclonal antibodies and streptavidins used are listed in supplementary table 2.

**Serum Ig detection**

The levels of serum Ig subclasses were measured by sandwich ELISA using biotinylated Ig isotype-specific antibodies (Southern Biotechnology) and streptavidin-coupled peroxidase (Jackson ImmunoResearch). Azino-bis-ethylbenzthiazolium sulfonic acid was used as the substrate. The procedure has been described previously (27).

**Immunohistochemistry**

The procedure used for preparing 6 μm cryostat sections and double labeling have been described earlier (27). For visualizing the staining, sections were embedded in Kaiser glycerol gelatin (Merck) and viewed under a DM LB light microscope (Leica) and a DFC500 camera (Leica). Micrographs were made using Imaging software for Windows Version 1.0 (Kodak). The primary antibodies used are listed in supplementary table 2.

**Statistical Analysis**
Significance of differences between control and experimental values were determined by the Student’s *t*-test.

## Results

### Inactivation of Sp1/Sp3 impedes B cell development

To investigate the effect of Sp1 and Sp3 deletion during B cell development, Sp1 and Sp3 floxed mice (Sp1*fl/fl::Sp3*fl/fl) (CT) were crossed with mice expressing the Cre recombinase exclusively in the B cell lineage, the *Mb1-Cre* mice (22, 28). Flow cytometric analyses revealed a severely decreased proportion of B220⁺CD19⁺ B-lineage cells in the BM of *Mb1-Cre::Sp1* *fl/*fl::Sp3* *fl/*fl* (Sp1/Sp3 KO) mice when compared to the controls (Fig. 1A). Residual cells consisted mainly of surface IgM/IgD-negative CD43⁺CD2⁻ pre-B cells, whereas CD2⁺ small pre-B, immature B, and re-circulating mature B cells were almost completely absent in the BM of Sp1/Sp3 KO mice, indicating a block in early B cell development (Fig. 1A and 1B). The spleen and peritoneal cavity of Sp1/Sp3 KO mice also contained reduced proportions of CD19⁺/B220⁺ B cells (Fig. 1C).

To examine the stage at which Sp1 and Sp3 deletion is initiated, we used YFP reporter mice. *Mb1-Cre::Sp1* *fl/*fl::Sp3* *fl/*fl* mice were crossed with mice carrying the reporter gene, YFP under the control of the ubiquitously expressed ROSA26 locus (29). Upon *Cre* activation, the floxed *Sp1* and *Sp3* genes will be deleted only in cells expressing the recombinase resulting in YFP expression. YFP expression was initiated at the pre/pro B cell stage indicating *Sp1/Sp3* deletion consistent with the reported stage at which *Mb1-Cre* starts to be active (22, 30) (Fig. 1D). Immunohistochemical analysis of CT and Sp1/Sp3 KO spleen confirmed the dramatic reduction of B cell numbers and revealed normal CD3⁺ T cell organization. Interestingly, one allele of *Sp1* or *Sp3* alone is able to rescue the observed phenotype (Fig. 1E). The severe reduction of splenic B cell numbers was associated with aberrant splenic architecture: the population of metallophilic marginal zone macrophages was no longer located at the borders of white pulp, but instead mostly within the red pulp (suppl. Fig. 2).

Collectively, these findings showed that during B cell development, Sp1 and Sp3 have redundant roles in the progression of large cycling (CD43⁺CD2⁻) into small resting (CD43⁻CD2⁺) pre-B cells. Although residual Sp1/Sp3 double deficient cells can undergo productive *Igh* rearrangement, they manifest an almost complete block at the large pre-B cell stage.
Figure 1. Defective B cell development in *mb1-Cre::Sp1*\textsuperscript{fli/}::*Sp3*\textsuperscript{fli/} mice. (A) Flow cytometry analysis of *Sp1*\textsuperscript{fli/}::*Sp3*\textsuperscript{fli/} (CT) and *Mb1-Cre::Sp1*\textsuperscript{fli/}::*Sp3*\textsuperscript{fli/} (Sp1/Sp3 KO) total bone marrow (BM) cells for surface expression of CD19 and B220 (top). IgD\textsuperscript{+}IgM\textsuperscript{+} was gated from the CD19+B220+ B cells (middle). CD19+B220+ B cell fractions was also gated and analyzed for CD43/CD2 (bottom). The numbers in the dot plots indicate percentage of cells in each gate. Data are representative of 16-20 mice per genotype. (B) Absolute cell numbers were calculated from proportion of cells in live gate for pro-B (CD2\textsuperscript{−}intracellular Igμ\textsuperscript{−}), large pre-B (CD2\textsuperscript{−}intracellular Igμ\textsuperscript{+}), total small pre-B, immature B and re-circulating mature B cells (CD2\textsuperscript{+} intracellular Igμ\textsuperscript{+}) in the BM of CT and Sp1/Sp3 KO mice (n=7-8 mice per group; mean and standard deviation (SD), *, P<0.001). (C) CD19\textsuperscript{+}B220\textsuperscript{+} flow cytometry profile of spleen (Sp) and peritoneal cavity (PC) from the lymphocyte gate of CT and Sp1/Sp3 KO mice. Dot plots are representative of 7-8 mice per genotype. (D) Flow cytometric analysis of YFP expression in pre/pro B cells of Sp1/Sp3 KO and CT. (E) Immunohistochemical analysis of CT, *mb1-Cre::Sp1*\textsuperscript{WT/}::*Sp3*\textsuperscript{fli/} and Sp1/Sp3 KO spleen. Sections were stained with anti-CD19 (blue) and anti-CD3 (red).

**Impeded pre-B cell proliferation and differentiation in *Mb1-Cre::Sp1*\textsuperscript{fli/}::*Sp3*\textsuperscript{fli/} mice**

Flow cytometric analyses revealed that cytoplasmic Igμ\textsuperscript{+} CD2-CD43+ large pre-B cells from Sp1/Sp3 KO mice had reduced cell size, compared with CT (Fig. 2A), suggesting a defect in proliferation. Next, defective proliferative capacity of Sp1/Sp3 KO pre-B cells was confirmed in vitro. Cytoplasmic Igμ\textsuperscript{+} pre-B cells undergo rapid cell division in response to IL-7 (11). When total BM cell suspensions of the CT and Sp1/Sp3 KO mice were cultured in the presence of IL-7...
for 5 days, the yield of cytoplasmic Igμ⁺ CD2-CD43+ large pre-B cells was severely reduced (Fig. 2B).

**Figure 2.** Defective pre-B cell maturation in Mb1-Cre::Sp1flo/flo::Sp3flo/flo mice. (A) Flow cytometric characterization of CT and Sp1/Sp3 KO B220⁺CD19⁺ CD2-Igμ⁺ pre-B cells for cell size (forward scatter, FSC). Data are displayed as histogram overlays and are representative for 6-8 mice analyzed per genotype. (B) CD43+ pre B cell proliferative response to 100U/ml IL-7 in CT, Sp1/Sp3 KO, VH81X and VH81X Sp1/Sp3 KO. Histograms are calculated from the percentages CD43⁺ cells in live lymphocyte gate as determined by flow cytometry. (C) Flow cytometry analysis of CD19⁺ B220⁺ BM B cells. CD19⁺ B220⁺ B cell fraction was gated and analyzed for immature B and re-circulating mature B cell (B220⁺/κλ⁺), pre/pro cells (B220⁻/κλ⁻). B220⁻/κλ⁻ cells were gated and analyzed for large pro-B (CD2- Igμ⁺), large pre-B cell (CD2⁺ Igμ⁺) and total small pre-B, immature B and re-circulating mature B cell (CD2⁺ Igμ⁺). B220⁻/κλ⁺ was gated for cell size (FSC) in the BM of CT and Sp1/Sp3 KO mice. Dot plots are representative of 4-6 mice per genotype. (D) Serum concentration of Ig subclass in CT, Sp1/Sp3 KO and Rag1 KO mice as determined by ELISA.

Next, we wondered whether the proliferation and/or survival defect could be corrected by accelerating B cell development in vivo, by providing developing B-lineage cells with the functionally pre-rearranged Igµ chain, VH81X (24). This would enable early pro-B cells to progress immediately to the pre-B cell stage. However, we noticed that the yield of VH81X transgenic Sp1/Sp3 KO pre-B cells in IL-7-driven BM cultures was also very low (Fig. 2B).

To further investigate the ability of pre-B cells to progress beyond the large pre-B cell stage and start rearrangement of the Ig light chain loci, total BM lymphocytes were gated and analyzed for CD19⁺B220⁺ cells that express κ or λ L chain on their cell surface (which indicate a successful light chain re-arrangement). Only a small proportion of Sp1/Sp3 KO B220⁺CD19⁺ cells expressed the κ L chain (~7%, compared to ~38% in CT cells) (Fig 2. C). The size of the L chain + fraction...
of Sp1/Sp3 KO cells was comparable to that observed in the CT cells. B220− Igμ− pre/pro B cells were also gated and analyzed for the CD2−Igμ− (pro B), CD2−Igμ+ (Large pre B) CD2+ Igμ+ (small pre B, immature and re-circulating). Most of Sp1/Sp3 KO pre/pro B cells were arrested in the large pre B cell stage revealing a differentiation defect in the Sp1/Sp3 KO B cells (Fig 2. C).

Next, we measured the Ig concentration in the serum of Sp1/Sp3 KO animals and in the littermate controls. As expected, Sp1/Sp3 KO B cells do not produce Ig in their serum which is in line with the defective B cell development observed in Sp1/Sp3 KO animals (Fig. 2 D).

Taken together, these data revealed that Sp1/Sp3 KO pre-B cells have a proliferation and differentiation defect both *in vivo* and *in vitro*.

**Expression of the V₇₈1X IgHC transgene enables Sp1/Sp3 KO cells to progress beyond the pre-B cell stage.**

The phenotype observed in Sp1/Sp3 KO B cells was partially rescued after the introduction of a functionally pre-arranged Igμ transgene V₇₈1X (24). A significant increase in the number of CD19+B220+ B cells in V₇₈1X Sp1/Sp3 KO BM indicated that more B cells were able to differentiate beyond the pre-B cell stage, thereby becoming CD2+ and surface IgM+ B cells (Fig. 3A) Even though the numbers of large pre-B cells *in vivo* were not significantly different in V₇₈1X and V₇₈1X Sp1/Sp3 KO BM, the V₇₈1X transgene was not able to rescue the defective proliferative response to IL-7 *in vitro* observed in Sp1/Sp3 KO pre B-cells (Fig 3B and Fig. 2B). This could explain why the number of CD2+ small pre-B, immature and re-circulating mature IgM+ cells were also reduced in V₇₈1X Sp1/Sp3 KO BM when compared to VH81X controls even though we cannot rule out defective light chain recombination or small pre-B cells survival (Fig. 3B). However, the expression of Igκ/λ light chain on B220+ B cells, the expression of CD2+cytoplasmic Igμ+ small pre-B cell and the size of these cells were similar in V₇₈1X and V₇₈1X Sp1/Sp3 KO BM (Fig. 3C).

