Zeb2 is essential for terminal differentiation of multiple hematopoietic lineages

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DECLARATION

I hereby declare, that this Doctoral Thesis, entitled "Zeb2 is essential for terminal differentiation of multiple hematopoietic lineages", is original research work and was written independently, using no other sources and aids than cited. I furthermore declare that wherever contributions of others are involved, this contribution is indicated, clearly acknowledged.

Bonn, September 2015

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Abrrevation

AGM	aorta-gonad-mesonephros
AML	acute myeloid leukemia
APC	allophycocyanin
BasoE	basophilic erythroblast
BFU-E	burst forming unit-erythroid
BrdU	bromdesoxyuridin
CFU-E	colony forming unit-erythroid
CLP	common lymphoid progenitor
DNMT	DNA methyltransferase
EMT	epithelia-mesenchymal transition
ETP-ALL	early T-cell precursor leukemia
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GO	gene ontology
GMP	granulocyte-macrophage progenitor
Hb	hemoglobin
HCT	hematocrit
HSC	hematopoietic stem cell
IHRES	internal ribosome entry site
IL	interleukin
iPS	induced pluripotent stem cell
JAK	janus kinase
KO	knockout
LKS	lineage negative, c-Kit positive, Sca-1 positive population
LMPP	lymphoid-primed multipotent progenitor
LT-HSC	long-term hematopoietic stem cell
MCV	mean corpuscular volume
MEP	megakaryocyte-erythroid progenitor
MDS	myelodysplastic syndrome
MPN	myeloproliferative neoplasm
MPP	multipotent progenitor cell
MWS	Mowat-Wilson syndrome
NuRD	nucleosome remodeling and histone deacetylation
OrthoE	orthochromatic erythroblast
PE	phycoerthrin
PerCP	peridinin chlorophyll protein
PFA	paraformaldehyde
PHZ	phenylhydrayine
PolyE	polychromatic erythroblast

Poly I:C	polyinosinic:polycytidylic acid
PRC2	polycomb repressor complex 2
ProE	proerythroblast
RBC	red blood cell
RIPA	radio-immunoprecipitation assay
RT	room temperature
TO	thiazole orange
qRT-PCR	quantitative real time polymerase chain reaction
RRBS	reduced representation bisulfite sequencing
ST-HSC	short-term hematopoietic stem cell
STAT	signal transducer and activator of transcription
UTR	untranslated region
WT	wild type

1. Introduction

1.1 Hematopoiesis

1.1.1 Sites of hematopoiesis

Hematopoiesis is the formation and developmental process of all types of peripheral blood and bone marrow cells. The primary sites of hematopoiesis change during the development of vertebrates. In various mammal species, it first takes place in the embryonic yolk sac blood islands. At later stages of development it moves to the aorta-gonad-mesonephros (AGM) region, placenta, fetal liver, spleen, and finally to the bone marrow, which then remains the major hematopoietic organ (Dzierzak and Speck, 2008).

In an adult organism, bone marrow is the main location of hematopoietic cell production. However, in case of hematopoietic stress or medullary insufficiency, hematopoiesis will relocate to other organs, like liver or spleen. This is called extramedullary hematopoiesis (Munker and Munker, 2006).
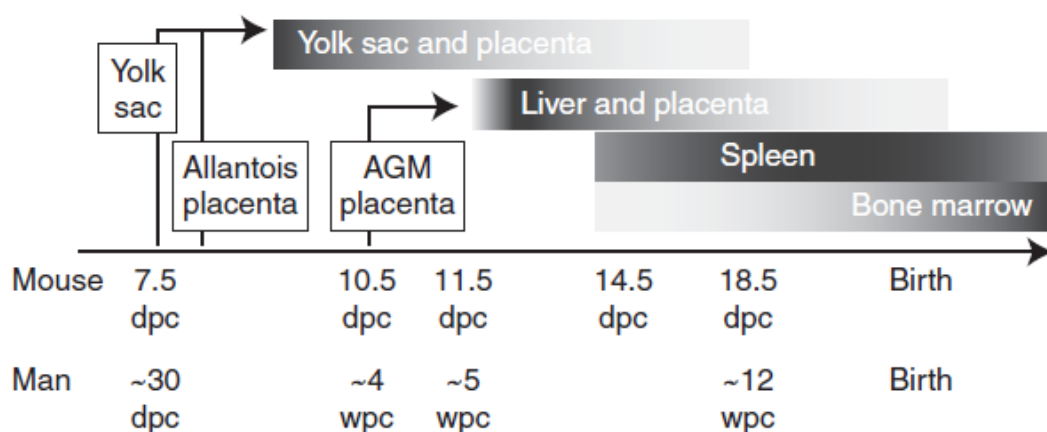
Figure 1.1 Timeline of embryonic mouse and human hematopoietic development (Rieger and Schroeder, 2012). The primitive wave takes place in the yolk sac to generate primitive erythroid cells, and rapidly replaced by second wave, which also occurs in yolk sac but produce definitive erythroid/ myeloid progenitors. The third wave emerges first functional HSC generation in AGM region. Those HSCs soon home to and expand in the fetal liver and eventually seed the bone marrow. dpc, days after conception; wpc, weeks after conception.

1.1.2 Hematopoietic stem cells

Hematopoiesis is maintained by a small population of hematopoietic stem cells (HSCs), which are capable of self-renewal and giving rise to all hematopoietic lineages. As shown in Fig. 1.1 long-term hematopoietic stem cells (LT-HSCs) can replenish themselves by one of the daughter cell by asymmetric division, and the other daughter cell will differentiate into short-term hematopoietic stem cells (ST-HSCs), which are also multipotent, but only retain self-renewal capacity for a limited time (≈8 weeks) (Morrison and Weissman, 1994). ST-HSCs then differentiate into multipotent progenitor cells (MPPs), which in turn generate oligopotent megakaryocyte-erythroid progenitors (MEPs), granulocyte-macrophage progenitors (GMPs), and common lymphoid progenitors (CLPs). Those progenitor cells are responsible for erythropoiesis and megakaryocytopoiesis, myelopoiesis, and lymphopythoiesis respectively, and finally can give rise to functionally mature cells, including erythrocytes (red blood cells), thrombocytes (platelets), monocytes, granulocytes, B- and T-lymphocytes, etc. Each progenitor and mature blood cell type can be

distinguished by their surface antigen expression on the cell surface by the so

called immunophenotype (Kiel et al., 2005; Passegue et al., 2003).
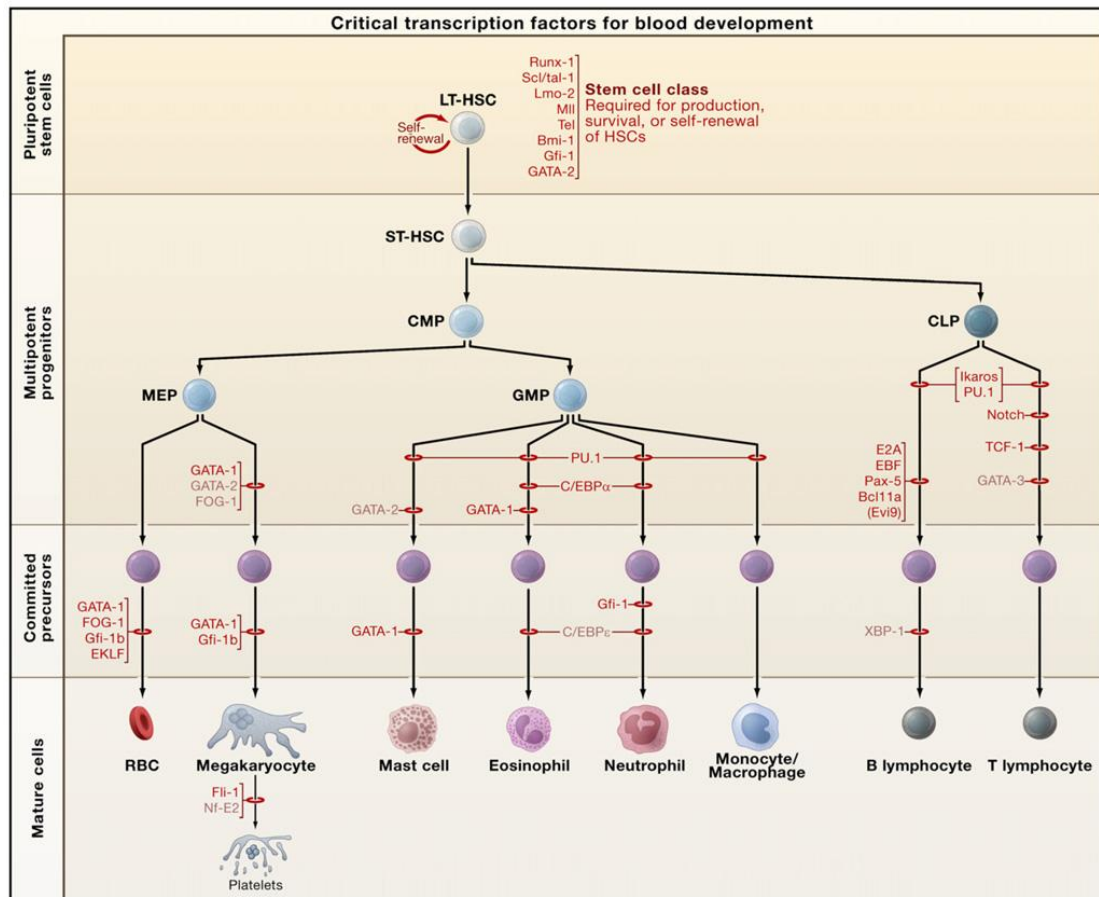
Figure 1.2 Overview of hematopoietic differentiation hierarchy (Orkin and Zon, 2008) HSC: hematopoietic stem cell, CLP: common lymphoid progenitor, CMP: common myeloid progenitor, MEP: Megakaryocyte-erythrocyte progenitor, GMP: granulocyte-macrophage progenitor

1.1.3 Transcription factors in hematopoietic development and lineage choice

To continuously generate an suitable amount of functional blood cells, HSCs

have to undergo cell-fate decisions to turn from quiescent state to proliferation,

self-renewal and differentiation. This processes are regulated by both extrinsic and intrinsic factors. As an important element of intrinsic regulatory network, many lineage specific nuclear regulators have been identified to govern HSC behavior (Fig. 1.1) (Orkin, 1995; Orkin and Zon, 2008; Zhu and Emerson, 2002).

The essential role of stem cell leukemia (SCL), also known as Tal1 in hematopoiesis was originally revealed by chromosomal translocation studies associated with T cell acute lymphoblastic leukemia coincidently (Begley et al., 1989). SCL deletion in mice is lethal due to incapabillity of generating erythroid cells in the yolk sac, and SCL-/- embryonic stem cells cannot give rise to all myeloid and lymphoid cells (Porcher et al., 1996; Shivdasani et al., 1995). As a protein partner of SCL, the LIM-finger protein LMO2 can functionally interacts with SCL, and plays an indispensable role in primitive and definitive hematopoiesis (Orkin and Zon, 2008; Yamada et al., 1998). Furthermore, SCL and Lmo2 can form a multiprotein complex with Ldb1, E2A and Gata1 in erythroid cells. This complex recognizes and binds to a GATA-E-box DNA motif in which the SCL-E2A component interacts with E-box site and Gata1 binds to the GATA motif (Wadman et al., 1997). Those interactions indicate a broad physiological role of SCL and Lmo2 at distinct stages of hematopoiesis.

The GATA family plays a crucial role during hematopoietic development. Gata2 is highly expressed in hematopoietic stem and progenitor cells (Orlic et al., 1995). Mice lacking Gata2 die from deficient primitive hematopoiesis and

severe anemia before embryonic day 10.5 (Tsai et al., 1994). Studies in Gata2-/+ animal model show its role in HSC generation and proliferation during embryo development, while HSC apoptosis and cellular quiescence in adult hematopoiesis respectively (Ling et al., 2004; Rodrigues et al., 2005). Many Gata2 mutations are identified in human disorders relative to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Dickinson et al., 2011; Hsu et al., 2011; Ostergaard et al., 2011). In contrast, Gata1 and Gata3 are responsible for lineage specific differentiation. Gain and loss of function studies have shown that Gata1 effects erythroid cells (Fujiwara et al., 1996; Leonard et al., 1993), megakaryocytes, mast cells (Martin et al., 1990) and eosinophils (Zon et al., 1993), while Gata3 is required for T-cell development (Ting et al., 1996; Yamagata et al., 2000).

Runx1 is a transcription factor that plays a critical role in the emergence of HSC by forming a core binding factor (CBF) complex with another non-DNA-binding protein CBFβ. Its disruption is embryonicaly lethal due to the absence of fetal liver hematopoiesis (Okuda et al., 1996). Using mice with Runx1-driven *LacZ* expression of Runx1 was first detected in mesenchymal cells from yolk sac where hematopoietic cells emerge: In addition, all HSCs show Runx1 expression in transplantation assays. Furthermore, Runx1 dosage could affect the number of CD45 molecules, as well as the distribution of HSCs between CD45+, endothelial and mesenchymal cell pools (North et al., 1999; North et al., 2002). However, inducible Runx1 deletion in adult

hematopoietic cells revealed that it is critical for megakaryocytic maturation and B-, T- lyphopoiesis, but only limited role in HSC maintenance (Ichikawa et al., 2004).

The differentiation of multipotent progenitors to committed precursor or mature cells involves numerous of lineage specific factors. They are not only promoting and maintaining their own lineage differentiation but also repressing factors favoring other lineages simultaneously.

It has been reported that the overexpression of Gata1 or the ETS factor PU.1 blocks myeloid or erythroid differentiation respectively. PU.1 is capable of suppressing erythroid gene transcription by inhibiting Gata1 DNA-binding capacity through direct protein-protein interactions, while Gata1 represses myeloid gene expression by directly binding to the PU.1 ETS domain (Nerlov et al., 2000; Zhang et al., 2000). This was later confirmed in vivo in zebrafish (Rhodes et al., 2005). Moreover, both PU.1 and Gata1 showed positive auto-regulation for their own expression (Fig 1.3.D) (Kobayashi et al., 2001; Okuno et al., 2005). Klf1 and Fli1 is another pair of cross-antagonistic transcription factors that antagonize each other in the regulation of erythroid versus megakaryocyte development (Starck et al., 2003). Recent study shows that Runx1 inhibits erythroid differentiation by shifting the balance between Klf1 and Fli1 in the direction of Fli1 (Kuvardina et al., 2015). Gfi1 blocks PU.1 induced macrophage differentiation but increases granulocyte differentiation simultaneously by physically interacting with PU.1 (Dahl et al., 2007).
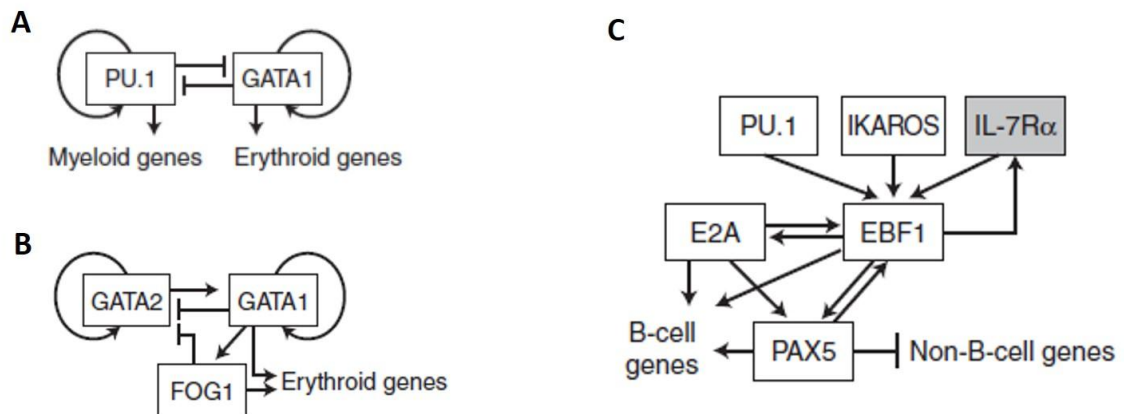
Figure 1.3 Network motifs in distinct lineage commitment (Vassiliou et al., 2012). Simplified examples of molecular activation and repression networks in myeloid (A), erythroid (B), and B-lymphoid (C) cells. Transcription factors are depicted in white, surface receptors in gray.

After the lineage choice has been made, a set of distinct genes are needed to maintain the lineage commitment and produce the right number and type of mature cells. During erythropoiesis, besides the Gata/SCL/Lmo2/Ldb1 transcriptional complex mentioned above, Gata1 binds Fog1 (friend of Gata1), TRAP220 (thyroid hormone receptor) and Sp1 by its N-terminal zinc finger as well, whereas binds CBP (CREB-binding protein), EKLF (erthroid Kruppel-like factor) and PU.1 by C-terminal zinc finger (Kim and Bresnick, 2007). As the cofactor of Gata1, Fog1 mediates the Gata2 displacement by interacting with Gata1 to promote terminal erythropoiesis (Fig. 1.3A) (Grass et al., 2003). EKLF is known as a determinant of hemoglobin switch, which binds CACCC motif of β–globin promoter directly (Miller and Bieker, 1993).

