The Role of Sp1 and Sp3 Transcription Factors in Hematopoiesis

Divine Ikome Kulu

ISBN: 978-94-6203-459-4

The work presented in this thesis was performed at the Department of Cell Biology at Erasmus MC, Rotterdam, The Netherlands. The Department is a member of the research schools Medisch Genetisch Centrum Zuid-West Nederland (MGC) and Molecular Medicine (MM)

The studies described in this thesis were supported financially by ZonMw

©Divine Ikome Kulu, 2013
All rights reserved. No part of this thesis may be reproduced, stored in a retrieval system, or transmitted in any form or by any means without the prior permission of the author.

Cover Design: Divine Ikome Kulu and Mariëtte van de Corput
Cover: B and T cells in the spleen.

Printed by Wöhrmann Print Service B.V.

The Role of Sp1 and Sp3 Transcription Factors in Hematopoiesis

De rol van Specificiteits transcriptie factoren Sp1 en Sp3 in hematopoiese

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H.G. Schmidt

and in accordance with the decision of the
Doctorate Board

The public defense shall be held on
Wednesday 9th of October 2013 at 15:30

by

Divine Ikome Kulu

born in Buea, Cameroon

Doctoral Committee

Promotor:	Prof.dr. J.N.J. Philipsen
Other members:	Prof.dr. R.W. Hendriks Prof.dr. J.H. Gribnau Dr. G. Jansen

Contents

List of abbreviations……………………….…………………………......6

Chapter 1 General Introduction…...…………………………….……....7

Scope of the thesis………..………………………..……….26

Chapter 2 Severe hematopoietic defects after
Mx1-Cre-mediated depletion of the
Sp1 and Sp3 transcription factors in mice…..……..43

Chapter 3 Sp1 and Sp3 co-operate to regulate
B cell development…..………………………………….....63

Chapter 4 Sp1 and Sp3 co-operate to regulate
megakaryopoiesis…...……………..……...…………….85

Chapter 5 General Discussion………………………….………113

Summary (Samenvatting)…………………...……..125

Curriculum Vitae……………………………….....…..132

List of Publications …………………………...……133

PhD portfolio ………………………………….……….134

Acknowledgements ……………………………...…136

List of abbreviations

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

Chapter 1

General Introduction

Hematopoiesis

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]

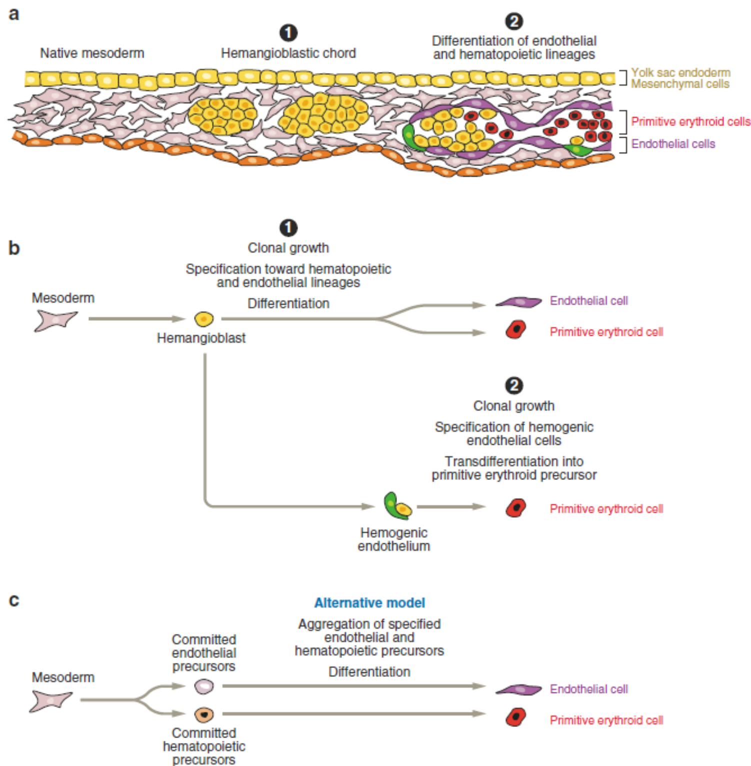


Figure 1. Mechanism of yolk sac (YS) blood island formation. a) Schematic view of the progressive evolution of blood island mesodermal cells to a functional vascular network and primitive erythroid cells. Two alternative mechanisms of the YS blood island formation are shown. b) The hemangioblast can 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]. *In vivo* cell lineage tracing and conditional deletion of transcription factors such as *Runx1 and 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

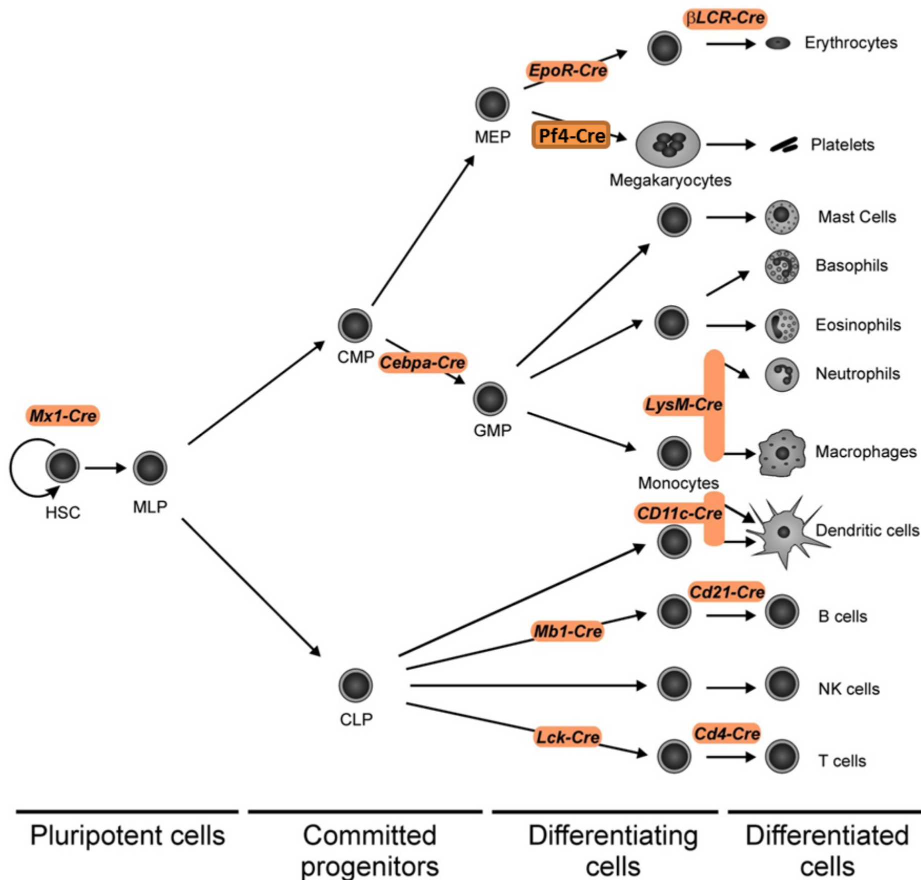


Figure 2. Hematopoietic Lineage specific Cre lines. The figure shows the classical hierarchical model of hematopoiesis and the lineages and cell types where various Cre recombinase are expressed. The hematopoietic stem cell (HSC) gives rise to multilineage progenitors (MLP), which differentiate into 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 distruction. 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 blot 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 Il7-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].

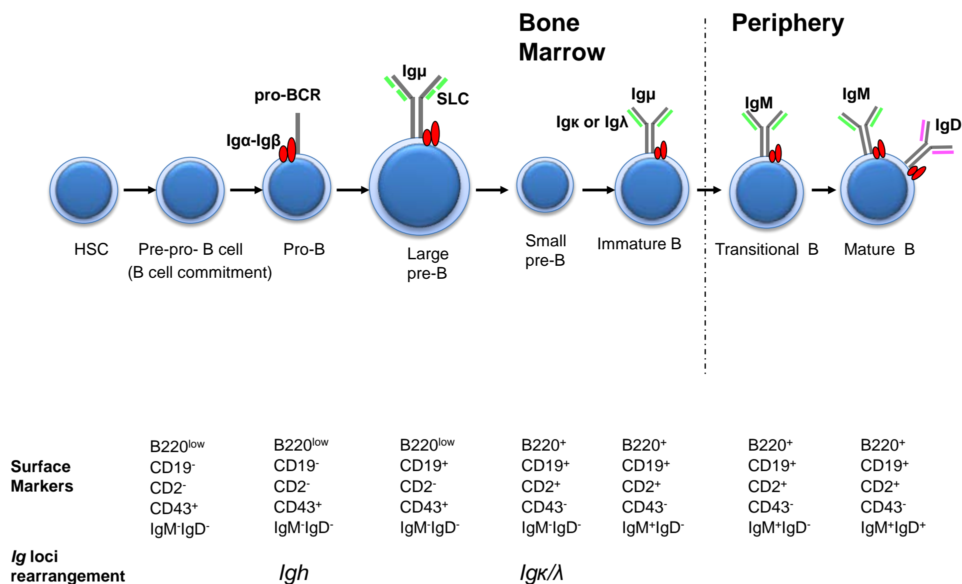


Figure 3.The development of B cells begins in the bone marrow (BM) from the hematopoietic stem cells (HSC). Committed B cells at the pro-B cell stage move to the large pre- B cell stage by successful expression of Igμ and the surrogate light chain (SLC) on the cell surface. After proliferating, these cells internalize their SLC and become the small pre-B cell. IgL re-arrangement 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, lef1, 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].

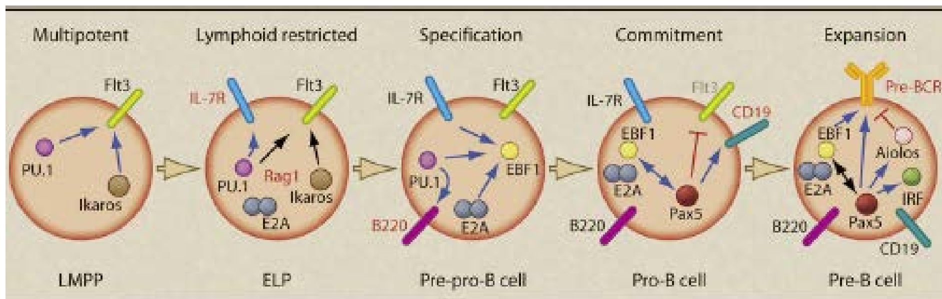


Figure 4: Transcription factors critical for B cell development. Transcription factors involved in various stages of B cell development, growth factor receptors and cell- surface markers are shown. From left to right the successive stages include LMPP (lymphoid-primed multipotent progenitor), ELP (early 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 FOG-1 (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]. FOG-1 binds to GATA protein and has

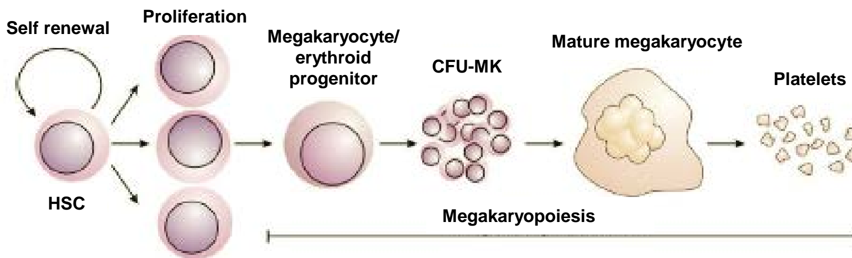


Figure 5: Megakaryopoiesis. Under the influence of TPO, HSC proliferate and differentiate into megakaryocytes [91].

similar expression pattern in erythroid cells and in MKs. Mice deficient of FOG-1 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[111-113]. In addition to TPO, other thrombopoietic cytokines include stem cell factor (SCF), erythropoietin, IL-3, IL-6 and IL-11[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 [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-terminis of the proteins (**Fig. 6)** [119, 123]. These krüppel-like zinc fingers were first identified in the *Drosophilia melanogaster* segmentation Krüppel (Kr) protein [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 [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 [125, 126]. Due to amino acid substitutions in the zinc fingers, Sp/KLF members recognize GC- (GGGGCGGGG) 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 [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 [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 [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].

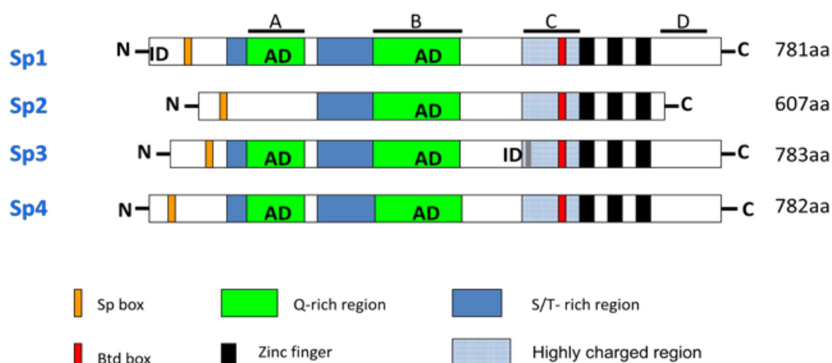


Figure 6 . Structural features of Sp-proteins. Schematic representation of the four human Sp-family members Sp1, Sp2, Sp3 and Sp4. Amino acid length is indicated on the right. Colors for Sp box, buttonhead (BTD) box, zinc-finger domain, highly charged region, serine/threonine (S/T)-rich, and

glutamine (Q)-rich domains are shown at the bottom. The black bars marked with A, B. C, and D denote the sub-domains which contribute to the transcriptional properties of Sp1. Activation domains (AD) and inhibitory domains (ID) are indicated. The amino acid (a.a.) lengths are also shown.

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 *Socs1* 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 wells 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**

6) 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-GlcNAcylation 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 (K_{551}) 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 K_{551} 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-typelittermates. 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 trangene 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

1. Morrison, S.J. and A.C. Spradling, *Stem cells and niches: mechanisms that promote stem cell maintenance throughout life.* Cell, 2008. **132**(4): p. 598-611.
2. Costa, G., V. Kouskoff, and G. Lacaud, *Origin of blood cells and HSC production in the embryo.* Trends Immunol, 2012. **33**(5): p. 215-23.
3. Qing, Y., Y. Lin, and S.L. Gerson, *An intrinsic BM hematopoietic niche occupancy defect of HSC in scid mice facilitates exogenous HSC engraftment.* Blood, 2012. **119**(7): p. 1768-71.
4. Fehling, H.J., et al., *Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation.* Development, 2003. **130**(17): p. 4217-27.
5. Ferkowicz, M.J. and M.C. Yoder, *Blood island formation: longstanding observations and modern interpretations.* Exp Hematol, 2005. **33**(9): p. 1041-7.
6. Lancrin, C., et al., *The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage.* Nature, 2009. **457**(7231): p. 892-5.
7. Huber, T.L., et al., *Haemangioblast commitment is initiated in the primitive streak of the mouse embryo.* Nature, 2004. **432**(7017): p. 625-30.
8. Palis, J., et al., *Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.* Development, 1999. **126**(22): p. 5073-84.
9. Kingsley, P.D., et al., *Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis.* Blood, 2004. **104**(1): p. 19-25.
10. Kingsley, P.D., et al., *"Maturational" globin switching in primary primitive erythroid cells.* Blood, 2006. **107**(4): p. 1665-72.
11. Cumano, A. and I. Godin, *Ontogeny of the hematopoietic system.* Annu Rev Immunol, 2007. **25**: p. 745-85.
12. Medvinsky, A. and E. Dzierzak, *Development of the hematopoietic stem cell: can we describe it?* Blood, 1999. **94**(10): p. 3613-4.
13. Boisset, J.C., et al., *In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium.* Nature, 2010. **464**(7285): p. 116-20.
14. Ivanovs, A., et al., *Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region.* J Exp Med, 2011. **208**(12): p. 2417-27.
15. Chen, M.J., et al., *Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter.* Nature, 2009. **457**(7231): p. 887-91.

16. Yokomizo, T., et al., *Requirement of Runx1/AML1/PEBP2alphaB for the generation of haematopoietic cells from endothelial cells.* Genes Cells, 2001. **6**(1): p. 13-23.
17. Nottingham, W.T., et al., *Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer.* Blood, 2007. **110**(13): p. 4188-97.
18. Yoder, M.C., et al., *Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac.* Immunity, 1997. **7**(3): p. 335-44.
19. Godin, I., F. Dieterlen-Lievre, and A. Cumano, *Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus.* Proc Natl Acad Sci U S A, 1995. **92**(3): p. 773-7.
20. Gekas, C., et al., *The placenta is a niche for hematopoietic stem cells.* Dev Cell, 2005. **8**(3): p. 365-75.
21. Gekas, C., et al., *Hematopoietic stem cell development in the placenta.* Int J Dev Biol, 2010. **54**(6-7): p. 1089-98.
22. Ottersbach, K. and E. Dzierzak, *The murine placenta contains hematopoietic stem cells within the vascular labyrinth region.* Dev Cell, 2005. **8**(3): p. 377-87.
23. Robin, C., et al., *Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development.* Cell Stem Cell, 2009. **5**(4): p. 385-95.
24. Kumaravelu, P., et al., *Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver.* Development, 2002. **129**(21): p. 4891-9.
25. Boisset, J.C. and C. Robin, *Imaging the founder of adult hematopoiesis in the mouse embryo aorta.* Cell Cycle, 2010. **9**(13): p. 2489-90.
26. Palis, J. and M.C. Yoder, *Yolk-sac hematopoiesis: the first blood cells of mouse and man.* Exp Hematol, 2001. **29**(8): p. 927-36.
27. Adolfsson, J., et al., *Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity.* Immunity, 2001. **15**(4): p. 659-69.
28. Adolfsson, J., et al., *Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment.* Cell, 2005. **121**(2): p. 295-306.
29. Kondo, M., I.L. Weissman, and K. Akashi, *Identification of clonogenic common lymphoid progenitors in mouse bone marrow.* Cell, 1997. **91**(5): p. 661-72.
30. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.* Nature, 2000. **404**(6774): p. 193-7.