Taken together, the expression of V₇₈1X transgene allows Sp1/Sp3-deficient cells to differentiate beyond the pre-B cell stage, resulting in the significant generation of surface Ig+ B cells.
Figure 3. Partial rescue of Sp1/Sp3 KO B cells carrying the VH81X transgene. (A) Flow cytometric characterization of VH81X and VH81X Sp1/Sp3 KO BM cells for the expression of CD19/B220 (top). Total CD19+B220+ B cell fractions were gated and analyzed for the expression of IgD/IgM (middle). IgD− IgD− cells were gated and analyzed for CD2/CD43 (bottom). Numbers in each gate represent percentages of cells. Data are representative of 8-12 mice per genotype. (B) Absolute cell numbers were calculated from proportions of cells in live gate for pro-B (CD2− intracellular Igμ−), large pre-B (CD2− Igμ+), small pre-B (CD2+ IgM−) and immature B/re-circulating mature B cell (CD2+ IgM+) fractions in BM of VH81X and VH81X Sp1/Sp3 KO mice (mean values and SD, * P<0.001). (C) Flow cytometry analysis of CD19+ B220+ BM B cells. CD19+ B220+ B cell fraction was gated and analyzed for Immature B and re-circulating mature B cell (B220+/κλ+) and pre/pro cells (B220−/κλ−). B220−/κλ− cells were gated and analyzed for pro-B (CD2− Igμ−), large pre-B cell (CD2− Igμ+) and total small pre-B, Immature B and re-circulating mature B cell (CD2+ Igμ+). B220−/κ− was gated for cell size (FSC) in VH81X and VH81X Sp1/Sp3 KO BM. Numbers are percentages in each gate. Dot plots are representative of 13-15 mice per genotype.

Sp1/Sp3 KO B cells are arrested at the G1 phase of the cell cycle with a defect in cell metabolism
In vitro stimulation of purified splenic B cells with anti-IgM revealed functional BCR signaling in the Sp1/Sp3 KO cells, irrespective of the presence of the VH81X transgene (Fig. 4A and 4B). This was evident from the up-regulation of the surface expression of the activation markers CD25 (IL-2R) and CD86, which was generally comparable to that observed in the CT cells (Fig 4A and 4B). In contrast, stimulation of Sp1/Sp3 KO splenic B cells did not result in appreciable upregulation of CD25, and induction of CD86 was reduced, compared with CT B cells. Introducing the V\textsubscript{H}81X transgene did not rescue the LPS-induced upregulation of CD25 or CD86 on the surface of Sp1/Sp3 KO B cells (Fig. 4B).

We observed that, even though Sp1/Sp3 KO B cells exhibited functional BCR signaling, their metabolism was defective as the cells were not able to progress beyond the S phase of the cell cycle (Fig. 4A).

The effect of Sp1/Sp3 on the progression of B cells through the cell cycle was further revealed by Propidium Iodide (PI) staining on the cultured splenic B cells stimulated with LPS (Fig 4C). PI intercalates into major groove of double-stranded DNA and produces fluorescent that can be measured by flow cytometry. The result showed that 43% of Sp1/Sp3 KO and 60% of V\textsubscript{H}81X Sp1/Sp3 KO B cells appeared in the sub-G1 phase compared to < 27% of CT or V\textsubscript{H}81X B cells. This revealed that a lot of Sp1/Sp3 KO and V\textsubscript{H}81X Sp1/Sp3 KO B cells go into apoptosis (Fig 4C). The appearance of cells at the early s-phase of the cell cycle will indicate that the cells have accumulated macromolecules and reached a certain size before initiating chromosome duplication. Our findings showed that < 12% of the Sp1/Sp3 KO or VH81X Sp1/Sp3 KO were able to enter the S-phase of the cell cycle compared to between 22-23% of the CT and VH81X B cells even though similar percentages of cells is observed at the G\textsubscript{1} phase (Fig 4C).

Taken together, our data revealed that Sp1/Sp3 KO B cells are arrested at the G\textsubscript{1} phase of the cell cycle with defective metabolism which was not corrected by the introduction of V\textsubscript{H}81X transgene.
Sp1/Sp3 regulate B cell development

Figure 4. Metabolic and cell cycle defect in Sp1/Sp3 KO B cells. (A) Flow cytometry analysis of anti-IgM or LPS activated CT and Sp1/Sp3 KO splenic B cells. Total CD19+B220+ B cell fraction where gated and analyzed for cell size (FSC) (Left), the expression of CD25 (middle) or CD86 (right). (B) Flow cytometry analysis of anti-IgM or LPS stimulated VH81X and VH81X Sp1/Sp3 KO splenic B cells. Total CD19+B220+ B cell fraction where gated and analyzed for cell size (FSC) (Left), the expression of CD25 (middle) or CD86 (right). Histogram overlays are representative of 4 mice per genotype. (C) Propidium iodide (PI) cell cycle analysis on LPS stimulated purified splenic B cell from CT, Sp1/Sp3 KO, VH81X and VH81X Sp1/Sp3 KO mice. Data are representative of 4 mice per genotype. Numbers are percentages of cells in the various stages of the cell cycle (sub-G1> G0/G1>S>G2M)
Discussion

The ubiquitous transcription factors Sp1 and Sp3 have been shown to be crucial for mouse development as Sp1 or Sp3 knockouts are embryonic lethal (17, 18). However, as opposed to a more general delay in developmental progression for Sp1, Sp3 knockout embryos exhibit specific hematopoietic defects including a delay in lymphopoiesis (20).

In this report, we used a B cell-specific Cre expressing strain to simultaneously inactivate Sp1 and Sp3 in the B cell lineage thereby unraveling their crucial role during B cell development. Signaling through the pre-BCR receptor checkpoint which monitors functional Igh rearrangement and IL-7R signaling, induces cell cycle entry of large cycling Igμ⁺ pre-B cells, and rapid down-regulation of the rearrangement machinery, thereby ensuring allelic exclusion (31, 32). Subsequently, large pre-B cell proliferation is terminated and further differentiation into small resting pre B and Ig κ or λ light chain rearrangement is initiated (11, 12, 33).

A key finding in our study is the demonstration that Sp1/Sp3 deficient B cells are arrested at the large pre-B cell stage of development. These cells showed a reduced size suggesting a proliferation defect. However, the residual B lineage cells were mainly pro-B cells expressing intracellular Igμ, indicating productive Igh rearrangement.

We demonstrated that after introduction of the pre-rearranged Igμ transgene VH81X (24), the block in B cell proliferation was partially rescued as a substantial amount of B cells were able to proliferate and differentiate to stages beyond the large cycling pre-B cell stage in vivo. However, the VH81X transgene did not rescue the proliferative defect seen in IL-7-stimulated Sp1/Sp3 KO pre-B cell cultures. This suggests that Sp1/Sp3 are required for the proliferation and differentiation of pre-B cells. Residual Sp1/Sp3 KO cells which were able to re-arrange their Igh chain were also able to re-arrange their L chain. Light chain re-arrangement became very obvious after the introduction of VH81X transgene. VH81X Sp1/Sp3 KO B cells were able to re-arrange their Ig κλ L chains. This further strengthens the notion that Sp1 and Sp3 are required for IgH recombination as the addition of the heavy chain transgene partially rescued the pre B cell phenotype.

Resting splenic B cells enter the G₁ phase of the cell cycle when their membrane immunoglobulins are stimulated with LPS or anti-IgM (34, 35). This is accompanied by up-regulation of activation markers including CD25 and CD86, and an increase in cell size linked to an increase in de novo macromolecule synthesis necessary for cellular processes like genome replication before progressing to the S-phase (36, 37). Sp1/Sp3 KO B cells up-regulate activation markers including CD25 and the T-cell co-stimulatory molecule CD86 upon IgM or to a lesser extend upon LPS stimulation, although not to the same extent as the CT. Our data revealed that BCR
receptor signaling in Sp1/Sp3 KO cells appears to be normal. However, the size of activated Sp1/Sp3 KO cells was drastically smaller than that of the CT cells, indicating a defect in cell metabolism. This might explain why the Sp1/Sp3 KO cells were arrested at the G1 phase of the cell cycle. A role of Sp1 in regulating the cell cycle has been reported in several cells. Epithelial cells lacking Sp1 down regulates cyclin D1 and fails to progress beyond the G1 phase of the cell cycle (38). The induction of cyclin D1 activity in vascular endothelial cells is mediated by Sp1 binding to it promoter (39). So it is possible that Sp1 regulates B cells entry into the cell cycle at least in part by regulating cyclin D1 activity. This needs to be verified.

To further examine the underlying cause of the cell cycle arrest and apoptosis observed in Sp1/Sp3 KO B cells, we performed genome-wide expression profiling of purified and 4 hours stimulated naïve splenic B cells. Our result revealed a list of differentially expressed genes in Sp1/Sp3 KO B cells. Examples include anti-apoptotic genes Bcl2l1 and Bcl2a1d, Stat5a, Il2ra and c-Myc (Data analysis is in progress). Bcl2l1 and Bcl2a1d are among the Bcl2 family of genes that regulate apoptosis and cell survival (40, 41). We found that a 4 fold increase in the expression of both Bcl2l1 and Bcl2a1d in CT but not in Sp1/Sp3 KO B cells. This could explain why the cells enter into apoptosis.

Il2 receptor signaling via the JAK/STAT pathway plays an important role in lymphocyte proliferation (42, 43). Our result revealed a 6 fold increase in Il2ra and a 3 fold increase in Stat5a expression the CT and not present in Sp1/Sp3 KO B cells. This might explain the impede proliferation observed in the knockout cells. During B cell development, c-Myc has previously been reported to enhance protein synthesis and cell size (36, 37). Two fold decrease in the expression of c-Myc was observed in Sp1/Sp3 deficient cells which might be attributed to the observed metabolic defect.

Further analysis of the mRNA sequence data could reveal metabolic and other important pathways involve in B cell development.

Interestingly, we found that the phenotypes observed in Sp1/Sp3 KO are not observed in either Sp1 or Sp3 single knockout mice and that a single allele of either Sp1 or Sp3 is sufficient to support B cell development. Redundancy between Sp1 and Sp3 have previously been reported in Sp1/Sp3 compound heterozygous mice (21). We could show for the first time that Sp1 and Sp3 functions redundantly during B cell development and are necessary for B cell proliferation, differentiation and metabolism.
References


Supplementary Figures and Tables

Supplementary Figure 1: Sp1 and Sp3 conditional knockout alleles. Cre activation mediates the deletion of exon 5 and 6 in Sp1 floxed allele (A) and exon 4 in Sp3 floxed allele (B) resulting to Sp1 and Sp3 knockout alleles respectively.

Supplementary Figure 2: Immunohistochemistry on spleen. Control Sp1fl/fl::Sp3fl/fl (CT), Mb1-Cre::Sp1wt/fl::Sp3fl/fl and Mb1-Cre::Sp1fl/fl::Sp3fl/fl (Sp1/Sp KO) splenic sections were stained with anti-IgM (red) for B cells, MOMA1 (blue) for staining metallophilic marginal zone macrophages. Splenic B cells and macrophages were almost completely absent in Sp1/Sp3 KO mice. One allele of Sp1 or Sp3 is sufficient to rescue the phenotype.
Supplementary Figure 3: The VH81X transgene partially rescues the defects observed in Mb1-Cre::Sp1fl/fl::Sp3fl/fl B cells. (A) Flow cytometry analysis of splenic cells for the expression of CD19^+B220^+ mature and transitional B cells from CT and Sp1/Sp3 KO mice (B) Flow cytometry analysis of splenic cells for the expression of CD19^+B220^+ mature and transitional B cells from VH81X and VH81X Sp1/Sp3 KO mice (* P<0.001).

**Supplementary Table 1: Primers used**

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**Supplementary Table 1**: Antibodies used in this study, grouped per experimental procedure.