The B-cell lineage commitment is mediated by a hierarchical network of key

transcription factors (Fig. 1.3B). Ikaros, E2A and PU.1 play critical roles in early lymphoid cell fate decision by functioning parallel or downstream of IL-7R signaling, and inducing early B-cell factor (EBF) and Pax5. E2A-deficient mice show a developmental block at the Prepro-B cell stage with dramatically reduced Rag1, mb-1, CD19 and lambda5 expression in fetal liver and compromised immunoglobulin D-J rearrangements (Bain et al., 1994; Borghesi et al., 2005). However, the B-cell differentiation arrest in the absence of E2A can be rescued with retroviral expression of EBF and ectopic Pax5 expression (Kwon et al., 2008; Seet et al., 2004). EBF is a major B cell transcriptional determinant that was suggested to act upstream of Pax5 and downstream of E2A, which binds to EBF promoter and activates its transcription (Smith et al., 2002). More recently, distinct EBF isoforms were found due to two promoters, distal promoter, which is activated by IL-7 signaling, E2A and EBF, and proximal promoter, which is activated by Pax5, Ets1 and PU.1 (Roessler et al., 2007). As the guardian of B cell identity and function, Pax5 is required for B lymphopoiesis in fetal and adult (Nutt et al., 1997). Interestingly, pro-B cells lacking Pax5 are able to differentiate into functional macrophages, granulocytes, dendritic cells, osteoclasts and natural killer cells in culture in the presence of lineage appropriate cytokines , suggesting that Pax5 plays a critical role in maintaining B cell fate by suppressing alternative lineage genes (Nutt et al., 1999). Signal transducer and activator of transcription 5 (STAT5) is activated by IL-7 signaling. Conditional deletion of Stat5 results in an early

block in B cell development, which could be rescued by EBF and Pax5 expression. Moreover, many other transcription factors, including Foxo1, MYB, MIZ1 are required for proper B cell development (Clark et al., 2014).

1.1.4 Epigenetic regulation of hematopoietic stem cell development

While the focus on investigation of hematopoietic stem cell regulation has been put predominantly on the transcription factors, recent studies have indicated that the maintenance and differentiation of HSCs is also regulated by another intrinsic factor, the epigenetic modifications, which include DNA methylation, histone modification, chromatin remodeling, as well as micro and noncoding RNAs.

DNA methylation

DNA methylation is a biochemical process that involves the addition of a methyl group to the cytosine or adenine nucleotides and mediates transcriptional silencing. It generally takes place at the C5 position of CpG dinucleotides and catalyzed by two classes of DNA methyltransferases (Dnmts), the maintenance methytransferase (Dnmt1), which preserves methylation patterns during DNA replication, and *de novo* methyltransferases (Dnmt3a and Dnmt3b), which sets up DNA methylation early in development (Chuang et al., 1997; Okano et al., 1999). In contrast, Tet methylcytosine

dioxygenase Tet1, Tet2 and Tet3 have been identified as DNA demethylation catalases to convert 5-methylcytosine (5-mC) into 5-hydroximethylcytosines (5-hmCs), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) through consecutive oxidation reactions (Ito et al., 2010; Koh et al., 2011).

Over the past decade, analyses of knockout mouse models helped better understand the roles of DNA modifiers in hematopoiesis (Table 1.1). HSC lacking Dnmt1 activity exhibited increased transcription of key myeloerythroid associated regulators, including Gata1, ID2, and Cepba, but not lymphoid related factors, which lead to imbalanced myeloid and lymphoid differentiation (Broske et al., 2009a). Conditional ablation of Dnmt3a expanded HSC numbers while simultaneously impaired HSC differentiation ability in serial transplantations. Both hyper- and hypo-methylation occured in Dnmt3a-null HSCs. Some multipotency genes were selectively upregulated, including Runx1 and Gata3 while some differentiation factors were suppressed, such as Flk2, Ikaros and PU.1(Challen et al., 2012).

Table1.1 Roles of DNA modifiers in hematopoiesis (Challen et al., 2012)

Gene	Type of mouse	Pre-Tp defect	Tp defect	Increased engraftment	Development	Self-renewal	Differentiation	Lineage specification	Cells tested	PMID
Dnmt1	cKO		++		+	+	+	+	BM	19801979
Dnmt3a	cKO			+	+	+	+		HSC	22138693
Tet2	KO, cKO	+		+		+			HSC, KSL	21723200
Tet2	KO	+		+					KSL	21873190
Tet2	GT, cKO	+		+					HSC, KSL	21723201
Dnmt3b[a]			nl						HSC	17420264
Tet1[a]		nl								21816367
Tet3[a]										

Summary of hematopoietic phenotypes in mouse models after mutation of genes encoding DNA modifiers. KO, knockout; cKO, conditional KO; GT, gene rap; Tp, transplantation; nl, apparently normal. Tp defect indicates a phenotype observed upon transplantation with ++ signifying a severe defect (reduction in PB output to less than 20% normal). Other columns indicate the processes the gene has been demonstrated to play a role in. BM, whole bone marrow; PMID, PubMed ID number.
[a]Indicates genes that require further investigation into hematopoietic-specific phenotypes.

<u>Histone modification</u>

As the basic unit of DNA packing, nucleosome consists of eight histone proteins and tightly wrapped DNA winds. The long tails of Histones can be modified by methylation, acetylation, phosphorylation and ubiquitination, which further alters the nucleosome structure either to be more compact or loose, which is associated with gene silencing or activation respectively. Acetylation and methylation are the most investigated histone modifications. Histone acetylation is executed by two classes of enzymes: histone acetyltransferases (HATs), which add acetylation marks to lysine residues, and histone deacetylases (HDACs), which remove acetyl group from histone tails. Similarly, histone methylation involves two opposing enzymes: histone methytransferases (HMTs) and histone demethylases (HDMs), leading to append or detach methyl groups respectively.

Most histone modifiers have been identified in hematopoietic progenitors, in particular activating H3K4me, H3K9ac and repressive H3K7me marks. Some "bivalent" genes bear both conflicting modifications and are poised for expression in undifferentiated ES cells. During differentiation, either silencing of some genes or activation of gene expression will be conducted according to the tissue specific development (Fig. 1.5) (Spivakov and Fisher, 2007).
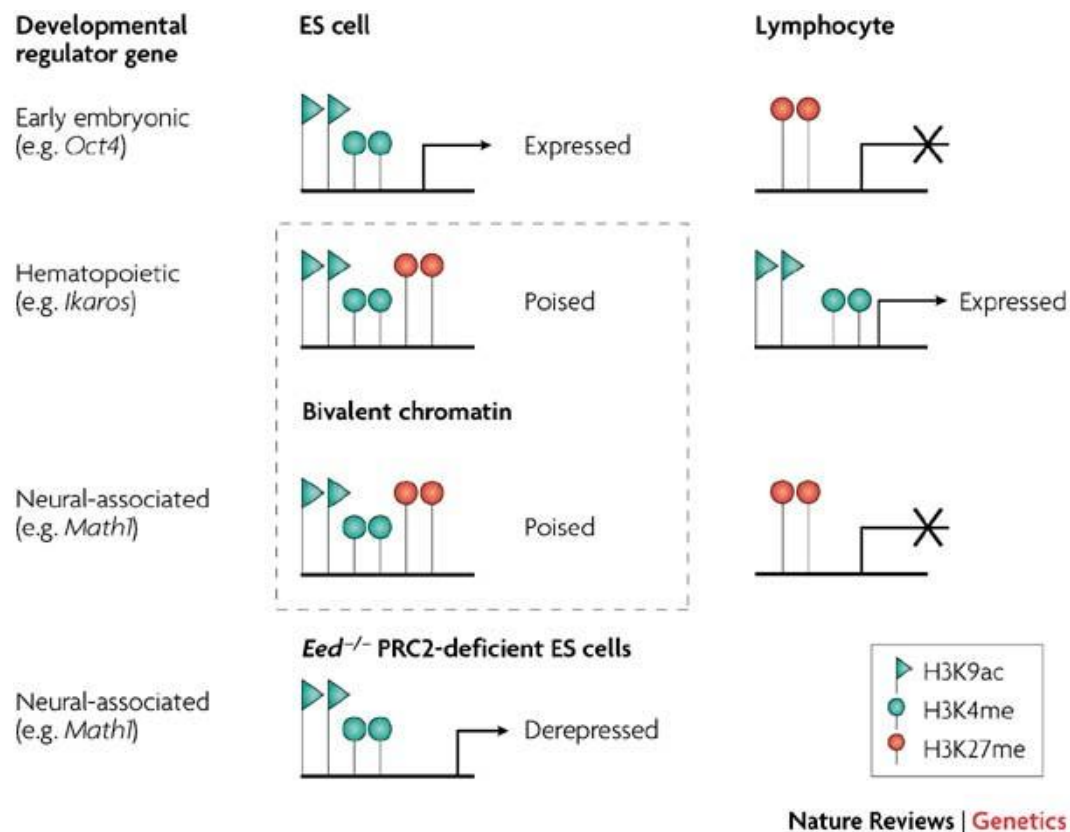
Figure 1.5 Bivalent epigenetic signatres in ES cells (Spivakov and Fisher, 2007).

Methylation of H3K27 is catalyzed by polycomb repressor complex 2 (PRC2), which consists of embryonic ectoderm development (Eed), suppressor of zeste12 (Suz12) and the HMTase enhancer of zeste homologue 2 (Ezh2). Mice lacking functional PRC2 components display enhanced hematopoietic stem and progenitor cell (HSPC) activity, suggesting that PRC2 is restricting hematopoietic stem and progenitor pool size (Majewski et al., 2010). Some evidence indicates that Ezh2 can recruit all three Dnmts to specific PRC2-target promoters for DNA methylation in vivo, providing a link between DNA methylation and histone modification (Vire et al., 2006).

1.2 Zeb2

1.2.1 Structure of Zeb2

Zeb2, the Zinc Finger E-Box-Binding Homeobox 2, also named Sip1 and ZFHX1B, is a DNA-binding transcriptional regulator, which dimerize with E-box motif in different promoters. ZEB2 protein consists of 1,214 amino acids encoding by this gene belong to 2-handed zinc finger/homeodomain proteins family Zfh1. These proteins are characterized by a homeodomain flanked by two separated, highly conserved zinc finger clusters: one N-terminal and one C-terminal, which contain four and three zinc fingers respectively. Each zinc finger cluster can bind independently to CACCT(G) sequences in promoter regions of genes involved in differentiation and development, such as the Xenopus Xbra2 promoter, the human a4-integrin promoter and the E-cadherin promoter (Remacle et al., 1999; Verschueren et al., 1999). The Smad interacting domain (SID) binds to phosphorylated receptor activated Smads (R-Smads), which regulate TGF-β and BMP signaling pathway. The CtBP interacting domain (CID) interacts with CtBP, which complex with histone deacetylases and methyltransferases (Sanchez-Tillo et al., 2011).
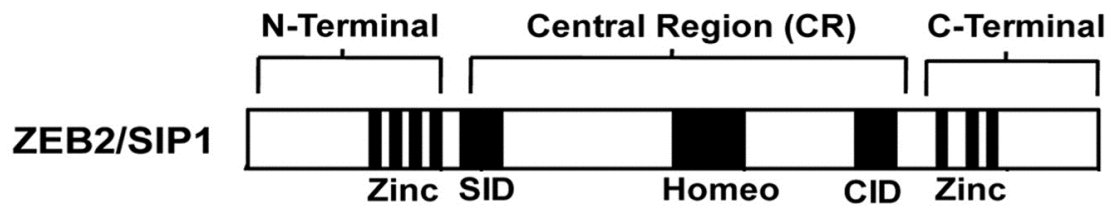
Figure 1.6 Motif of ZEB2 protein structure. SID, Smad-interacting domain; CID, CtBP-interacting domain.

1.2.2 ZEB2 and MWS

Mutations and deletions of ZEB2 may link to severe diseases, such as Mowat-Wilson syndrome (MWS), which characterized by a recognizable facial phenotype: widely spaced eyes, broad medial eyebrows, low hanging columella, prominent or pointed chin, uplifted earlobes with a central depression, open-mouthed expression. And structural anomalies which may include: Hirschsprung disease (Wakamatsu et al., 2001), congenital heart defects (particularly abnormalities of the pulmonary arteries and/or valves), urogenital anomalies (particularly hypospadias in males), agenesis or hypogenesis of the corpus callosum, Structure eye defects (microphthalmia and Axenfeld anomaly). In addition, functional differences including: moderate to severe intellectual disability, severe speech impairment with relative preservation of receptive language, seizures, growth retardation with microcephaly are common (Ariss et al., 2012; Meral et al., 2012; Saunders et al., 2009).

Recently, Linda Pons et al report that spleen hypo-/aplasia is a part of the MWS phenotype and makes Zeb2 a possible candidate gene for primary

asplenia (Pons et al., 2014). Mowat-Wilson is caused by de novo heterozygous loss of function mutations including nonsense mutations, frameshift mutations, and deletions in Zeb2 at 2q22. Over 100 distinct mutations of ZEB2 have been described. By sequencing analyses, approximately 80% of Mowat-Wilson syndrome patients have a nonsense or frameshift mutation detectable, with the rest having gross deletions necessitating a dosage sensitive assay (Dastot-Le Moal et al., 2007; Saunders et al., 2009; Yamada et al., 2014).

1.2.3 Zeb2, a repressor of SMADs

TGFbeta/BMP signaling is highly conserved in different animal species. Ligands from extracellular environment transmit the signals to the cell by binding to type II receptors, which form heterodimers with type I receptors. This activates the receptor's serine/threonine kinase activity to phosphorylate and activate SMAD transcription factors, leads to nuclear translocation of Smad proteins, which activate or inhibit transcription of specific target genes (Massague, 2000).

ZEB family of zinc finger factors were found to be involved in regulation of TGF-β/BMP signaling pathway. Both, ZEB-1 and ZEB-2 effect in opposite ways: ZEB-2 represses Smad-mediated transcriptional activation, while ZEB-1 synergize with it. These antagonistic effects by the ZEB proteins arise from the differential recruitment of transcriptional coactivators and corepressors to the

Smads. In detail, ZEB-1 binds to p300 and promotes the formation of a p300-Smad transcriptional complex, while ZEB-2 acts as a repressor by recruiting CtBP (Postigo et al., 2003).

1.2.4 ZEB2 induce EMT

Epithelial to mesenchymal transition (EMT), a process of the transdifferentiation of epithelial cells into motile mesenchymal cells, is integral in development, wound healing and stem cell behaviour, and contributes pathologically to fibrosis and cancer progression. This switch in cell differentiation and behaviour is mediated by key transcription factors, including SNAIL, zinc-finger E-box-binding (ZEB) and basic helix-loop-helix transcription factors, the functions of which are finely regulated at the transcriptional, translational and post-translational levels.(Lamouille et al., 2014)

Loss of E-cadherin expression is a hallmark of EMT. E-cadherin is a central component of cell-cell adhesion and is required for the formation of epithelia in the embryo and to maintain epithelial homeostasis in the adult. Loss of E-cadherin is consistently observed at sites of EMT during development and cancer (Thiery, 2002). The loss of E-cadherin increases tumor cell invasiveness in vitro and contributes to the transition of adenoma to carcinoma in animal models (Thiery, 2002). ZEB2 can bind to the E-cadherin promoter and downregulate E-cadherin and other tight junction related genes' transcription. Thus ZEB2 plays an important role in inducing the EMT

process (Comijn et al., 2001; Vandewalle et al., 2005).

Mechanistically, ZEB2 have been found to upregulate integrin alpha5 expression through cooperation with Sp1 to induce invasion during the EMT of human cancer cells (Nam et al., 2012). Meanwhile, ZEB2 was found to be regulated by MicroRNAs, which eventually can affect the EMT process. MicroRNAs are a large family of small (21–23-nt) RNAs which can regulate gene expression by interacting with multiple mRNAs and inducing either translational suppression or degradation of mRNA. They have been implicated in regulating complex physiological processes such as embryogenesis (Wienholds et al., 2005), organ development (Yi et al., 2006), and oncogenesis (Esquela-Kerscher and Slack, 2006).The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and ZEB2. These microRNAs was found to be lost in invasive breast cancer cell lines with mesenchymal phenotype. Expression of the miR-200 family was also lost in regions of metaplastic breast cancer specimens lacking E-cadherin (Gregory et al., 2008). The miR-200 family inhibits EMT and cancer cell migration by direct targeting of E-cadherin transcriptional repressors Zeb1 and Zeb2 (Korpal et al., 2008; Park et al., 2008). Beyond that, in Snail1-induced EMT, synthesis of Zeb2 is up-regulated, Snail1 does not affect the synthesis of Zeb2 mRNA, but prevent the processing of a large intron located in its 5'-untranslated region (UTR). This intron contains an internal ribosome entry site (IRES) necessary for the

expression of Zeb2. Maintenance of 5'-UTR Zeb2 intron is dependent on the expression of a natural antisense transcript (NAT) that overlaps the 5' splice site in the intron. Ectopic overexpression of this NAT in epithelial cells prevents splicing of the Zeb2 5'-UTR, increases the levels of Zeb2 protein, and consequently down-regulates E-cadherin mRNA and protein (Beltran et al., 2008).

miR-200a/ZEB2 pathway not only regulates EMT but also stem-like transition in nasopharyngeal carcinoma cells. Stable knockdown of miR-200a promotes the transition of epithelium-like CNE-1 cells to the mesenchymal phenotype. More importantly, it also induced several stem cell-like traits, including CD133(+) side population, sphere formation capacity, in vivo tumorigenicity in nude mice, and stem cell marker expression (Xia et al., 2010). For induced pluripotent stem cell (iPS),members of the miR-200 family are unique mediators of the reprogramming factors Oct4/Sox2, the miR-200/ZEB2 pathway as one critical mechanism of Oct4/Sox2 to induce somatic cell reprogramming at the early stage (Wang et al., 2013).

1.2.5 ZEB2 in cancer

As EMT is also a widespread biological phenomena in cancers, ZEB2 was found to play an important role in initiation and progression in several kinds of cancer (De Craene and Berx, 2013). Overexpression of ZEB2 at the invasion front of colorectal cancer is an independent prognostic marker and regulates

tumor invasion in vitro (Kahlert et al., 2011). By transactivating ZEB2 and VersicanV1 expression, Forkhead box Q1 will promotes hepatocellular carcinoma metastasis (Xia et al., 2014; You et al., 2011). In melanoma cell, ZEB2-MITF-ZEB1 transcriptional network controls melanogenesis and melanoma progression (Denecker et al., 2014). In prostate cancer, ZEB2 expression was regulate by Androgen receptor and its implications in epithelial-to-mesenchymal transition (Jacob et al., 2014).