31. Serke, S. and D. Huhn, *Identification of CD71 (transferrin receptor) expressing erythrocytes by multiparameter-flow-cytometry (MP-FCM): correlation to the quantitation of reticulocytes as determined by conventional microscopy and by MP-FCM using a RNA-staining dye.* Br J Haematol, 1992. **81**(3): p. 432-9.
32. Dong, H.Y., S. Wilkes, and H. Yang, *CD71 is selectively and ubiquitously expressed at high levels in erythroid precursors of all maturation stages: a comparative immunochemical study with glycophorin A and hemoglobin A.* Am J Surg Pathol, 2011. **35**(5): p. 723-32.
33. Koulnis, M., et al., *Identification and analysis of mouse erythroid progenitors using the CD71/TER119 flow-cytometric assay.* J Vis Exp, 2011(54).
34. Palis, J., *Developmental biology: no red cell is an island.* Nature, 2004. **432**(7020): p. 964-5.
35. Schatz, D.G., M.A. Oettinger, and D. Baltimore, *The V(D)J recombination activating gene, RAG-1.* Cell, 1989. **59**(6): p. 1035-48.
36. Oettinger, M.A., et al., *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.* Science, 1990. **248**(4962): p. 1517-23.
37. Jung, D. and F.W. Alt, *Unraveling V(D)J recombination; insights into gene regulation.* Cell, 2004. **116**(2): p. 299-311.
38. O'Riordan, M. and R. Grosschedl, *Transcriptional regulation of early B-lymphocyte differentiation.* Immunol Rev, 2000. **175**: p. 94-103.
39. Li, Y.S., et al., *Identification of the earliest B lineage stage in mouse bone marrow.* Immunity, 1996. **5**(6): p. 527-35.
40. Gounari, F., et al., *Tracing lymphopoiesis with the aid of a pTalpha-controlled reporter gene.* Nat Immunol, 2002. **3**(5): p. 489-96.
41. Rumfelt, L.L., et al., *Lineage specification and plasticity in CD19- early B cell precursors.* J Exp Med, 2006. **203**(3): p. 675-87.
42. Hendriks, R.W. and S. Middendorp, *The pre-BCR checkpoint as a cell-autonomous proliferation switch.* Trends Immunol, 2004. **25**(5): p. 249-56.
43. Herzog, S., M. Reth, and H. Jumaa, *Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling.* Nat Rev Immunol, 2009. **9**(3): p. 195-205.
44. Casola, S., et al., *B cell receptor signal strength determines B cell fate.* Nat Immunol, 2004. **5**(3): p. 317-27.
45. Nutt, S.L. and B.L. Kee, *The transcriptional regulation of B cell lineage commitment.* Immunity, 2007. **26**(6): p. 715-25.
46. Miller, J.P., et al., *The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7.* J Exp Med, 2002. **196**(5): p. 705-11.

47. Johnson, K., et al., *IL-7 functionally segregates the pro-B cell stage by regulating transcription of recombination mediators across cell cycle.* J Immunol, 2012. **188**(12): p. 6084-92.
48. von Freeden-Jeffry, U., et al., *Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine.* J Exp Med, 1995. **181**(4): p. 1519-26.
49. Sitnicka, E., et al., *Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis.* J Exp Med, 2003. **198**(10): p. 1495-506.
50. Dias, S., et al., *Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors.* J Exp Med, 2005. **201**(6): p. 971-9.
51. Kikuchi, K., et al., *IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF.* J Exp Med, 2005. **201**(8): p. 1197-203.
52. Vosshenrich, C.A., et al., *Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development.* Nat Immunol, 2003. **4**(8): p. 773-9.
53. Scott, E.W., et al., *Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages.* Science, 1994. **265**(5178): p. 1573-7.
54. DeKoter, R.P., H.J. Lee, and H. Singh, *PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors.* Immunity, 2002. **16**(2): p. 297-309.
55. Georgopoulos, K., et al., *The Ikaros gene is required for the development of all lymphoid lineages.* Cell, 1994. **79**(1): p. 143-56.
56. Wang, J.H., et al., *Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation.* Immunity, 1996. **5**(6): p. 537-49.
57. Yoshida, T., et al., *Early hematopoietic lineage restrictions directed by Ikaros.* Nat Immunol, 2006. **7**(4): p. 382-91.
58. Dias, S., et al., *E2A proteins promote development of lymphoid-primed multipotent progenitors.* Immunity, 2008. **29**(2): p. 217-27.
59. Satterwhite, E., et al., *The BCL11 gene family: involvement of BCL11A in lymphoid malignancies.* Blood, 2001. **98**(12): p. 3413-20.
60. Liu, P., et al., *Bcl11a is essential for normal lymphoid development.* Nat Immunol, 2003. **4**(6): p. 525-32.
61. Yu, Y., et al., *Bcl11a is essential for lymphoid development and negatively regulates p53.* J Exp Med, 2012. **209**(13): p. 2467-83.
62. Hagman, J. and K. Lukin, *Early B-cell factor 'pioneers' the way for B-cell development.* Trends Immunol, 2005. **26**(9): p. 455-61.

63. Sigvardsson, M., M. O'Riordan, and R. Grosschedl, *EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes.* Immunity, 1997. **7**(1): p. 25-36.
64. Bain, G., et al., *E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements.* Cell, 1994. **79**(5): p. 885-92.
65. Lin, H. and R. Grosschedl, *Failure of B-cell differentiation in mice lacking the transcription factor EBF.* Nature, 1995. **376**(6537): p. 263-7.
66. Zhuang, Y., P. Soriano, and H. Weintraub, *The helix-loop-helix gene E2A is required for B cell formation.* Cell, 1994. **79**(5): p. 875-84.
67. O'Riordan, M. and R. Grosschedl, *Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A.* Immunity, 1999. **11**(1): p. 21-31.
68. Maier, H. and J. Hagman, *Roles of EBF and Pax-5 in B lineage commitment and development.* Semin Immunol, 2002. **14**(6): p. 415-22.
69. Cobaleda, C., et al., *Pax5: the guardian of B cell identity and function.* Nat Immunol, 2007. **8**(5): p. 463-70.
70. Nutt, S.L., et al., *Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments.* EMBO J, 1998. **17**(8): p. 2319-33.
71. Carotta, S., et al., *Pax5 maintains cellular identity by repressing gene expression throughout B cell differentiation.* Cell Cycle, 2006. **5**(21): p. 2452-6.
72. Holmes, M.L., et al., *Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment.* Genes Dev, 2006. **20**(8): p. 933-8.
73. Ingram, R.M., et al., *Differential regulation of sense and antisense promoter activity at the Csf1R locus in B cells by the transcription factor PAX5.* Exp Hematol, 2011. **39**(7): p. 730-40 e1-2.
74. Souabni, A., et al., *Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1.* Immunity, 2002. **17**(6): p. 781-93.
75. Rolink, A.G., et al., *Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors.* Nature, 1999. **401**(6753): p. 603-6.
76. Schebesta, A., et al., *Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function.* Immunity, 2007. **27**(1): p. 49-63.
77. Pongubala, J.M., et al., *Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5.* Nat Immunol, 2008. **9**(2): p. 203-15.
78. Maier, H., et al., *Early B cell factor cooperates with Runx1 and mediates epigenetic changes associated with mb-1 transcription.* Nat Immunol, 2004. **5**(10): p. 1069-77.

79. Jackson, C.W., et al., *Two-color flow cytometric measurement of DNA distributions of rat megakaryocytes in unfixed, unfractionated marrow cell suspensions.* Blood, 1984. **63**(4): p. 768-78.
80. Semple, J.W. and J. Freedman, *Platelets and innate immunity.* Cell Mol Life Sci, 2010. **67**(4): p. 499-511.
81. Fujiwara, Y., et al., *Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12355-8.
82. Shivdasani, R.A., et al., *A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development.* EMBO J, 1997. **16**(13): p. 3965-73.
83. Ferreira, R., et al., *GATA1 function, a paradigm for transcription factors in hematopoiesis.* Mol Cell Biol, 2005. **25**(4): p. 1215-27.
84. Stachura, D.L., S.T. Chou, and M.J. Weiss, *Early block to erythromegakaryocytic development conferred by loss of transcription factor GATA-1.* Blood, 2006. **107**(1): p. 87-97.
85. Fujiwara, Y., et al., *Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development.* Blood, 2004. **103**(2): p. 583-5.
86. Fischer, K.D., A. Haese, and J. Nowock, *Cooperation of GATA-1 and Sp1 can result in synergistic transcriptional activation or interference.* J Biol Chem, 1993. **268**(32): p. 23915-23.
87. Hou, C.H., et al., *Involvement of Sp1/Sp3 in the activation of the GATA-1 erythroid promoter in K562 cells.* Cell Res, 2008. **18**(2): p. 302-10.
88. Block, K.L., Y. Shou, and M. Poncz, *An Ets/Sp1 interaction in the 5'-flanking region of the megakaryocyte-specific alpha IIb gene appears to stabilize Sp1 binding and is essential for expression of this TATA-less gene.* Blood, 1996. **88**(6): p. 2071-80.
89. Nerlov, C., et al., *GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription.* Blood, 2000. **95**(8): p. 2543-51.
90. Chou, S.T., et al., *Graded repression of PU.1/Sfpi1 gene transcription by GATA factors regulates hematopoietic cell fate.* Blood, 2009. **114**(5): p. 983-94.
91. Geddis, A.E., *Megakaryopoiesis.* Semin Hematol, 2010. **47**(3): p. 212-9.
92. Chang, A.N., et al., *GATA-factor dependence of the multitype zinc-finger protein FOG-1 for its essential role in megakaryopoiesis.* Proc Natl Acad Sci U S A, 2002. **99**(14): p. 9237-42.
93. Tsang, A.P., et al., *Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG.* Genes Dev, 1998. **12**(8): p. 1176-88.

94. Holmes, M.L., et al., *Cloning and analysis of the thrombopoietin-induced megakaryocyte-specific glycoprotein VI promoter and its regulation by GATA-1, Fli-1, and Sp1.* J Biol Chem, 2002. **277**(50): p. 48333-41.
95. Lemarchandel, V., et al., *GATA and Ets cis-acting sequences mediate megakaryocyte-specific expression.* Mol Cell Biol, 1993. **13**(1): p. 668-76.
96. Eisbacher, M., et al., *Protein-protein interaction between Fli-1 and GATA-1 mediates synergistic expression of megakaryocyte-specific genes through cooperative DNA binding.* Mol Cell Biol, 2003. **23**(10): p. 3427-41.
97. Starck, J., et al., *Functional cross-antagonism between transcription factors FLI-1 and EKLF.* Mol Cell Biol, 2003. **23**(4): p. 1390-402.
98. Hart, A., et al., *Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia.* Immunity, 2000. **13**(2): p. 167-77.
99. Kawada, H., et al., *Defective megakaryopoiesis and abnormal erythroid development in Fli-1 gene-targeted mice.* Int J Hematol, 2001. **73**(4): p. 463-8.
100. Ichikawa, M., et al., *Runx1/AML-1 ranks as a master regulator of adult hematopoiesis.* Cell Cycle, 2004. **3**(6): p. 722-4.
101. Irvin, B.J. and S.W. Hiebert, *AML-1 steps up to the platelets.* Nat Med, 2004. **10**(3): p. 238-9.
102. Ichikawa, M., et al., *AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis.* Nat Med, 2004. **10**(3): p. 299-304.
103. Putz, G., et al., *AML1 deletion in adult mice causes splenomegaly and lymphomas.* Oncogene, 2006. **25**(6): p. 929-39.
104. Lecine, P., et al., *Hematopoietic-specific beta 1 tubulin participates in a pathway of platelet biogenesis dependent on the transcription factor NF-E2.* Blood, 2000. **96**(4): p. 1366-73.
105. Patel, S.R., et al., *Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes.* Blood, 2005. **106**(13): p. 4076-85.
106. Lecine, P. and R.A. Shivdasani, *Cellular and molecular biology of megakaryocyte differentiation in the absence of lineage-restricted transcription factors.* Stem Cells, 1998. **16 Suppl 2**: p. 91-5.
107. Lecine, P., et al., *Mice lacking transcription factor NF-E2 provide in vivo validation of the proplatelet model of thrombocytopoiesis and show a platelet production defect that is intrinsic to megakaryocytes.* Blood, 1998. **92**(5): p. 1608-16.
108. Schlaeger, T.M., et al., *Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (SCL/tal1)-dependent window during hematopoietic stem-cell development.* Blood, 2005. **105**(10): p. 3871-4.

109. Chagraoui, H., et al., *SCL-mediated regulation of the cell-cycle regulator p21 is critical for murine megakaryopoiesis.* Blood, 2011. **118**(3): p. 723-35.
110. Gurney, A.L., et al., *Thrombocytopenia in c-mpl-deficient mice.* Science, 1994. **265**(5177): p. 1445-7.
111. Kaushansky, K., et al., *Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin.* Nature, 1994. **369**(6481): p. 568-71.
112. Murone, M., D.A. Carpenter, and F.J. de Sauvage, *Hematopoietic deficiencies in c-mpl and TPO knockout mice.* Stem Cells, 1998. **16**(1): p. 1-6.
113. Fox, N., et al., *Thrombopoietin expands hematopoietic stem cells after transplantation.* J Clin Invest, 2002. **110**(3): p. 389-94.
114. Quesenberry, P.J., et al., *Multifactor stimulation of megakaryocytopoiesis: effects of interleukin 6.* Exp Hematol, 1991. **19**(1): p. 35-41.
115. Broudy, V.C., N.L. Lin, and K. Kaushansky, *Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro.* Blood, 1995. **85**(7): p. 1719-26.
116. Broudy, V.C. and K. Kaushansky, *Thrombopoietin, the c-mpl ligand, is a major regulator of platelet production.* J Leukoc Biol, 1995. **57**(5): p. 719-25.
117. Kaushansky, K., *Thrombopoietin: the primary regulator of megakaryocyte and platelet production.* Thromb Haemost, 1995. **74**(1): p. 521-5.
118. Bouwman, P. and S. Philipsen, *Regulation of the activity of Sp1-related transcription factors.* Mol Cell Endocrinol, 2002. **195**(1-2): p. 27-38.
119. Philipsen, S. and G. Suske, *A tale of three fingers: the family of mammalian Sp/XKLF transcription factors.* Nucleic Acids Res, 1999. **27**(15): p. 2991-3000.
120. Lomberk, G. and R. Urrutia, *The family feud: turning off Sp1 by Sp1-like KLF proteins.* Biochem J, 2005. **392**(Pt 1): p. 1-11.
121. Suske, G., *The Sp-family of transcription factors.* Gene, 1999. **238**(2): p. 291-300.
122. Suske, G., E. Bruford, and S. Philipsen, *Mammalian SP/KLF transcription factors: bring in the family.* Genomics, 2005. **85**(5): p. 551-6.
123. Kadonaga, J.T., et al., *Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain.* Cell, 1987. **51**(6): p. 1079-90.
124. Schuh, R., et al., *A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene.* Cell, 1986. **47**(6): p. 1025-32.

125. Saegusa, N., et al., *Different contributions of three zinc fingers of transcription factor Sp1 to DNA recognition: novel binding mode of N-terminal finger 1.* Nucleic Acids Symp Ser, 1997(37): p. 151-2.
126. Song, J., et al., *Two consecutive zinc fingers in Sp1 and in MAZ are essential for interactions with cis-elements.* J Biol Chem, 2001. **276**(32): p. 30429-34.
127. Brandeis, M., et al., *Sp1 elements protect a CpG island from de novo methylation.* Nature, 1994. **371**(6496): p. 435-8.
128. Macleod, D., et al., *Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island.* Genes Dev, 1994. **8**(19): p. 2282-92.
129. Wimmer, E.A., et al., *A Drosophila homologue of human Sp1 is a head-specific segmentation gene.* Nature, 1993. **366**(6456): p. 690-4.
130. Athanikar, J.N., H.B. Sanchez, and T.F. Osborne, *Promoter selective transcriptional synergy mediated by sterol regulatory element binding protein and Sp1: a critical role for the Btd domain of Sp1.* Mol Cell Biol, 1997. **17**(9): p. 5193-200.
131. Cagen, L.M., et al., *Insulin activates the rat sterol-regulatory-element-binding protein 1c (SREBP-1c) promoter through the combinatorial actions of SREBP, LXR, Sp-1 and NF-Y cis-acting elements.* Biochem J, 2005. **385**(Pt 1): p. 207-16.
132. Yieh, L., H.B. Sanchez, and T.F. Osborne, *Domains of transcription factor Sp1 required for synergistic activation with sterol regulatory element binding protein 1 of low density lipoprotein receptor promoter.* Proc Natl Acad Sci U S A, 1995. **92**(13): p. 6102-6.
133. Courey, A.J. and R. Tjian, *Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif.* Cell, 1988. **55**(5): p. 887-98.
134. Baur, F., et al., *Specificity protein 2 (Sp2) is essential for mouse development and autonomous proliferation of mouse embryonic fibroblasts.* PLoS One, 2010. **5**(3): p. e9587.
135. Matera, A.G. and D.C. Ward, *Localization of the human Sp1 transcription factor gene to 12q13 by fluorescence in situ hybridization.* Genomics, 1993. **17**(3): p. 793-4.
136. Milona, M.A., J.E. Gough, and A.J. Edgar, *Expression of alternatively spliced isoforms of human Sp7 in osteoblast-like cells.* BMC Genomics, 2003. **4**: p. 43.
137. Kingsley, C. and A. Winoto, *Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression.* Mol Cell Biol, 1992. **12**(10): p. 4251-61.
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*

SP2 gene to human chromosome bands 17q21.3-->q22 by in situ hybridization. Cytogenet Cell Genet, 1998. **81**(3-4): p. 273-4.

139. Harrison, S.M., et al., *Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury.* Dev Biol, 2000. **227**(2): p. 358-72.
140. Kalff-Suske, M., et al., *Human Sp3 transcriptional regulator gene (SP3) maps to chromosome 2q31.* Genomics, 1996. **37**(3): p. 410-2.
141. Supp, D.M., et al., *Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility.* Dev Biol, 1996. **176**(2): p. 284-99.
142. Hagen, G., et al., *Functional analyses of the transcription factor Sp4 reveal properties distinct from Sp1 and Sp3.* J Biol Chem, 1995. **270**(42): p. 24989-94.
143. Wimmer, E.A., et al., *buttonhead and D-Sp1: a novel Drosophila gene pair.* Mech Dev, 1996. **59**(1): p. 53-62.
144. Santoro, C., et al., *A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs.* Nature, 1988. **334**(6179): p. 218-24.
145. Kadonaga, J.T., et al., *Distinct regions of Sp1 modulate DNA binding and transcriptional activation.* Science, 1988. **242**(4885): p. 1566-70.
146. Dynan, W.S. and R. Tjian, *The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter.* Cell, 1983. **35**(1): p. 79-87.
147. Dynan, W.S. and R. Tjian, *Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II.* Cell, 1983. **32**(3): p. 669-80.
148. Courey, A.J., et al., *Synergistic activation by the glutamine-rich domains of human transcription factor Sp1.* Cell, 1989. **59**(5): p. 827-36.
149. Hagen, G., et al., *Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes.* Nucleic Acids Res, 1992. **20**(21): p. 5519-25.
150. Pascal, E. and R. Tjian, *Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism.* Genes Dev, 1991. **5**(9): p. 1646-56.
151. Lee, J.A., et al., *Transcriptional activity of Sp1 is regulated by molecular interactions between the zinc finger DNA binding domain and the inhibitory domain with corepressors, and this interaction is modulated by MEK.* J Biol Chem, 2005. **280**(30): p. 28061-71.
152. Moorefield, K.S., S.J. Fry, and J.M. Horowitz, *Sp2 DNA binding activity and trans-activation are negatively regulated in mammalian cells.* J Biol Chem, 2004. **279**(14): p. 13911-24.