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Chapter 4

*Sp1 and Sp3 co-operate to regulate megakaryopoiesis*
Sp1 and Sp3 co-operate to regulate megakaryopoiesis

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*,# equal contribution

(Manuscript in preparation)
Abstract

The ubiquitous zinc finger transcription factors Sp1 and Sp3 play critical roles in embryonic development. While deficiency of Sp1 is lethal in utero at E10.5, deficiency of Sp3 results in postnatal lethality. However, compound heterozygous mice die in utero at around E18.5 displaying severe developmental defects including anemia resulting from impaired erythropoiesis. This suggests that their function is important for the development of many tissues including the fetal hematopoietic system. Simultaneous ablation of Sp1 and Sp3 in the hematopoietic system in adult mice has a major impact on the platelet compartment. Here we study megakaryocyte-specific Sp1/Sp3 double knockout mice and show that they are a phenocopy of Bernard Soulier Syndrome. While in humans this disease is caused by mutation of a single gene, encoding one of the subunits of the vWF receptor, in the Sp1/Sp3 double knockout mice, several genes regulated by Sp1 and Sp3 are able to recapitulate all the phenotypic features of the disease. Further characterization pointed to a general defect in downstream signaling molecules including those involved in cytoskeletal dynamics.
Introduction

Sp1 and Sp3 belong to the family of Specificity protein/Krüppel-like Factor (SP/KLF) characterized by three highly conserved zinc fingers of the C2H2-type related to those found in the *Drosophila melanogaster* regulator protein Krüppel [1-3]. These three zinc fingers form the sequence-specific DNA binding domain recognizing the widely distributed G-rich promoter elements such as the GC (GGGGCGGGG)-box and the related GT (GGGGTGTGGG)-box present in the regulatory regions of many ubiquitous, tissue-specific and viral genes. Some of these genes control critical biological processes such as the cell cycle, apoptosis and developmental patterning. Unlike Sp1, which is a transcriptional activator, Sp3 can activate or repress transcription driven by Sp1 or other transcription factors [4]. Sp1 and Sp3 are expressed in most mammalian cell types while other family members such as Sp4 have a more restricted expression pattern including neuronal tissues [1, 5].

The overall structural similarity and ubiquitous expression patterns shared by Sp1 and Sp3 suggest that these two proteins are functionally equivalent. However, classical gene knockout studies have revealed they have different functions as they exhibit distinct phenotypes. Sp1 knockout embryos are severely retarded in development and do not survive beyond embryonic day (E) 10.5 [6]. Sp3 null embryos develop throughout gestation but die shortly after birth due to a series of complications which include a delay in lung-, tooth- and heart development and a defect in hematopoiesis affecting the lymphoid and myeloid lineages [7-9]. Sp1/Sp3 compound heterozygous mice are not viable and are retarded in development, suggesting that a critical threshold of Sp1 and Sp3 activity is required for normal embryonic development and that these two proteins have additive effects in regulating downstream target genes [10].

To understand the role of Sp1 and Sp3 in the adult hematopoietic system, we generated mice with conditional knockout alleles for Sp1 and Sp3 which we crossed with the well characterized inducible pan-hematopoietic Cre line, Mx1-Cre [11]. Surprisingly, the compound knockout animals showed a dramatic defect in the adult hematopoietic system affecting the lymphoid and myeloid lineages [12]. More strikingly, we observed a severe reduction of circulatory platelets in the compound knockout animals.

Based on these findings we used the Pf4-Cre line, which drives Cre expression exclusively in the megakaryocytic lineage, to investigate the role of Sp1 and Sp3 in adult megakaryopoiesis [12].

Megakaryopoiesis is the process by which platelets are formed. Hematopoietic stem cells (HSC), which are responsive to thrombopoietin (TPO), already commit to platelet-biased HSC with the capacity to differentiate into megakaryocytes and erythroid precursors. Megakaryocytes follow an intriguing differentiation program in
which they go through several rounds of endomitosis in order to become polyploid mature megakaryocytes. Maturation entails the development of a membrane demarcation system, which characterizes the latest stages of megakaryopoiesis prior to proplatelet formation and platelet release. Inherited or acquired defects in megakaryopoiesis can lead to either thrombocytopenia or thrombocytosis, resulting in platelet hemostatic dysfunction.

Different platelet receptors have been identified which are all involved in the maintenance of hemostasis and the formation of a thrombus. At the site of vessel wall injury, collagen is being exposed which causes a conformational change in von Willebrand factor (vWF), enabling interaction between vWF and the GPIb/V/IX complex. Platelets are able to bind to collagen through their GPVI and α2β1 receptors. This leads to platelet activation, release of thromboxane A2 and ADP and engagement of the αIIb/β3 receptor which assures platelet-platelet binding through fibrinogen bridges. Recently, a novel platelet receptor called CLEC-2 has been identified, also known as Clec1b in mouse [13, 14]. This receptor is activated by rhodocytin and podoplanin and plays an essential developmental role by separating the blood and lymphatic vessels and probably a minor role in hemostasis [15, 16]. While CLEC-2, GPVI and GPIb/V/IX have different structures and agonists, they share striking similarities in signaling pathways and they undergo cluster formation in lipid rafts necessary for proper signaling [17]. Although some platelet disorders have been linked to mutations in a specific set of genes, including GPIIbIIIa in Glanzmann Thrombasthenia, and GPIb/V/IX complex in Bernard Soulier syndrome (BSS) [18, 19], not all congenital platelet pathologies are fully understood. For instance, Bernard Soulier syndrome is also characterized by macrothrombocytopenia, without changes in plasma TPO levels. Megakaryocytes from Bernard Soulier patients have defects in membrane demarcation system formation, and their platelets are unable to respond to activation via the GPIb/V/IX complex.

By simultaneously deleting Sp1 and Sp3 specifically in the megakaryocytic lineage, we observed that these mice are a phenocopy of the Bernard Soulier syndrome. Although the phenotypic aspects of these mice share many similarities with BSS which is usually caused by single-gene mutations, we found that the Sp1/Sp3 transcriptional program in megakaryocytes regulates key CLEC-2, GPVI and GPIb/V/IX downstream signaling molecules.

Material and Methods

Mice
Mice were generated and maintained in the Erasmus MC animal care facility (EDC) under specific pathogen-free conditions. All the animal experiments were approved
by the Erasmus MC Animal Ethics Committee. The strategy for the generation of Sp1 and Sp3 conditional knockout mice has been described in chapter 2. Animals carrying floxed Sp1 and Sp3 alleles were bred to homozygosity and crossed with the Pf4-Cre line [12]. Additional rounds of breeding resulted in compound mice homozygous for the floxed Sp1 and Sp3 alleles, with or without the Pf4-Cre transgene.

**Blood analysis**

Blood parameters were analyzed on the scil Vet abc Plus+ hematology analyzer. Plasma TPO levels were measured by ELISA Quantikine recombinant human TPO kit from Roche Diagnostics.

**In vitro analysis - flow chamber perfusion**

Whole blood was reconstituted to normalize the platelet count to 250-500 10^6 platelets/mL. 200 μl was perfused over a μ-slide (0.1 Luer, Ibidi) coated with 100 μg/mL collagen (Horm, Nycomed Arzneimittel) under arterial shear conditions (shear rate 1500/s) for 5 minutes. After washing with 200μl 2nM CSFE/PBS (Invitrogen) images were taken at 20X magnification with an EVOS fluorescent microscope (AMG) [20].

**Flow chamber aggregation assay**

The procedure used in the aggregation assay has been described previously [21]. In brief, CD9 APC and CD9 PE (Abcam) labeled platelets were mixed 1:1 and pre-incubated 10 minutes at 37°C. As agonists, we used 100 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich), Botrocetin 10 μg/mL (sigma), 10 μg/mL collagen, or 30 nM AggretinA (a kind gift of Prof. Dr. Johannes A. Eble) in the presence of 3 mM CaCl2. Time samples fixed in 0.5% formaldehyde/PBS were measured on an LSRII + HTS flow cytometer and analyzed for double-colored events by FACSDiva Version 6.1 software (BD Biosciences) [21].

**Integrin activation phenotyping**

Platelets were stimulated for 5 minutes with 100 ng/mL PMA, 10 μg/mL collagen. Total expression of β3 was measured with CD61 (BD Pharmingen). The high-affinity conformation of integrin β3 was measured with JON/A (Efmret). The extent of activated integrin was determined relative to total integrin expression after background correction with isotype controls and normalizing ratios of unstimulated platelets to 1.
**α-Granule release**

Platelets were stimulated for 5 minutes with 100 ng/mL PMA. Release of α-granules was measured by surface expression of p-selectin (Emfret) on platelets.

**Electron microscopic analysis of platelets**

Platelets were fixed in Karnovsky’s fixative containing paraformaldehyde, 1M sodium hydroxide, 50% glutaraldehyde and 0.2M cacodylate buffer, pH 7.4. Post-fixation was done with 1% Osmiumtetraoxide in 0.1M cacodylate buffer. After washing the pellets were stained and blocked with Ultrastain 1 (Leica,), followed by an ethanol dehydration series. Finally, the platelets were embedded in a mixture of DDSA/NMA/Embed-812 (EMS), sectioned and stained with Ultrastain 2 (Leica,) and analyzed with a Philips CM10 electron microscope (FEI,).

**Flow cytometry**

*Reagents* - All fine chemicals were from Merck (Merck KGaA, Darmstadt, Germany) unless otherwise stated. The antibodies CD62P-APC, CD42b-APC, CD41-PerCPCy5.5 and CD49b-PE were purchased from BD Biosciences (San Jose, CA, USA). CD31-Pacific Blue and CD36-APCCy7 from Biolegend (San Diego, CA, USA), CD61-FITC from R&D (Basel, Switzerland) and CD63-PE was obtained from Sanquin Reagents (Amsterdam, The Netherlands). The PAR1 activating peptide (SFLLRN-NH2; Par-3676-PI) was purchased from Peptides International (Louisville, Kentucky, USA).

Femurs and tibiae were crushed and subsequently filtered through a 40µm cell strainer to obtain bone marrow single-cell suspensions. Splenic single-cell suspensions were obtained by mincing through 40µm cell strainers. Cells were washed with FACS buffer (PBS, 0.25% (w/v) BSA, 0.5mM EDTA, 0.05% (w/v) NaN₃) and incubated with monoclonal antibodies (mAbs) at room temperature for 10 minutes. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and the data analyzed with Tree Star FlowJo software.

**Analysis of platelet degranulation by flow cytometry and flow cytometry based platelet aggregation assay (FCA)**

Washed platelets were activated with increasing concentrations of PAR1-stimulating peptide (10, 100 and 1000µM), at 37°C while shaking at 1000 rpm. 0.5% paraformaldehyde fixed samples were stained with Allophycocyanin (APC) labeled anti-CD62P antibody (CD62P/APC) and Phycoerythrin (PE) labeled anti-CD63. The procedure for FCA has been described previously [21] As agonists we used 100 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, St Louis, MO), 10 µg/ml type-I collagen (Horm, Nycomed Arzneimittel GmbH, München, Germany), and 1.5 mg/ml Ristocetin (Biopool, Trinity Biotech Plc, Bray, Co Wincklow, Ireland).
Samples were measured by flow cytometry (LSR II + HTS, BD Biosciences) and analyzed using FlowJo software.