ZEB2 protein protects cells from DNA damage-induced apoptosis and has independent prognostic value in bladder cancer (Sayan et al., 2009). ZEB2 overexpression (examined by IHC) is also an independent biomarker for the poor prognosis of patients with renal cell carcinoma (Fang et al., 2013).

1.2.6 Zeb2 in hematopoietic system

In hematopoietic system, high Zeb2 mRNA expression levels was detected in HSCs and hematopoietic progenitor cells (HPCs). Previously, Tie2-Cre and Vav-iCre based conditional Zeb2 deletion were used to study the role of Zeb2 in hematopoietic system. Zeb2$^{-/\Delta \text{Tie2-Cre}}$ displayed an early lethality around E12.5-E13.5 due to intracerebral hemorrhages. However, no obvious loss of HSCs were observed in knockout mice before E11.5 compared with littermate controls, suggesting that Zeb2 is not essential for HSC formation. Vav-iCre mediated aproach of gene deletion, wich is more HSC specific, revealed similar defects in hematopoiesis with early letality as Zeb2$^{-/\Delta \text{Tie2-Cre}}$ knockouts.

In addition, Zeb2-ablated hematopoietic precursor cells showed severe block in multiple hematopoietic lineages. After colonizing the fetal liver Zeb2-deficient hematopoietic stem/progenitor cells exhibited altered adhesive and homing properties and an inability to reenter the blood circulation and colonize the bone marrow cavity. These results indicate critical roles of Zeb2 in embryonic hematopoiesis and suggest that Zeb2 may also be important for adult hematopoiesis (Goossens et al., 2011). Moreover, there is increasing evidence suggesting that overexpression of Zeb2 is involved in hematopoietic malignancies. Retroviral insertional mutagenesis identified Zeb2 activation as a novel leukemogenic collaborating event in CALM-AF10 transgenic mice. 89% of mice with a Zeb2 integration developed B-cell acute lymphoblastic leukemia (ALL) (Caudell et al., 2010). A recent study has reported a recurrent t(2;14)(q22;q32) chromosomal translocation, targeting Zeb2, in early T-cell precursor leukemia (ETP-ALL) indicating that sustained Zeb2 expression initiates T-cell leukaemia. Moreover, Zeb2-driven mouse leukaemia exhibits an enhanced leukaemia-initiation potential and activated Janus kinase (JAK)/ STAT signalling through transcriptional activation of IL7R (Goossens et al., 2015). Above studies point to a possible importance of Zeb2 for maintenance of normal hematopoiesis.

1.2.7 Preliminary work

In order to investigate the physiological role of Zeb2 in the regulation of

homeostasis in adult hematopoietic system, we use the interferon sensitive Mx1-Cre based inducible knockout model to generate Zeb2 deficient mice, where Zeb2 was deleted in adult hematopoietic cells upon Poly (I:C) administration. Our previous work revealed that eight weeks following Zeb2 deletion mice exhibited significantly lower number of B-lymphocytes, monocytes, platelets and erythrocytes in peripheral blood, whereas the number of granulocytes was slightly increased and T-lymphocytes was not different to controls. However, further analyses of the immature bone marrow compartment revealed an accumulation of HSPC (defined as lineage-, cKit+, Sca1+, LKS) and HSC (LKSCD48-CD150+) populations. Those changes were not due to altered apoptotic events or proliferation rates. Within the different myeloid restricted progenitor subpopulations we observed a shift towards increased frequency of MEP and reduced GMP. However, no change in the percentage of CLP was detectable. Similar but more pronounced phenotype were observed in competitive and non-competitive transplant settings, indicating that the hematopoietic lineage impairment is not due to self-renewal but intrinsic differentiation defects of stem cells. Moreover, Zeb2 ablation in the hematopoietic compartment led to a phenotype with several features reminiscent of myeloproliferative disorders in human, such as bone marrow fibrosis, splenomegaly and extramedullary hematopoiesis.

To determine downstream targets that might be responsible for the complex differentiation impairment phenotype in most hematopoietic lineages in the

absence of Zeb2 a global gene expression analyzes focusing on the HSPC containing bone marrow subset were performed. Several molecules involved in the Wnt signaling pathway stood out and suggested increased Wnt signaling activity in Zeb2-deficient HSPC. Surprisingly, when Zeb2 and β-catenin were deleted simultaneously in the hematopoietic compartment using the same conditional knockout strategy, all double knockout mice died very shortly after Poly (I:C) administration. No rescue or difference was observed in Zeb2 deletion mice with or without haploinsufficiency of β-catenin (data not published).

1.3 research goals

Our previous results demonstrated that Zeb2 is essential for normal hematopoietic stem cell differentiation. However, given the complex phenotype of insufficient blood cell maturation in multiple lineages at different stages of differentiation and the inability to rescue the phenotype by affecting the expression level a single putative downstream targets we reasoned, that the observed changes would probably not be caused by one downstream target, but rather due to a broad spectrum of epigenetic changes.

The goal of this thesis is to understand the role of Zeb2 in differentiation process of different hematopoietic lineages at a molecular level. We first aimed to define at which stage of maturation the bone marrow cells of different lineages exhibited a differentiation block. Next we investigated the gene expression profile of putative target genes known to be essential at the particular developmental stage. Thirdly, we aimed to test whether Zeb2 is involved in in epigenetic regulation by gene expression analyses of known epigenetic modulators specifically in sell subsetts where the differentiation was blocked. Finally we performed functional analyses to confirm alterations in cell signaling pathways.

2. Materials and Methods

2.1 Materials

2.1.1 Laboratory equipments

Equipment	Model	Supplier
Autoclave	CX-150	Systec, Wettenberg, Germany
Centrifuges	5810R, 5430R, miniSpin plus	Eppendorf, Hamburg, Germany
	Mini Star	VWR, Radnor, PA
	Varifuge 3.0R	Heraeus, Hanao, Germany
CO2 Incubator	MCO-18AIC (UV)	SANYO, Moriguchi, Japan
Electrophoresis chamber	PerfectBlue Gel System Mini	Peqlab, Erlangen, Germany
Flow cytometer	FACS CantoII	BD, Heidelberg, Germany
Gel imaging system	ChemiDoc XRS+	Bio-Rad, Hercules, CA
Hemocytometer	Hemavet 950	Drew Scientific, USA
Heating Magnetic stirrer	Aluminum Hot Plate Stirrer - ARE	VELP Scientifica, Usmate, Italy
Laminar flow hood	MSC-Advantage	Thermo Scientific, Waltham, MA
Light microscope	Eclipse TS100	Nikon, Tokyo, Japan
MACS Separator	QuadroMACS^{TM} Separator	Miltenyi, Gladbach, Germany
Microwave	NN-E245W	Panasonic, Kadoma, Japan
Microplate reader	Infinite 200	TECAN, Mannedorf, Switzerland
PCR cycler	Mastercycler pro	Eppendorf, Hamburg, Germany
Power supply	EV202	Consort, Turnhout, Belgium
Electrophoresis chamber	XCell SureLock Mini-Cell	Thermo Scientific, Waltham, MA
Electrophoresis chamber	Mini-PROTEAN Tetra Cel	Bio-Rad, Hercules, CA
Real-Time PCR cycler	Mastercycler Realplex	Eppendorf, Hamburg, Germany
Roller mixer	STR9	Stuart, Staffordshire, UK

Scale	SBA 53	Scaltec, Heiligenstadt, Germany
Shaker-Incubator	ES-20	BioSan, Riga, Latvia
Spectrophotometer	Nanodrop 2000	Thermo Scientific, Waltham, MA
Thermomixer	Thermomixer comfort	Eppendorf, Hamburg, Germany
Vortexer	ZX3 Vortex mixer	VELP Scientifica, Usmate, Italy
Waterbath	WNB 7-45	Memmert, Nuremberg, Germany
X-ray generator	RS-2000 biological irradiator	Rad source, Suwanee, GA

2.1.2 Consumable materials

Item	Supplier
Alcohol pads	Braun
Cell culture dishes (10cm)	Greiner Bio-one
Cell culture flasks (25/ 75/ 175 cm)	Greiner Bio-one
Cell culture plates (6-well, 12-well, 24-well, 96-well)	BD
Cell strainer 70µm Nylon	Corning
Disposable scalpel	Feather
Filter tips (10 µL, 200 µL, 1000 µL)	Nerbe Plus
Falcon tubes 15mL	Corning
Falcon tubes 50mL	Greiner Bio-one
Falcon tube with cell stainer snap cap	Corning
Falcon tube with snap cap	Corning
FACS tubes	Röhren
Hypodermic safety needle	Magellan
LD Columns	Miltenyi Biotec
Leukosilk	BSN medical
Micro-fine U-40 insulin	BD
Microtainer K2E Tubes	BD
Microtubes (1mL, 1.5mL)	Sarstedt
Microtubes (0.6mL)	Axygen
Needles	BD
Parafilm	Pechiney
PCR reaction tubes	Biozym Scientific
Pipette (5mL, 10mL)	Greiner Bio-one

real-time PCR film	Eppendorf
real-time PCR plate	Applied Biosystems
Refill pipette tips (10µL, 200µL,)	Nerbe Plus
Safe-lock tubes (0.5mL)	Eppendorf
Sterile filter 0.22 µm	Millipore
Syringe (2ml, 5ml, 10 mL)	BD

2.1.3 Chemicals, reagents and kits

Item	Supplier
2 x M-PCR OPTI^{TM} Mix (Dye Plus)	Biotool
2x SYBR Green qPCR Master Mix	Biotool
4-12% Bis-Tris Protein Gels	Life Technologies
Agarose	Peqlab
β-Mercaptoethanol	Sigma
BCA Protein Assay Kit	Life Technologies
BSA (Bovine serum albumin)	Molecular Probes, Invitrogen
BrdU Flow Kit	BD
cDNA Reverse Transcription Kit	Life Technologies
CellTrace™ CFSE Cell Proliferation Kit	Life Technologies
Dexamethasone	Sigma
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Distilled water	Gibco, Life technologies
ECL Prime Western Blotting Detection Reagent	GE Healthcare
Ethylendiamidtetraacetat (EDTA)	Sigma
Ethanol (70%, 100%)	Merck
Ethidium bromide	Sigma
FACS Lysing Solution/Buffer	BD
Fetal bovine serum (FBS)	PAA
Formaldehyde (4%, 10%)	Merck
G-CSF	Amgen
Glycin	Roch
LDS Sample Buffer (4X)	Life Technologies
Methanol	Merk
Mouse IGF-1	eBioscience
Mouse IL-3	Miltenyi Biotec
Mouse IL-6	R&D Systems
Mouse IL-7	Miltenyi Biotec
Mouse SCF	Peprotech

Non-fat milk powder	Roche
PCR Tail Lysing Reagent	Peqlab
Pre-Separation Filter, 30 µm	Miltenyi Biotec
Penicillin-streptavidin, 100x	Gibco, Life Technologies
Perm/Wash Buffer	BD
Perm/Wash Buffer I	BD
Perm/Wash Buffer III	BD
Phenylhydrazine (PHZ)	Sigma
Phosphate-buffered saline (PBS)	Gibco, Life Technologies
Poly(I) Poly (C) Double Strand	GE healthcare
Proteinase K	Roch
Pure Link RNA mini Kit	Life Technologies
SDS Running Buffer (20X)	Life Technologies
Sodium chloride 0.9%	Ecotainer
Streptavidin Microbeads	Miltenyi
Thiazol orange (TO)	Sigma Aldrich
Tris	Sigma Aldrich
Tris-HCl	Sigma Aldrich
Tween-20	Sigma Aldrich

2.1.4 Antibodies

Antibodies for flow cytometry

Specificity	Fluorochrome	Dilution	Suplier
7-AAD			BD
anti-rabbit	FITC	1:1000	
BrdU	FITC	1:50	BD
CD11b/Mac1	eFluor450	1:500	eBioscience
CD19	APC	1:500	eBioscience
CD24	PE-Cy7	1:200	eBioscience
CD34	FITC	1:200	eBioscience
CD43	PE	1:500	eBioscience
CD44	PE	1:500	eBioscience
	Biotin	1:200	eBioscience
CD45	PE	1:1000	eBioscience
CD45.1	PE	1:200	eBioscience
CD45.2	FITC	1:200	eBioscience
CD45R/B220	PerCP-Cyanine5.5	1:500	eBioscience
CD71	APC	1:500	eBioscience
CD117/c-Kit	APC	1:100	eBioscience
CD127/IL-7R	APC-eFluor780	1:200	eBioscience

CD135/Flt-3	PE		1:500	eBioscience
DAPI			1:2000	eBioscience
Gr1	eFluor660		1:500	eBioscience
Ki67	FITC		1:50	eBioscience
Lineage cocktail	Biotin		1:50	BD
IgD	FITC		1:500	eBioscience
IgM	PE		1:500	eBioscience
Phospho-Akt (Ser473)			1:100	Cell Signaling
Phospho-Erk1/2 (Thr202/Tyr204)			1:200	Cell Signaling
Phospho-Stat3 (Tyr705)			1:200	Cell Signaling
Phospho-Stat5 (Tyr694)			1:400	Cell Signaling
Sca-1	PE-Cy7		1:300	eBioscience
	eF450		1:500	eBioscience
	PE		1:1000	eBioscience
Streptavidin	PerCP-Cy5.5		1:500	eBioscience
Ter119	APC-eFluor780		1:500	eBioscience

Antibodies for western blot

Antigen	coupling	Host	Dilution	Suplier
β-actin		Mouse	1:1000	Cell Signaling
Erk		Rabbit	1:1000	Cell Signaling
GAPDH		Rabbit	1:1000	Cell Signaling
Phospho-Akt (Ser473)		Rabbit	1:1000	Cell Signaling
Phospho-Erk1/2 (Thr202/Tyr204)		Rabbit	1:1000	Cell Signaling
Phospho-Stat3 (Tyr705)		Rabbit	1:2000	Cell Signaling
Phospho-Stat5 (Tyr694)		Rabbit	1:1000	Cell Signaling
Mouse IgG	HRP	Rabbit	1:5000	Jackson ImmunoResearch
Rabbit IgG	HRP	Rabbit	1:5000	Jackson ImmunoResearch
Stat3		Rabbit	1:2000	Cell Signaling
Stat5		Rabbit	1:1000	Cell Signaling

2.1.5 Primers

Primers for qRT-PCR

Oligoname	Sequence 5' -> 3'
β-Actin-F	AGTGTGACGTTGACATCCGTA
β-Actin-R	GCCAGAGCAGTAATCTCCTTCT
Bcl-2-F	TGAGTACCTGAACCGGCATCT
Bcl-2-R	GCATCCCAGCCTCCGTTAT
Blimp1-F	ACACACAGGAGAGAAGCCACATGA
Blimp1-R	TCGAAGGTGGGTCTTGAGATTGCT
CDK4-F	TCGAAAGCCTCTCTTCTGTG
CDK4-R	TACATCTCGAGGCCAGTCAT
Cxcr4-F	GAAGTGGGGTCTGGAGACTAT
Cxcr4-R	TTGCCGACTATGCCAGTCAAG
Dnmt1-F	CCTAGTTCCGTGGCTACGAGGAGAA
Dnmt1-R	TCTCTCCTCTGCAGCCGACTCA
Dnmt3a-F	GAGGGAACTGAGACCCCAC
Dnmt3a-R	CTGGAAGGTGAGTCTTGGCA
Dnmt3b-F	AGCGGGTATGAGGAGTGCAT
Dnmt3b-R	GGGAGCATCCTTCGTGTCTG
E2A-F	CCCGGATCACTCCAGCAATAA
E2A-R	TGGAGACCTGCATCGTAGTTG
EBF-F	CTTGCTAACACTTCGGTCCAT
EBF-R	ACCTTGATTGGTGGCTTGTG
Eed-F	AATGTGGCGCGGCTATTC
Eed-R	CGAAGTCGATCCCATCGC
Ezh2-F	CCAGACTGGTGAAGAGTTGTTTT
Ezh2-R	CAAGGGATTTCCATTTCTCG
Fli1-F	GGACTGATCGTCACTCACCA
Fli1-R	AATCTGAAGGGGCTACGAGG
Fog1-F	CTTGGGGCTGCTGTCCAT
Fog1-R	GTCCAGGAGGAAACAGAGCA
Foxo1-F	AGGAACTGAGGAGCAGTCCA
Foxo1-R	ATGATTCCCAATGGCACAGT
GAPDH-F	AGGTTGTCTCCTGCGACTTCA
GAPDH-R	GGTGGTCCAGGGTTTCTTACTC
Gata1-F	ACTGGCCTACTACAGAGAAGC
Gata1-R	GTAGAGTGCCGTCTTGCCATA
Gata2-F	CACCCCGCCGTATTGAATG
Gata2-R	CCTGCGAGTCGAGATGGTTG
Gata3-F	TTTACCCTCCGGCTTCATCCTCCT
Gata3-R	TGCACCTGATACTTGAGGCACTCT