153. Terrados, G., et al., *Genome-wide localization and expression profiling establish Sp2 as a sequence-specific transcription factor regulating vitally important genes.* Nucleic Acids Res, 2012. **40**(16): p. 7844-57.
154. Letourneur, M., et al., *Sp2 regulates interferon-gamma-mediated socs1 gene expression.* Mol Immunol, 2009. **46**(11-12): p. 2151-60.
155. Bakovic, M., K.A. Waite, and D.E. Vance, *Functional significance of Sp1, Sp2, and Sp3 transcription factors in regulation of the murine CTP:phosphocholine cytidylyltransferase alpha promoter.* J Lipid Res, 2000. **41**(4): p. 583-94.
156. Phan, D., et al., *Identification of Sp2 as a transcriptional repressor of carcinoembryonic antigen-related cell adhesion molecule 1 in tumorigenesis.* Cancer Res, 2004. **64**(9): p. 3072-8.
157. Galvagni, F., S. Capo, and S. Oliviero, *Sp1 and Sp3 physically interact and co-operate with GABP for the activation of the utrophin promoter.* J Mol Biol, 2001. **306**(5): p. 985-96.
158. Ding, H., et al., *Functional interactions between Sp1 or Sp3 and the helicase-like transcription factor mediate basal expression from the human plasminogen activator inhibitor-1 gene.* J Biol Chem, 1999. **274**(28): p. 19573-80.
159. Feng, X., et al., *Sp1/Sp3 and PU.1 differentially regulate beta(5) integrin gene expression in macrophages and osteoblasts.* J Biol Chem, 2000. **275**(12): p. 8331-40.
160. Bigger, C.B., I.N. Melnikova, and P.D. Gardner, *Sp1 and Sp3 regulate expression of the neuronal nicotinic acetylcholine receptor beta4 subunit gene.* J Biol Chem, 1997. **272**(41): p. 25976-82.
161. Zhang, X., et al., *Sp1 and Sp3 transcription factors synergistically regulate HGF receptor gene expression in kidney.* Am J Physiol Renal Physiol, 2003. **284**(1): p. F82-94.
162. Xu, R., et al., *Sp1 and Sp3 regulate basal transcription of the survivin gene.* Biochem Biophys Res Commun, 2007. **356**(1): p. 286-92.
163. Kwon, H.S., et al., *Sp3 and Sp4 can repress transcription by competing with Sp1 for the core cis-elements on the human ADH5/FDH minimal promoter.* J Biol Chem, 1999. **274**(1): p. 20-8.
164. Valin, A., J. Ouyang, and G. Gill, *Transcription factor Sp3 represses expression of p21CIP(1) via inhibition of productive elongation by RNA polymerase II.* Mol Cell Biol, 2013. **33**(8): p. 1582-93.
165. Ghayor, C., et al., *Sp3 represses the Sp1-mediated transactivation of the human COL2A1 gene in primary and de-differentiated chondrocytes.* J Biol Chem, 2001. **276**(40): p. 36881-95.
166. Dennig, J., M. Beato, and G. Suske, *An inhibitor domain in Sp3 regulates its glutamine-rich activation domains.* EMBO J, 1996. **15**(20): p. 5659-67.

167. Stielow, B., et al., *SUMO-modified Sp3 represses transcription by provoking local heterochromatic gene silencing.* EMBO Rep, 2008. **9**(9): p. 899-906.
168. Chu, C., et al., *Transcription factors Sp1 and Sp4 regulate TRPV1 gene expression in rat sensory neurons.* Mol Pain, 2011. **7**: p. 44.
169. Ramos, B., et al., *Sp4-dependent repression of neurotrophin-3 limits dendritic branching.* Mol Cell Neurosci, 2009. **42**(2): p. 152-9.
170. Ishimaru, N., et al., *Regulation of neurotrophin-3 gene transcription by Sp3 and Sp4 in neurons.* J Neurochem, 2007. **100**(2): p. 520-31.
171. Wei, S., et al., *Thiazolidinediones mimic glucose starvation in facilitating Sp1 degradation through the up-regulation of beta-transducin repeat-containing protein.* Mol Pharmacol, 2009. **76**(1): p. 47-57.
172. Guo, L., T. Eviatar-Ribak, and R. Miskimins, *Sp1 phosphorylation is involved in myelin basic protein gene transcription.* J Neurosci Res, 2010. **88**(15): p. 3233-42.
173. Rojo, A.I., et al., *Regulation of heme oxygenase-1 gene expression through the phosphatidylinositol 3-kinase/PKC-zeta pathway and Sp1.* Free Radic Biol Med, 2006. **41**(2): p. 247-61.
174. Zhang, Y., M. Liao, and M.L. Dufau, *Phosphatidylinositol 3-kinase/protein kinase Czeta-induced phosphorylation of Sp1 and p107 repressor release have a critical role in histone deacetylase inhibitor-mediated derepression [corrected] of transcription of the luteinizing hormone receptor gene.* Mol Cell Biol, 2006. **26**(18): p. 6748-61.
175. Chen, X., et al., *Transcriptional regulation of ATP-binding cassette transporter A1 expression by a novel signaling pathway.* J Biol Chem, 2011. **286**(11): p. 8917-23.
176. Tan, N.Y., et al., *Angiotensin II-inducible platelet-derived growth factor-D transcription requires specific Ser/Thr residues in the second zinc finger region of Sp1.* Circ Res, 2008. **102**(4): p. e38-51.
177. Tsai, P.F., et al., *Interplay between PKCdelta and Sp1 on histone deacetylase inhibitor-mediated Epstein-Barr virus reactivation.* J Virol, 2011. **85**(5): p. 2373-85.
178. Rohlff, C., et al., *Modulation of transcription factor Sp1 by cAMP-dependent protein kinase.* J Biol Chem, 1997. **272**(34): p. 21137-41.
179. Chu, S. and T.J. Ferro, *Identification of a hydrogen peroxide-induced PP1-JNK1-Sp1 signaling pathway for gene regulation.* Am J Physiol Lung Cell Mol Physiol, 2006. **291**(5): p. L983-92.
180. Chuang, J.Y., et al., *Phosphorylation by c-Jun NH2-terminal kinase 1 regulates the stability of transcription factor Sp1 during mitosis.* Mol Biol Cell, 2008. **19**(3): p. 1139-51.
181. Jackson, S.P., et al., *GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase.* Cell, 1990. **63**(1): p. 155-65.

182. Zhang, L., et al., *Rat pancreatic level of cystathionine gamma-lyase is regulated by glucose level via specificity protein 1 (SP1) phosphorylation.* Diabetologia, 2011. **54**(10): p. 2615-25.
183. Moon, S.K., S.Y. Jung, and C.H. Kim, *Transcription factor Sp1 mediates p38MAPK-dependent activation of the p21WAF1 gene promoter in vascular smooth muscle cells by pyrrolidine dithiocarbamate.* Biochem Biophys Res Commun, 2004. **316**(3): p. 605-11.
184. Fojas de Borja, P., et al., *Cyclin A-CDK phosphorylates Sp1 and enhances Sp1-mediated transcription.* EMBO J, 2001. **20**(20): p. 5737-47.
185. Moon, S.K., et al., *Platelet-derived growth factor induces p21/WAF1 promoter in vascular smooth muscle cells via activation of an Sp1 site.* FEBS Lett, 2003. **552**(2-3): p. 130-4.
186. Pages, G., *Sp3-mediated VEGF regulation is dependent on phosphorylation by extra-cellular signals regulated kinases (Erk).* J Cell Physiol, 2007. **213**(2): p. 454-63.
187. Jackson, S.P. and R. Tjian, *O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation.* Cell, 1988. **55**(1): p. 125-33.
188. Li, L. and J.R. Davie, *The role of Sp1 and Sp3 in normal and cancer cell biology.* Ann Anat, 2010. **192**(5): p. 275-83.
189. Majumdar, G., et al., *O-glycosylation of Sp1 and transcriptional regulation of the calmodulin gene by insulin and glucagon.* Am J Physiol Endocrinol Metab, 2003. **285**(3): p. E584-91.
190. Majumdar, G., et al., *Insulin stimulates and diabetes inhibits O-linked N-acetylglucosamine transferase and O-glycosylation of Sp1.* Diabetes, 2004. **53**(12): p. 3184-92.
191. Slawson, C. and G.W. Hart, *Dynamic interplay between O-GlcNAc and O-phosphate: the sweet side of protein regulation.* Curr Opin Struct Biol, 2003. **13**(5): p. 631-6.
192. Comer, F.I. and G.W. Hart, *O-Glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate.* J Biol Chem, 2000. **275**(38): p. 29179-82.
193. Roos, M.D., et al., *O glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions.* Mol Cell Biol, 1997. **17**(11): p. 6472-80.
194. Yang, X., et al., *O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6611-6.
195. Ross, S., et al., *SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization.* Mol Cell, 2002. **10**(4): p. 831-42.
196. Sapetschnig, A., et al., *Transcription factor Sp3 is silenced through SUMO modification by PIAS1.* EMBO J, 2002. **21**(19): p. 5206-15.

197. Stielow, B., et al., *Epigenetic silencing of spermatocyte-specific and neuronal genes by SUMO modification of the transcription factor Sp3.* PLoS Genet, 2010. **6**(11): p. e1001203.
198. Valin, A. and G. Gill, *Regulation of the dual-function transcription factor Sp3 by SUMO.* Biochem Soc Trans, 2007. **35**(Pt 6): p. 1393-6.
199. Braun, H., et al., *Transcription factor Sp3 is regulated by acetylation.* Nucleic Acids Res, 2001. **29**(24): p. 4994-5000.
200. Kou, X.X., et al., *Acetylated Sp1 inhibits PTEN expression through binding to PTEN core promoter and recruitment of HDAC1 and promotes cancer cell migration and invasion.* Carcinogenesis, 2013. **34**(1): p. 58-67.
201. Black, A.R., et al., *Growth/cell cycle regulation of Sp1 phosphorylation.* J Biol Chem, 1999. **274**(3): p. 1207-15.
202. Grinstein, E., et al., *Sp1 as G1 cell cycle phase specific transcription factor in epithelial cells.* Oncogene, 2002. **21**(10): p. 1485-92.
203. Kavurma, M.M. and L.M. Khachigian, *Sp1 inhibits proliferation and induces apoptosis in vascular smooth muscle cells by repressing p21WAF1/Cip1 transcription and cyclin D1-Cdk4-p21WAF1/Cip1 complex formation.* J Biol Chem, 2003. **278**(35): p. 32537-43.
204. Koutsodontis, G., A. Moustakas, and D. Kardassis, *The role of Sp1 family members, the proximal GC-rich motifs, and the upstream enhancer region in the regulation of the human cell cycle inhibitor p21WAF-1/Cip1 gene promoter.* Biochemistry, 2002. **41**(42): p. 12771-84.
205. Martino, A., et al., *Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-2.* J Immunol, 2001. **166**(3): p. 1723-9.
206. Tapias, A., et al., *Regulation of Sp1 by cell cycle related proteins.* Cell Cycle, 2008. **7**(18): p. 2856-67.
207. Wei, M., et al., *Stat6 cooperates with Sp1 in controlling breast cancer cell proliferation by modulating the expression of p21(Cip1/WAF1) and p27 (Kip1).* Cell Oncol (Dordr), 2013. **36**(1): p. 79-93.
208. Marin, M., et al., *Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation.* Cell, 1997. **89**(4): p. 619-28.
209. Moorefield, K.S., et al., *Sp2 localizes to subnuclear foci associated with the nuclear matrix.* Mol Biol Cell, 2006. **17**(4): p. 1711-22.
210. Bouwman, P., et al., *Transcription factor Sp3 is essential for post-natal survival and late tooth development.* EMBO J, 2000. **19**(4): p. 655-61.
211. van Loo, P.F., et al., *Transcription factor Sp3 knockout mice display serious cardiac malformations.* Mol Cell Biol, 2007. **27**(24): p. 8571-82.
212. Gollner, H., et al., *Impaired ossification in mice lacking the transcription factor Sp3.* Mech Dev, 2001. **106**(1-2): p. 77-83.
213. Van Loo, P.F., et al., *Impaired hematopoiesis in mice lacking the transcription factor Sp3.* Blood, 2003. **102**(3): p. 858-66.

214. Kruger, I., et al., *Sp1/Sp3 compound heterozygous mice are not viable: impaired erythropoiesis and severe placental defects.* Dev Dyn, 2007. **236**(8): p. 2235-44.
215. Mao, X., et al., *Glutamate receptor activation evokes calpain-mediated degradation of Sp3 and Sp4, the prominent Sp-family transcription factors in neurons.* J Neurochem, 2007. **100**(5): p. 1300-14.
216. Ramos, B., et al., *Transcription factor Sp4 regulates dendritic patterning during cerebellar maturation.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9882-7.
217. Zhou, X., et al., *Impaired postnatal development of hippocampal dentate gyrus in Sp4 null mutant mice.* Genes Brain Behav, 2007. **6**(3): p. 269-76.

Chapter 2

Sp1 and Sp3 regulate adult hematopoiesis

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

Divine I. Kulu[1], Marjolein J. W. de Bruijn[2], Ulrike Jaegle[1], Nynke Gillemans[1], Rita Ferreira[1], Laura Gutiérrez[1], Imme Krüger[3], Guntram Suske[3], Rudi W. Hendriks[2], Sjaak Philipsen[1]

[1]Department of Cell Biology, Erasmus MC, Dr. Molewaterplein 50-60, 3015 GE Rotterdam, The Netherlands
[2]Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50-60, 3015 GE Rotterdam, The Netherlands
[3]Institute for Molecular Biology and Tumor Research, Philipps University Marburg, 35032 Marburg Germany

Correspondence:
Sjaak Philipsen
Erasmus MC-Cell Biology, room Ee720
P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
Tel: +31-10-7044282 (office), +31-10-7043169 (department),
Fax: +31-10-7044743
Email: j.philipsen@erasmusmc.nl

(Manuscript in preparation)

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 *Sp1wt/ko::Sp3wt/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.

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]. $Sp1^{wt/ko}$::$Sp3^{wt/ko}$ 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*^{Flp} 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´-GAGGCCTTGTTGCAAAAGTAAG-3´) and antisense primer 2 (5´-ACACCACCAGATTCAAAGACTCT-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 recombined allele, an additional antisense primer (5´-TTGGACCCATGCTACCTTGC -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'-AAGTCTCAGTGCCAAACGCA-3'. This yields a 4.2 kb band for the floxed *Sp1* allele, and a 3.7 band for the recombined *Sp1* allele.
PCR Genotyping of *Sp3* alleles. To distinguish the wildtype and floxed alleles, we used a forward primer in exon 4 (5'-TGTTCAGACACTCACGCTTGGTCA-3') and a reverse primer in intron 4 (5'-GTCTACATAGCAAGTTCCAG-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 recombined allele, we used a three primer PCR strategy with forward primers located in intron 3 (5'-GGAGGGGCTTTAATATTACC-3') and exon 4 (5'-TGTTCAGACACTCACGCTTGGTCA-3'), and a common reverse primer in intron 4 (5'-GTCTACATAGCAAGTTCCAG-3'). This yields a product size of 592 bp for the *Sp3* floxed allele, and 629 bp for the *Sp3* recombined 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'-AGGCCTGGAGATTTGCAGTGGGT-3' and 5'-CTGAGCACCAAGGAATCTGTATGATCC-3'. This yields a 6.6 kb band for the floxed *Sp3* allele, and a 5.2 kb band for the recombined allele.

Induction of *Mx1-Cre* by pIpC treatment

Animals were injected intraperitoneally with poly (I:C) (Sigma) dissolved in PBS (pIpC/PBS; 25 µg pIpC 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

pIpC 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 $MgCl_2$, 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 $MgCl_2$, 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) NaN_3) 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

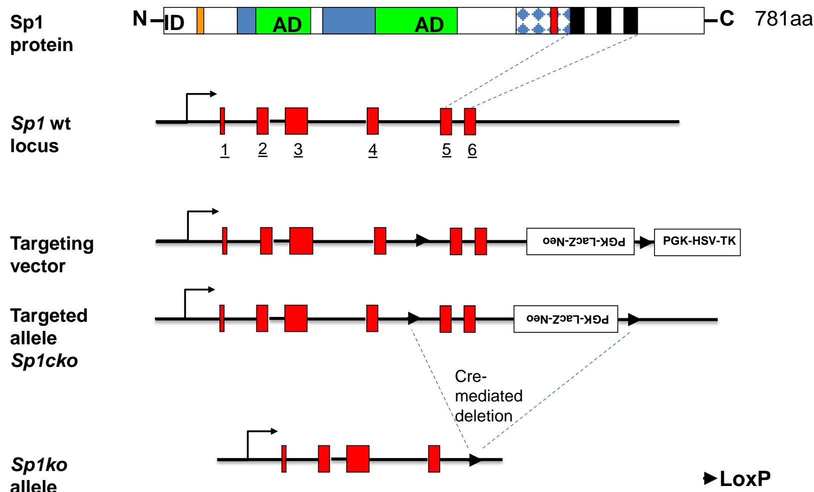
Generation of mice with Sp1 and Sp3 conditional knockout alleles

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 *β-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$^{fl/fl}$ with *Sp3*$^{fl/fl}$ animals resulted in mice with homozygous conditional knockout alleles for both Sp1 and Sp3 (*Sp1*$^{fl/fl}$*::Sp3*$^{fl/fl}$). Mice of all three genotypes (*Sp1*$^{fl/fl}$, *Sp3*$^{fl/fl}$ and *Sp1*$^{fl/fl}$*::Sp3*$^{fl/fl}$) 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*$^{fl/fl}$*::Sp3*$^{fl/fl}$ (double KO, dKO) and *Sp1*$^{fl/fl}$*::Sp3*$^{fl/fl}$ (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*$^{fl/fl}$*::Sp3*$^{fl/fl}$ 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*$^{fl/fl}$*::Sp3*$^{fl/fl}$ animals.