**In vitro culture of bone marrow derived megakaryocytes and analysis of megakaryocytes**

Bone marrow was isolated from femurs and single cell suspensions were made by crushing the bones and straining them through a 40 µm filter (BD falcon). Cells were cultured in Stemspan medium (Stemcell Technologies) supplemented with 10% heat-inactivated fetal calf serum, Penicillin/Streptomycin, 2% low density lipoprotein at a concentration of 2 X10^6 complemented with stem cell factor, 20 ng/ml TPO, 1 unit/mL erythropoietin (Eprex) and Flit3L. Cells were washed at day 2 and 4 and an increasing concentration of TPO was added, day 2 50 ng/mL and day 4 100 ng/mL. At day 7 the cells were ready for harvesting.

**FACS sorting**

CD61 CD41 and CD49b triple positive cells were sorted on the ARIA (BD Bioscience).

**RNA isolation**

Cell pellets were re-suspended in Trizol (Ambion Life Technologies) to lyse the cells. Chloroform was added and after centrifugation, the aqueous layer was transferred into a fresh tube and isopropanol added to precipitate the RNA. It was later washed with 75% EtOH. Pellet was re-suspended in RNase-free water.

**RT-QPCR and mRNA sequencing**

Total RNA isolated from FACS sorted cultured megakaryocytes was used to synthesize cDNA. cDNA was synthesized by the SuperScript III First-strand synthesis system for RT-PCR kit (Invitrogen) and amplified with the CFX96 TouchTM Real-Time PCR detection system (Bio-rad). Each sample was amplified in triplicate. SYBR green (Fermentas) was used to quantify the amplified products and gene expression was analyzed using the CFX manager software version 2.0 (Bio-rad). Cycle threshold levels were calculated for each gene and normalized to values obtained for the endogenous house-keeping gene, Gapdh. For genome-wide expression analysis, at least 10 ng of total RNA isolated from BSA gradient purified cultured megakaryocytes was used in Illumina Next Generation Sequencing (Erasmus Center for Biomics).

**Proplatelet formation**

Bone marrows from Pf4-Cre::Sp1fl/fl::Sp3fl/fl and Sp1fl/fl::Sp3fl/fl mice were obtained by flushing femora with Hepes buffer. The bone marrows were cut in transverse
sections of 1 mm and placed in an incubation chamber containing Hepes buffer (Life Technologies). Each chamber contained 4 fragments and was maintained at 37°C for 6 h. Megakaryocytes at the periphery of the tissue were observed under a phase contrast microscope (Axiove) (20x objective) coupled to a camera. Tile scans were taken every hour. Cells were classified according to megakaryocytes without proplatelets and megakaryocytes with proplatelets. Three mice of each genotype were analyzed.

**Bone marrow histology**
Bone marrow was harvested by flushing mouse femora with PBS. The samples were immediately embedded in Tissue-Tek®, EMS, Hatfield, USA and frozen with liquid nitrogen to prepare cryosections. Sections with a thickness of 5 μm were cut. Cryosections were stained with CD41 PE (BD Pharmingen), CD31 FITC (R&D systems) and Hoechst. Pictures (40X) were taken with confocal LSM510

**Mass spectrometry analysis**
Proteins in the lysate of the platelets were separated on SDS-PAGE and processed for mass spectrometry analysis as described [22]. Peptides were separated using a reverse-phase C18 Acclaim PepMap RSLC (75 μm × 150 mm, 2 μm particles) at a flow rate of 300 nl/min using a one-hour linear gradient from 0.05% acetic acid (v/v) to 0.05% (v/v) acetic acid and 35% (v/v) acetonitrile employing a Dionex Ultimate 3000 RSLC. Once separated, the peptides were directly sprayed into the LTQ Orbitrap XL mass spectrometer. Peptides were identified using the Sequest search algorithm using proteome discoverer 1.2 as described [23]. A maximum false discovery rate of 5% was allowed. The mass spectrometer, the nanoelectrospray source and emitters, the C18 column, nanoLC system, and analysis software were from Thermo Fisher Scientific Inc, (Bremen, Germany). Normalized spectral count of the proteins was obtained employing Scaffold (version Scaffold_4.0.4, Proteome Software Inc., Portland, OR).
Results

*Sp1/Sp3* dKO mice are macrothrombocytopenic with normal plasma TPO levels

Peripheral blood analysis was performed on *Pf4-Cre::Sp1*\(^{fl/fl}\)::*Sp3*\(^{fl/fl}\) mice (*Sp1/Sp3* dKO) and *Sp1*\(^{fl/fl}\)::*Sp3*\(^{fl/fl}\) (CT) littermate control mice. The result revealed that the mean blood platelet count was 341.7 ± 21.87 (n=13) in the *Sp1/Sp3* dKO mice, compared to 879.1 ± 50.18 (N=13; *P*<0.001) in control mice, which is a reduction of 61.1% (Figure 1A), while other blood parameters such as red blood cells or white blood cells were not affected (Figure 1A and Supplementary Table 1). In addition to a defect in platelet count, an increase in mean platelet volume was observed whereby the *Sp1/Sp3* dKO platelets had a larger volume when compared to the CT platelets (Figure 1A and Supplementary Table 1). These data show that *Sp1/Sp3* dKO mice suffered from macrothrombocytopenia. When examining the platelets by electron microscopy, we could confirm the larger size of *Sp1/Sp3* dKO platelets, and the higher frequency of vacuolization, although alpha granules appeared normal and in normal numbers (Figure 1C).

Thrombopoietin (TPO) is the most potent regulator of megakaryopoiesis. TPO is produced in the liver and its plasma levels are directly linked to the megakaryocyte-platelet mass. TPO levels are commonly increased when the production of platelets is reduced and in situations when the total megakaryocyte-platelet mass is reduced [23]. We therefore analyzed the plasma levels of TPO in *Sp1/Sp3* dKO mice by ELISA. We observed that TPO levels were normal and not different from those detected in CT littermates (Figure 1B). This suggests that despite the thrombocytopenia, the megakaryocyte-platelet mass is not altered.
Sp1/Sp3 regulate megakaryopoiesis

Figure 1. Peripheral blood and structural analysis of platelets. (A) Standard blood measurement of circulatory platelets revealed that Sp1/Sp3 dKO mice are thrombocytopenic with larger platelets. Mean and standard deviation (SD), *** P<0.001. (PLT = platelet, MPV = mean platelet volume, WBC = White blood cell, RBC = Red blood cell). (B) Normal plasma TPO levels are measured by ELISA. (C) Representative electron microscopy image showing normal alpha granules and higher vacuolization in Sp1/Sp3 dKO platelets.

Sp1/Sp3 dKO mice display impaired platelet function

We proceeded to examine the capacity of the platelets to form thrombi by perfusing them over collagen-coated slides at physiological flow rates. Platelets were reconstituted in whole blood after normalization of the platelet counts in Sp1/Sp3 dKO and WT samples, and the same volumes were perfused. After washing with PBS, platelets were stained with CFSE and thrombi were observed under the fluorescent microscope. Sp1/Sp3 dKO platelets had a reduced capacity to form thrombi when compared to WT platelets as measured by CFSE coverage (Figure 2A). This suggests that Sp1/Sp3 dKO platelets are impaired to form thrombi under...
flow conditions. However, which pathways are dysfunctional cannot be detected with this assay.

Furthermore, we used a flow cytometry based platelet aggregation assay (FCA) to determine the capacity of platelets to form small aggregates upon stimulation with different agonists [21]. Isolated platelets were stimulated with the following agonists, PMA (which activates the fibrinogen receptor GPIIb/IIIa or αIbb3 integrin), Collagen (which activates the collagen receptors GPVI and α1β2 integrin), Botrocetin (which activates the vWF receptor/GPIb-IX-V complex) and AggretinA (which activates the Clec1b receptor and signals via Syk to activate both αIbb3 and α1β2 integrins) [24]. Upon AggretinA (Clec1b) and Botrocetin (GPIb-V-IX) stimulation, the aggregation capacity of Sp1/Sp3 dKO platelets was severely decreased when compared to WT control platelets. We also observed a slight decrease in aggregation of Sp1/Sp3 dKO platelets upon collagen activation while PMA mediated platelet aggregation was not affected (Figure 2B).

When platelets become activated they switch their integrins from an inactive to an active conformation thereby releasing their granular content such as the α-granule. The ability to release α-granules can be measured by the appearance of P-selectin (CD62P) on the platelet membrane and activation of GPIIb/IIIa integrin can be measured by the ratio of active GPIIb/IIIa on the platelet membrane (with JON/A antibody) compared to the basal GPIIb/IIIa levels (CD61 antibody). We found that Sp1/Sp3 dKO platelets have no defect in either their degranulation capacity or the ability to switch their fibrinogen receptor from an inactive to an active form (data not shown), which is in line with our findings using PMA stimulation by FCA.

Taken together, our data revealed that Sp1/Sp3 dKO platelets have a general aggregation defect which is not caused by integrin malfunction. These findings suggest a defect in either receptor trafficking to the cell surface, intracellular signaling or receptor clustering specifically affecting the Clec1b and vWF receptors.
Sp1/Sp3 regulate megakaryopoiesis

Figure 2. Functional analysis of platelets (A) Sp1/Sp3 dKO display a decreased capacity to form thrombi. (B) Sp1/Sp3 dKO platelets responded normally to PMA and slightly lower to collagen. Their response to Botrocetin and Aggretin A were impeded. Mean and standard deviation (SD), * P<0.05, *** P<0.001.

Normal expression of GPIb-IX-V in Sp1/Sp3 dKO mice

The aggregation capacity of Sp1/Sp3 dKO platelets upon stimulation with different agonists was impaired. Therefore surface expression of some of the most relevant platelet receptors was analyzed by flow cytometry on fixed resting platelets. Although the aggregation upon stimulation with PMA was not impaired, we saw a slight but consistent decrease in receptor expression of GPIIβIIIα using CD61 and CD41 antibodies, but also a modest decrease in CD49b and an increase in CD9 (Figure 3A). In contrast, aggregation was severely impaired upon stimulation with Botrocetin, which activates the GPIb-V-IX complex. However, all the different
subunits of this receptor were significantly upregulated (Figure 3B). We conclude that the signaling pathway activated upon stimulation with Botrocetin via vWF receptor (and AggretinA via Clec1b) was affected in these mice, rather than a deficiency of their receptors.

![Image of flow cytometry analysis of platelet receptors](image)

**Figure 3. Flow cytometry analysis of platelet receptors** (A) Sp1/Sp3 dKO platelets expressed lower levels of fibrinogen receptor subunits (CD61/CD41) and CD49b but a higher level of CD9. (B) Increased expression of the various vWF receptor subunits in Sp1/Sp3 dKO platelets.