G-CSFR-F	GTTTTGTGGGGAGTGGGGAT
G-CSFR-R	CCACATGCTGGAGACCAGAG
Hdac1-F	CTGTCCGGTATTTGATGGCT
Hdac1-R	CACGAACTCCACACACTTGG
Hdac2-F	GGCGGCAAGAAGAAAGTGTGC
Hdac2-R	GGCATCATGTAGTCCTCCAGC
Id2-F	GACCCGATGAGCCTGCTATAC
Id2-R	GGTGCTGCAGGATTTCCATCT
Id3-F	CTCTTAGCCTCTTGGACGACAT
Id3-R	GATCGAAGCTCATCCATGCCCT
Ikaros-F	TGAGGGTCAAGACATGTCCCAAGT
Ikaros-R	TCACTCTTGGAGTTCTGCTGTGCT
IL-3Rα-F	TGGGCGCCACTCAGGAAC
IL-3Rα-R	GTCACAGCAGTGGGTGGAG
IL-6Rα-F	TGAATGATGACCCCAGGCAC
IL-6Rα-F	ACACCCATCCGCTCTCTACT
IL-7R-F	ACGATCACTCCTTCTGGTGC
IL-7R-R	GCATTTCACTCGTAAAAGAGCC
Pbx1-F	AGGACATCGGGGACATTTTAC
Pbx1-R	CATTAAACAAGGCAGGCTTCA
Klf1-F	GACGATGTCCAGTGTGCTTC
Klf1-R	CTACTCCAAGAGCTCGCACC
Lmo2-F	AGGAGAGACTATCTCAGGCTTTT
Lmo2-R	TTGAAACACTCCAGGTGATACAC
LSD1-F	GCCTGTTTCCCAGACATCAT
LSD1-R	CAGCTGGATCTTTGGGTTGT
LRF-F	TCTTCTCGGAAGAGAGGGAA
LRF-R	CAGAAGGCCTCAGAATCCTC
Mi-2β-F	CCTCATTGTGGATGAAGCC
Mi-2β-R	GAAAGTTGAGCAGATGAAACAG
Mpl-F	ACCTTTGGAACCCGGTATGTG
Mpl-R	CTGTGGAGAGCAGCTGAATG
Nr4a2-F	ATTGCTGCCCTGGCTATGGTCA
Nr4a2-R	CAGGTAGTTGGGTCGGTTCAAACC
Pax5-F	AGCAGCCCCCCAATCAG
Pax5-R	TGCGTCACGGAGCCTGTA
Prdm16-F	AGATGAACCAGGCATCCACTCGAA
Prdm16-R	ACTTCCCGGCTAAGCTGTCATCAT
Runx1-F	GCAGGCAACGATGAAAACTACT
Runx1-R	GCAACTTGTGGCGGATTTGTA
Runx3-F	ACCACGAGCCACTTCAGCAG
Runx3-R	CGATGGTGTGGCGCTGTA
SCL-F	CATTCACATTCTGCTGCCTC
SCL-R	AACAACAACCGGGTGAAGAG

Sip1-F	GGCAAGGCCTTCAAGTACAA
Sip1-R	AAGCGTTTCTTGCAGTTTGG
Suz12-F	CTCAGGATATACATCGCCAACCT
Suz12-R	TGTTCTTTTTGGCCTGCAAAC
Tet-1-F	ACACAGTGGTGCTAATGCAG
Tet-1-R	AGCATGAACGGGAGAATCGG
Tet2-F	TGTTGTTGTCAGGGTGAGAATC
Tet2-R	TCTTGCTTCTGGCAAACTTACA
Tet-3-F	TCTCTGAAGGGTGGATTGTCC
Tet-3-R	CCCAGCACCGAGTAGCTTTC
TBP-F	TCTATTTTGGAAGAGCAACAAAGAC
TBP-R	GAGGCTGCTGCAGTTGCTA
YWHAZ-F	AGACGGAAGGTGCTGAGAAA
YWHAZ-R	TCAAGAACTTTTCCAAAAGAGACA

Primers for genotyping

Oligoname	Sequence 5' -> 3'
Zeb2-Intron6F	GAACTAGTTGAATTGGTAGAATCAATGGGG
Zeb2-Intron6R	GTAAAGGCTCTCTACGCCTTTTTCAGTTAG
Zeb2-Intron7F	AAGCATGTCGGTAAGCTGACCAACTACTAG
MxCreF	CATGTGTCTTGGTGGGCTGAG
MxCreR	CGCATAACCAGTGAAACAGCAT

2.1.6 Buffers and solutions

Item	Composition	Storage
IL-7 10ng/µL	2µg IL-7 200µL dd H2O	-80°C
PHZ 1.1g/mL	11mg phenylhydrazine 10mL H2O	4°C
Poly(I) Poly(C)	265mg Poly(I) Poly(C) 180mL 0.9% sodium chloride	-80°C
Proteinase K 20mg/mL	100mg Proteinase K 5mL 10mM Tris buffer	-20°C
Staining buffer	500µL FBS 49.5mL PBS	4°C
Tris buffer 10mM	0.08g Tris-HCl 50mL H2O	RT
TAE buffer 50x	242g Tris 57,1 ml Acetic Acid	RT

	100 ml EDTA (0,5 M, pH 8,0) fill up to 1000 ml with water.	
TAE buffer 1x	40mL TAE buffer 50x 1960mL H2O	RT
TBS-T stock 10x	24.2g Tris 80g NaCl adjust pH to 7.6 700ml H2O	RT
TBS-T1x	100ml TBS-T stock 10x 2ml Tween-20 fill up to 1000 ml with water.	RT
TO stock 10000x	10mg thiazol orange 10mL methanol	-20°C stable for 2m
TO 1x	5µL TO stock 10000x 50mL PBS	fresh prepared
Transfer buffer stock 10x	150.14g Glycin 30.285g Tris adjust pH to 8.3 fill up to 1000 ml with water.	RT
Transfer buffer 1x	100ml Transfer buffer stock 10x 200ml methanol fill up to 1000 ml with water.	RT

2.1.7 Softwares

Software	Purpose
BD FACSDiva	Application setup, data acquisition, and data analysis on BD FACSCanto II
Flowjow	Viewing and analyzing flow cytometric data
GraphPad Prism 5	Graphing, statistical analyses and data organization
Image Lab	Image acquisition and analyzing gel and blot features. Runs with gel ChemiDoc XRS+ imaging system.
Nanodrop	Quantification of RNA
qBase plus	real-time PCR data analysis
Realplex	Thermal cycling program setup and data acquision
TierBase	a database for recording, raisiong and breeding laboratory animals as well as the documentation of animal experiments

2.2 Methods

2.2.1 Mice

Mice harboring a floxed Zeb2 allele were described previously (Higashi et al., 2002). Zeb2 fl/fl mice were mated to Mx1-Cre mice, which expressing an interferon-inducible Cre recombinase, to generate Zeb2 fl/fl harboring or not the Mx1-Cre transgene. Genotyping was performed by PCR, the temperature cycle conditions of which was described below. Primers are listed above. Conditional Zeb2 knockout mice were induced 3 times by intraperitoneal injection with 12.5 ug polyinosinic-polycytidylic acid (poly I:C) dissolved in 0.9% NaCl/ g body weight on alternate days in 6-8 weeks old mice. Mice were analyzed 2-3 months after the first injection. To induce a hemolytic anemia, mice were injected subcutaneously with 30mg/kg body weight phenylhydrazine (PHZ; Sigma-Aldrich) for 2 consecutive days. Mice were bled on days 0, 2, 4, 7, 10, 14 for hematocrit and reticulocyte count measurements. The C57BL/6J (CD45.2) mice were purchased from Charles River. All animal experiments were approved by the Federal office for Nature, Environment and Consumer protection North Rhine Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz NRW).
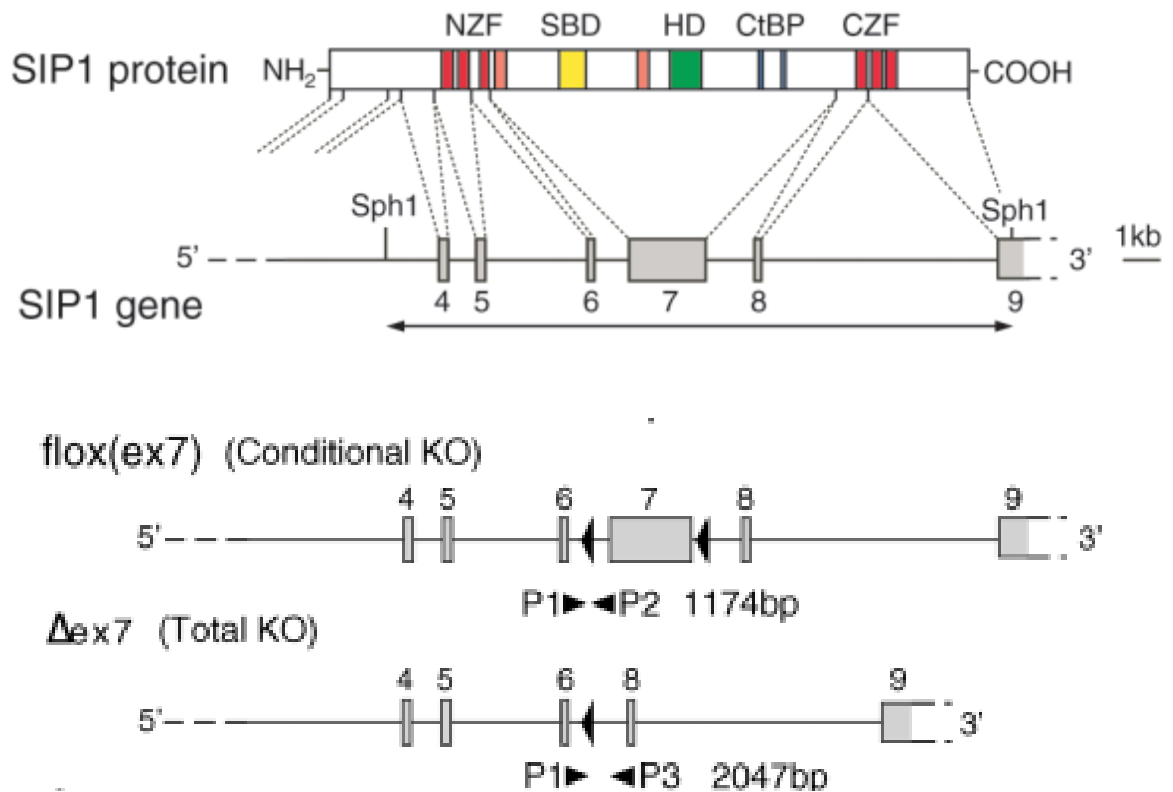
Figure 2.1 Schematic representation of Zeb2 locus and strategy for Zeb2 conditional knockout (Higashi et al., 2002). NZF: N-terminal zinc finger clusters, CZF: C-terminal zinc finger clusters, HD: homeodomain-like sequence, CtBP: CtBP binding sites, SB: Smad-binding domain. The gray boxes represent coding exons.

Table 2.1 PCR program for genotyping Mx1-Cre and Zeb2 allels

Temperature (°C)	Time	Cycles
94	5 min	1
94 (Denaturing)	20 sec	
58 (Mx1-Cre)/ 52(Zeb2) (Annealing)	30 sec	
72 (Elongation)	40 sec	38
72	5 min	1
4	--	1

2.2.2 Blood

Peripheral blood was obtained from the tail vein and collected in EDTA-coated

microtainers (BD Biosciences). Blood parameters were counted by an

automated hemocytometer (Hemavet 950; Drew Scientific). The reticulocyte counts were measured by thiazole orange (TO, sigma) staining as previously described.(Lee et al., 1986)

2.2.3 Bone marrow transplantation

2×10^6 bone marrow mononuclear cells from 8 to 12 week old control (CD45.1) and Zeb2-deficient (CD45.1) littermates were isolated and transplanted into female C57BL/6 (CD45.2) recipients. Recipients were lethally irradiated with 12-Gy in split doses. Hematologic parameters were monitored by analysis of peripheral blood at 4, 8, 12 weeks after transplantation. 12 weeks posttransplantation, hemolytic anemia was induced as described above.

2.2.4 Flow cytometry and cell sorting

A single-cell suspension of bone marrow or spleen was prepared by staining buffer and incubated with specific cell surface markers for 20 minutes at 4 degree. The antibodies for different panels were described above. 7-Aminoactinomycin D (7-AAD; BD Biosciences) was used to exclude dead cells before measurement. For BrdU incorporation assay, mice were injected intraperitoneally with BrdU (BD) at a dosage of 150ug/g of body mass 1 hour before sacrifice. The intracellular BrdU was stained using a BrdU-FITC flow kit (BD Biosciences) according to the manufacturer´s protocol after erythroid cells were staged as described above. The DNA content was assessed by 7-AAD.

Cells were measured by FACS CantoII (BD Biosciences) and analyzed by Flowjo. Ter119$^+$CD71^{high}, Prepro-B, LKS, progenitor and myeloid population were sorted by FACSAria III (BD Biosciences).

2.2.5 CFU-E and BFU-E

Freshly isolated bone marrow and spleen cells were counted by Hemavet. For CFU-E detection and quantification, $1*10^5$ Bone marrow or $1*10^6$ spleen cells were mixed with M3334 MethoCult methylcullulose media and seeded into one well of 12-well plates. Colonies were counted two days after incubation. For BFU-E detection, whole bone marrow cells and spleen cells were seeded into 12-well plates at a density of $1*10^4$ cells/well and $1*10^5$ cells/well in M3434 MethoCult methylcullulose media. Following incubation for 7 days at a 37°C humidified incubator with 5% CO_2, BFU-E were quantified.

2.2.6 Quantitative Real Time Polymerase Chain Reactions (qRT-PCR)

<u>Reagents and kits</u>

All steps and procedures have been established following the MIQE guidelines. Total RNA was extracted using the Ambion PureLink RNA mini Kit. RNA quality and concentration was measured using a Nanodrop machine. cDNA was obtained by reverse transcription using the high capacity cDNA

transcription kit (life technologies). The qPCR was carried out using the TaKaRa Syber Premix Ex Taq kit on an Eppendorf Mastercycler ep Realplex machine. Primers sequences were either found in the literature, obtained from the RTPrimerDB database or developed in our lab. All sequences are exon-spanning and were blasted in order to ascertain specificity. Secondary structure formation was also analysed. They were chosen to work in a temperature range of 59 to 61°C, resulting in an amplicon of 80 to 200 bases. Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

RNA and cDNA preparation

After RNA was extracted according to the manufacturer's instructions, its quality and concentration was measured using the Nanodrop. If samples were contaminated with DNA, they were subjected to DNase digestion using the PureLink Amplification grade DNase I according to the manufacturer's instructions. Aliquots of the samples were then diluted to 10 ng/µl working concentration. Stock solutions were stored immediately after dilution at -80°C. Working solutions were stores immediately at -20°C. For the reverse transcription, 5 µl of RNA per sample were used in a reaction volume of 20 µl according to the manufacturer's instructions. The reactions were carried out using an eppendorf cycler. cDNA was then either immediately used for qPCR or stored at -20°C.

<u>qPCR</u>

qPCR reactions were done in 96 well twin.tec plates covered by a Masterclear *real-time* PCR adhesive film, both supplied by Eppendorf. The program is listed in table 2.2. In each well were pipeted 2 µl of cDNA and 18 µl of Syber Premix reaction mix containing forward and reverse primers. All Samples were measured at least in duplicates. The amplification program used always included a melting curve analysis at the end to ascertain that the amplification was target specific.

For each new type of sample, a panel of 10 reference genes were analysed using the geNorm software included in the software package qBase Plus from Biogazelle (Ghent, Belgium) in order to determine the minimal number of reference genes necessary, as well as which genes are the most stable. For each primer pair used, a dilution curve in triplicate was established to determine amplification efficiency. In cases were all samples could not be fitted onto the same plate, inter-run calibrators were used.

Data was analysed using the qBase Plus software package from Biogazelle (Ghent, Belgium). Results are expressed as a ration of the mean expression level of the KO compared to the WT. Statistical analysis was done using the two-tailed student t-test. Primer sequences are listed in material part 2.1.5.

Table 2.2 qPCR program

Step	Temperature (°C)	Time	Cycles
Hot-Start DNA Polymerase activation	94	5 mins	1

	Denature	95	15 secs	
PCR	Anneal/Extend	60	40 secs	40
		95	15 secs	
		60	60 secs	
Melt Curve		95	15 secs	1

2.2.7 Reduced Representation Bisulfite Sequencing (RRBS)

The genome DNA methylation analysis were performed by Zymo Research. Libraries were prepared from 20 ng of genomic DNA from HSC, digested with 60 units of TaqαI and 30 units of MspI (NEB) sequentially and then extracted with Zymo Research (ZR) DNA Clean & ConcentratorTM-5 kit (Cat#: D4003). Fragments were ligated to pre-annealed adapters containing 5'-methyl-cytosine instead of cytosine according to Illumina's specified guidelines (www.illumina.com). Adaptor-ligated fragments of 150–250 bp and 250–350 bp in size were recovered from a 2.5% NuSieve 1:1 agarose gel (ZymocleanTM Gel DNA Recovery Kit, ZR Cat#: D4001). The fragments were then bisulfite-treated using the EZ DNA Methylation-LightningTM Kit (ZR, Cat#: D5020). Preparative-scale PCR was performed and the resulting products were purified (DNA Clean & ConcentratorTM - ZR, Cat#D4005) for sequencing on an Illumina HiSeq.

Sequence reads from bisulfite-treated EpiQuest libraries were identified using standard Illumina base-calling software and then analyzed using a Zymo Research proprietary analysis pipeline, which is written in Python and used

Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/) to perform the alignment. Index files were constructed using the bismark_genome_preparation command and the entire reference genome. The --non_directional parameter was applied while running Bismark. All other parameters were set to default. Filled-in nucleotides were trimmed off when doing methylation calling. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Fisher's exact test was performed for each CpG site which has at least five reads coverage, and promoter, gene body and CpG island annotations were added for each CpG included in the comparison.