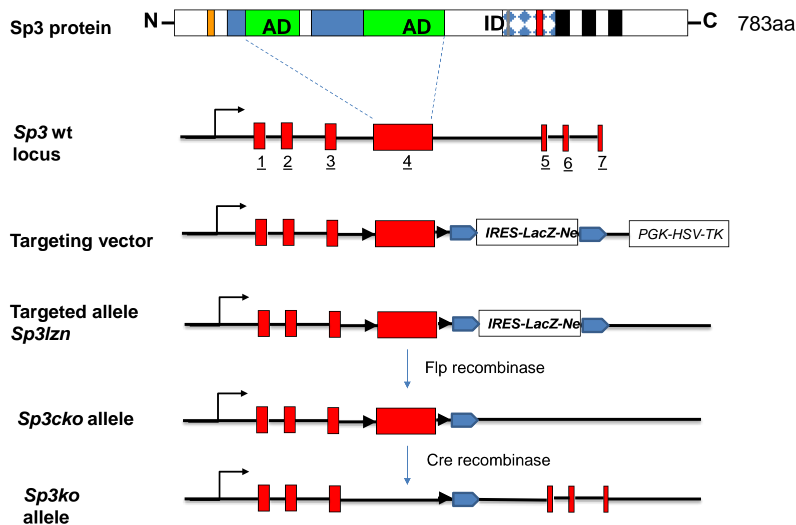


Figure 1: Generation of *Sp1* and *Sp3* mutant alleles. Schematic drawings of the strategy to create the alleles used for the *Mx1-Cre*-mediated conditional knockout of the *Sp1* and *Sp3* genes. The *PGK-HSV-TK* cassette is deleted upon homologous recombination and was used for counter-selection to 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.

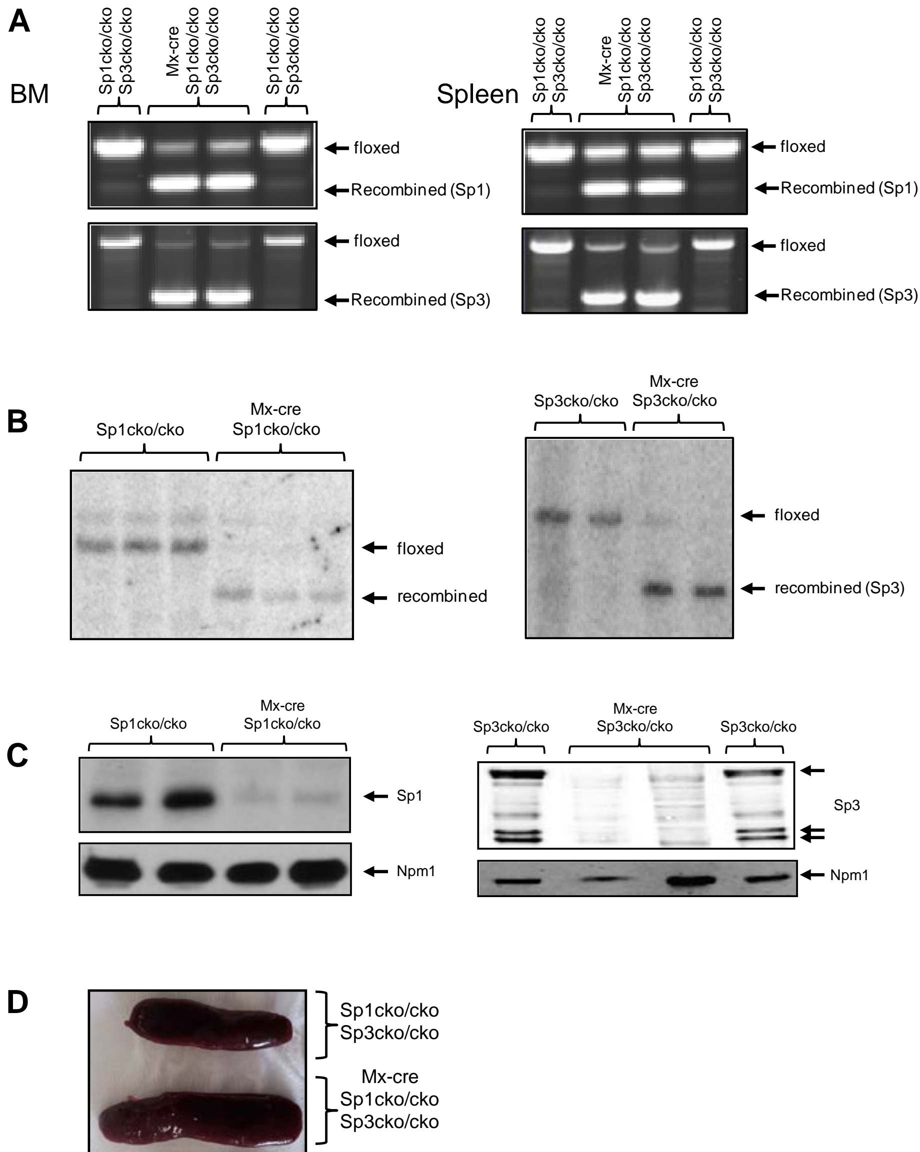


Figure 2: *Mx1-Cre* mediated deletion of floxed *Sp1* and *Sp3* alleles in hematopoietic tissues.
(A) PCR analysis on bone marrow and spleen. **(B)** Southern blot analysis of genomic DNA isolated from the bone marrow after pIpC treatment course. **(C)** Western blot analysis of nuclear extracts from 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.

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::Sp1*$^{fl/fl}$ (Sp1 sKO), *Mx1-Cre::Sp3*$^{fl/fl}$ (Sp3 sKO) and their littermate controls, *Sp1*$^{fl/fl}$ and *Sp3*$^{fl/fl}$ 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).

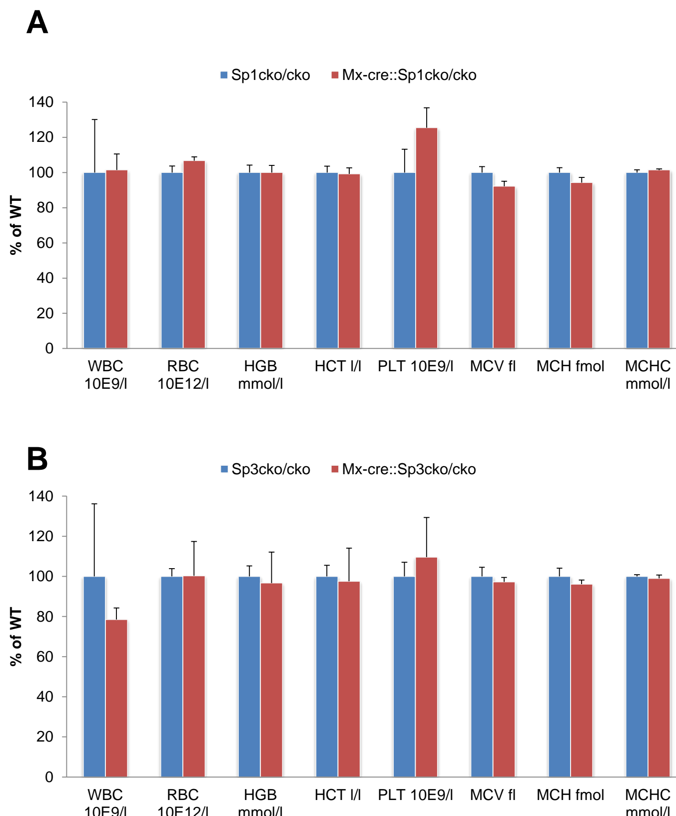


Figure 3. No obvious hematopoietic defects in Sp1 or Sp3 mutants. Peripheral blood analysis of Sp1KO (n=12), Sp3KO (n=16) and their corresponding littermates *Sp1fl/fl* (n=12), *Sp3fl/fl* (n=14). The values for the white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), 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::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* animals

After pIpC 7 treatment course and recovery, peripheral blood was collected from Mx1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$ animals and littermate controls, Sp1$^{fl/fl}$::Sp3$^{fl/fl}$. 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.

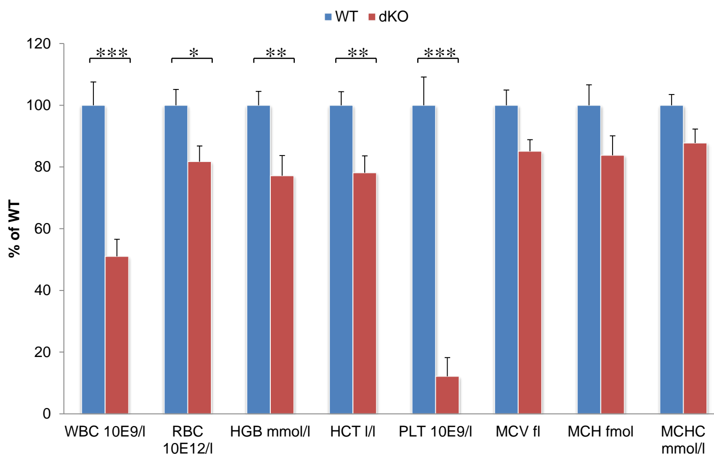
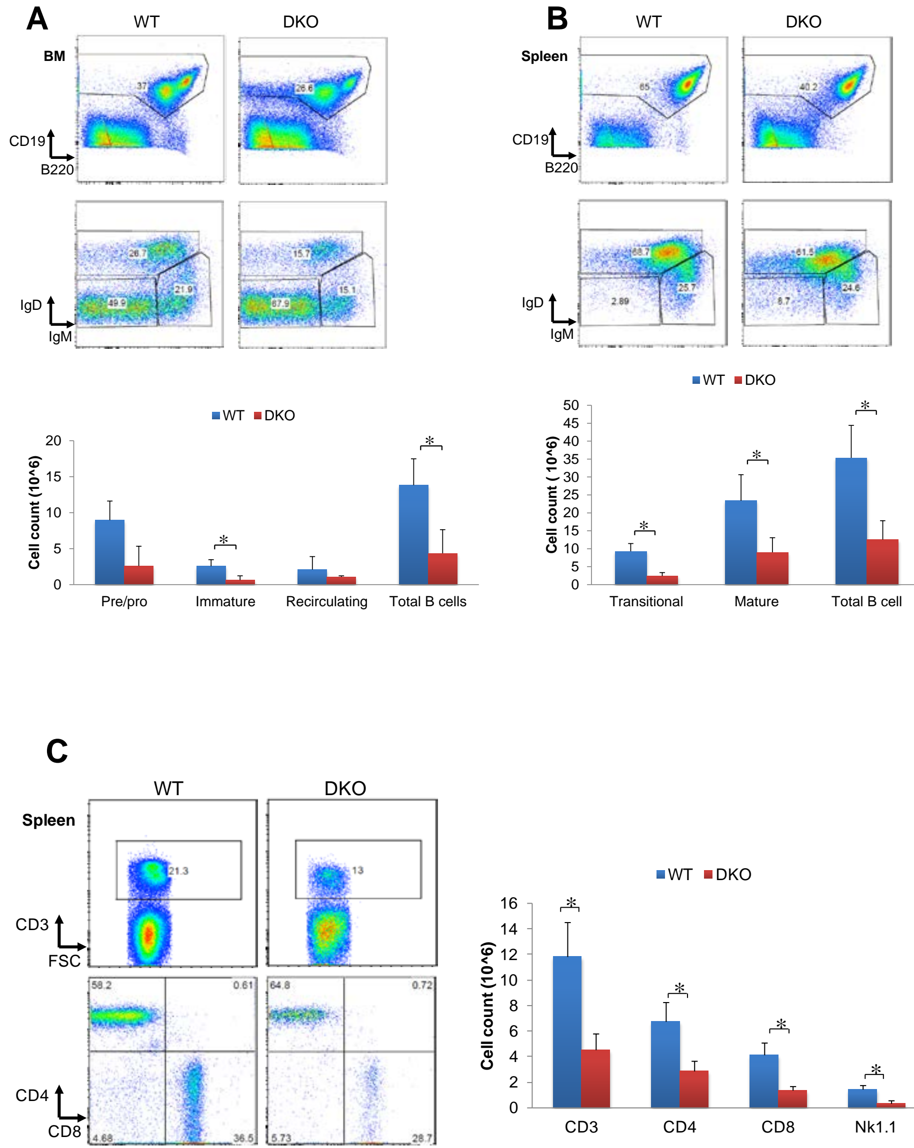


Figure 4. Hematopoietic defects in Sp1/Sp3 double mutant mice. Peripheral blood analysis of DKO mice (n=14) and littermate CT mice (n=12). Counts for white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), mean cell volume (MCV), mean corpuscular volume (MCV) 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^+ IgD^{low}) and mature (IgM^+ IgD^{high})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)**.


A
WT
DKO
BM
CD19
B220
IgD
IgM
WT DKO
Cell count (10^6)
Pre/pro
Immature
Recirculating
Total B cells
B
WT
DKO
Spleen
CD19
B220
IgD
IgM
WT DKO
Cell count (10^6)
Transitional
Mature
Total B cell
C
WT
DKO
Spleen
CD3
FSC
CD4
CD8
WT DKO
Cell count (10^6)
CD3
CD4
CD8
Nk1.1

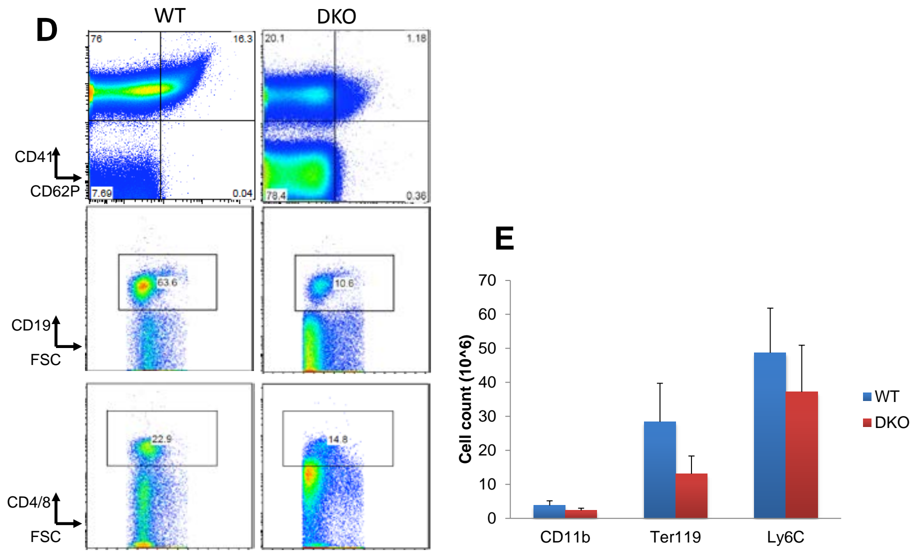


Figure 5. Impeded myelopoiesis and lymphopoieisis in *Mx1-Cre::Sp1fl/fl::Sp3fl/fl* mice. Hematopoietic tissues from *Mx1-Cre::Sp1fl/fl::Sp3fl/fl* (DKO) (n=16) and *Sp1fl/fl::Sp3fl/fl* (CT) (n=14) litters were analysed by flow cytometry after the pIpC 3 treatment course. **(A&B)** B cell analysis in the 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 pIpC 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 IIb/IIIa) 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 extramedullar erythropoiesis occured 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

1. Orkin, S.H. and L.I. Zon, *Hematopoiesis: an evolving paradigm for stem cell biology.* Cell, 2008. **132**(4): p. 631-44.
2. Van Loo, P.F., et al., *Impaired hematopoiesis in mice lacking the transcription factor Sp3.* Blood, 2003. **102**(3): p. 858-66.
3. Kruger, I., et al., *Sp1/Sp3 compound heterozygous mice are not viable: impaired erythropoiesis and severe placental defects.* Dev Dyn, 2007. **236**(8): p. 2235-44.
4. Marin, M., et al., *Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation.* Cell, 1997. **89**(4): p. 619-28.
5. Drissen, R., et al., *The erythroid phenotype of EKLF-null mice: defects in hemoglobin metabolism and membrane stability.* Mol Cell Biol, 2005. **25**(12): p. 5205-14.
6. Nuez, B., et al., *Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene.* Nature, 1995. **375**(6529): p. 316-8.
7. Kuo, C.T., M.L. Veselits, and J.M. Leiden, *LKLF: A transcriptional regulator of single-positive T cell quiescence and survival.* Science, 1997. **277**(5334): p. 1986-90.
8. Philipsen, S. and G. Suske, *A tale of three fingers: the family of mammalian Sp/XKLF transcription factors.* Nucleic Acids Res, 1999. **27**(15): p. 2991-3000.
9. Suske, G., *The Sp-family of transcription factors.* Gene, 1999. **238**(2): p. 291-300.
10. Suske, G., E. Bruford, and S. Philipsen, *Mammalian SP/KLF transcription factors: bring in the family.* Genomics, 2005. **85**(5): p. 551-6.
11. Bouwman, P. and S. Philipsen, *Regulation of the activity of Sp1-related transcription factors.* Mol Cell Endocrinol, 2002. **195**(1-2): p. 27-38.
12. Gollner, H., et al., *Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene.* Genes Cells, 2001. **6**(8): p. 689-97.
13. Bouwman, P., et al., *Transcription factor Sp3 is essential for post-natal survival and late tooth development.* EMBO J, 2000. **19**(4): p. 655-61.
14. van Loo, P.F., et al., *Transcription factor Sp3 knockout mice display serious cardiac malformations.* Mol Cell Biol, 2007. **27**(24): p. 8571-82.
15. Kuhn, R., et al., *Inducible gene targeting in mice.* Science, 1995. **269**(5229): p. 1427-9.
16. Srinivas, S., et al., *Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus.* BMC Dev Biol, 2001. **1**: p. 4.

17. Sakai, K. and J. Miyazaki, *A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission.* Biochem Biophys Res Commun, 1997. **237**(2): p. 318-24.
18. Buchholz, F., P.O. Angrand, and A.F. Stewart, *A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs.* Nucleic Acids Res, 1996. **24**(15): p. 3118-9.
19. Farley, F.W., et al., *Widespread recombinase expression using FLPeR (flipper) mice.* Genesis, 2000. **28**(3-4): p. 106-10.
20. Sjobring, U., U. Ringdahl, and Z.M. Ruggeri, *Induction of platelet thrombi by bacteria and antibodies.* Blood, 2002. **100**(13): p. 4470-7.
21. Boisset, J.C., et al., *Integrin alphaIIb (CD41) plays a role in the maintenance of hematopoietic stem cell activity in the mouse embryonic aorta.* Biol Open, 2013. **2**(5): p. 525-32.