**Sp1/Sp3 dKO mice have normal numbers of megakaryocytes in bone marrow and spleen, which fail to mature and produce proplatelets**

The normal TPO levels in plasma suggest that megakaryocyte numbers should not be altered in the bone marrow and spleen, in case the thrombocytopenia is caused by macrothrombocytes. To corroborate this, we next examined the megakaryocytic compartment in Sp1/Sp3 dKO mice. We measured the percentage of megakaryocytes in single cell suspensions of bone marrow and spleen, since both tissues host megakaryopoiesis in the adult mice. As shown in Figure 4A, the percentage of megakaryocytes in bone marrow and spleen were not affected, supporting our hypothesis.
Megakaryocytes are located in the mouse bone marrow and spleen, where they migrate upon maturation from the osteoblastic niche (in bone marrow specifically) to the vessel wall where they are able to shed the platelets directly into the bloodstream. During maturation and migration the megakaryocyte undergoes several biomolecular changes including polyploidization of the nucleus, the development of a demarcation membrane and the formation of proplatelets. Analyzing the ploidy status of these megakaryocytes (CD61+) by staining DNA with Hoechst dye, we could not detect significant changes (Figure 4B). However, it is known that the ploidy status does not necessarily correlate with full megakaryocyte maturation, and we cannot discard a maturation arrest leading to deficient platelet production. To circumvent this, we next looked at the mature megakaryocyte population by performing acetylcholinesterase stainings in spleen cryosections. We observed a significant reduction in the acetylcholinesterase+ megakaryocyte number relative to splenic surface (Figure 5A). This suggests that despite the normal numbers of megakaryocytes found in the spleen, the mature population is reduced, and this could be one of the reasons for the thrombocytopenia. Another reason why platelet production could be affected is a dysfunctional migration of the megakaryocytes in the bone marrow niche towards the sinusoids. The vessel wall secretes the chemoattractant CXCL12, which recruits
Figure 4. Analysis of megakaryocytes in bone marrow and Spleen (A) Similar numbers of megakaryocytes in the BM and spleen of Sp1/Sp3 dKO and CT mice. (B) Similar ploidy status between Sp1/Sp3 dKO and the controls.

megakaryocytes to migrate towards it. Since some of the relevant integrins were downregulated on platelets (Figure 3A), we wondered whether
Figure 5. Visualization of Megakaryocytes in the Bone marrow and Spleen (A) Acetylcholinesterase staining of splenic section showing reduced numbers of mature megakaryocytes in the Sp1/Sp3 dKO mice. (B) IHC on bone marrow section showing normal migration of Sp1/Sp3 dKO megakaryocytes to the vessel wall were they subsequently release platelets into circulation. Sp1/Sp3 dKO megakaryocytes are smaller in size.

Megakaryocyte location next to vessels would be affected. In order to investigate this, we performed immunocytochemistry of bone marrow cryosections, staining the vessel wall with CD31 (which also stains megakaryocytes), and CD41 as a megakaryocyte marker. We observed no major differences in the location of megakaryocytes within the bone marrow niche and megakaryocytes were also found next to the vessel wall in Sp1/Sp3 dKO mice (Figure 5B). A representative example is shown, which reveals a slight morphological difference of the Sp1/Sp3 dKO megakaryocytes, which appear slightly smaller and with fewer proplatelet protrusions.

In order to investigate proplatelet formation capacity of dKO megakaryocytes, we performed bone marrow explants, whereby the bone marrow was flushed from the femur and cut in 1 mm slices, and counted the megakaryocytes forming proplatelets after 4 and 6 hours of explant culture. Although we were able to
Figure 6. Analysis of cultured megakaryocyte (A) Proplatelet forming megakaryocytes were significantly reduced in BM culture from Sp1/Sp3 dKO mice when compared to the CT. The arrowheads indicates proplatelet filaments protruding from the megakaryocytes (B) Electron microscopy showing increased vacuolization and diffuse demarcation membrane system in Sp1/Sp3 megakaryocytes when compared to the controls.

detect megakaryocytes forming proplatelets after 6 hours (Figure 6A), the number was significantly reduced when compared to CT, while the megakaryocytes numbers at the periphery of the explant were not altered (data not shown). The demarcation membrane develops in the final stage of megakaryocyte maturation to ensure sufficient membrane capacity to produce platelets[25]. Coordinated interactions between the membrane, cytoskeletal and signaling system is crucial for proper platelet function [26, 27].
Electron microscopy of bone marrow derived megakaryocytes cultured for 7 days revealed that Sp1/Sp3 dKO megakaryocytes had increased vacuolization and diffuse demarcation membrane system when compared to the CT cells (Figure 6B). These data altogether show that the megakaryocyte-mass is not altered in Sp1/Sp3 dKO mice. The macrothrombocytopenia observed could be attributed to a defect in megakaryocyte maturation which includes impeded demarcation membrane system and proplatelet formation, suggesting that these defects could be the cause of the aberrant production of reduced numbers and giant platelets.

Mass spectrometry analysis of Sp1/Sp3 dKO platelets reveals deficiency of proteins related to cytoskeletal rearrangements and signal transduction

In order to dissect the deregulated genes responsible for the phenotype of Sp1/Sp3 dKO mice, we performed mass spectrometry analysis of platelet lysates from dKO and CT mice. Two samples from each genotype were analyzed. In order to verify the mass spectrometry data, we used the peptide ratio of dKO vs CT lysates of proteins (surface markers) we previously analysed by flow cytometry (Figure 3). We could corroborate the expression levels of all the investigated
Figure 6. Mass spectrometry analysis of platelet lysate (A) Mostly proteins involved in cytoskeleton network were reduced in Sp1/Sp3 dKO platelets (B) The reduction of Filamin A level in Sp1/Sp3 dKO platelets was confirmed by western blotting.

surface markers (Figure 7A). We could also analyze the expression levels of Clec1b, and observed no changes in its expression levels between the two genotypes.
Interestingly, some of the proteins that were reduced with higher significance in Sp1/Sp3 dKO platelets regulate cytoskeletal rearrangements, such as FlnA, Coro1a, Myh9, Was and tubulins.

One of the targets identified by proteomics was Filamin A. Filamin A is a cross-linker between actin filaments, it tethers membrane glycoproteins and serves as a scaffold for signaling [28]. It is known that FilamenA has a critical structural role in attaching the GPIb/V/IX complex to the underlying actin cytoskeleton and the interaction between FilaminA and GPIbα has been reported to influence vWF receptor function [29]. Mouse platelets lacking FilaminA are large, and have a severe functional impairment in signaling responses downstream of GPVI and Clec-2 which is a result from the loss of CA/Syk interaction [28].

We validated the mass spectrometry results by performing western blot analysis of VA expression on platelet lysates, and showed that FilaminA levels were reduced in Sp1/Sp3 dKO mice.

A reduction in these identified proteins does not necessarily mean that they are directly controlled by Sp1/Sp3 transcription factors, that is, due to deregulated transcription of their genes. It is possible that due to defective megakaryocyte maturation, the released platelets are not supplied with the normal proportions of all intracellular components.

To analyze more comprehensively changes in gene expression profiling, we performed genome wide profiling of purified cultured megakaryocytes. Preliminary analysis revealed down-regulation of several genes encoding cytoskeletal proteins including Coro1a, Myh9, Was and tubulins. These genes could be targets of Sp1 and Sp3.
Discussion

The ubiquitous zinc finger transcription factors Sp1 and Sp3 play critical roles in embryonic development. While deficiency of Sp1 is lethal in utero at E10.5 [6], deficiency of Sp3 results in postnatal lethality [7-9, 30]. However, compound heterozygous mice die in utero at around E18.5 displaying severe developmental defects including anemia resulting from impaired erythropoiesis [10]. This results suggest that their redundant functions are crucial for the development and function of many cells and processes including the fetal hematopoietic system.

Analyzing the effect of simultaneously deleting Sp1 and Sp3 from the adult hematopoietic system we showed that the adult hematopoietic system was severely disrupted in Mx1-Cre:: Sp1^{fl/fl}::Sp3^{fl/fl} mice (Chapter 2) [11] particularly affecting the megakaryocytic lineage.

In order to investigate whether the platelet phenotype observed was due to an intrinsic megakaryocyte/platelet defect, we crossed Sp1^{fl/fl}::Sp3^{fl/fl} mice with Pf4-Cre mice [12] which express Cre recombinase exclusively in the megakaryocytic lineage. We found that Sp1/Sp3 dKO mice suffered from macrothrombocytopenia.

TPO together with its receptor c-Mpl are critical for megakaryocyte growth and development, as knockout mice displayed reduced numbers of megakaryocytes and platelets [31-33]. By measuring the level of TPO in the serum of Sp1/Sp3 dKO and the CT we showed that the levels were similar indicating that the macrothrombocytopenia observed is not due to the inefficient production of TPO by the liver of Sp1/Sp3 dKO mouse.

We reasoned that if the macrothrombocytopenia is not caused by defective TPO level, then the number of megakaryocytes in the bone marrow and spleen should not be altered. By measuring the percentage of megakaryocytes in single cell suspension of bone marrow and spleen we showed that this was indeed the case.

After ruling out a defective TPO production, we went further to examine maturation defects. Mature megakaryocytes reach high ploidy levels through endomitosis [34]. Analyzing the ploidy status of bone marrow and spleen megakaryocytes revealed that the proportion of megakaryocytes with higher ploidy in Sp1/Sp3 dKO tissues was comparable to the controls. This is in contrast to what has been reported to occur in patients with Bernard Soulier syndrome who display higher ploidy megakaryocytes [35]. Since ploidy status does not necessarily correlate to megakaryocyte maturation, it remains possible that Sp1/Sp3 deficient megakaryocytes exhibit maturation defects leading to deficient platelet production. The formation of a demarcation membrane, which is independent of endomitosis and precedes proplatelet formation, also marks megakaryocyte maturation [36, 37].

We showed that Sp1/Sp3 dKO megakaryocytes display a specific defect at the demarcation membrane system stage which could explain the impeded proplatelet formation. However, migration towards the vessels does not seem to be affected.
Next, we examined the degranulation capacity of the platelets by measuring the appearance of P-selectin on the platelet membrane and activation of integrin by measuring the ratio of active GPIIbIIIa (JON/A antibody) compared to the basal GPIIbIIIa levels (CD61 antibody) on the platelet membrane. We found that Sp1/Sp3 dKO platelets have no defect in neither their degranulation capacity or the ability to switch their fibrinogen receptor from an inactive to an active form. This could explain why we did not observe differences in aggregation upon PMA activation. However, we observed impaired aggregation via the vWF receptor and Clec1b receptor. Surprising, mass spectrometry analysis revealed a normal level of Clec1b in the Sp1/Sp3 dKO platelets.

Interestingly, proteins involved in cytoskeletal re-organization including FilaminA, Coro1a, Myh9, Was and tubulins were all reduced in Sp1/Sp3 dKO platelets. For example, FilaminA crosslink actin filaments, tethers membrane glycoproteins and serves as a scaffold for signaling [28, 38]. FilaminA has a critical structural role in attaching the GPIb/V/IX complex to the underlying actin cytoskeleton and the interaction between FilaminA and GPIbα has been reported to influence vWF receptor function by stabilizing and enhancing its surface expression [39-41]. Mouse platelets lacking FilaminA are larger, and have a severe functional impairment in signaling responses downstream of GPVI and Clec 1b resulting from the loss of FilaminA/Syk interaction[28].

To ascertain whether the reduction in the identified proteins are a result of decreased gene expression, we preformed gene-wide expression analysis on cultured megakaryocytes. Our data revealed down-regulation of a number of cytoskeletal genes which included Coro1a, Myh9, Was and tubulins[42]. Myh9 deficient mice has a reduce DMS affecting the capacity to extend proplatelets [43].