2.2.8 Cell treatments

To evaluate the effects of different cytokines to control and Zeb2-KO hematopoietic cells, freshly isolated whole bone marrow cells or purified myeloid and Lin- cells were firstly starved in 200μL IMDM medium with 0.5% FBS at 37°C for 30 minutes. After starvation, 10ng/ml of IL-3, IL-6, IL-7 or G-CSF were added to the cells for 15 minutes. The dilution strategy was listed below (Table 2.3). To stop the simulation process, cells for intracellular staining were added 200μL of 4% PFA, while cells for western blot were centrifuged and lysed with RIPA buffer. The ruxolitinib was preincubated for 15 minutes before G-CSF treatment.

Table 2.3 Cytokine dilution stragegy

Cytokines	Stock conc.	Final conc.	Dilution
IL-3	10ng/ul	10ng/mL	1/1000
IL-6	10ng/ul	10ng/mL	1/1000
IL-7	10ng/ul	10ng/mL	1/1000
G-CSF	600ng/ul	10ng/mL	1/600000
Ruxolitinib	30mM	80nM	1/375000

2.2.9 Intracellular protein staining

To detect intracellular phosphorylated proteins, stimulated and control cells were fixed with 2% paraformaldehyde at 37°C for 10min. Samples were washed with staining buffer and followed by permeabilization with cold BD Perm/Wash III reagent. Cells were then frozen at -80°C for 30min or overnight. Next, thawed cells were labeled with anti-phospho and surface antibodies. FITC-conjugated anti-rabbit secondary antibody was used in the end to detect phosphor signal.

2.2.10 Western blot

Protein preparation

Protein was extracted from cell lysates by RIPA lysis buffer with 1% of phosphorylases inhibitors (Sigma). To quantify the total protein content, BCA kit (Pierce) was used. According to the concentration measured by spectrophotometer, we were able to calculate the volume containing 30µg protein of each sample and diluted it with loading buffer (Pierce). The mixtures

were heated at 70°C for 15min to denature the high structure.

Gel electrophoresis

In this study, NuPAGE 4-12% Bis-Tris Protein Gels (Invitrogen) were used, which give optimal separation of small-sized proteins. Two gels were clamped in the electrophoresis chamber and the chambers were filled with SDS running buffer (Invitrogen). 30μg of denatured proteins were loaded into the gel, including 2 lanes with 5μL of molecular weight marker. The gel was then connected to the power supply and run at 80V for 30min, followed by 120V, until the blue dye reaches the bottom.

Blotting

Blotting is to immobilize proteins out of the gel and transfer to the membrane for immunostaining. Here we use polyvinylidene difluoride (PVDF) membrane, which was pre-incubated and activate with methanol. Then the transfer sandwich was prepared. A sponge, two whatman filter papers, the gel, the PVDF membrane, another two whatman filter papers and a second sponge were placed on the black site of the blot cassette, which was then closed and placed in the transfer tank, filled with cold transfer buffer. The gels were electrotransferred at constant voltage 65V for 1.5 hours at 4°C.

Blocking, staining and developing

The membrane was blocked with 5% milk powder in TBS-T buffer for one hour at room temperature under gentle agitation. According to the sizes of different proteins, we cut the membrane to small pieces and incubate them with primary antibodies at 4°C overnight on an orbital shaker. The primary antibodies were diluted with 5% BSA in TBS-T buffer. After 4 times of 5 minutes washing with TBS-T, horseradish peroxidase (HRP) conjugated secondary antibody was added and incubate for 1 hour. Membrane was again washed 3 times for 5min with TBS-T. For detecting HRP activity, ECL reagent (equal amount of solution A and B, GE Healthcare) was used and generated chemiluminescence was measured by ChemiDoc imaging system.

2.2.11 Statistical analyses

Statistical analysis was done using the unpaired two-tailed student t-test; significance was set at p values less than 0.05. The tests were performed either with Microsoft Excel or GraphPad Prism. Fisher's exact test was performed for each CpG site which has at least five reads coverage in the RRBS assay.

3. Results

3.1 Zeb2 expression in adult murine hematopoietic cells is required for terminal erythroid differentiation

Terminal erythroid differentiation is the process by which nucleated proerythroblasts (ProE) mature into enucleated red blood cells (RBCs). ProE undergo 3-4 mitotic cell divisions and further generate sequentially basophilic erythroblasts (BasoE), polychromatic erythroblasts (PolyE), and orthochromatic erythroblasts (OrthoE). Subsequently, OrthoE exit the cell cycle and enucleate to become reticulocytes (Retic), and then mature RBCs are developed and circulate in the blood stream. Erythroid terminal differentiation is tightly and precisely controlled by differentiation divisions, which is highly coupled to the irreversible cell cycle exit. Our previous data showed that Zeb2 deletion is embryonic lethal and Zeb2-deficient ES cells failed to produce hemoglobin producing erythoid cells (Goossens et al., 2011). When Zeb2 was deleted in adult age, mice develop anemia, which was progressive over time. So we firstly investigated the erythroid differentiation upon Zeb2 ablation.

3.1.1 Zeb2-deficiency leads to an impaired terminal differentiation of the erythroid lineage

First we performed peripheral blood analysis at 8 weeks after Poly (I:C) administration and evaluated different erythrocyte specific parameters. Zeb2$^{\Delta/\Delta Mx1-Cre}$ animals displayed significant reductions of red blood cell counts (RBC), hemoglobin (Hb) and hematocrit (HCT) values, but increased level of mean corpuscular volume (MCV) than control mice, indicating increased size of erythrocytes. The frequency of reticulocytes was analyzed using Thiazole orange (TO), which is a nucleic acid binding dye. We detected a two fold increase in the percentage of reticulocytes in Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice compared to controls (Table 3.1). This data indicate, that in absence of Zeb2 there is an augmented erythropoiesis, but it is still not sufficient to compensate and balance the number of red blood cells over time.

Table 3.1 Hematologic parameters in control and Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice

		control	Zeb2$^{\Delta/\Delta Mx1-Cre}$	p	
RBC	M/uL	10.46±0.54	7.90±0.45	0.003	**
HB	g/dL	13.52±0.49	11.15±0.56	0.006	**
HCT	%	45.30±2.19	38.05±2.28	0.037	*
MCV	fL	43.40±0.52	48.09±0.58	<0.001	***
MCH	Pg	13.08±0.51	14.16±0.46	0.137	
MCHC	g/dL	30.14±1.02	29.50±0.89	0.644	
RDW	%	18.36±0.60	19.18±0.47	0.307	
Reticulocyte	%	2.55±0.11	5.70±1.00	0.040	*

To investigate what accounts for the anemia in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice, bone marrow and spleen cells were collected and analyzed using erythroid surface markers by flow cytometry. During erythroid maturation, progenitor cells are losing CD71 expression while gaining Ter119 markers. We noted that more ProE (CD71- Ter119-) and BasoE (CD71+ Ter119+) were accumulated in both bone marrow and spleen, while late BasoE and PolyE (CD71med Ter119+) and OrthoE (CD71- Ter119+) populations were dramatically reduced, compared to values in control animals (Fig. 3.1, 3.2). These data indicate an affected differentiation at the transition from early erythroid progenitors to later stages of maturation.

We then investigated the earliest erythroid progenitor burst forming unit-erythroid (BFU-E) and more mature colony forming unit-erythroid (CFU-E), which gives rise to proerythroblasts. There was no difference in the frequency of BFU-E and CFU-E colonies between bone marrow of Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ and controls, while CFU-E cells were increased in spleen after Zeb2 deletion (Fig. 3.3). These data suggest that the partial differentiation block is not at the stage of BFU-E and CFU-E cells, but at the transition from BasoE to PolyE stage of differentiation and the increase in CFU-U in spleens of Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice might be due to general increase of spleen erythropoiesis in Zeb2-deficient mice.
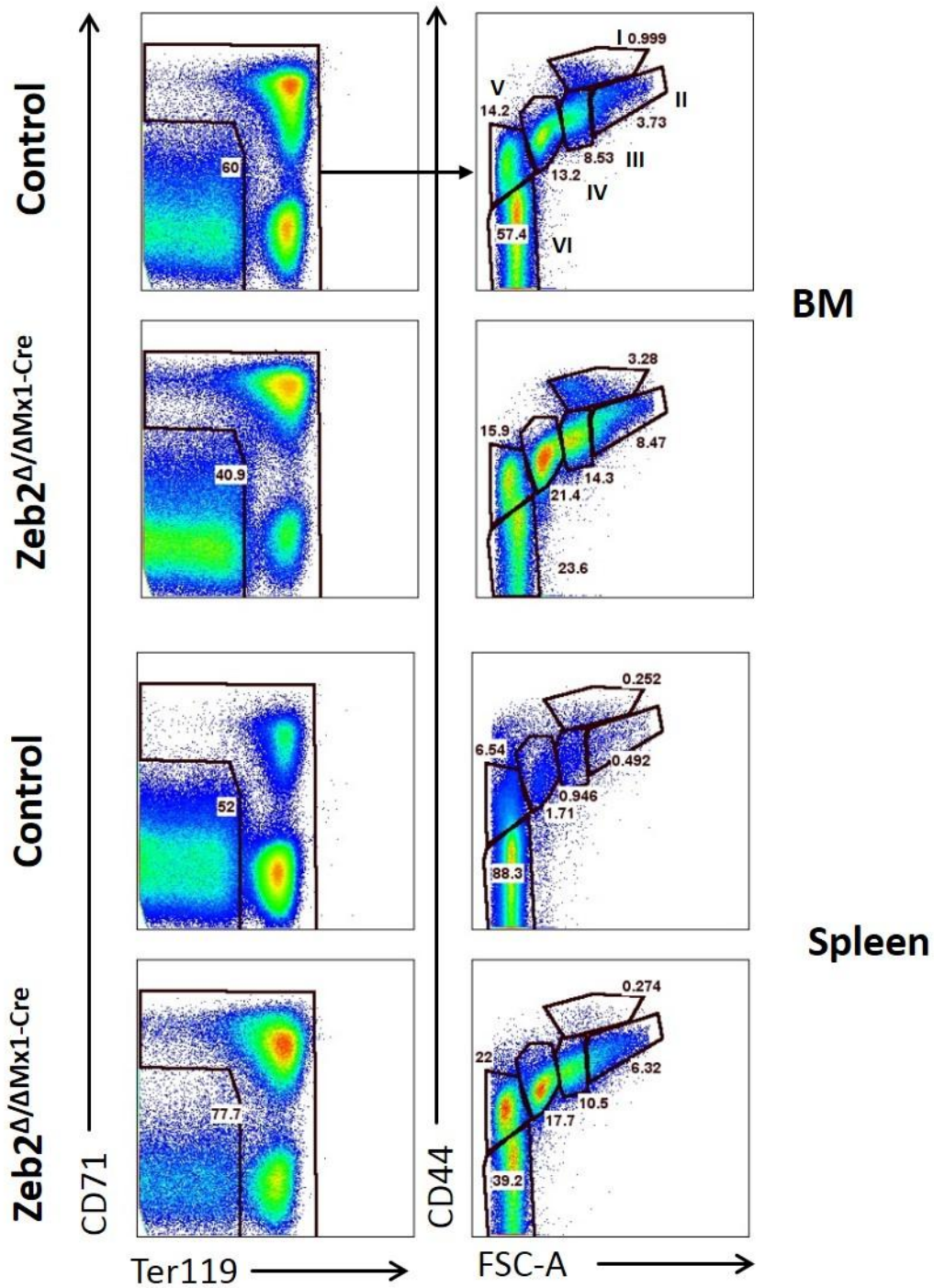
Figure 3.1. Representative flow cytometric patterns of erythroid differentiation in vivo. Regions I-VI define distinct stages of erythroid differentiation and corresponded to pro E, basophilic E, polychromatophilic E, orthochromatophilic E, reticulocyte and mature RBC respectively.
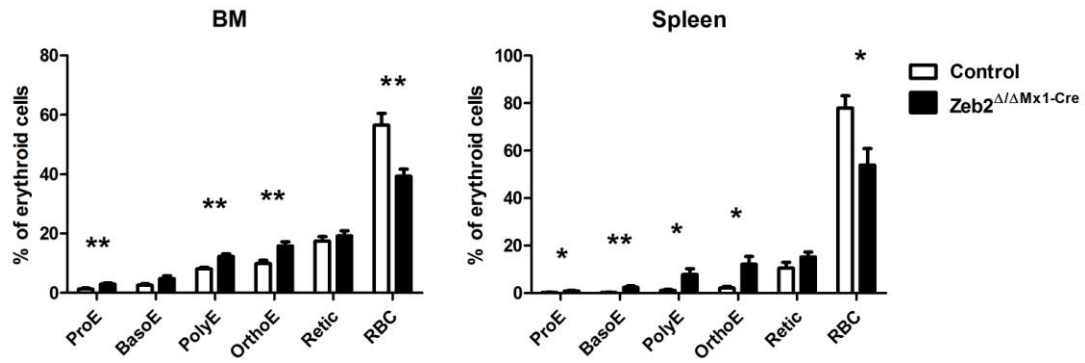
Figure 3.2 The percentage of indicated erythroid fractions in BM and spleen respectively. Data are shown as means ± SEM. *, P< 0.05, **, P < 0.01.
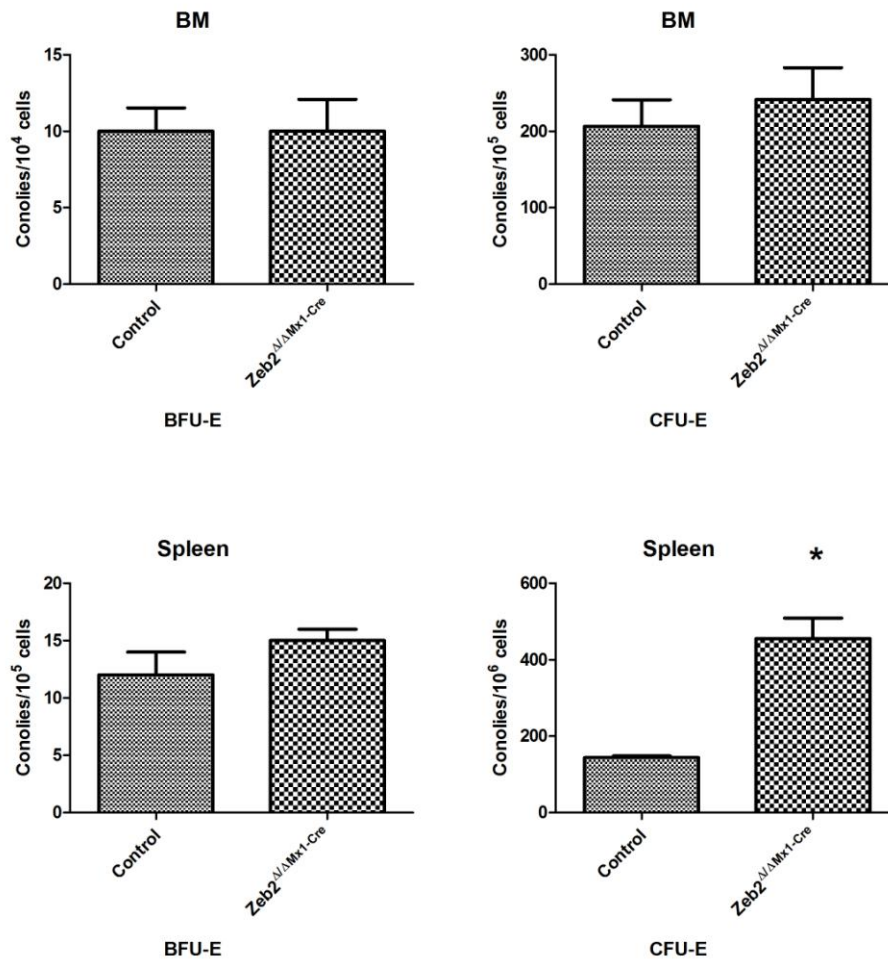

Fig 3.3 Erythroid colony-forming counts (BFU-E and CFU-E) from bone marrow and spleen cells of control and Zeb2-KO mice. Data are shown as the means ± SE of three independent experiments. *, P< 0.05.