Supplemental Table 1

The following antibodies were used for flow cytometry

Antigen	**Conjugation**	**clone**	**source**
B220	AlexaFluor 700	RA3-6B2	eBioscience
CD3	FITC	UCHT1	eBioscience
CD3	APC Cy7	SK7	BD biosciences
CD4	FITC	GK1.5	BD biosciences
CD4	PerCP-Cy5.5	SK3	BD biosciences
CD8a	PE	53-6.7	eBioscience
CD8a	PerCP-Cy5.5	-	eBioscience
CD19	PE	MB19-1	eBioscience
CD19	APC Cy7	ID3	eBioscience
CD41	FITC	eBiomwReg30	eBioscience
CD11b	AF700	-	BD bioscience
CD62P	bio	RB40.34	BD bioscience
IgD	FITC	11-26c2a	BD bioscience
IgM	PE-Cy7	11/41	eBioscience
Ly6C	bio	AL-21	BD bioscience
Nk1.1	APC	PK136	BD bioscience
Ter119	PE	TER-119	ebioscience
Streptavidin	PE-Cy7	-	BD bioscience

Chapter 3

Sp1 and Sp3 co-operate to regulate B cell development

Sp1 and Sp3 co-operate to regulate B cell development

Divine I. Kulu[1], Marjolein J. W. de Bruijn[2], Imme Krüger[3], Guntram Suske[3], Rudi W. Hendriks[2] and Sjaak Philipsen[1]

[1]Department of Cell Biology, Erasmus MC, Dr. Molewaterplein 50-60, 3015 GE Rotterdam, The Netherlands
[2]Department of Pulmonary Medicine, Erasmus MC, Dr. Molewaterplein 50-60, 3015 GE Rotterdam, The Netherlands
[3]Institute for Molecular Biology and Tumor Research, Philipps University Marburg, 35032 Marburg Germany

Correspondence:
Sjaak Philipsen
Erasmus MC-Cell Biology, room Ee720
P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
Tel: +31-10-7044282 (office), +31-10-7043169 (department),
Fax: +31-10-7044743
Email: j.philipsen@erasmusmc.nl

(Manuscript in preparation)

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.

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 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 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 Cys_2-His_2 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 >1X10^6 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% NaN_3) 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-ethylbenzthiazoline 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- pro-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.

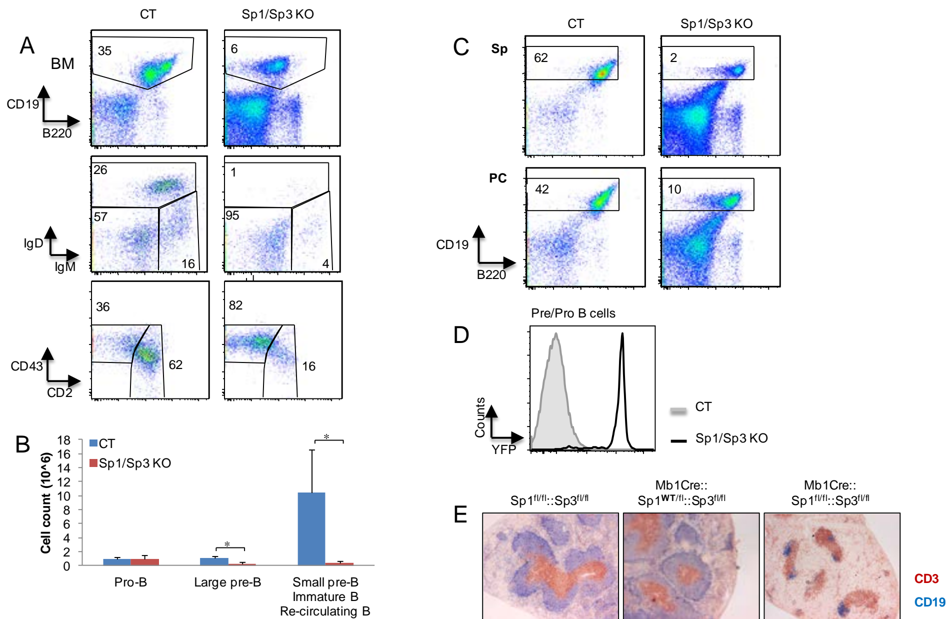


Figure 1. Defective B cell development in *mb1*-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$ mice. (A) Flow cytometry analysis of *Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* (CT) and *Mb1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* (Sp1/Sp3 KO) total bone marrow (BM) cells for surface expression of CD19 and B220 (top). IgD$^+$IgM$^+$ 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$^-$ intracellular Igμ$^-$), large pre-B (CD2$^-$ intracellular Igμ$^+$), total small pre-B, Immature B and re-circulating mature B cell (CD2$^+$ intracellular Igμ$^+$) 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$^+$/B220$^+$ 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$^{WT/fl}$::Sp3$^{fl/fl}$ 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$^{fl/fl}$::Sp3$^{fl/fl}$* mice

Flow cytometric analyses revealed that cytoplasmic Igμ+ 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$^+$ 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).**

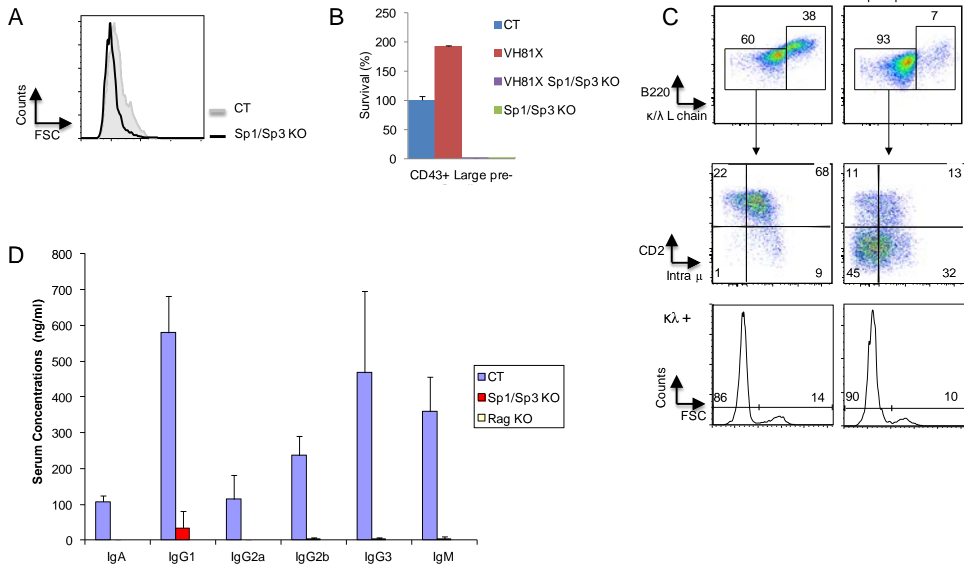


Figure 2. Defective pre-B cell maturation in *Mb1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* mice. (A) Flow cytometric characterization of CT and Sp1/Sp3 KO B220$^+$CD19$^+$ CD2$^-$Igμ$^+$ pre-B cells for cell size (forward scatter, FSC). Data are displayed as histogram overlays anjd are representative for 6-8 mice analyzed per genotype. **(B)** CD43+ pre B cell proliferative response to 100U/ml IL-7 in CT, Sp1/Sp3 KO, V_H81X and V_H81X 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$^+$/κ$^+$ 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. $B220^-$ $Ig\mu^-$ pre/pro B cells were also gated and analyzed for the $CD2^-Ig\mu^-$ (pro B), $CD2^-Ig\mu^+$ (Large pre B) $CD2^+$ $Ig\mu^+$ (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 V_H81X 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 V_H81X (24). A significant increase in the number of $CD19^+B220^+$ B cells in V_H81X 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 V_H81X and V_H81X Sp1/Sp3 KO BM, the V_H81X 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 V_H81X 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 $Ig\mu^+$ small pre-B cell and the size of these cells were similar in V_H81X and V_H81X Sp1/Sp3 KO BM **(Fig. 3C)**

Taken together, the expression of V_H81X transgene allows Sp1/Sp3-deficient cells to differentiate beyond the pre-B cell stage, resulting in the significant generation of surface Ig+ B cells.

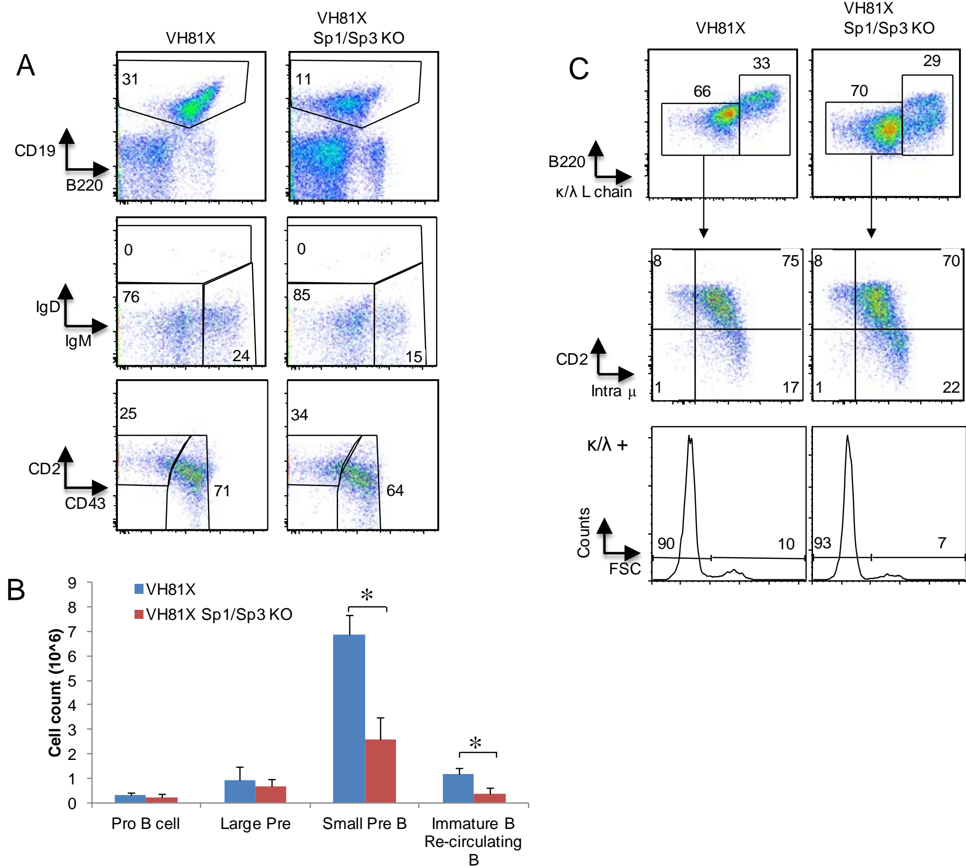


Figure 3. Partial rescue of Sp1/Sp3 KO B cells carrying the V_H81X transgene. (A) Flow cytometric characterization of V_H81X and V_H81X Sp1/Sp3 KO BM cells for the expression of CD19/B220 (top). Total CD19$^+$B220$^+$ B cell fractions were gated and analyzed for the expression of IgD/IgM (middle) . IgD$^-$ 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$^-$ intracellular Igμ$^+$), small pre-B (CD2$^+$IgM$^-$) and immature B/re-circulating mature B cell (CD2$^+$IgM$^+$) fractions in BM of V_H81X and V_H81X 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 V_H81X and V_H81X 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 G_1 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_H81X 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_H81X Sp1/Sp3 KO B cells appeared in the sub-G1 phase compared to < 27% of CT or V_H81X B cells. This revealed that a lot of Sp1/Sp3 KO and V_H81X 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_1 phase **(Fig 4C)**.

Taken together, our data revealed that Sp1/Sp3 KO B cells are arrested at the G_1 phase of the cell cycle with defective metabolism which was not corrected by the introduction of V_H81X trangene.

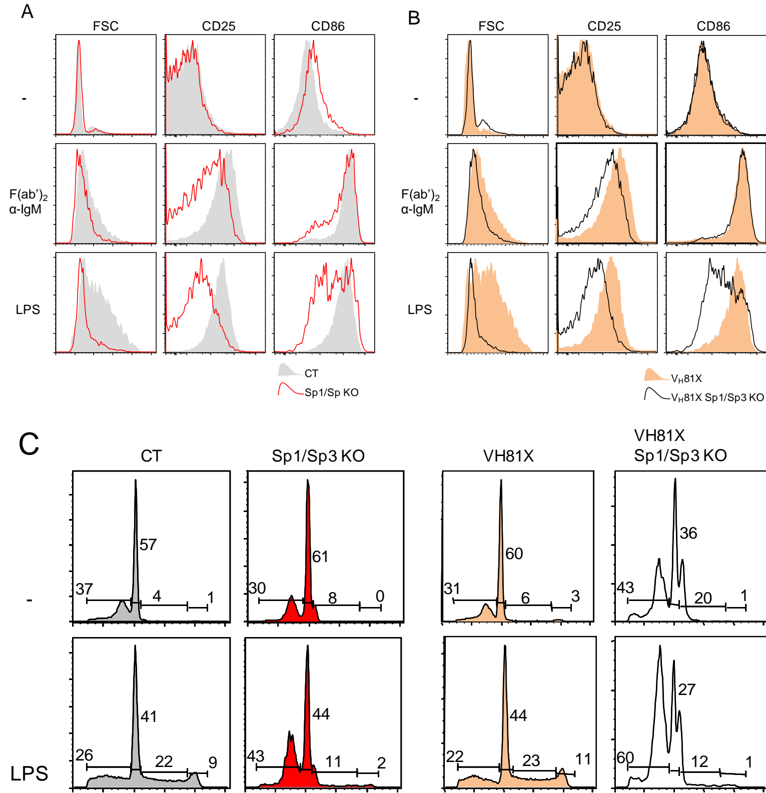


Figure 4. Metabolic and cell cycle defect in Sp1/Sp3 KO B cells. (A) Flow cytometry analysis of anti-IgM or LPS activated CT and Sp1/Sp3 KO splenic B cells. Total CD19+B220+ B cell fraction where gated and analyzed for cell size (FSC) (Left), the expression of CD25 (middle) or CD86 (right). **(B)** Flow cytometry analysis of anti-IgM or LPS stimulated V_H81X and V_H81X 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, V_H81X and V_H81X 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> G_0/G_1>S>G_2M)

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 $Ig\mu^{+}$ pre-B cells, and rapid down-regulation of the re-arrangement 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 V_H81X (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 V_H81X 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 V_H81X transgene. V_H81X 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 G_1 phase of the cell cycle when their membrane immunoglobins 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 G_1 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 G_1 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 be 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

1. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. Cell. 1989;59(6):1035-48. Epub 1989/12/22.
2. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science. 1990;248(4962):1517-23. Epub 1990/06/22.
3. Jung D, Alt FW. Unraveling V(D)J recombination; insights into gene regulation. Cell. 2004;116(2):299-311. Epub 2004/01/28.
4. Nutt SL, Kee BL. The transcriptional regulation of B cell lineage commitment. Immunity. 2007;26(6):715-25. Epub 2007/06/22.
5. Vilagos B, Hoffmann M, Souabni A, Sun Q, Werner B, Medvedovic J, et al. Essential role of EBF1 in the generation and function of distinct mature B cell types. J Exp Med. 2012;209(4):775-92. Epub 2012/04/05.
6. Cobaleda C, Schebesta A, Delogu A, Busslinger M. Pax5: the guardian of B cell identity and function. Nat Immunol. 2007;8(5):463-70. Epub 2007/04/19.
7. Carotta S, Holmes ML, Pridans C, Nutt SL. Pax5 maintains cellular identity by repressing gene expression throughout B cell differentiation. Cell Cycle. 2006;5(21):2452-6. Epub 2006/11/15.
8. Mandel EM, Grosschedl R. Transcription control of early B cell differentiation. Curr Opin Immunol. 2010;22(2):161-7. Epub 2010/02/11.
9. O'Riordan M, Grosschedl R. Transcriptional regulation of early B-lymphocyte differentiation. Immunol Rev. 2000;175:94-103. Epub 2000/08/10.
10. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. 1991. J Immunol. 2012;189(7):3271-83. Epub 2012/09/22.
11. Hendriks RW, Middendorp S. The pre-BCR checkpoint as a cell-autonomous proliferation switch. Trends Immunol. 2004;25(5):249-56. Epub 2004/04/22.
12. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. Nat Rev Immunol. 2009;9(3):195-205. Epub 2009/02/26.
13. Casola S, Otipoby KL, Alimzhanov M, Humme S, Uyttersprot N, Kutok JL, et al. B cell receptor signal strength determines B cell fate. Nat Immunol. 2004;5(3):317-27. Epub 2004/02/06.
14. Suske G, Bruford E, Philipsen S. Mammalian SP/KLF transcription factors: bring in the family. Genomics. 2005;85(5):551-6. Epub 2005/04/12.
15. Philipsen S, Suske G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res. 1999;27(15):2991-3000. Epub 1999/08/24.

16. Bouwman P, Philipsen S. Regulation of the activity of Sp1-related transcription factors. Mol Cell Endocrinol. 2002;195(1-2):27-38. Epub 2002/10/02.
17. Marin M, Karis A, Visser P, Grosveld F, Philipsen S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell. 1997;89(4):619-28. Epub 1997/05/16.
18. Bouwman P, Gollner H, Elsasser HP, Eckhoff G, Karis A, Grosveld F, et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. EMBO J. 2000;19(4):655-61. Epub 2000/02/17.
19. van Loo PF, Mahtab EA, Wisse LJ, Hou J, Grosveld F, Suske G, et al. Transcription factor Sp3 knockout mice display serious cardiac malformations. Mol Cell Biol. 2007;27(24):8571-82. Epub 2007/10/10.
20. Van Loo PF, Bouwman P, Ling KW, Middendorp S, Suske G, Grosveld F, et al. Impaired hematopoiesis in mice lacking the transcription factor Sp3. Blood. 2003;102(3):858-66. Epub 2003/04/05.
21. Kruger I, Vollmer M, Simmons DG, Elsasser HP, Philipsen S, Suske G. Sp1/Sp3 compound heterozygous mice are not viable: impaired erythropoiesis and severe placental defects. Dev Dyn. 2007;236(8):2235-44. Epub 2007/06/23.
22. Hobeika E, Thiemann S, Storch B, Jumaa H, Nielsen PJ, Pelanda R, et al. Testing gene function early in the B cell lineage in mb1-cre mice. Proc Natl Acad Sci U S A. 2006;103(37):13789-94. Epub 2006/08/31.
23. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell. 1992;68(5):869-77. Epub 1992/03/06.
24. Martin F, Chen X, Kearney JF. Development of VH81X transgene-bearing B cells in fetus and adult: sites for expansion and deletion in conventional and CD5/B1 cells. Int Immunol. 1997;9(4):493-505. Epub 1997/04/01.
25. Middendorp S, Dingjan GM, Hendriks RW. Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice. J Immunol. 2002;168(6):2695-703. Epub 2002/03/09.
26. Kil LP, de Bruijn MJ, van Nimwegen M, Corneth OB, van Hamburg JP, Dingjan GM, et al. Btk levels set the threshold for B-cell activation and negative selection of autoreactive B cells in mice. Blood. 2012;119(16):3744-56. Epub 2012/03/03.
27. Dingjan GM, Maas A, Nawijn MC, Smit L, Voerman JS, Grosveld F, et al. Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. EMBO J. 1998;17(18):5309-20. Epub 1998/09/16.
28. Hagman J, Travis A, Grosschedl R. A novel lineage-specific nuclear factor regulates mb-1 gene transcription at the early stages of B cell differentiation. EMBO J. 1991;10(11):3409-17. Epub 1991/11/01.