Taken together, the phenotypes observed in Sp1/Sp3 dKO mice make these mice a phenocopy of BS syndrome. BS is caused by single gene mutations while in the Sp1/Sp3 dKO megakaryocytes the expression of many genes involved in megakaryopoiesis is modulated. This mouse model could therefore help to guide understanding of cases of congenital or acquired thrombocytopenia with unknown cause.
Reference


Sp1/Sp3 regulate megakaryopoiesis


Sp1/Sp3 regulate megakaryopoiesis

Supplementary Table

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CT (n = 13)</th>
<th>Sp1/Sp3 dKO (n = 13)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>879.1 ± 50.18</td>
<td>341.7 ± 21.87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MPV (fl)</td>
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<td>8.485 ± 0.09257</td>
<td>&lt;0.01</td>
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<tr>
<td>RBC</td>
<td>9.247 ± 0.1443</td>
<td>9.592 ± 0.1256</td>
<td>NS</td>
</tr>
<tr>
<td>WBC</td>
<td>4.046 ± 0.3576</td>
<td>5.077 ± 0.6351</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 1: Blood parameters in Sp1::Sp3 dKO and WT mice at 12 weeks of age. Average and standard deviation are indicated (n = 13). Significance is indicated as p-value. MPV, mean platelet volume; RBC, red blood cells; WBC, white blood cells
Hematopoiesis

Hematopoiesis is the generation of distinct mature blood cells from pluripotent hematopoietic stem cells (1). Unraveling the molecular mechanisms that govern hematopoietic lineage commitment and differentiation would guide the development of new therapies for diseases caused by defects in hematopoiesis. To study the roles of Sp1 and Sp3 transcription factors in the adult hematopoietic system, we generated mice with conditional knockout allele for Sp1 and Sp3 and used various Cre lines to inactivate Sp1 and Sp3 in distinct hematopoietic cell types and lineages.

Sp1 and Sp3 regulate the adult hematopoietic system

Members of the Specificity protein/Krüppel-Like Factor (Sp/KLF) family play a crucial role in regulating the expression of many genes, including developmental specific, tissue specific, and cell cycle regulated genes. Previous gene knockout studies have shown that Sp1 and Sp3 knockout embryos are not viable. Sp1 knockout embryos are severely retarded in development and do not survive beyond embryonic day (E) 10.5 (2). Analysis of Sp3 deficient embryos revealed a series of developmental abnormalities including cardiac malformation, delayed tooth and lung development and defects in hematopoiesis (3-6). Considering the importance of Sp/KLF family members in regulating the expression of many genes (7-12) and their vital role in embryogenesis, we hypothesized that they are crucial for the proper functioning of the adult hematopoietic system. To prove this hypothesis, we generated mice with Sp1 and Sp3 conditional knockout alleles which we crossed with various Cre lines to inactivate Sp1 and Sp3 in several hematopoietic lineages and cell types starting from hematopoietic stem cells.

Chapter 2: To characterize the in vivo function of Sp1 and Sp3 in the hematopoietic system, we crossed Sp1fl/fl or Sp3fl/fl mice with mice expressing Cre recombinase in the entire hematopoietic system, Mx1-Cre (13). Surprisingly, efficient deletion of Sp1 or Sp3 from the adult hematopoietic system displayed no obvious phenotype which is in contrast to the severe phenotypes observed in knockout embryos (6). A reason for the lack of phenotype in the Mx1-Cre::Sp1fl/fl or Mx1-Cre::Sp3fl/fl mice could be that Sp1 and Sp3 exert their functions specifically in the embryonic hematopoietic system and not in the adult or that redundancy exists between Sp/KLF family members, as previously suggested (14-16). Since Sp1 and Sp3 are structurally closely related to each other and are also expressed ubiquitously, we proposed that redundancy exists between Sp1 and Sp3 in the adult hematopoietic system similar to what was recently reported to occur during embryogenesis (17). To further study the redundancy of Sp1 and Sp3 in the adult hematopoietic system, we crossed the Mx1-Cre::Sp1fl/fl, Mx1-Cre::Sp3fl/fl and Mx1-
Cre mice together resulting in Mx1-Cre::Sp1^{fl/fl}::Sp3^{fl/fl} double conditional knockout mice. We showed that Mx1-Cre mediated simultaneous deletion of Sp1 and Sp3 resulted in severe disruption of the adult hematopoietic system as the double knockout animals suffered from defective myelopoiesis, lymphopoiesis and were thrombocytopenic. The broad range of defects observed in Mx1-Cre::Sp1^{fl/fl}::Sp3^{fl/fl} animals might suggest that Sp1/Sp3 have a more general function in regulating proliferation and differentiation in the adult hematopoietic system.

Crossing Sp1/Sp3 conditional knockout mice with lineage or cell-specifically expressed Cre will reveal the cell or lineage specific functions of Sp1 and Sp3. To further unravel the molecular roles of Sp1 and Sp3 in the affected hematopoietic lineage or cell types, mRNA sequencing and ChIP will be used to identify target genes and pathways regulated by Sp1 and Sp3. This could lead to the discovery of promising intervention targets for serious diseases like cancer. A similar approached has been used to identify Sp3 as a repressor of a critical growth inhibitory pathway, the transforming growth factor β (TGFβ) signaling pathway in breast cancer cells (18). Also, anti-apoptotic gene, FLIP, signaling pathway regulates growth in many mammalian cells. Sp1 activates, while Sp3 represses FLIP gene promoter activity. Targeting this pathway has been predicted to be a new therapeutic approach for prostate cancer (19, 20).

Sp1 and Sp3 cooperate to regulate B cell development

One of the lineages affected upon pan-hematopoietic deletion of Sp1 and Sp3 was the B cell lineage. B cell development is regulated by a series of transcription factors including EBF1, E2A and Pax5 which regulate commitment and differentiation of HSCs towards the B cell lineage (21-27).

As discussed in Chapter 3, in order to further examine the in vivo function of Sp1/Sp3 during B cell development we crossed Sp1^{fl/fl}::Sp3^{fl/fl} mice with Mb1-Cre (28) mice which express Cre recombinase at the pre/pro stage of B cell development. We demonstrated that B cell development is impaired in Mb1-Cre::Sp1^{fl/fl}::Sp3^{fl/fl} mice due to a remarkable defect in proliferation and differentiation of CD2^-cytoplasmic Igµ^+ large pre B cells.

To monitor the deletion of Sp1 and Sp3 in the B cell lineage, we analyzed YFP expression, a reporter gene present in the Sp1^{fl/fl}::Sp3^{fl/fl} allele and under the control of the ubiquitously expressed ROSA26 locus (29). YFP can only be expressed in cells were Cre recombinase is active. During B cell development, the deletion of Sp1^{fl/fl}::Sp3^{fl/fl} by Mb1-Cre occurred very early (at the pre/pro B cell stage) and was very efficient.

During B cell development, signaling through the pre-BCR receptor checkpoint which monitors functional IgH rearrangement and IL-7R signaling, induces cell cycle entry of large cycling Igµ^+ pre-B cells, and rapid down-regulation of the re-arrangement machinery, thereby ensuring allelic exclusion (30, 31). Subsequently,
large pre-B cell proliferation is terminated and further differentiation into small resting pre B and Ig κ or λ light chain rearrangement is initiated (32-34).

Further analysis of the pre-B cells revealed that \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) large pre-B cells displayed a reduced cell size indicating a proliferative defect. However, residual cells expressed intracellular Igμ suggesting a productive \( Igh \) re-arrangement. These results suggest that Sp1/Sp3 specifically regulate proliferation and differentiation of large pre-B cell but not V(D)J recombination (35, 36).

Since a functional \( Igh \) chain is required for large pre-B cells to progress to small pre-B cell, we introduced a functionally pre-rearranged \( \text{Igμ} \) heavy chain, \( \text{VH81X} \) (37), to determine whether the developmental arrest observed in \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) large pre-B cells was as a consequence of defective \( Igh \) re-arrangement. We showed that a substantial number of \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) B cells were able to progress beyond the large pre-B cell stage albeit not at the level observed for \( \text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) cells. These suggest that in addition to a productive heavy chain re-arrangement, Sp1/Sp3 deficient cells can also undergo Ig light chain rearrangement.

Interestingly, we found that the phenotypes observed in \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) B cells were not present in either \( \text{Sp1} \) or \( \text{Sp3} \) single knockout mice and that a single allele of either Sp1 or Sp3 is sufficient to support B cell development. We showed for the first time that Sp1 and Sp3 redundantly regulate B cell development.

To further characterize the function of Sp1/Sp3 in B cells, we cultured BM pre-B and mature B cells from the spleen. We showed that Sp1/Sp3 deficient pre-B cells do not proliferate in response to IL-7. Furthermore, we determined the proliferation block of splenic B cell to be at the S phase of the cell cycle accompanying defects in metabolism (38, 39).

Sp1 regulates cell cycle by directly binding and regulating the expression of cyclin D1 in epithelial cells. Epithelial cells lacking Sp1 down regulates cyclin D1 and fails to progress beyond the G1 phase of the cell cycle (40). Therefore, it is likely that Sp1 regulates B cells progression through the cell cycle at least in part by regulating cyclin D1 activity.

To investigate whether changes in gene expression profile of many genes could be involved in the defects observed in \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) cells, we performed genome-wide RNA expression analysis of naive \( \text{Mb1-Cre}::\text{VH81X}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) and \( \text{VH81X}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) splenic B cells since \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) cells did not survive. Preliminary analysis has identified hundreds of genes differentially expressed in \( \text{Mb1-Cre}::\text{VH81X}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) mature B cells compared to the controls. Some of the genes identified regulate critical biological processes such as proliferation, metabolism, signal transduction and apoptosis. The deregulation of these genes could explain the proliferation, differentiation and survival defects observed in \( \text{Mb1-Cre}::\text{Sp1}\text{fl/fl}::\text{Sp3}\text{fl/fl} \) B cells. As an example, we observed the down regulation of the IL2 receptor and of components of the JAK/STAT signaling pathway crucial for lymphocyte proliferation (41, 42).
Another very interesting observation was the down-regulation of the Myc gene in \( \text{Mb1-Cre::VH81X::Sp1}^{+/+}::\text{Sp3}^{+/+} \) B cells. Myc is a proto-oncogene previously reported to enhance protein synthesis, cell size and growth in B cells \((43, 44)\). We showed that Sp1/Sp3 deficient B cells were smaller in size, accumulate at the G0/G1 stage of the cell cycle and were not able to enter the S phase. This is consistent with a previous report where similar defects were observed in Myc-deficient B cells \((45)\). Also, c-myc interacts with Sp1 to repress the cyclin-dependent kinase inhibitor, p21 which inhibits proliferation both \textit{in vivo} and \textit{in vitro} \((46)\).

Performing ChIP sequencing analysis will ascertain whether Sp1 or Sp3 directly regulate Myc and other target genes in B cells and provide insight into the molecular regulation of B lymphopoiesis.

**Sp1 and Sp3 regulate megakaryopoiesis**

Using the platelet-specific Cre mouse strain \( \text{PF4-Cre} \) we efficiently deleted both Sp1 and Sp3 from the megakaryocytic lineage \((47)\). In line with our earlier observation, Sp1/Sp3 dKO mice suffered from macrothrombocytopenia. Interestingly, Sp1/Sp3 dKO mice displayed a normal TPO level which is a hormone crucial for megakaryocyte development \((48)\). Since the TPO level in plasma was normal, we hypothesized that the mice would contain similar numbers of megakaryocytes in BM and spleen. Indeed we observed a similar numbers of megakaryocytes in the BM and spleen of Sp1/Sp3 dKO and control animals indicating that the defect in platelet production was not due to a defect in TPO or its receptor \((48-50)\). Proper functioning of the platelet demarcation membrane system is necessary for proplatelet formation \((51, 52)\). We showed that Sp1/Sp3 dKO platelets displayed a defective demarcation membrane system resulting in a defect in proplatelet formation. However, migration towards the vessels does not appear to be affected.