As we observed increase in reticulocytes, we were wondering whether Zeb2 deficiency was only affecting steady state erythropoiesis or is also involved in stress erythropoiesis. We therefore induced hemolytic anemia by phenylhydrazine (PHZ) treatment, which leads to denaturation of erythrocytes and results in decreased HCT and increased reticulocytes. After 2 consecutive days of PHZ administration, Zeb2-deficient mice displayed a similar recovery kinetic in hematocrit, and were able to recover to the initial hematocrit and hemoglobin values as the control animals (Fig. 3.4). To exclude the different capacity of splenic erythropoiesis between control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice, we compared responses of transplanted recipients reconstituted with control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ donor cells to PHZ challenge. Again, we did not observe a significant difference in the kinetic of recovery between control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ bone marrow recipients (Fig. 3.4 bottom panel). Since the steady state erythropoiesis is mainly restricted to the bone marrow, while adult spleen is the primary effector to stress erythropoiesis (Paulson et al., 2011), our data suggest that Zeb2 is mainly involved in steady state erythropoiesis, while it is dispensable stress erythropoiesis.
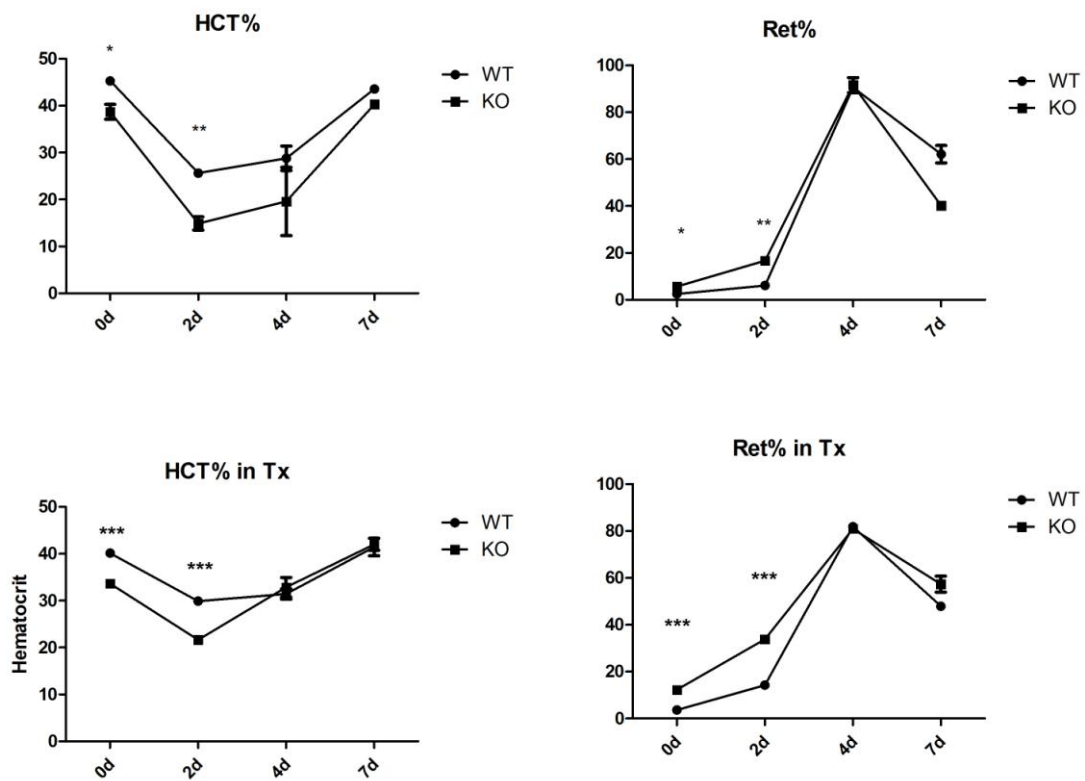
Figure 3.4 Hematocrit and reticulocyte assessments in the steady-state (upper) and control and Zeb2-KO hematopoietic cells transplanted mice (bottom) after induction of hemolytic anemia with PHZ. Data are shown as means ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

3.1.2 Impaired erythropoiesis in Zeb2 deletion mice is due to a primary intrinsic defect

To test whether defects in erythropoiesis in $Zeb2^{\Delta/\Delta Mx1-Cre}$ mice are caused by an intrinsic effect of marrow cells themselves or are influenced by the affected microenvironment, a bone marrow transplant was performed. The peripheral blood from recipients was collected and analyzed 4, 8, 12, 16 weeks post-transplantation. A consistent but more pronounced anemia was observed

in recipients with Zeb2$^{\Delta/\Delta Mx1-Cre}$ donor cells throughout the course of engraftment (Fig. 3.5), recapitulating the phenotype of donor mice. To further exclude a possible nice effect we performed an additional niche transplant assay by transplanting wild type bone marrow cells (CD45.2) into lethally irradiated control and Zeb2$^{\Delta/\Delta Mx1-Cre}$ (CD45.1) recipients. After 16 weeks, both recipient groups did not display abnormal parameters in the erythroid lineage (Fig. 3.6). This data confirms that the abnormalities are intrinsic to the marrow cells themselves and independent from bone marrow microenvironment.
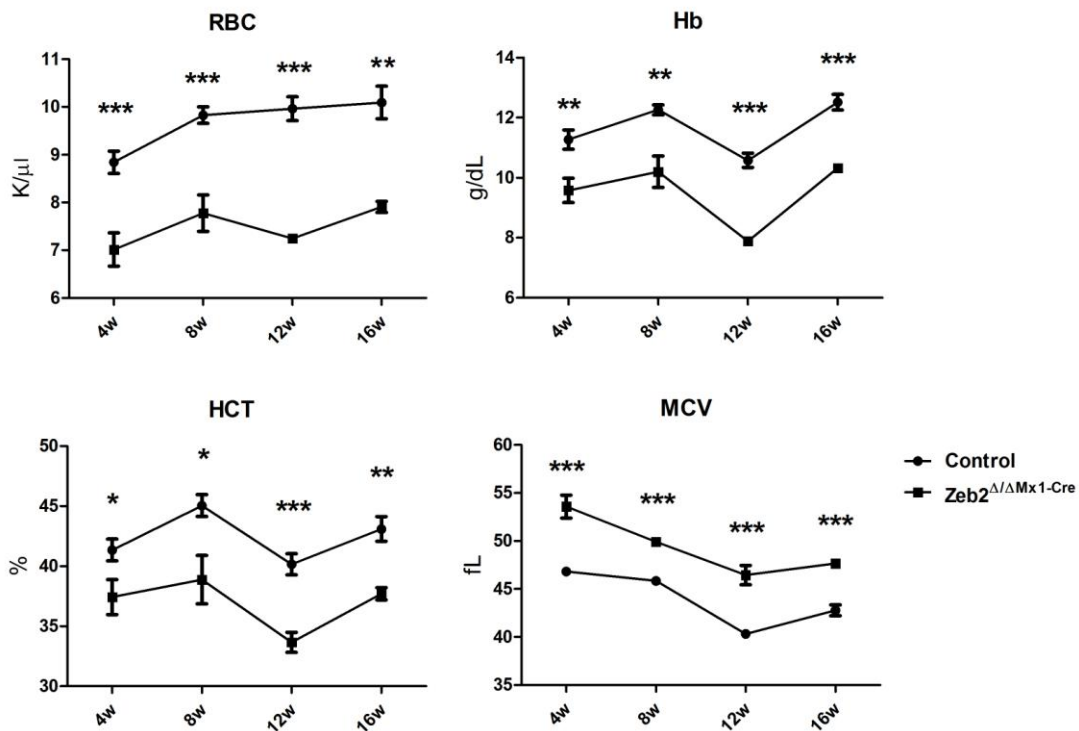
Figure 3.5 Assessment of RBC, hemaglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) in peripheral blood 4, 8, 12 and 16 weeks posttransplantation. Data are shown as means ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.
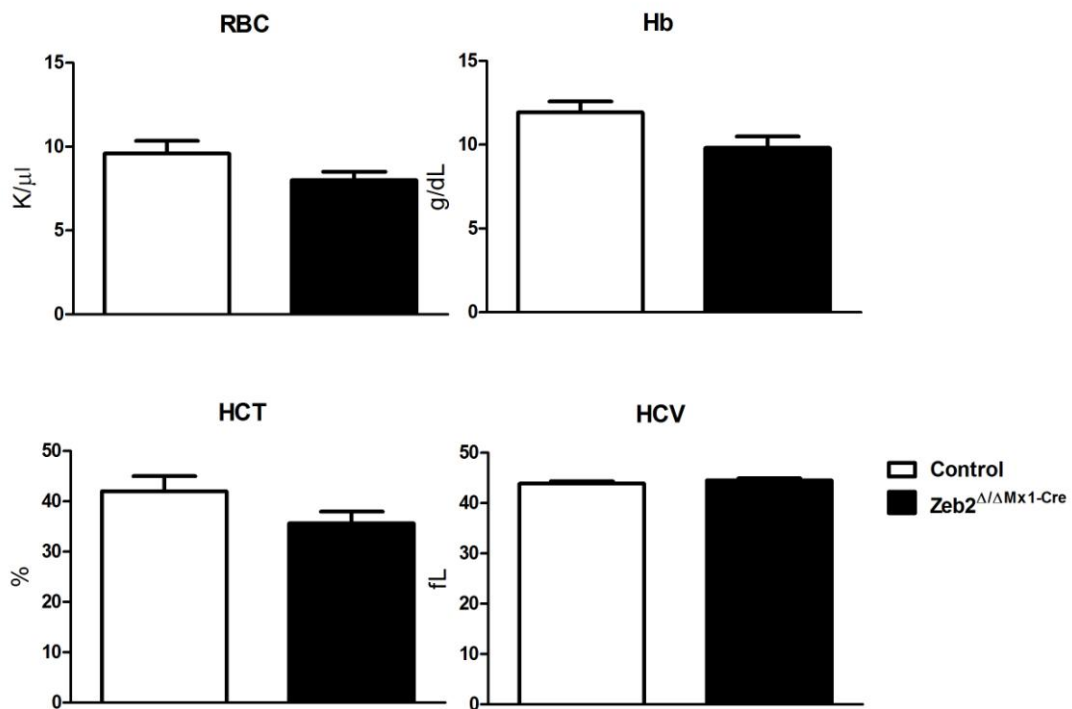
Figure 3.6 Analysis of donor cells repopulation and reconstitution in peripheral blood 16w after niche transplant. Data are shown as means ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

3.1.3 Zeb2 does not affect cell cycle activity but impact the expression of key transcription factors in erythroid progenitors

Since the terminal erythroid differentiation is tightly associated with cell cycle, we next thought to investigate whether cell cycle defects are responsible for the differentiation block in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice. The cell cycle analysis in different erythroid subsets of BM compartment by Ki67 detection revealed that the distribution of cells in quiescent (G0), growth (G1), and cell cycle (S/G2/M) phases had no difference between Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ and control mice. However,

when we analyzed the erythroid subsets in the spleens there was significant decrease in proportions of BasoE and PolyE cells that were in G0 status of the cell cycle, while increase in dividing cells (S/G2/M phases) in Zeb2-KO mice compared to controls (Fig. 3.7). Furthermore, in vivo BrdU incorporation assay was performed by giving Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ and littermate controls a single injection of BrdU, which can detect new DNA synthesis. Mice were sacrificed and analyzed 1h later. Consistent with Ki67 analysis, the data did not show different cell cycle distribution of erythroid population in the BM between control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice. Although we observed a similar trend in BrdU incorporation in the spleens, these were not statistically significant (Fig. 3.7). These data indicated that Zeb2 has no effect on cell cycle activity in bone marrow erythroid cells. However, there is accelerated proliferation in erythroid cells from Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ spleen, which may be responsible for constant stress hematopoiesis due to anemia caused by insufficient bone marrow erythropoiesis and contribute to increased reticulocyte number.
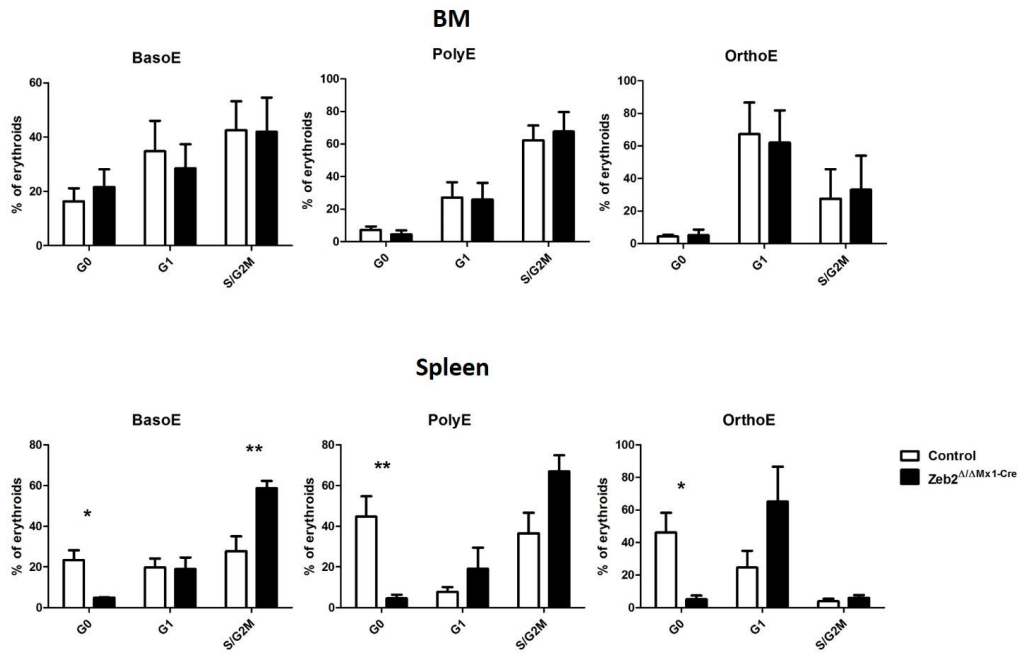
Figure 3.7 Cell cycle analysis by Ki-67 assays. Data show the distribution of indicated erythroid subsets in BM and spleen from control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$. Data are shown as means ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.
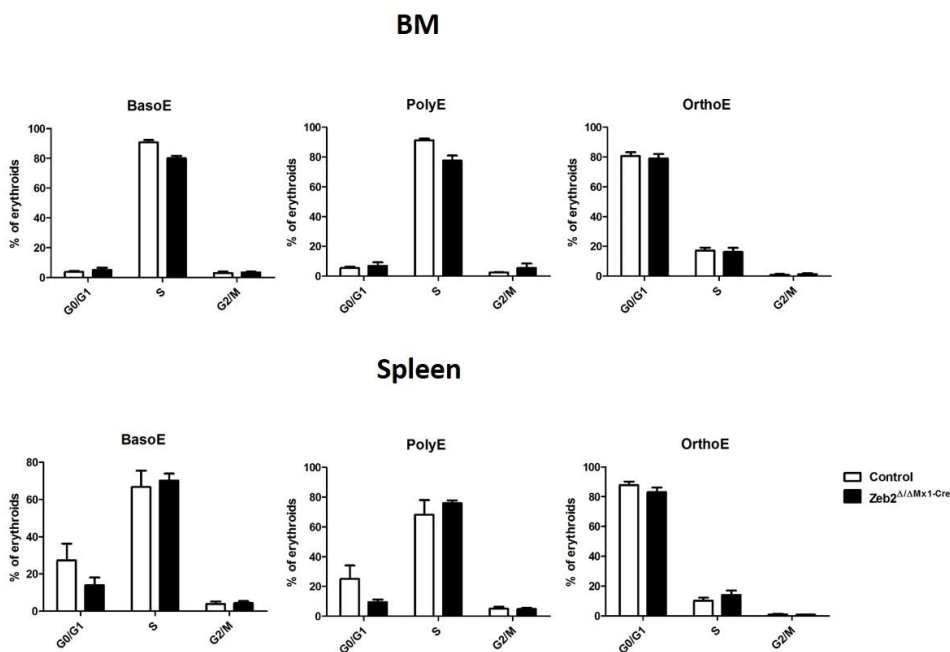

Figure 3.8 Cell cycle analysis by BrdU incorporation assay. Data show the distribution of indicated erythroid subsets in BM and spleen from control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$. Data are shown as means ± SEM.

We next examined the expression of putative transcription factors that contribute to erythropoiesis in sorted CD71^{high}TER119$^{+/-}$ population from BM of Zeb2$^{\Delta/\Delta Mx1-Cre}$ and control mice by real-time PCR analysis. The result showed that Zeb2 loss led to downregulation of Gata1, Fli2 and Klf2, which are mostly associated with erythroid differentiation. The balance between Klf1 and Fli1 plays an antagonistic role in the regulation of erythroid versus megakaryocyte development. Our previous data also indicated that in the absence of Zeb2, megakaryocytes are developing but show maturation impairment at the stage of terminal differentiation. In conclusion, the dysregulation of Klf1 and Fli1, as well as Gata1 in Zeb2-deficient early erythroid progenitors, may be due to dysregulation of key transcription factors that are promoting terminal differentiation in the erythroid lineage.
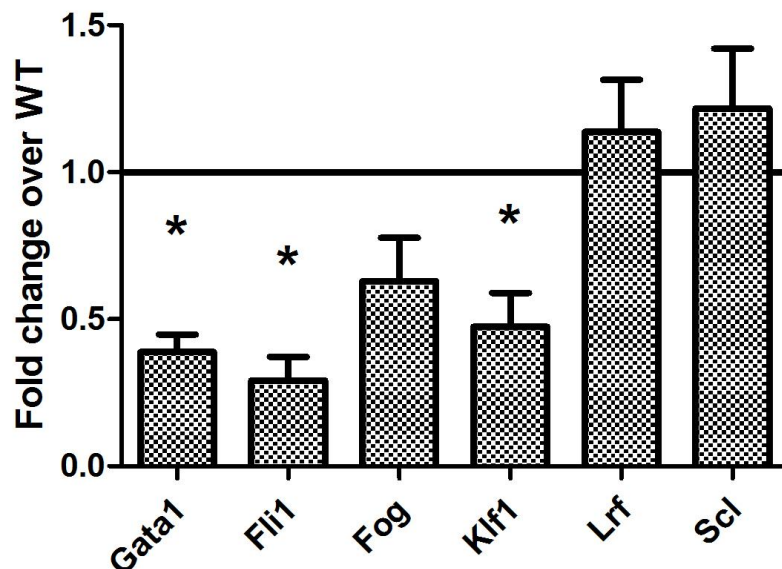

Figure 3.9 Potential target genes of Zeb2 in erythropoiesis. Folds change of mRNA levels (Zeb2$^{\Delta/\Delta Mx1-Cre}$ over controls) of indicated genes in sorted CD71highTER119+/- population. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

3.2 Zeb2 is essential for B-cell differentiation and orchestrates the transcriptional network by epigenetic regulation

The hierarchical network of transcription factors and epigenetic regulators mediate different stages of B-cell development, which starts from lymphoid-primed multipotent progenitors (LMPPs). LMPPs give rise to common lymphoid progenitors (CLPs), which further differentiate into early B-cell precursors: Prepro-B, Pro-B and Pre-B cells, and terminally differentiated mature B cells. Our previous data showed significantly lower number of B-lymphocytes in both peripheral blood (less than 20% of control) and bone marrow (5% of control) after Zeb2 deletion. However, at which stage of differentiation B lineage exhibited a differentiation block was not clear.