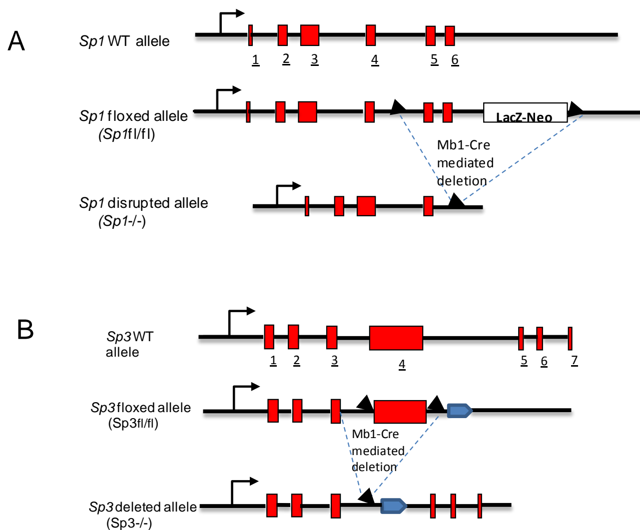
29. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001;1:4. Epub 2001/04/12.
30. Liu H, Schmidt-Supprian M, Shi Y, Hobeika E, Barteneva N, Jumaa H, et al. Yin Yang 1 is a critical regulator of B-cell development. Genes & development. 2007;21(10):1179-89. Epub 2007/05/17.
31. Melchers F, ten Boekel E, Seidl T, Kong XC, Yamagami T, Onishi K, et al. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. Immunol Rev. 2000;175:33-46. Epub 2000/08/10.
32. Grawunder U, Leu TM, Schatz DG, Werner A, Rolink AG, Melchers F, et al. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity. 1995;3(5):601-8. Epub 1995/11/01.
33. Melchers F. The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire. Nat Rev Immunol. 2005;5(7):578-84. Epub 2005/07/07.
34. DeFranco AL, Raveche ES, Paul WE. Separate control of B lymphocyte early activation and proliferation in response to anti-IgM antibodies. J Immunol. 1985;135(1):87-94. Epub 1985/07/01.
35. Sieckmann DG. The use of anti-immunoglobulins to induce a signal for cell division in B lymphocytes via their membrane IgM and IgD. Immunol Rev. 1980;52:181-210. Epub 1980/01/01.
36. Schuhmacher M, Staege MS, Pajic A, Polack A, Weidle UH, Bornkamm GW, et al. Control of cell growth by c-Myc in the absence of cell division. Current biology : CB. 1999;9(21):1255-8. Epub 1999/11/11.
37. Iritani BM, Eisenman RN. c-Myc enhances protein synthesis and cell size during B lymphocyte development. Proc Natl Acad Sci U S A. 1999;96(23):13180-5. Epub 1999/11/11.
38. Grinstein E, Jundt F, Weinert I, Wernet P, Royer HD. Sp1 as G1 cell cycle phase specific transcription factor in epithelial cells. Oncogene. 2002;21(10):1485-92. Epub 2002/03/16.
39. Nagata D, Suzuki E, Nishimatsu H, Satonaka H, Goto A, Omata M, et al. Transcriptional activation of the cyclin D1 gene is mediated by multiple cis-elements, including SP1 sites and a cAMP-responsive element in vascular endothelial cells. J Biol Chem. 2001;276(1):662-9. Epub 2000/10/12.
40. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 1993;74(4):597-608. Epub 1993/08/27.
41. Xiang Z, Ahmed AA, Moller C, Nakayama K, Hatakeyama S, Nilsson G. Essential role of the prosurvival bcl-2 homologue A1 in mast cell survival

after allergic activation. J Exp Med. 2001;194(11):1561-69. Epub 2001/12/26.

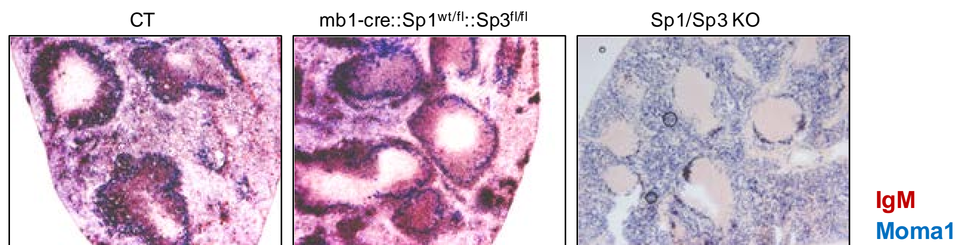
42. Burchill MA, Yang J, Vang KB, Farrar MA. Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. Immunol Lett. 2007;114(1):1-8. Epub 2007/10/16.

43. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. Immunity. 2010;33(2):153-65. Epub 2010/08/25.

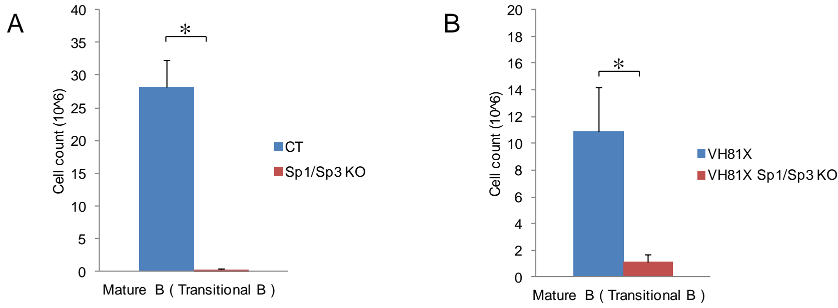
Supplementary Figures and Tables



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 metallophillic 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

Gene		Sequence	Purpose
Sp1cko	Forward	GAGGCCTTGTTGCAAAAGTAAG	Genotyping
	Reverse	ACACCACCAGATTCAAAGACTCT	
	Reverse	TTGGACCCATGCTACCTTGC	
Sp3cko	Forward	TGTTCAGACACTCACGCTTGGTCA	Genotyping
	Reverse	GTCTACATAGCAAGTTCCAG	
V_H81X	Forward	CGCGCGGCCGCGTGGAGTCTGGGGGAG GCT	Genotyping
	Reverse	CCCAGACATCGAAGTACCAGCTACTACCATG	
Rosa26YFP	Forward	AAAGTCGCTCTGAGTTGTTAT	Genotyping
	Reverse	GCGAAGAGTTTGTCCTCAACC	
	Reverse	GGAGCGGGAGAAATGGATATG	
Rag 1	Forward	AGGCCTGTGGAGCAAGGTA	Genotyping
	Reverse	GCTCAGGGTAGACGGCAAG	

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

Antigen	Clone	Conjugate	source
Flow cytometry			
B220	RA3-6B2	AlexaFluor®700	eBioscience
B220	RA3-6B2	FITC	eBioscience
B220	RA3-6B2	APC	eBioscience
CD2	LFA	PE	BD biosciences
CD19	ID3	PerCP-Cy™5.5	eBioscience
CD19	ID3	APC	eBioscience
CD25	PC61.5	PE-Cy™7	ebioscience
CD43	S7	PE	BD biosciences
CD86	GI1	APC	ebioscience
IgA	-	-	-
IgD	11-26c	PE	BD biosciences
IgM	II/41	FITC	eBioscience
Igλ		PE	
Igκ	187.1	PE	
Igμ	-	FITC	Jackson Immunoresearch Laboraties
Streptavidin	-	APC	eBiosciences
MACS purification			
CD3	145-2c11	biotin	BD biosciences
CD4	L3T4	biotin	BD biosciences
CD8	Ly-2	biotin	BD biosciences
CD11b	M1/70	biotin	BD biosciences
CD11c	N418	biotin	BD biosciences
CD43	S7	Biotin	BD biosciences
Gr-1	RB6-8C5	biotin	BD biosciences
Nk1.1	PK136	biotin	BD biosciences
Ter119	PK136	biotin	BD biosciences
Immunohistochemistry			
CD3	145-2c11	biotin	BD biosciences
CD19	ID3	PE	BD biosciences
IgM	-	biotin	BD Pharmingen
Moma 1	-	-	-
Anti-rat IgG	-	Alkaline phosphatase	Jackson ImmunoResearch
Anti-PE	-	peroxidase	Rockland Immunochemicals
Streptavidin	-	Peroxidase	Sigma-Aldrich

Chapter 4

Sp1 and Sp3 co-operate to regulate megakaryopoiesis

Sp1 and Sp3 co-operate to regulate megakaryopoiesis

Divine I. Kulu[1,*], Marjolein Meinders[2,*], Hans Janssen[3], Mark Hoogenboezem[4], Erik Mul[4], Tomasz Poplonsky[4], Iris M. De Cuyper[2], Sander Meijer[5], Imme Krüger[6], Guntram Suske[6], Laura Gutiérrez[2,#], Sjaak Philipsen[1,#$]

[1]Department of Cell Biology, ErasmusMC, Rotterdam, The Netherlands.
[2]Department of Blood Cell Research, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands.
[3]Division of Cell Biology, The Netherlands Cancer Institute (NKI), Amsterdam, The Netherlands.
[4]Department of Molecular and Cellular Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands.
[5]Department of Plasma Proteins, Sanquin Research, The Netherlands.
[6]Institute for Molecular Biology and Tumor Research, Philipps University Marburg, 35032 Marburg Germany

$ Correspondence:
Sjaak Philipsen
Erasmus MC-Cell Biology, room Ee720
P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
Tel: +31-10-7044282 (office), +31-10-7043169 (department),
Fax: +31-10-7044743
Email: j.philipsen@erasmusmc.nl

*,# 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 C_2H_2-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 (GGGGTGTGG)-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 lymphoid and myeloid lineage cells. 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 megakayopoiesis 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 $CaCl_2$. 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% Osmiumtetroxide 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) NaN_3) 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 (LSRII + 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::Sp1*$^{fl/fl}$*::Sp3*$^{fl/fl}$ and *Sp1*$^{fl/fl}$*::Sp3*$^{fl/fl}$ 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 ìm × 150 mm, 2 ìm 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).

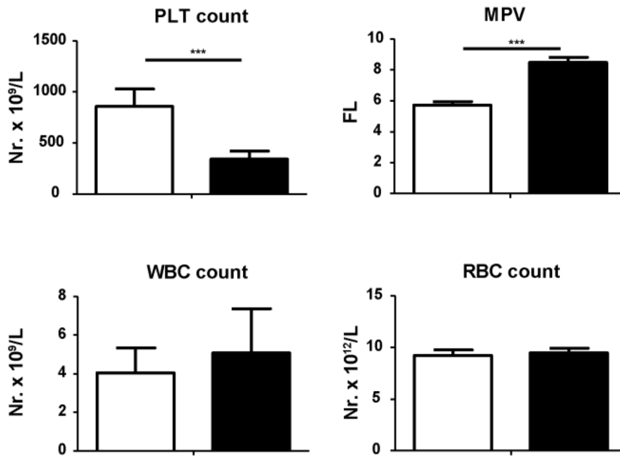
Results

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

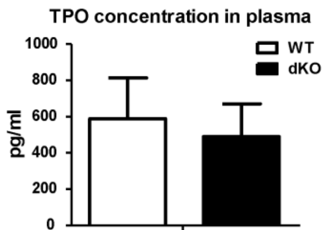
Peripheral blood analysis was performed on *Pf4-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* mice (*Sp1/Sp3* dKO) and *Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* (CT) littermate control mice. The result revealed that the mean blood platelet count was 341.7 ± 21.87 (n=13) in the *Sp1/Sp3* dKO mice, compared to 879.1 ± 50.18 (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.

A



B



C

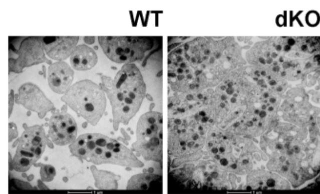


Figure 1. Peripheral blood and structural analysis of platelets. (A) Standard blood measurement of circulatory platelets revealed that Sp1/Sp3 dKO mice are thrombocytopenic with larger platelets. Mean and standard deviation (SD), *** $P<0.001$. (PLT = platelet, MPV = mean platelet volume, WBC = White 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 GPIIbIIIa or αIIbβ3 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 αIIbβ3 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 GPIIβIIIα integrin can be measured by the ratio of active GPIIβIIIα on the platelet membrane (with JON/A antibody) compared to the basal GPIIβIIIα 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.

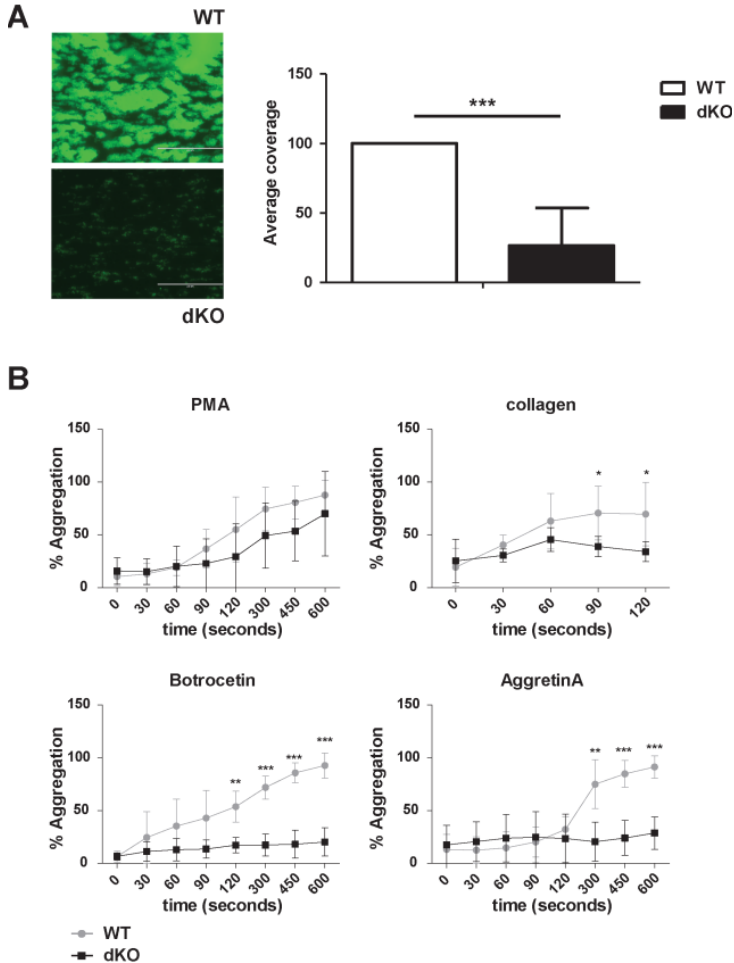


Figure 2. Functional analysis of platelets (A) Sp1/Sp3 dKO display a decreased capacity to form thrombi. **(B)** Sp1/Sp3 dKO platelets responded normally to PMA and slightly lower to collagen. Their response to Botrocetin and Aggretin A were impeded. Mean and standard deviation (SD), * *P<0.05,* ***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

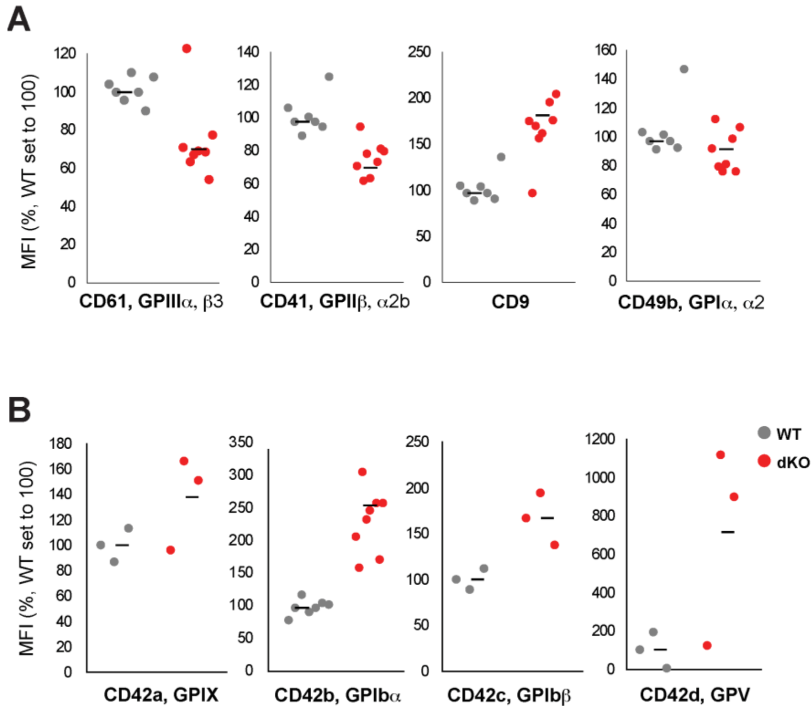


Figure 3. Flow cytometry analysis of platelet receptors (A) Sp1/Sp3 dKO platelets expressed lower levels of fibrinogen receptor subunits (CD61/CD41) and CD49b but a higher level of CD9. **(B)** Increased expression of the various vWF receptor subunits in Sp1/Sp3 dKO platelets.

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.