Furthermore, the platelets do not show degranulation defects or integrin activation defects (outside-in inside-out signaling) However, Sp1/Sp3 dKO platelets showed impede aggregation upon stimulation with Botrocetin or Aggretin A which activates platelets via vWF and Clec1b receptors respectively. This suggests a defect in either receptor trafficking to the cell surface, intracellular signaling or receptor clustering specifically affecting the Clec1b and vWF receptors.

Mass spectrometry analysis revealed a reduction in proteins that play an important role in cytoskeletal rearrangements including Filamin A (FlnA), Coro1a, Myh9, Was and tubulins. Filamin A has been reported to function as a scaffold for signaling proteins and also to cluster membrane glycoproteins at lipid rafts in the membrane \((53, 54)\). We confirmed by western blotting that Filamin A was indeed undetectable in Sp1/Sp3 dKO platelets. The absence of Filamin A could explain the improper
DMS, proplatelet formation and release and lower expression of vWF and Clec1b receptor on the platelet membrane surface observed in the Sp1/Sp3 dKO mice. One of the vWF receptor subunits, GPVI, has been reported to bind collagen and requires functional Filna for its signaling (53). Future analysis of GPVI might reveal its contribution to this phenotype.

In order to identify Sp1 and Sp3 target genes, we performed RNA sequencing which we found a large number of genes that were down-regulated including Coro1a, Myh9, Was and Tubulins (55, 56), which are important for megakaryopoiesis. These findings validate our mass spectrometry results and suggest that these genes could be novel targets for Sp1 and Sp3 in megakaryocytes. Interestingly, Sp1 has been reported to regulate Filamin A in fibroblast (57), so it is likely that it also regulates Filna A in megakaryocytes. RT-QPCR needs to be performed to confirm this hypothesis. Also, we are still confirming which identified proteins are direct targets of the Sp1/Sp3 transcriptional program in order to understand the specificity of platelet function defects observed in this mouse model.

Bernard Soulier syndrome (BSS) is caused by mutation of GP1b and its characterized by deficient ristocetin-dependent platelet agglutination. Sp1/Sp3 KO platelets displayed a similar phenotype. Interestingly, BSS is caused by single-gene mutations, and we modulated the expression levels of many genes in megakaryocytes. These data indicated that Sp1/Sp3 dKO mice are a phenocopy of patients with Bernard Soulier syndrome. This mouse model may provide a useful tool for deeper understanding of congenital and acquired thrombocytopenias with unknown causes.

Other Cre lines available for further studies are listed in table 1. CD4-Cre (58) mediated deletion of Sp1 and Sp3 from the T cell lineage resulted in reduced circulatory CD4+ and CD8+ T cells in CD4-Cre::Sp1fl/fl::Sp3fl/fl animals. Experiments to determine the role of Sp1 and Sp3 in T cells differentiation, by differentiating naïve CD4+ T-cells in vitro into various T helper cell subsets mimicking in vivo immune response and their role in cytokine production has been performed. Preliminary analysis revealed a defect in cytokine production and in Th2 subset. However a more detail analysis still needs to be performed.

Cebpa-Cre mediated deletion of Sp1 and Sp3 from early myeloid progenitors is embryonic lethal (59). Further analysis is needed to determine the cause of the of the embryonic lethality. Cre mediated deletion of Sp1/Sp3 from early erythroid progenitors and macrophages/granulocytes displayed no obvious phenotypes.
<table>
<thead>
<tr>
<th>Cre line</th>
<th>Specificity</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Cd4-cre</em></td>
<td>Double positive T cells</td>
<td>transgene</td>
</tr>
<tr>
<td>2</td>
<td><em>LysM-cre</em></td>
<td>Macrophages and granulocytes</td>
<td>Knock-in line</td>
</tr>
<tr>
<td>3</td>
<td><em>EpoR-cre</em></td>
<td>Early erythroid progenitors</td>
<td>Knock-in line</td>
</tr>
<tr>
<td>4</td>
<td><em>Cebpa-cre</em></td>
<td>Early myeloid progenitors, liver, lung</td>
<td>Knock-in line</td>
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Table 1. Cre lines used in this study. The cells and tissues where they are expressed are also indicated.
References


Summary
(Samenvatting)
Summary

Hematopoietic disorders are a serious burden to societies worldwide requiring detailed studies on the hematopoietic system. The hematopoietic system is a self-renewal system and serves as an important system to study hematopoietic disorders and provide the molecular basis for the development of new therapies to combat many diseases. Transcription factors play a crucial role in regulating this system. These studies focus on the role of Specificity (Sp) transcription factors in adult hematopoiesis.

CHAPTER 2: The approach was to start with a pan-hematopoietic Cre line, the Mx-cre and study the effect of Sp1 and Sp3 in the entire Hematopoietic system. We will use lineage specific Cre-lines to study their roles in the affected lineages. In this chapter, we reveal the effect of simultaneously deleting Sp1 and Sp3 from the adult hematopoietic system. Since Sp1 or Sp3 knockouts are embryonic lethal, we generated mice with conditional knock alleles for Sp1 (Sp1\(^{fl/fl}\)) and Sp3 (Sp3\(^{fl/fl}\)). Crossing Mx1-Cre mice with Sp1\(^{fl/fl}\) or Sp3\(^{fl/fl}\) mice, we were able to efficiently delete the genes from the entire hematopoietic system. Surprisingly, both Mx1-Cre::Sp1\(^{fl/fl}\) and Mx1-Cre::Sp3\(^{fl/fl}\) mice displayed no obvious phenotype. Due to redundancy that exist between Sp family members, particularly Sp1 and Sp3, we hypothesized that simultaneous in-activation Sp1 and Sp3 from the adult hematopoietic system will produce a severe phenotype. Indeed we found that Mx1-Cre::Sp1\(^{fl/fl}\)::Sp3\(^{fl/fl}\) animals suffered from a defective myelopoiesis, lymphopoiesis and were severely thrombocytopenic. This demonstrated for the first time the redundant role of Sp1 and Sp3 in regulating the adult hematopoietic system.

CHAPTER 3: This chapter describes the role of Sp1/Sp3 during B cell development. In Chapter 2, one of the hematopoietic lineage affected was the B cell lineage. So we used B cell Specific transgenic Cre (Mb1-Cre) mice to inactivate both Sp1 and Sp3 in the B cell lineage. Flow cytometry revealed a developmental blockage of mb1-Cre::Sp1\(^{fl/fl}\)::Sp3\(^{fl/fl}\) (KO) B cells at the large pre B cell stage. Further analysis of the revealed a proliferation and differentiation
defect in $Mb1-Cre::Sp1^{fl/fl}::Sp3^{fl/fl}$ large pre-B cell which could not be rescued by the introduction of a pre-rearranged Igµ transgene, $V_{H}81X$ but could progress beyond the large pre B cell stage. Our data revealed that B cell receptor signaling in splenic B cells from $Mb1-Cre::Sp1^{fl/fl}::Sp3^{fl/fl}$ mice appeared to be normal but these cells displayed a defect in cell metabolism. Analyzing genome-wide expression profile we found hundreds of deregulated genes of which genes involved in critical biological functions including proliferation, differentiation and survival were down-regulated in $Mb1-Cre::Sp1^{fl/fl}::Sp3^{fl/fl}$ B cells. In addition, $mb1-Cre::Sp1^{fl/fl}$ or $mb1-Cre::Sp3^{fl/fl}$ B cells displayed no obvious phenotype further strengthening the fact that redundancy exist between Sp1 and Sp3 in the hematopoietic system particular in the B cell lineage. Taken together, we concluded that, during B cell development, co-operation between Sp1 and Sp3 regulates B cell proliferation, differentiation and metabolism. The can do so either by directly regulating genes involves in those biological processes or indirectly via different pathways.

CHAPTER 4: In this chapter, we unraveled the effect of inactivating Sp1 and Sp3 from megakaryocytes/platelet lineage. We showed that crossing $Sp1^{fl/fl}::Sp3^{fl/fl}$ mice with megakaryocyte/platelet specific Cre ($pf4-Cre$) mice we efficiently deleted Sp1 and Sp3 from those cells. Blood analysis revealed that $pf4-Cre::Sp1^{fl/fl}::Sp3^{fl/fl}$ (Sp1/Sp3 dKO) mice had macrothrombocytopenia, normal thrombopoietin levels, normal numbers of megakaryocytes (MKs) in the bone marrow and spleen. However, these MK do not mature normally, and display specifically a defect at the demarcation membrane system stages, which could have resulted in the defect in proplatelet formation. Furthermore, migration towards the vessels does not seem to be affected. We demonstrated that Sp1/Sp3 dKO platelets do not exhibit defect in degranulation and in integrin activation (invagination signaling) suggesting instead a defect in receptor trafficking, intracellular signaling or receptor clustering which affect specifically Clec1b and vWF receptors.

Mass spectrometry data analysis showed a reduction of proteins crucial for cytoskeletal rearrangements. Performing a genome-wide expression profiling, we could show the reduction of their corresponding genes suggesting novel
targets of Sp1 and Sp3 in megakaryocytes. Collectively, our data revealed that mice deficient in both Sp1 and Sp3 displayed phenotypes consistent to patients with Bernard Soulier syndrome (BSS). BSS is caused by mutation in GP1b but by deleting Sp1 and Sp3 in megakaryocytes we modulated the expression of many genes involved in megakaryopoiesis to observe a similar phenotype. This mouse model could therefore facilitate our understanding of congenital or acquired thrombocytopenia of which the cause is not yet known.
Samenvatting

Hematopoëtische stoornissen vormen wereldwijd een grote last voor de samenleving en dit vereist grondige bestudering van het hematopoëtische systeem. Het hematopoëtische systeem is een systeem dat zichzelf in stand houdt en dient als een belangrijk systeem voor het bestuderen van hematopoëtische stoornissen en het ontwikkelen van nieuwe therapieën tegen deze stoornissen. Transcriptie factoren spelen een belangrijke rol in het hematopoëtische systeem. Deze studie richt zich op de rol die Specificiteits (Sp) transcriptie factoren spelen in hematopoëse bij volwassenen.

HOOFDSTUK 2: De aanpak was om te beginnen met de pan-hematopoëtische Cre cellijn Mx-cre en om hiermee het gevolg van Sp1 en Sp3 in het gehele hematopoëtische systeem te bestuderen. We maken gebruik van celltype specifieke Cre-cellijnen om hun rol in beïnvloede celtypes te bestuderen. In dit hoofdstuk onthullen wij het gevolg van het gelijktijdig verwijderen van Sp1 en Sp3 uit het volwassen hematopoëtische systeem. Aangezien knockout van Sp1 en Sp3 dodelijk is in het embryonale stadium, hebben wij voorwaardelijk knockout allelen vervaardigd voor Sp1 (Sp1^fl/fl) en Sp3 (Sp3^fl/fl). Door het kruizen van de Mx1-Cre muis met de Sp1^fl/fl of Sp3^fl/fl muis, waren wij in staat om de genen doeltreffend te verwijderen van het gehele hematopoëtische systeem. Tot onze verrassing vertoonden beide de Mx1-Cre::Sp1^fl/fl en de Mx1-Cre::Sp3^fl/fl geen opvallend fenotype. Doordat leden van Sp transcriptie factor familie vaak gedeeltelijk functioneel uitwisselbaar zijn, veronderstelden wij dat het gelijktijdige uitschakelen van Sp1 en Sp3 zou leiden tot een ernstig fenotype. Dit bleek inderdaad het geval te zijn en Mx1-Cre::Sp1^fl/fl ::Sp3^fl/fl muizen hadden ernstig verstoorde myleopoëse en lymphopoëse en leden aan hevige trombocytopenie. Dit toonde voor het eerst de onderling uitwisselbare rol van Sp1 en Sp3 aan in het hematopoëtische systeem.