3.2.1 Loss of Zeb2 results in arrested B-cell development at Prepro-B stage

We first investigated different stages of B-cell maturation in the bone marrow of WT and Zeb2$^{\Delta/\Delta Mx1-Cre}$ animals based on surface marker expression profile using flow cytometry. Deletion of Zeb2 in the hematopoietic system did not change the relative frequency of LMPPs within the LKS compartment (Fig. 3.10). However, due to increased number of LKS in the BM, the absolute cell numbers of LMPPs were significantly increased in the BM of Zeb2-deficient

mice compared with littermate controls (Fig. 3.10). CLPs were found at similar frequency in the BM of Zeb2$^{\Delta/\Delta Mx1-Cre}$ and controls. Those data demonstrated that impaired B-cell differentiation in mice which lacking Zeb2 were not due to impaired lymphoid lineage commitment.

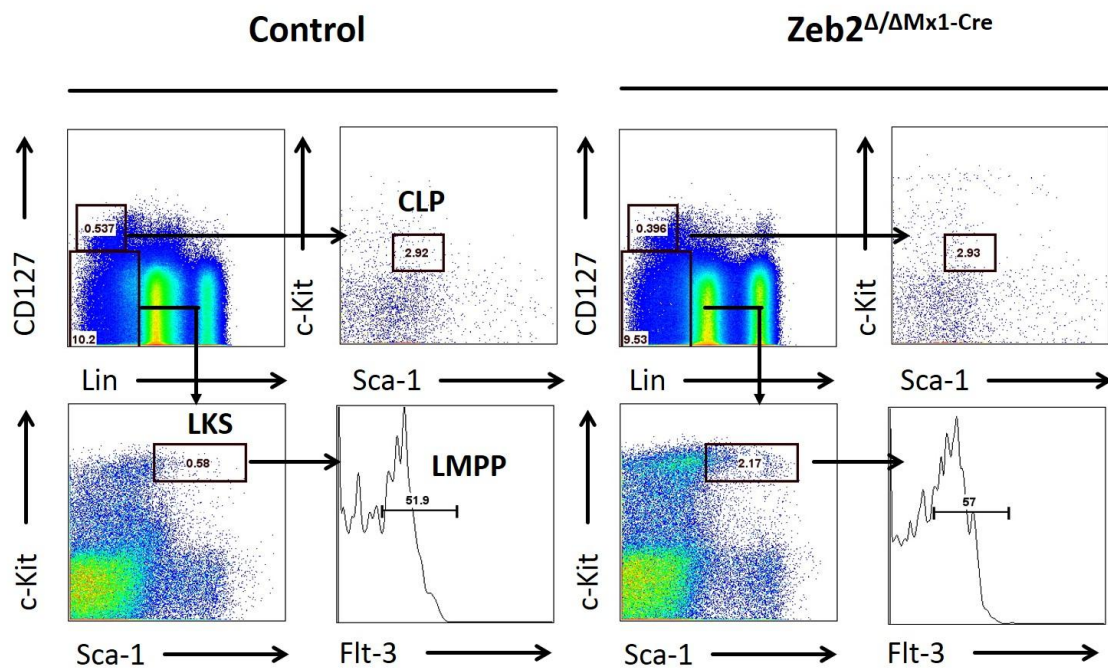
Control   **Zeb2$^{\Delta/\Delta Mx1-Cre}$**

Figure 3.10 FACS analysis of whole bone marrow cells to detect lymphoid-primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs) in control and Zeb2$^{\Delta/\Delta Mx1-Cre}$, as the gating strategy indicated. LMPPs are characterized by their LKS (Lineage$^-$ c-Kit$^+$ Sca-1$^{+)}$) Flt-3+ surface marker phenotype, while CLP (c-Kit^{med} Sca-1^{med}) were gated from Lineage-, CD127+ compartment.

We further analyzed specified B-cell subsets from B220+ compartment. Prepro-B is recognized as the first specified B-cell precursor and can developmentally progress to progenitor B-cells (Pro-B and Pre-B). All these three populations could be identified from B220$^+$IgM$^-$IgD$^-$ population by the

determination of c-Kit and CD24 expression (Fig. 3.11). The analyses revealed that Zeb2-deficient cells exhibited an apparent accumulation of Prepro-B but massively diminished Pro-B and Pre-B population. However, other late stages of B-cell subpopulations showed similar relative frequencies within the B220+ BM cells in both genotypes.
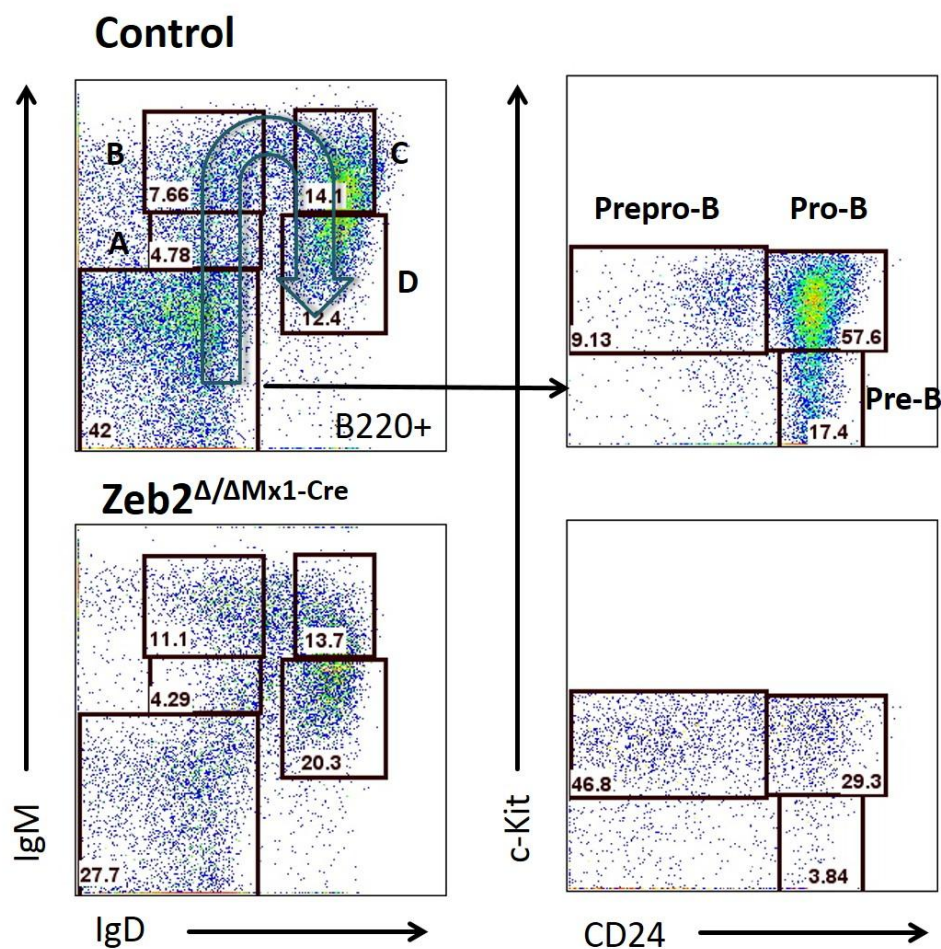

Figure 3.11 Representative immunophenotypic analysis of B220+ B lymphocytes in control and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice. The IgM$^-$IgD$^-$ cells in the B220$^+$ were gated out for Pre-Pro-B cell analysis. Curved arrow represents the B-cell differentiation transition from immature- to mature-B cells. A: Immature B; B: Transitional B; C: early mature B (Marginal Zone B and Regulatory B); D: late mature B (Follicular B, Germinal-Center B and plasma B).

In order to confirm the differentiation block at the transition from Prepro-B to Pro-B in Zeb2-deficient BM cells, we examined B-cell subsets by an additional panel, which is based on CD19 and CD 24 expression. CD19 is a cell surface antigen that is broadly expressed beginning at the Pro-B cells until mature B cells, which allowed us define the Prepro-B population. As depicted in a representative FACS plot, a notable differentiation block at the transition from Prepro-B to Pro-B stage was observed after Zeb2 deletion (Fig. 3.12).
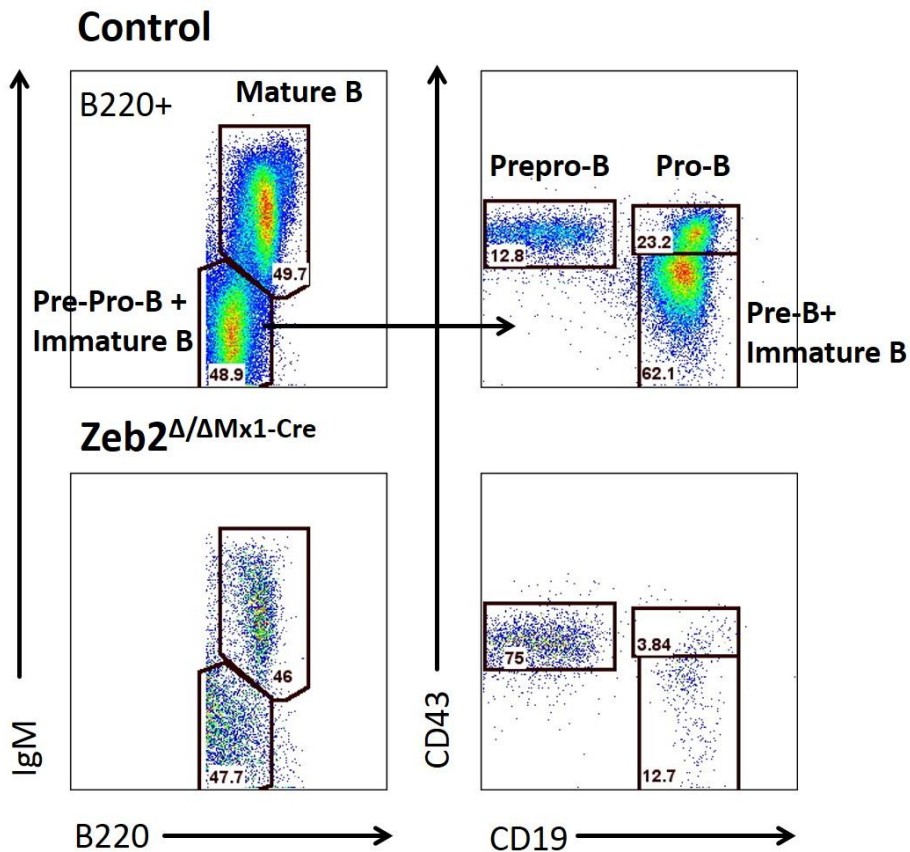

Figure 3.12 Representative staining profiles for Prepro-B (B220$^+$IgM$^-$CD19$^-$CD43$^+$), Pro-B (B220$^+$IgM$^-$CD19$^+$CD43$^+$), Pre- and immature B (B220$^+$IgM$^-$CD19$^+$CD43$^-$), and mature B (B220$^+$IgM$^+$) fractions in the BM of Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice and controls.

The statistical analyses of at least 3 biological replicates calculating different B-cell subsets within B220$^+$ bone marrow population further supported the developmental block at Prepro-B stage (38% in B220+ in Zeb2-deficient mice compared to 6% in controls) (Fig. 3.13). This data indicate that Zeb2 is essential for specification of B-cell development. However, since at later stages no significant relative differences between more mature B-cell subsets were detectable, we conclude that Zeb2 is not required for later stages of differentiation or maintenance.
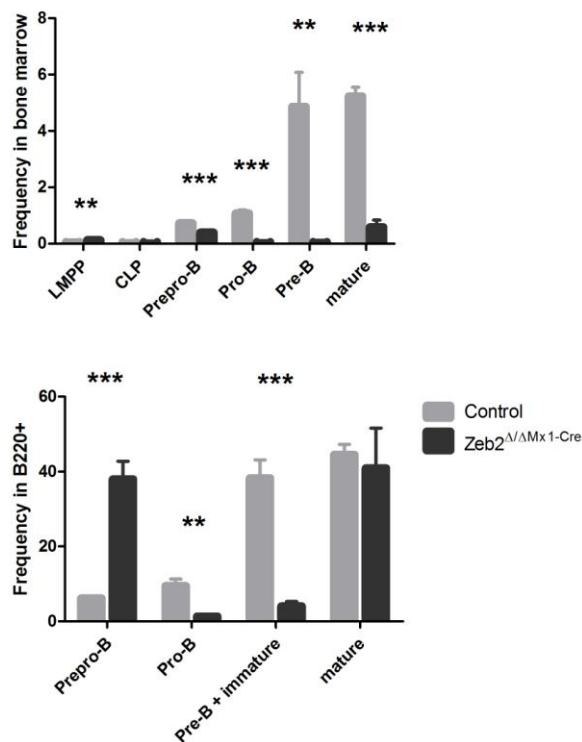

Figure 3.13 B-subsets distribution in control and Zeb2-deficienct cells. Shown are the percentages of indicated B fractions (based on the CD19 staining) in BM and B220 respectively. Data are shown as means ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

3.2.2 Zeb2-deficiency leads to impaired IL-7 receptor signaling and transcriptional network in Prepro-B

B-lymphopoiesis is precisely controlled by hierarchical network of key transcription factors. We analyzed mRNA expression of B-specific regulators in sorted Prepro-B population, in which the differentiation block was asserted. We found numerous genes to be down regulated upon Zeb2 deletion, including IL-7R, and its target EBF and Pax5 (Fig. 3.14). In addition, we found some key regulators of early B-cell development upregulated in Prepro-B cells, like E2A (1.8-fold increase), PU.1 (1.9-fold increase), Runx1 (1.4-fold increase), and Id2 (2.6-fold increase), while Id3 was strikingly inhibited.
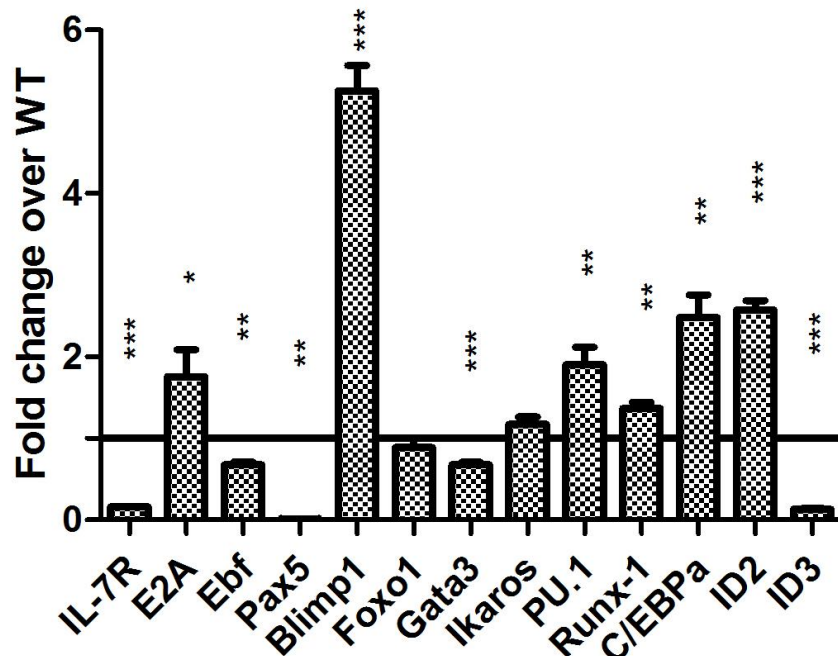

Figure 3.14 Potential target genes of Zeb2 in B-cell development. Folds change of mRNA levels (Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ over controls) of indicated genes. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

It has been suggested that IL-7 receptor signaling pathway can stimulate B-cell differentiation through up-regulation of Ebf and its target genes (Kikuchi et al., 2005). IL-7Rα$^{-/-}$ mice displayed a similar B differentiation block at Prepro-B stage as we observed in Zeb2 deficient mice. It is known that STAT5 could be activated by IL-7 signaling, so we further evaluated the phosphorylation of STAT5 in Prepro-B cells in response to IL-7 administration. Upon stimulating BM cells with 20ng/ml recombinant murine IL-7 for 15 min, the expression level of activated pSTAT5 was highly increased in WT Prepro-B cells, however, no detectable response was observed in corresponding Zeb2 deficient cells (Fig. 3.15). Thus, in the absence of Zeb2, early B-cell progenitors do not respond to IL-7 signaling, most likely due to lack of corresponding receptor expression. This may contribute to an impaired transcription factor network that is initiated upon IL7 signaling in Prepro-B cell subset.
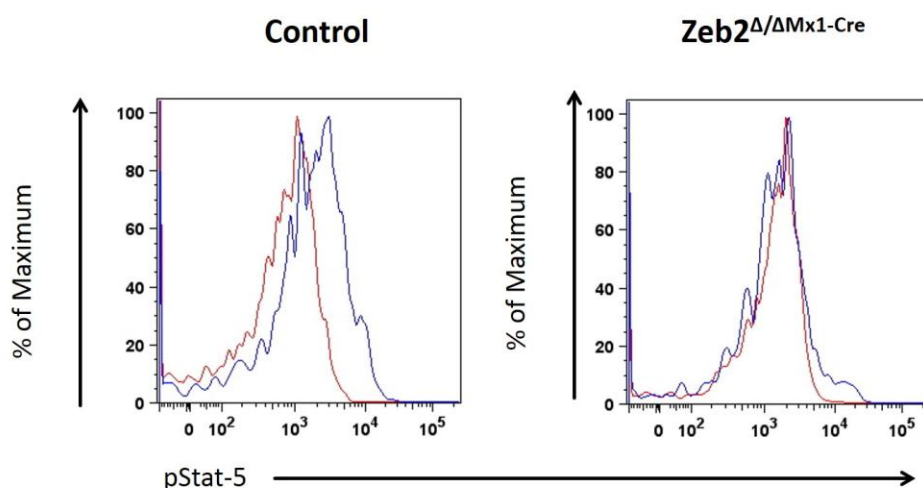

Fig. 3.15 Flow cytometry analysis of intracellular phosphorylated STAT5 in Prepro-B-cell population of Zeb2$^{Δ/ΔMx1-Cre}$ mice and controls. BM cells were stimulated (blue line) or not stimulated (red line) with IL-7 for 15 min. Prepro-B population has been gated on B220^{+}IgM^{-}CD19^{-}CD43^{+} subset.