Megakayocytes 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 blood stream. 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

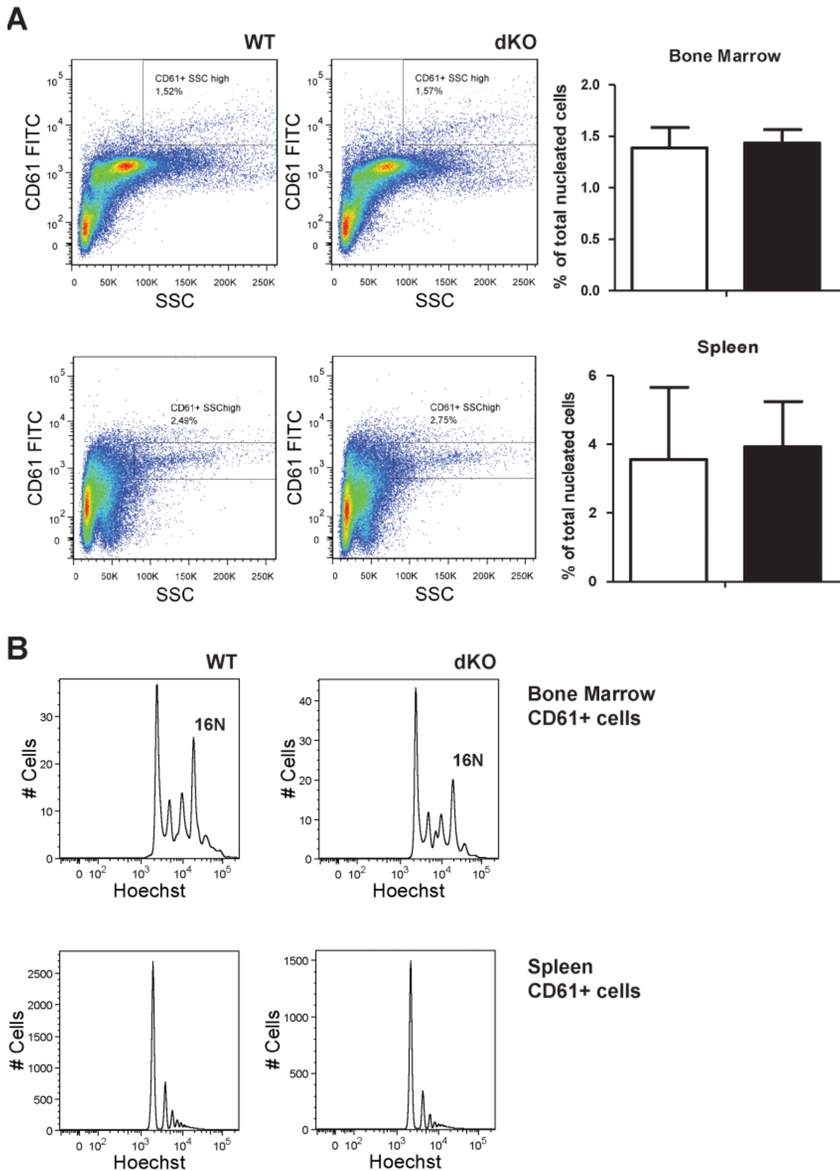


Figure 4. Analysis of megakaryocytes in bone marrow and Spleen (A) Similar numbers of megakaryocytes in the BM and spleen of Sp1/Sp3 dKO and CT mice.**(B)** Similar ploidy status between Sp1/Sp3 dKO and the controls.

megakaryocytes to migrate towards it. Since some of the relevant integrins were downregulated on platelets (Figure 3A), we wondered whether

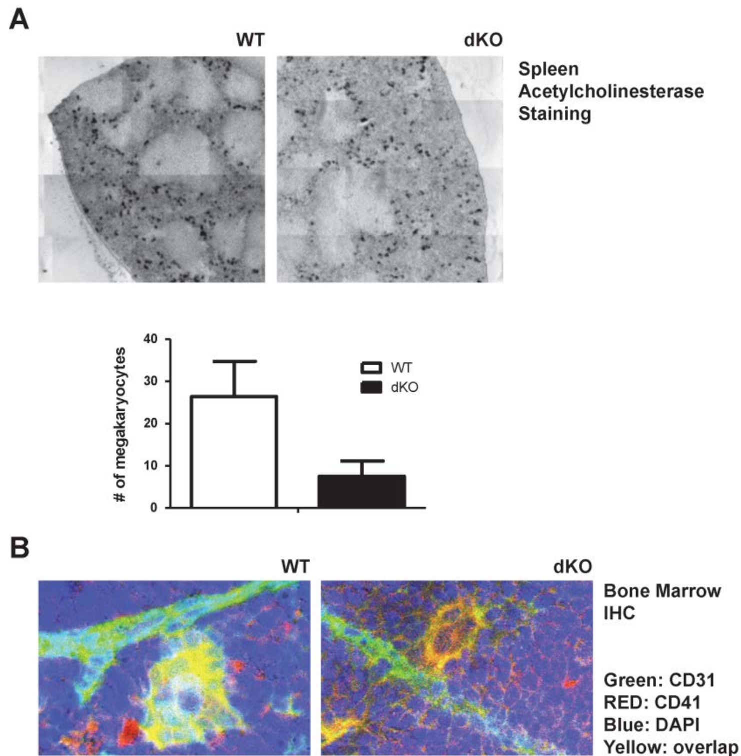


Figure 5. Visualization of Megakaryocytes in the Bone marrow and Spleen (A) Acetylcholinerase staining of splenic section showing reduced numbers of mature megakaryocytes in the Sp1/Sp3 dKO mice. **(B)** IHC on bone marrow section showing normal migration of Sp1/Sp3 dKO megakaryocytes to the vessel wall 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

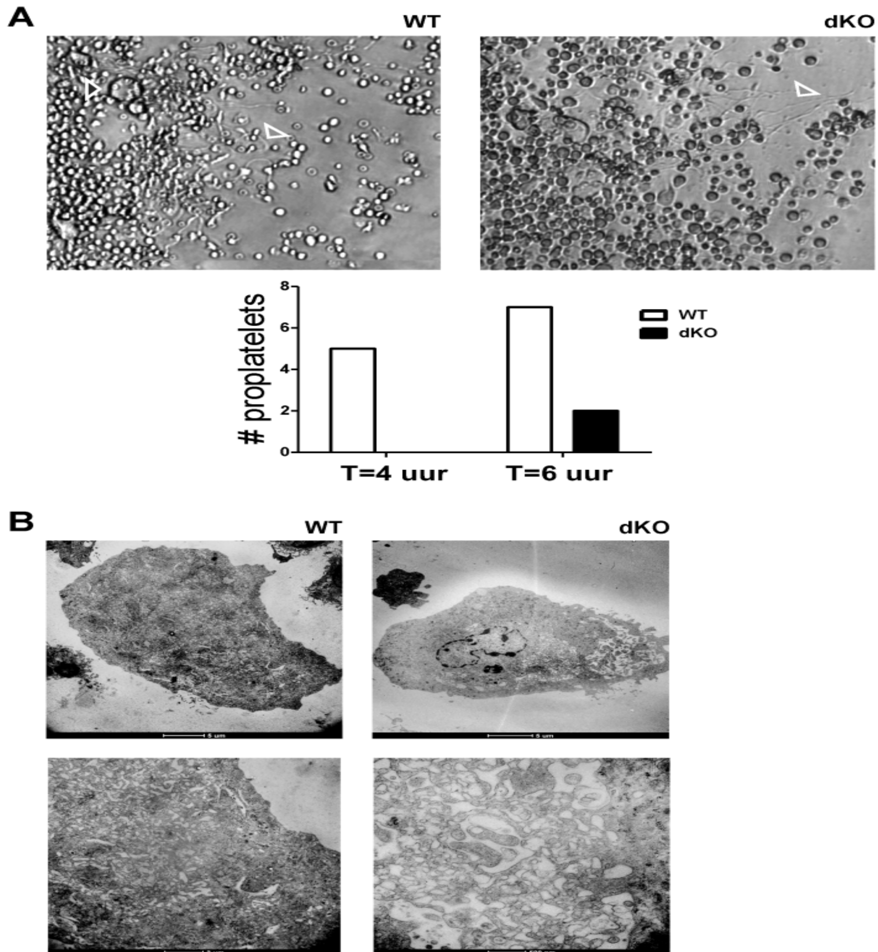


Figure 6. Analysis of cultured megakaryocyte (A) Proplatelet forming megakaryocytes were significantly reduced in BM culture from Sp1/Sp3 dKO mice when compared to the CT. The arrowheads indicates proplatelet filaments protruding from the megakaryocytes **(B)** Electron microscopy showing increased vacuolization and diffuse demarcation membrane system in Sp1/Sp3 megakaryocytes when compared to the controls.

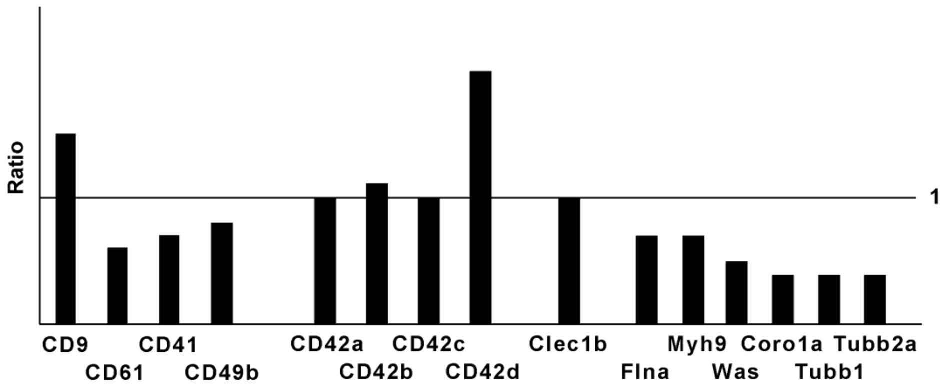
detect megakaryocytes forming proplatelets after 6 hours (Figure 6A), the number was significantly reduced when compared to CT, while the megakaryocytes numbers at the periphery of the explant were not altered (data not shown).

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



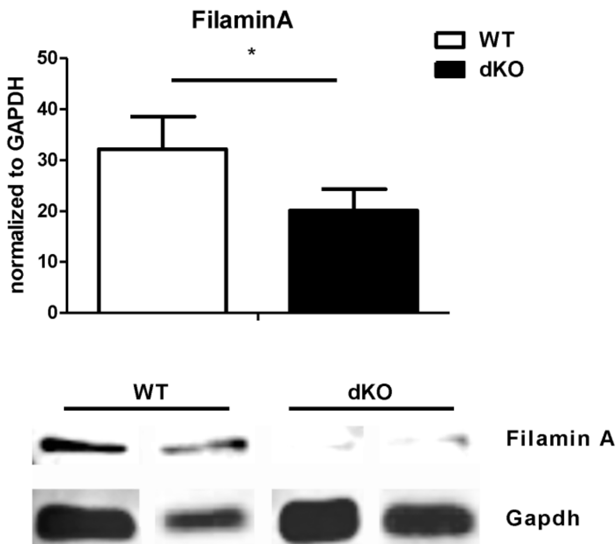


Figure 6. Mass spectrometry analysis of platelet lysate (A) Mostly proteins involved in cytoskeleton network were reduced in Sp1/Sp3 dKO platelets **(B)** The reduction of Filamin A level in Sp1/Sp3 dKO platelets was confirmed by western blotting.

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 GPIIβIIIα (JON/A antibody) compared to the basal GPIIβIIIα 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

1. Philipsen, S. and G. Suske, *A tale of three fingers: the family of mammalian Sp/XKLF transcription factors.* Nucleic Acids Res, 1999. **27**(15): p. 2991-3000.
2. Suske, G., E. Bruford, and S. Philipsen, *Mammalian SP/KLF transcription factors: bring in the family.* Genomics, 2005. **85**(5): p. 551-6.
3. Suske, G., *The Sp-family of transcription factors.* Gene, 1999. **238**(2): p. 291-300.
4. Birnbaum, M.J., et al., *Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3.* Biochemistry, 1995. **34**(50): p. 16503-8.
5. Bouwman, P. and S. Philipsen, *Regulation of the activity of Sp1-related transcription factors.* Mol Cell Endocrinol, 2002. **195**(1-2): p. 27-38.
6. Marin, M., et al., *Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation.* Cell, 1997. **89**(4): p. 619-28.
7. Bouwman, P., et al., *Transcription factor Sp3 is essential for post-natal survival and late tooth development.* EMBO J, 2000. **19**(4): p. 655-61.
8. Gollner, H., et al., *Impaired ossification in mice lacking the transcription factor Sp3.* Mech Dev, 2001. **106**(1-2): p. 77-83.
9. van Loo, P.F., et al., *Transcription factor Sp3 knockout mice display serious cardiac malformations.* Mol Cell Biol, 2007. **27**(24): p. 8571-82.
10. Kruger, I., et al., *Sp1/Sp3 compound heterozygous mice are not viable: impaired erythropoiesis and severe placental defects.* Dev Dyn, 2007. **236**(8): p. 2235-44.
11. Kuhn, R., et al., *Inducible gene targeting in mice.* Science, 1995. **269**(5229): p. 1427-9.
12. Tiedt, R., et al., *Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo.* Blood, 2007. **109**(4): p. 1503-6.
13. Suzuki-Inoue, K., et al., *A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2.* Blood, 2006. **107**(2): p. 542-9.
14. Suzuki-Inoue, K., et al., *Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells.* J Biol Chem, 2007. **282**(36): p. 25993-6001.
15. Bertozzi, C.C., et al., *Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling.* Blood, 2010. **116**(4): p. 661-70.
16. Suzuki-Inoue, K., et al., *Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by*

blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem, 2010. **285**(32): p. 24494-507.

17. Ozaki, Y., K. Suzuki-Inoue, and O. Inoue, *Platelet receptors activated via mulitmerization: glycoprotein VI, GPIb-IX-V, and CLEC-2.* J Thromb Haemost, 2013. **11 Suppl 1**: p. 330-9.
18. Nurden, A.T., K. Freson, and U. Seligsohn, *Inherited platelet disorders.* Haemophilia, 2012. **18 Suppl 4**: p. 154-60.
19. Salles, II, et al., *Inherited traits affecting platelet function.* Blood Rev, 2008. **22**(3): p. 155-72.
20. van de Vijver, E., et al., *Defects in Glanzmann thrombasthenia and LAD-III (LAD-1/v) syndrome: the role of integrin beta 1 and beta 3 in platelet adhesion to collagen.* Blood, 2012. **119**(2): p. 583-586.
21. De Cuyper, I.M., et al., *A novel flow cytometry-based platelet aggregation assay.* Blood, 2013. **121**(10): p. e70-80.
22. Piersma, S.R., et al., *Proteomics of the TRAP-induced platelet releasate.* J Proteomics, 2009. **72**(1): p. 91-109.
23. van Breevoort, D., et al., *Proteomic screen identifies IGFBP7 as a novel component of endothelial cell-specific Weibel-Palade bodies.* J Proteome Res, 2012. **11**(5): p. 2925-36.
24. Chang, C.H., et al., *A novel mechanism of cytokine release in phagocytes induced by aggretin, a snake venom C-type lectin protein, through CLEC-2 ligation.* J Thromb Haemost, 2010. **8**(11): p. 2563-70.
25. Schulze, H., et al., *Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis.* Blood, 2006. **107**(10): p. 3868-75.
26. Rojnuckarin, P. and K. Kaushansky, *Actin reorganization and proplatelet formation in murine megakaryocytes: the role of protein kinase calpha.* Blood, 2001. **97**(1): p. 154-61.
27. Tablin, F., M. Castro, and R.M. Leven, *Blood platelet formation in vitro. The role of the cytoskeleton in megakaryocyte fragmentation.* J Cell Sci, 1990. **97 (Pt 1)**: p. 59-70.
28. Falet, H., et al., *A novel interaction between FlnA and Syk regulates platelet ITAM-mediated receptor signaling and function.* J Exp Med, 2010. **207**(9): p. 1967-79.
29. Cranmer, S.L., et al., *High shear-dependent loss of membrane integrity and defective platelet adhesion following disruption of the GPIbalpha-filamin interaction.* Blood, 2011. **117**(9): p. 2718-27.
30. Van Loo, P.F., et al., *Impaired hematopoiesis in mice lacking the transcription factor Sp3.* Blood, 2003. **102**(3): p. 858-66.
31. Gurney, A.L., et al., *Thrombocytopenia in c-mpl-deficient mice.* Science, 1994. **265**(5177): p. 1445-7.
32. Geddis, A.E., *Megakaryopoiesis.* Semin Hematol, 2010. **47**(3): p. 212-9.

33. Kaushansky, K., et al., *Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin.* Nature, 1994. **369**(6481): p. 568-71.
34. Battinelli, E.M., J.H. Hartwig, and J.E. Italiano, Jr., *Delivering new insight into the biology of megakaryopoiesis and thrombopoiesis.* Curr Opin Hematol, 2007. **14**(5): p. 419-26.
35. Tomer, A., et al., *Bernard-Soulier syndrome: quantitative characterization of megakaryocytes and platelets by flow cytometric and platelet kinetic measurements.* Eur J Haematol, 1994. **52**(4): p. 193-200.
36. Cramer, E.M., *Megakaryocyte structure and function.* Curr Opin Hematol, 1999. **6**(5): p. 354-61.
37. Zucker-Franklin, D., *Megakaryocyte and platelet structure in thrombocytopoiesis: the effect of cytokines.* Stem Cells, 1996. **14 Suppl 1**: p. 1-17.
38. Hartwig, J.H. and J.E. Italiano, Jr., *Cytoskeletal mechanisms for platelet production.* Blood Cells Mol Dis, 2006. **36**(2): p. 99-103.
39. Feng, S., et al., *Filamin A binding to the cytoplasmic tail of glycoprotein Ibalpha regulates von Willebrand factor-induced platelet activation.* Blood, 2003. **102**(6): p. 2122-9.
40. Feng, S., X. Lu, and M.H. Kroll, *Filamin A binding stabilizes nascent glycoprotein Ibalpha trafficking and thereby enhances its surface expression.* J Biol Chem, 2005. **280**(8): p. 6709-15.
41. Williamson, D., et al., *Interaction between platelet glycoprotein Ibalpha and filamin-1 is essential for glycoprotein Ib/IX receptor anchorage at high shear.* J Biol Chem, 2002. **277**(3): p. 2151-9.
42. Schwer, H.D., et al., *A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets.* Curr Biol, 2001. **11**(8): p. 579-86.
43. Eckly, A., et al., *Abnormal megakaryocyte morphology and proplatelet formation in mice with megakaryocyte-restricted MYH9 inactivation.* Blood, 2009. **113**(14): p. 3182-9.

Supplementary Table

Parameter	CT (n = 13)	Sp1/Sp3 dKO (n = 13)	P-value
Platelets	879.1 ± 50.18	341.7 ± 21.87	<0.01
MPV (fl)	5.715 ± 0.06081	8.485 ± 0.09257	<0.01
RBC	9.247 ± 0.1443	9.592 ± 0.1256	NS
WBC	4.046 ± 0.3576	5.077 ± 0.6351	NS

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

Chapter 5

General Discussion

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 $Sp1^{fl/fl}$ or $Sp3^{fl/fl}$ 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::Sp1*$^{fl/fl}$ or *Mx1-Cre::Sp3*$^{fl/fl}$ 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::Sp1*$^{fl/fl}$, *Mx1-Cre::Sp3*$^{fl/fl}$ *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 $Ig\mu^+$ 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 $Ig\mu^+$ 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 *Mb1-Cre::Sp1$^{fl/fl:}$:Sp3$^{fl/fl}$* 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 *Igµ* heavy chain, V_H81X (37), to determine whether the developmental arrest observed in *Mb1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* large pre-B cells was as a consequence of defective *Igh* re-arrangement. We showed that a substantial number of *Mb1-Cre::Sp1$^{fl/fl:}$:Sp3$^{fl/fl}$* B cells were able to progress beyond the large pre-B cell stage albeit not at the level observed for *Sp1$^{fl/fl:}$:Sp3$^{fl/fl}$* 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 *Mb1-Cre::Sp1$^{fl/fl:}$:Sp3$^{fl/fl}$* B cells were not present 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. 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 *Mb1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* cells, we performed genome-wide RNA expression analysis of naïve *Mb1-Cre::VH81X::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* and *VH81X::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* splenic B cells since *Mb1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* cells did not survive. Preliminary analysis has identified hundreds of genes differentially expressed in *Mb1-Cre::VH81X::Sp1$^{fl/fl}$::Sp3$^{fl/f}$* 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 *Mb1-Cre::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* 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 *Mb1-Cre::VH81X::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* 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 *in vivo* and *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 *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 FlnA 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 Fln 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. Interesting, 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::Sp1$^{fl/fl}$::Sp3$^{fl/fl}$* 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 erthroid progenitors and macrophages/granulocytes displayed no obvious phenotypes.