HOOFDSTUK 3: Dit hoofdstuk omschrijft de rol van Sp1/Sp3 tijdens de ontwikkeling van B-cellen. In hoofdstuk 2 was één van de aangetaste cel types die van de B-cellen, daarom hebben we een B-cell specifieke transgene Cre (Mb1-Cre) muis gebruikt om gelijktijdig Sp1 en Sp3 uit te schakelen in B-cellen.
Flowcytometrie onthulde dat ontwikkeling van Mb1-Cre::Sp1^{fl/fl} ::Sp3^{fl/fl} (KO) geblokkeerd raakt tijdens het grote pre B-cell stadium. Verdere analyse onthulde defecten in de proliferatie en differentiatie van Mb1-Cre::Sp1^{fl/fl} ::Sp3^{fl/fl} grote pre B-cellen. Deze defecten konden niet worden verholpen door de introductie van een van te voren herschikt transgen V_{H}81X, maar stelde de cellen wel in staat om te vorderen voorbij het grote pre B-cell stadium. Onze data onthulde dat B-cell receptor signalering van B-cellen uit de milt van de Mb1-Cre::Sp1^{fl/fl} ::Sp3^{fl/fl} muis normaal leek, maar dat deze cellen defecten vertoonden in hun metabolisme. Na analyse van het genoom brede expressieprofiel vonden wij honderden onregelde genen, waarvan genen die betrokken zijn bij essentiële biologische functies waaronder proliferatie, differentiatie en het overleven van cellen, down-gereguleerd waren. Daarnaast vertoonden Mx1-Cre::Sp1^{fl/fl} en Mx1-Cre::Sp3^{fl/fl} geen duidelijk fenotype, wat ons verder overtuigde van het feit dat Sp1 en Sp3 functioneel uitwisselbaar zijn in het hematopoëtisch systeem, met name in de B-cell. Tezamen genomen concludeerden wij dat tijdens de ontwikkeling van de B-cell, Sp1 en Sp3 B cell proliferatie, differentiatie en metabolisme reguleren. Zij kunnen dit doen door rechtstreeks genen te reguleren die betrokken zijn bij deze biologische processen of door indirecte regulatie via verschillende ‘paden’.

**HOOFDSTUK 4:** In dit hoofdstuk ontrafelen wij het effect van het uitschakelen van Sp1 en Sp3 in megakaryocyten/bloedplaatjes. We hebben aangetoond dat we met het kruizen van de Mb1-Cre::Sp1^{fl/fl} ::Sp3^{fl/fl} muis met megakaryocyte/bloedplaatje specifieke Cre (Pf4-Cre) muis, Sp1 en Sp3 efficiënt hebben verwijderd uit deze cellen. Analyse van het bloed onthulde dat de Pf4-Cre::Sp1^{fl/fl} ::Sp3^{fl/fl} (Sp1/Sp3 dKO) muis macrothrombocytopenia, normale thrombopoetine waarden en normale aantallen megakaryocyten in het beenmerg en de milt had. Echter, de megakaryocyten volgroeien niet normaal en vertonen specifieke defecten tijdens de fase van het afbakenen van de membranen, wat resulteerde in defecten tijdens de formering van pro-bloedplaatjes. Daarnaast bleek migratie naar de bloedvaten niet te zijn beïnvloed. Wij tonen aan dat Sp1/Sp3 dKO bloedplaatjes geen mankementen bevatten in degranulatie en integrine activatie, wat daarom duid op een defect in ‘receptor trafficking’, intracellulaire signalering of het clusteren van receptoren. Dit leek vooral van invloed te zijn op Clec1b en vWF receptoren.
Massaspectrometrie toonde een vermindering van eiwitten die noodzakelijk zijn voor de reorganisatie van het cytoskelet. Na het opstellen van een genoombreed expressieprofiel, konden wij een afname in hun bijbehorende genen aantonen, wat een wijst op nieuwe doelwitten voor beide Sp1 en Sp3 in megakaryocyten. Gezamenlijk toont onze data aan dat muizen die zowel in Sp1 als in Sp3 defect zijn, een fenocopie zijn van het Bernard Soullier syndroom. BSS wordt veroorzaakt door een mutatie in GP1b, maar door het verwijderen van Sp1 en Sp3 in megakaryocytes veranderen we de expressie van veel genen die betrokken zijn bij megakaryopoëse, zodat we een soortgelijk fenotype observeren. Dit muismodel kan ons daarom helpen bij het begrijpen van aangeboren of verworven thrombocytopenia, waarvan de oorzaak nog niet bekend is.
Curriculum Vitae

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Place of Birth: Buea, Cameroon

Education
2009 - 2013 PhD student
Department of Cell Biology, Erasmus Medical Centre, Rotterdam, The Netherlands

2005 - 2008 MSc Biomolecular science, Specialization: Molecular Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands

2000 - 2003 BSc. Microbiology and Medical Laboratory Technology, University of Buea, Cameroon

1999 - 2000 High School; General Certificate of Education, Advanced Level, Government Bilingual High School, Muyuka, Cameroon


Research
2009 -2013 PhD research project
“Role of Sp transcription factors in adult hematopoiesis”
Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands
Prof. dr. Sjaak Philipsen (promoter & supervisor)

2008 Master internship research project
“Transforming a non-encapsulated Porphyromonas gingivalis K-strain to a capsulated one using the capsular polysaccharide locus”
Academic Centre for Dentistry Amsterdam, The Netherlands
Prof. Wim Crielaard (principal investigator), Dr. Jorg Brunner (supervisor)

2007 -2008 Master internship research project
“Subcellular localisation of novel AMPA receptor interacting proteins and proteins up-regulated in Fragile X mental retardation syndrome”
Department of Cellular and Molecular Neurobiology, Vrije Universiteit Amsterdam, The Netherlands
Prof. dr. A.B. Smit (principal investigator), Dr. Ka Wan Li (supervisor)
List of publications

- Kulu DI, de Bruijn JW, Jaegle U, Gillemans N, Ferreira R, Krüger I, Gutiérrez L, Suske G, Hendriks RW and Philipsen S: *Severe hematopoietic defects after Mx-Cre-mediated depletion of Sp1 and Sp3 transcription factors in mice*  
  *Manuscript in preparation*

- Kulu DI, de Bruijn JW, Suske G, Hendriks RW and Philipsen S: *Sp1 and Sp3 co-operate to regulate B cell development.*  
  *Manuscript in preparation*

  *Manuscript in preparation*

  *Manuscript submitted*

  *Exp Hematol. 2011;39(7):730-40*

# PhD Portfolio

## Summary of PhD training and teaching

<table>
<thead>
<tr>
<th>Name PhD student: <strong>Divine Ikome Kulu</strong></th>
<th>PhD period: Sept 2009 – Sept 2013</th>
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</thead>
<tbody>
<tr>
<td>Erasmus MC Department: Cell Biology</td>
<td>Promoters: prof. dr. Sjaak Philipsen</td>
</tr>
<tr>
<td>Research School: Molecular Medicine</td>
<td>Supervisors: prof. dr. Sjaak Philipsen, prof. dr. Rudi Hendriks</td>
</tr>
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### 1. PhD training

<table>
<thead>
<tr>
<th>General courses</th>
<th>Year</th>
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<tr>
<td>- Laboratory animal science (Article 9)</td>
<td>2010</td>
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<tr>
<td>- Cell and Developmental Biology</td>
<td>2010</td>
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<tr>
<td>- Biochemistry and Biophysics</td>
<td>2010</td>
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<tr>
<td>- Safely working in the laboratory</td>
<td>2010</td>
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<tr>
<td>- Epigenetic regulation</td>
<td>2010</td>
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<tr>
<td>- Technology Facilities</td>
<td>2010</td>
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<tr>
<td>- Transgenesis, gene targeting and <em>in vivo</em> imaging</td>
<td>2011</td>
</tr>
<tr>
<td>- Biomedical Research Techniques</td>
<td>2011</td>
</tr>
<tr>
<td>- Innovative mouse models</td>
<td>2011</td>
</tr>
<tr>
<td>- The Advanced Course “Molecular Immunology“</td>
<td>2012</td>
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</tbody>
</table>

**Specific courses (e.g. Research school, Medical Training)**

| 8th Winter School of the international Graduiertenkolleg GRK767 "Transcriptional Control in Developmental Processes" Kleinwalsertal, Austria (Oral presentation) | 2010 |
| Literature course                                    | 2010 |
| 1st & 3rd Winter School of the Collaborative Research Centre TRR81, "Chromatin Changes In Differentiation and Malignancies" Kleinwalsertal, Austria (Oral presentation) | 2011, 2013 |

### Seminars and workshops

| The 19th MGC-Symposium, Rotterdam, The Netherlands | 2009 |
| The 20th MGC-Symposium, Leiden, The Netherlands | 2010 |
| The 17th MGC PhD workshop, Cologne, Germany     | 2010 |
| The 21st MGC-Symposium, Leiden, The Netherlands | 2011 |
| The 18th MGC PhD workshop, Maastricht, The Netherlands (Poster presentation) | 2011 |
| The 22nd MGC-Symposium, Leiden, The Netherlands | 2012 |
| Erasmus guest lectures on cell and developmental biology | 2009-2013 |
| Erasmus guest lectures on stem cell and regenerative medicines | 2009-2013 |
| Monday Morning Meetings                           | 2009-2013 |

### Presentations

| Monday Morning Meetings                           | 2009-2013 |
| Work discussions                                 | 2009-2013 |
### International conferences
- Chromatin changes in differentiation and malignancies, Giessen, Germany (poster presentation) 2011
- XXIV Congress of the International Society on Thrombosis and Hemostasis, Amsterdam, The Netherlands (ePoster Presentation) 2013

### 2. Teaching

#### Supervising practicals and excursion, Tutoring
- High school student 2012

#### Other
- PhD students 2013
Acknowledgements

Even though I am defending my thesis today, this Journey was by no means a one person’s journey. I will like to thank the follow colleagues, friends and family who were by my side all alone.

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To my paronymphs Maria and Johan: Maria, you are so wonderful, always smiling and fun to be around. Johan thank you for always giving me headline on dutch news every morning😊, thank you both for being true friends and colleagues for the past years and accepting to be my paronymphs. I will miss the nice discussions we use to have that made our office a very conducive environment to study. I wish you all success in completing the Journey that you have already started. Past members, Sahar, Pavlos, Sylvia, and Teus thank you very much for all your assistance. To Laura M, Laura S and Laura G, good luck with your experiments.

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Special thanks to my Master thesis supervisor, Dr. Jorg Brunner for your great help and the training that I got from you during my masters internship.

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To Delphine, thank so much for being the backbone of my success. The care and support I received from you gave me the strength to complete this thesis. I was blessed the day I found you.

To my Family and friends here especially my son Joel, Elisabeth, Martin, Valeri, and Karen, thank you all for your support during my studies. It meant a lot to me.

Finally, to my family back home especially my dad, Franklin, Elvis and Sammy, thank you all for your support and prayers throughout these studies. Mummy Doris and my sister Solange, even though you are not here with us, I am sure that I have made you proud. R.I.P