	Cre line	Specificity	Remarks	Reference
1	*Cd4-cre*	Double positive T cells	transgene	(58)
2	*LysM-cre*	Macrophages and granulocytes	Knock-in line	(60)
3	*EpoR-cre*	Early erythroid progenitors	Knock-in line	(61)
4	*Cebpa-cre*	Early myeloid progenitors, liver, lung	Knock-in line	(59)

Table 1. Cre lines used in this study. The cells and tissues where they are expressed are also indicated.

References

1. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132(4):631-44. Epub 2008/02/26.
2. Marin M, Karis A, Visser P, Grosveld F, Philipsen S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell. 1997;89(4):619-28. Epub 1997/05/16.
3. Bouwman P, Gollner H, Elsasser HP, Eckhoff G, Karis A, Grosveld F, et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. EMBO J. 2000;19(4):655-61. Epub 2000/02/17.
4. Gollner H, Dani C, Phillips B, Philipsen S, Suske G. Impaired ossification in mice lacking the transcription factor Sp3. Mech Dev. 2001;106(1-2):77-83. Epub 2001/07/27.
5. van Loo PF, Mahtab EA, Wisse LJ, Hou J, Grosveld F, Suske G, et al. Transcription factor Sp3 knockout mice display serious cardiac malformations. Mol Cell Biol. 2007;27(24):8571-82. Epub 2007/10/10.
6. Van Loo PF, Bouwman P, Ling KW, Middendorp S, Suske G, Grosveld F, et al. Impaired hematopoiesis in mice lacking the transcription factor Sp3. Blood. 2003;102(3):858-66. Epub 2003/04/05.
7. Baur F, Nau K, Sadic D, Allweiss L, Elsasser HP, Gillemans N, et al. Specificity protein 2 (Sp2) is essential for mouse development and autonomous proliferation of mouse embryonic fibroblasts. PLoS One. 2010;5(3):e9587. Epub 2010/03/12.
8. Birnbaum MJ, van Wijnen AJ, Odgren PR, Last TJ, Suske G, Stein GS, et al. Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. Biochemistry. 1995;34(50):16503-8. Epub 1995/12/19.
9. Rose S, Misharin A, Perlman H. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. Cytometry Part A : the journal of the International Society for Analytical Cytology. 2012;81(4):343-50. Epub 2012/01/04.
10. Kishikawa S, Murata T, Kimura H, Shiota K, Yokoyama KK. Regulation of transcription of the Dnmt1 gene by Sp1 and Sp3 zinc finger proteins. Eur J Biochem. 2002;269(12):2961-70. Epub 2002/06/20.
11. Terrados G, Finkernagel F, Stielow B, Sadic D, Neubert J, Herdt O, et al. Genome-wide localization and expression profiling establish Sp2 as a sequence-specific transcription factor regulating vitally important genes. Nucleic Acids Res. 2012;40(16):7844-57. Epub 2012/06/12.
12. Deniaud E, Baguet J, Chalard R, Blanquier B, Brinza L, Meunier J, et al. Overexpression of transcription factor Sp1 leads to gene expression perturbations and cell cycle inhibition. PLoS One. 2009;4(9):e7035. Epub 2009/09/16.

13. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. Science. 1995;269(5229):1427-9. Epub 1995/09/08.
14. Middendorp S, Dingjan GM, Hendriks RW. Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice. J Immunol. 2002;168(6):2695-703. Epub 2002/03/09.
15. Hu T, Ghazaryan S, Sy C, Wiedmeyer C, Chang V, Wu L. Concomitant inactivation of Rb and E2f8 in hematopoietic stem cells synergizes to induce severe anemia. Blood. 2012;119(19):4532-42. Epub 2012/03/17.
16. Battinelli EM, Hartwig JH, Italiano JE, Jr. Delivering new insight into the biology of megakaryopoiesis and thrombopoiesis. Curr Opin Hematol. 2007;14(5):419-26. Epub 2007/10/16.
17. Kruger I, Vollmer M, Simmons DG, Elsasser HP, Philipsen S, Suske G. Sp1/Sp3 compound heterozygous mice are not viable: impaired erythropoiesis and severe placental defects. Dev Dyn. 2007;236(8):2235-44. Epub 2007/06/23.
18. Safe S, Abdelrahim M. Sp transcription factor family and its role in cancer. Eur J Cancer. 2005;41(16):2438-48. Epub 2005/10/08.
19. Ganapathy M, Ghosh R, Jianping X, Zhang X, Bedolla R, Schoolfield J, et al. Involvement of FLIP in 2-methoxyestradiol-induced tumor regression in transgenic adenocarcinoma of mouse prostate model. Clin Cancer Res. 2009;15(5):1601-11. Epub 2009/02/19.
20. Bedolla RG, Gong J, Prihoda TJ, Yeh IT, Thompson IM, Ghosh R, et al. Predictive value of Sp1/Sp3/FLIP signature for prostate cancer recurrence. PLoS One. 2012;7(9):e44917. Epub 2012/10/03.
21. Hagman J, Lukin K. Early B-cell factor 'pioneers' the way for B-cell development. Trends Immunol. 2005;26(9):455-61. Epub 2005/07/20.
22. Liu P, Keller JR, Ortiz M, Tessarollo L, Rachel RA, Nakamura T, et al. Bcl11a is essential for normal lymphoid development. Nat Immunol. 2003;4(6):525-32. Epub 2003/04/30.
23. Maier H, Hagman J. Roles of EBF and Pax-5 in B lineage commitment and development. Semin Immunol. 2002;14(6):415-22. Epub 2002/11/30.
24. Nutt SL, Morrison AM, Dorfler P, Rolink A, Busslinger M. Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. EMBO J. 1998;17(8):2319-33. Epub 1998/05/26.
25. Zhuang Y, Soriano P, Weintraub H. The helix-loop-helix gene E2A is required for B cell formation. Cell. 1994;79(5):875-84. Epub 1994/12/02.
26. Ingram RM, Valeaux S, Wilson N, Bouhlel MA, Clarke D, Kruger I, et al. Differential regulation of sense and antisense promoter activity at the Csf1R locus in B cells by the transcription factor PAX5. Exp Hematol. 2011;39(7):730-40 e1-2. Epub 2011/05/10.

27. O'Riordan M, Grosschedl R. Transcriptional regulation of early B-lymphocyte differentiation. Immunol Rev. 2000;175:94-103. Epub 2000/08/10.
28. Hobeika E, Thiemann S, Storch B, Jumaa H, Nielsen PJ, Pelanda R, et al. Testing gene function early in the B cell lineage in mb1-cre mice. Proc Natl Acad Sci U S A. 2006;103(37):13789-94. Epub 2006/08/31.
29. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001;1:4. Epub 2001/04/12.
30. Melchers F, ten Boekel E, Seidl T, Kong XC, Yamagami T, Onishi K, et al. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. Immunol Rev. 2000;175:33-46. Epub 2000/08/10.
31. Grawunder U, Leu TM, Schatz DG, Werner A, Rolink AG, Melchers F, et al. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity. 1995;3(5):601-8. Epub 1995/11/01.
32. Hendriks RW, Middendorp S. The pre-BCR checkpoint as a cell-autonomous proliferation switch. Trends Immunol. 2004;25(5):249-56. Epub 2004/04/22.
33. Melchers F. The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire. Nat Rev Immunol. 2005;5(7):578-84. Epub 2005/07/07.
34. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. Nat Rev Immunol. 2009;9(3):195-205. Epub 2009/02/26.
35. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. Cell. 2002;109 Suppl:S45-55. Epub 2002/05/02.
36. Jung D, Alt FW. Unraveling V(D)J recombination; insights into gene regulation. Cell. 2004;116(2):299-311. Epub 2004/01/28.
37. Martin F, Chen X, Kearney JF. Development of VH81X transgene-bearing B cells in fetus and adult: sites for expansion and deletion in conventional and CD5/B1 cells. Int Immunol. 1997;9(4):493-505. Epub 1997/04/01.
38. DeFranco AL, Raveche ES, Paul WE. Separate control of B lymphocyte early activation and proliferation in response to anti-IgM antibodies. J Immunol. 1985;135(1):87-94. Epub 1985/07/01.
39. Sieckmann DG. The use of anti-immunoglobulins to induce a signal for cell division in B lymphocytes via their membrane IgM and IgD. Immunol Rev. 1980;52:181-210. Epub 1980/01/01.

40. Grinstein E, Jundt F, Weinert I, Wernet P, Royer HD. Sp1 as G1 cell cycle phase specific transcription factor in epithelial cells. Oncogene. 2002;21(10):1485-92. Epub 2002/03/16.
41. Burchill MA, Yang J, Vang KB, Farrar MA. Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. Immunol Lett. 2007;114(1):1-8. Epub 2007/10/16.
42. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. Immunity. 2010;33(2):153-65. Epub 2010/08/25.
43. Iritani BM, Eisenman RN. c-Myc enhances protein synthesis and cell size during B lymphocyte development. Proc Natl Acad Sci U S A. 1999;96(23):13180-5. Epub 1999/11/11.
44. Schuhmacher M, Staege MS, Pajic A, Polack A, Weidle UH, Bornkamm GW, et al. Control of cell growth by c-Myc in the absence of cell division. Current biology : CB. 1999;9(21):1255-8. Epub 1999/11/11.
45. de Alboran IM, O'Hagan RC, Gartner F, Malynn B, Davidson L, Rickert R, et al. Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity. 2001;14(1):45-55. Epub 2001/02/13.
46. Gartel AL, Ye X, Goufman E, Shianov P, Hay N, Najmabadi F, et al. Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci U S A. 2001;98(8):4510-5. Epub 2001/03/29.
47. Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. Blood. 2007;109(4):1503-6. Epub 2006/10/13.
48. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature. 1994;369(6481):568-71. Epub 1994/06/16.
49. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. Science. 1994;265(5177):1445-7. Epub 1994/09/02.
50. Geddis AE. Megakaryopoiesis. Semin Hematol. 2010;47(3):212-9. Epub 2010/07/14.
51. Cramer EM. Megakaryocyte structure and function. Curr Opin Hematol. 1999;6(5):354-61. Epub 1999/09/01.
52. Zucker-Franklin D. Megakaryocyte and platelet structure in thrombocytopoiesis: the effect of cytokines. Stem Cells. 1996;14 Suppl 1:1-17. Epub 1996/01/01.
53. Falet H, Pollitt AY, Begonja AJ, Weber SE, Duerschmied D, Wagner DD, et al. A novel interaction between FlnA and Syk regulates platelet ITAM-

mediated receptor signaling and function. The Journal of experimental medicine. 2010;207(9):1967-79. Epub 2010/08/18.

54. Hartwig JH, Italiano JE, Jr. Cytoskeletal mechanisms for platelet production. Blood Cells Mol Dis. 2006;36(2):99-103. Epub 2006/02/09.
55. Eckly A, Strassel C, Freund M, Cazenave JP, Lanza F, Gachet C, et al. Abnormal megakaryocyte morphology and proplatelet formation in mice with megakaryocyte-restricted MYH9 inactivation. Blood. 2009;113(14):3182-9. Epub 2008/11/06.
56. Schwer HD, Lecine P, Tiwari S, Italiano JE, Jr., Hartwig JH, Shivdasani RA. A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. Curr Biol. 2001;11(8):579-86. Epub 2001/05/23.
57. D'Addario M, Arora PD, Ellen RP, McCulloch CA. Interaction of p38 and Sp1 in a mechanical force-induced, beta 1 integrin-mediated transcriptional circuit that regulates the actin-binding protein filamin-A. J Biol Chem. 2002;277(49):47541-50. Epub 2002/09/27.
58. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity. 2001;15(5):763-74. Epub 2001/12/01.
59. Wolfler A, Danen-van Oorschot AA, Haanstra JR, Valkhof M, Bodner C, Vroegindeweij E, et al. Lineage-instructive function of C/EBPalpha in multipotent hematopoietic cells and early thymic progenitors. Blood. 2010;116(20):4116-25. Epub 2010/09/03.
60. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 1999;8(4):265-77. Epub 2000/01/06.
61. Heinrich AC, Pelanda R, Klingmuller U. A mouse model for visualization and conditional mutations in the erythroid lineage. Blood. 2004;104(3):659-66. Epub 2004/04/20.

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 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 *w*e found that *Mx1-Cre::Sp1fl/fl::Sp3fl/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_H81X 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 *Sp1fl/fl::Sp3fl/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_H81X, maar stelde de cellen wel in staat om te vorderen voorbij het grote pre B-cell stadium. Onze data onthulde dat B-cel 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 ontregelde 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 hematopoëtische 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 thrombopoietine 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

Personal details

Name: Divine Ikome Kulu
Birthdate: 24th January 1981
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

1995 - 2000 Secondary School; General Certificate of Education, Ordinary 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 Denstitry 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

- Kulu DI, Meinders M, Janssen H, Hoogenboezem M, Mul E, Poplonsky T De Cuyper, Meijer S, Krüger I, Suske G, Gutiérrez L and Philipsen S: **Sp1 and Sp3 co-operate to regulate megakaryopoiesis**
  Manuscript in preparation

- Riepsaame J, Bruens ST, Jaegle UE, Kulu DI, van Oudenaren A, van IJcken WF, Philipsen S and Leenen PJM: **MicroRNA-mediated expression of transcription factor Sp1 controls early stage dendritic cell differentiation.**
  Manuscript submitted

- Ingram RM, Valeaux S, Wilson N, Bouhlel MA, Clarke D, Krüger I, Kulu DI, Suske G, Philipsen S, Tagoh H and Bonifer C: **Differential regulation of sense and antisense promoter activity at the Csf1R locus in B cells by the transcription factor PAX5.**
  Exp Hematol. 2011;39(7):730-40

- Bergink S, Theil AF, Toussaint W, De Cuyper IM, Kulu DI, Clapes T, van der Linden R, Demmers JA, Mul EP, van Alphen FP, Marteijn JA, van Gent T, Maas A, Robin C, Philipsen S, Vermeulen W, Mitchell JR, Gutiérrez L: **Erythropoietic Defect Associated with Reduced Cell Proliferation in Mice Lacking the 26S Proteasome Shuttling Factor Rad23b.**
  Manuscript accepted in Mol. Cell. Biol. Doi:10.1128/MCB.05772-11 (2013)

PhD Portfolio

Summary of PhD training and teaching

Name PhD student : **Divine Ikome Kulu** Erasmus MC Department : Cell Biology Research School : Molecular Medicine	PhD period: Sept 2009 – Sept 2013 Promotors : prof.dr. Sjaak Philipsen Supervisors : prof.dr. Sjaak Philipsen Prof.dr. Rudi Hendriks

1. PhD training	
	Year
General courses	
- Laboratory animal science (Article 9)	2010
- Cell and Developmental Biology	2010
- Biochemistry and Biophysics	2010
- Safely working in the laboratory	2010
- Epigenetic regulation	2010
- Technology Facilities	2010
- Transgenesis, gene targeting and *in vivo* imaging	2011
- Biomedical Research Techniques	2011
- Innovative mouse models	2011
- The Advanced Course “Molecular Immunology“	2012
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 21th MGC-Symposium, Leiden, The Netherlands	2011
- The 18th MGC PhD workshop, Maastricht, The Netherlands (Poster presentation)	2011
- The 22th 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 2009-2013
- Monday Morning Meetings	
Presentations	
- Monday Morning Meetings	2009-2013
- Work discussions	2009-2013

International conferences - Chromatin changes in differentiation and malignancies, Giessen, Germany (poster presentation) - XXIV Congress of the International Society on Thrombosis and Hemostasis, Amsterdam, The Netherlands (ePoster Presentation)	 2011 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.

I will start by thanking the one and only one person who made this day possible, my boss, Prof. Dr. Sjaak Philipsen. Dear Sjaak, words cannot explain how grateful I am to you for giving me the opportunity to develop as a researcher in your laboratory. Thank you very much for accepting me as the newest member of the Sp transcription factor family, a family that will always be part of me☺. Thank you for your patience and stimulation to work on those interesting experiments.

Prof. Dr. Frank Grosveld, i cannot think of any other better department to do my research other than the department of cell biology. Thank you for your superb managerial skills and critical questions doing the Monday morning meetings. It always made me to go back, think and work even harder.

Prof. R.W. Hendriks, I want to thank you for allowing me to do experiments in your lab. Your guidance and meetings as well as the useful discussions were very much appreciated.

Prof. dr. G. Suske, as one of the fore fathers of Sp transcription factors, I want to thank for the collaborations that we had over the years and for accepting to be part of my PhD defense committee member.

To members of my small committee, Prof.dr. R.W. Hendriks, Prof.dr. J.H. Gribnau, Dr.ir. Gert Jansen, thank you very much for creating the time to read my thesis in such a short time. Your useful comments were very much appreciated.

My lab colleagues at lab 716 both past and present, you were amazing to work with. Nynke, thank you so much for always willing to assist me if i need anything in the lab, Thank you for the support and the experiments that we did together. Thamar, thank you for many assistance in the lab. Anna, you were very talented in the lab. I wish you success in your future carrier. Ileana and Silvia, I wish you both successes in completing your phD.

To my paranymphs 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 paranymphs. 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.

Marjolein de Bruijn, I am very sure that this thesis could not have been the same without you. Beside your technical expertise, you were very fun to work with. Thank very much for all the times that we spent together doing experiments. I appreciate every moment of it.

Laura and Marjolein, thank you so much for the collaborations that we had together. I learned a lot from our joint experiments and resulting publications.

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.

Mariëtte thank you so much for your many assistance especially with the thesis cover design.

Lab 710, Alex, Rick, Dubi, thank you for being very nice people. Maaike, Martí and Raymond from Lab 706, I enjoyed your company. Anita, Andrea, Petros and Xiao from lab 702, I had wonderful times with you all. Xiao I will never forget the day we miss the train en route to Kleinwarsetal, you were a nice company☺.

PC-support team, Pim, Ton, Leo and Sjozef, thank you all for always responding to my PC problems. Melle, Leo, Enno and Koos thanks for the ordering our reagents and making sure that travelling expenses were always re-imbursed ☺. Bep, Jasperina and Marike you were very efficient in performing you secretarial duties. Marike, thank you for signing the portfolio for my inburgeringscursus.

To my buddy Joshua, thank you for being a true friend and a brother. We had many good times together. Your idea and critical thinking helped me complete my thesis. I will always remember the 10th floor because of you ☺

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