3.2.3 Zeb2 loss results in altered epigenetic regulation

In recent years, investigators have shown that epigenetic regulator-transcription factor complexes play critical roles during B-cell development. It has been demonstrated previously, that Zeb2 can affect gene expression by its ability to bind to the NuRD corepressor complex (Verstappen et al., 2008). To further investigate whether Zeb2 affects the expression level of key members of the nucleosome remodeling machinery, we performed qPCR analyses on sorted Prepro-B subset. We found 1.9 folds increase in Ezh2 and 1.7 folds increase in Suz12 expression, which are crucial members of the polycomb repressive complex 2 (PRC-2). PRC-2 is involved in histone modification, specifically in methylation of the lysine residue at the position 27 of the Histone 3 (H3K27). In addition, we found a slight but significant increase in mRNA expression in Lsd1 and Mi-2β, the components of the Mi-2/NuRD complex, while no difference in the expression level of HDACs were detectable (Fig. 3.16).
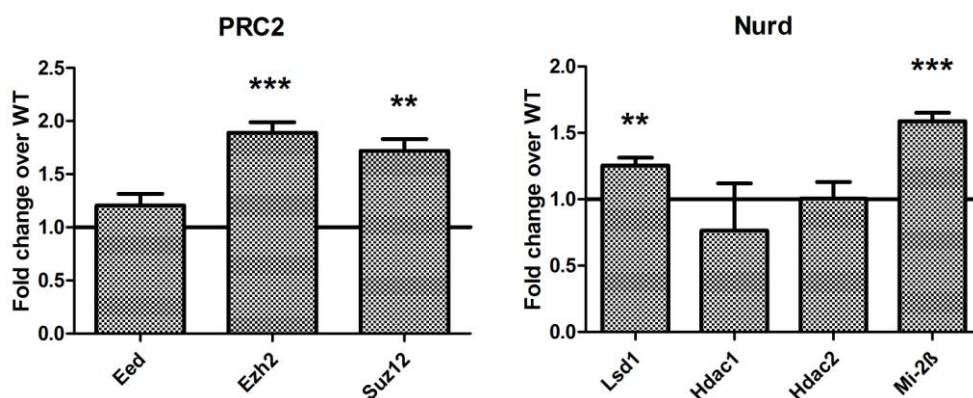

Fig. 3.16. Relative expression levels of selected PRC-2 and Mi-2/NuRD components

measured by real-time PCR analysis. Values are in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ Prepro-B cells normalized to the expression levels in controls. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

Histone acetylation and methylation are the best characterized epigenetic modifications. Lysine residues of histone 3 can be mono-, di-, or tri-methylated, each of which can differentially regulate chromatin structure and modulate transcription. In general, acetylation at H3K9, H3K14 and H3K18, or tri-methylation at H3K4, H3K36 and H3K79 are associated with open chromatin structure and considered as activating histone marks, whereas tri-methylation at H3K9 and H3K27 have been characterized as repression marks. In order to investigate whether altered histone remodeling complexes will lead to changed expression levels of different H3 modifications in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ cells, we next quantified 21 of H3 modifiers by colorimetric based ELISA-like assay. B220^{low} population, which is enriched with early B-cell subsets, was sorted for the measurement instead of Prepro-B population, since a certain amount of histone protein were needed. Strikingly, the majority of the H3 modification levels was elevated in absence of Zeb2, including H3K4me3 and H3K27me3, which is consistent with higher expression level of PRC-2 and Mi-2/NuRD complexes. However, when we analyzed whole bone marrow (WBM) cells of Zeb2-deficient and control mice only little differences were detected between the genotypes, implicating that the differences were only in cells with block in differentiation and diluted by other hematopoietic

lineages, like granulocytes, wich is the largest bone marrow population (Fig. 3.17). In summary, loss of Zeb2 can cause altered histone modification, which is cell population specific.
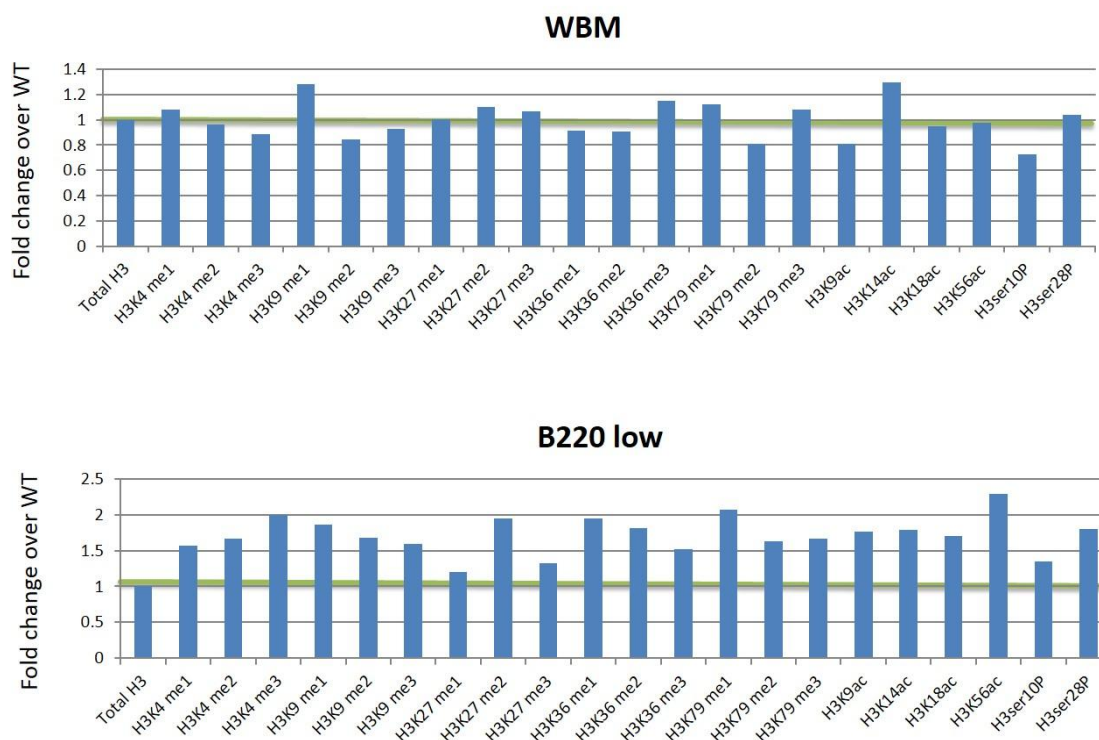
Fig 3.17 Relative change of each histone H3 modification between Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ and control WBM (top) and B220^{low} cells (bottom). Values were normalized to total H3 expression level

It has been demonstrated that histone modification and DNA methylation can influence or be dependent on each other. A recent study of *Dnmt3a*-null HSCs identified some increased HSC fingerprint genes without decreased methylation level, but within increased H3K4me3 expression level, this suggests two distinct mechanisms being involved in the upregulation of HSC

genes in Dnmt3a-null HSCs: hypomethylating directly binding targets (Runx1 and Gata3), and indirect histone modifications (Nr4a2) (Challen et al., 2012). Since Runx1, Gata3 and PU.1 are all significantly altered in their expression upon Zeb2 deletion as well, we next examined the mRNA levels of DNA methyltransferases (Dnmt1, Dnmt3a and Dnmt3b) and Tet methylcytosine dioxygenases (Tet1, Tet2 and Tet3) in sorted Prepro-B cells by real-time PCR. We found that Dnmt1 and Dnmt3b had 2.3- and 1.4-fold increase respectively, but Dnmt3a was slightly decreased, while all three Tets were consistently down regulated after Zeb2 deletion (Fig. 3.18). Thus, the impaired transcription factor network in Prepro-B population may be caused by altered epigenetic regulation mechanisms, including histone modification and DNA methylation.
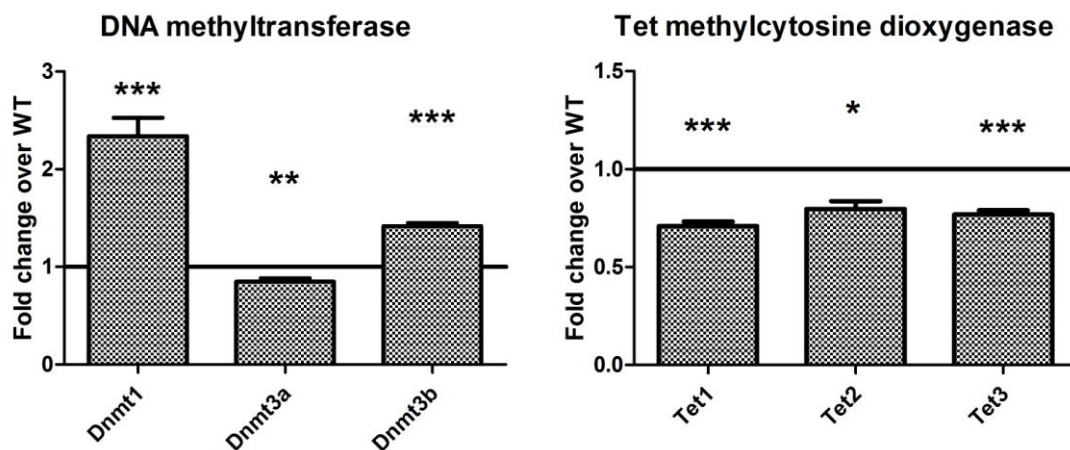

Figure 3.18 Relative expression levels of selected genes measured by real-time PCR analysis. Values are in sorted $Zeb2^{\Delta/\Delta Mx1-Cre}$ Prepro-B cells normalized to the expression levels in controls. Data are shown as mean ± SEM. *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$.

3.2.4 Zeb2 is affecting the transcriptional and epigenetic programs also at the stem- and progenitor level

It has been demonstrated that epigenetic marks play a pivotal role in HSC development and orchestrate the fate decision between stemness and lineage commitment. The link between the abnormal epigenetic code and B-cell differentiation failure upon Zeb2 deletion gave us a hint to hypothesize that defective epigenetic marks may take place in progenitor or even stem cell population of Zeb2-deficient mice. If so, this may provide an explanation for the complex Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ phenotype.

In order to challenge this hypothesis, we examined the expression levels of proteins involved in epigenetic regulation in HSPC population (LKS) by real-time PCR. Consistent with the major differences, we observed in Prepro-B cells, Eed and Ezh2 of PRC2 complex were highly upregulated in Zeb2-deficient LKS cells, whereas analyses of different components of the Mi-2/NuRD complex revealed only a significant upregulation of LSD1. In addition, Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ LKS cells showed higher expression of three DNA methyltransferases Dnmt-1 (2-fold), Dnmt-3a (2.1-fold) and Dnmt-3b (2-fold), while no difference in the expression level of Tet methylcytosine dioxygenases was detectable (Fig. 3.19). Taken together, the observed changes in expression for epigenetic factors, mainly Dnmts, in LKS cells may be responsible or contribute to the phenotype in Zeb2-deficient hematopoiesis.
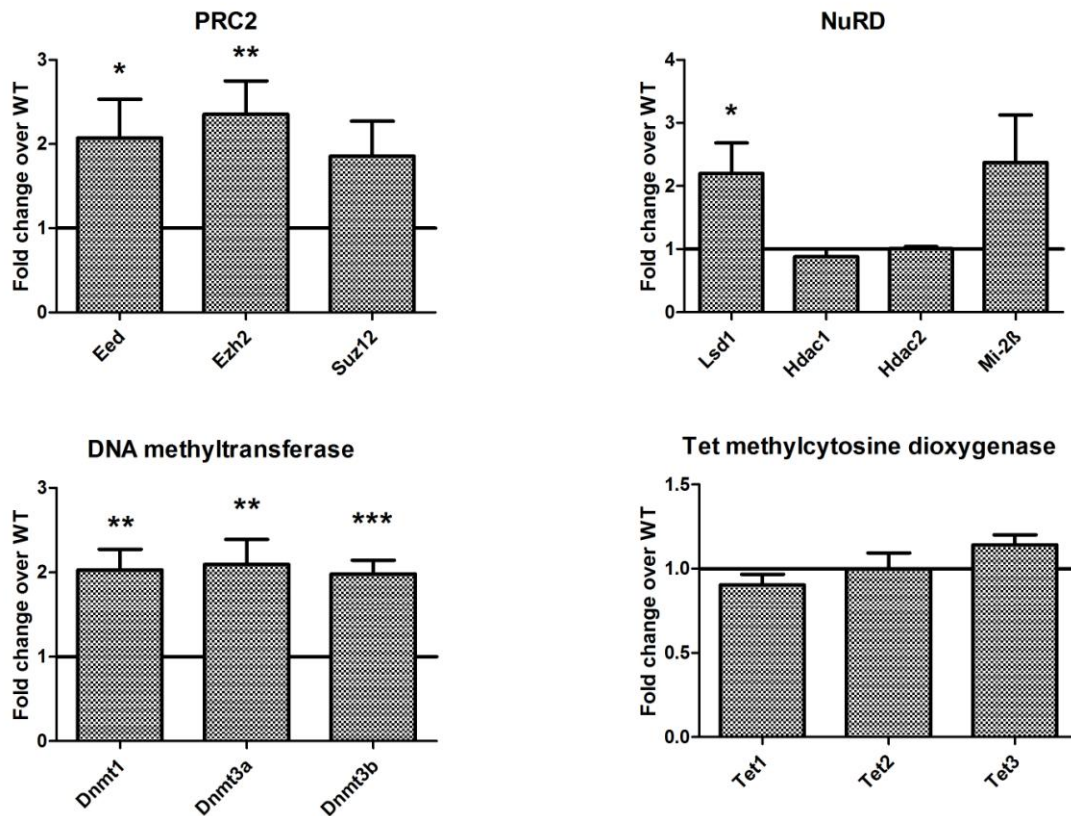
Figure 3.19 Relative expression levels of epigenetic factors measured by real-time PCR analysis. Values are in sorted Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ LKS (Lin⁻Sca-1⁺c-Kit⁺) cells normalized to the expression levels in controls. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

We next wondered if changes in expression of epigenetic regulators affect the expression of key multipotency and differentiation transcription factors in LKS population. We found an upregulation of Runx1 (2.1-fold increase) and Gata-2 (2.1-fold increase) in Zeb2-deficient LKS cells, which is known to be essential for preservation of stemness and thus might contribute to differentiation inhibition. On the other hand we saw an overexpression of PU.1 (1.5-fold increase) and E2A (1.7-fold increase), which might lead to impaired B-lineage

commitment (Fig. 3.20). It has been reported that Dnmt3a-null HSCs could upregulate HSC multipotency factors (including Runx-1 and Foxo-1) and inhibit differentiation genes (including PU.1 and E2A) through affecting the DNA methylation level (Challen et al., 2012). As Zeb2 deficient cells display increased expression of PU.1 and E2A, and we observe an increase in the expression level of these genes supports our hypothesis, that Zeb2 may act by modifying DNA methylation. This indicated that the changes observed in Zeb2-deficient LKS cells would probably be caused by a broad spectrum of epigenetic changes, mainly via changed DNA methylation.
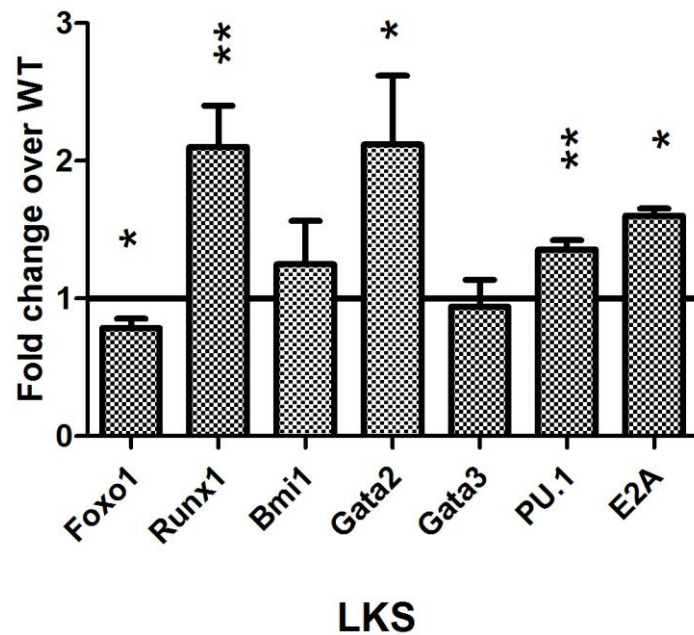

Figure 3.20 Relative expression levels of select key multipotency and differentiation genes measured by real-time PCR analysis. Values are in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ LKS cells normalized to the expression levels in controls. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01.

3.2.5 Zeb2 loss in HSCs leads to perturbed DNA methylation

To investigate the impact of Zeb2 on DNA methylation, we performed genome-wide RRBS analysis, which enables quantitative DNA methylation measurements using low amount of genomic DNA and therefore enabling to perform this assay within a relatively small population like HSC. Thus, LKS-SLAM (LKS CD48⁻CD150⁺) populations were sorted from Zeb2$^{\Delta/\Delta Mx1-Cre}$ and control mice, and two biological replicates for each genotype were analyzed. In the RRBS analysis, 4.4 million CpG sites were covered with average 16X and 28X CpG coverage in control and Zeb2$^{\Delta/\Delta Mx1-Cre}$ samples respectively (Table 3.2). About 95% of CpG islands (CGIs), 85% of gene bodies and 75% of promoters had more than 10 fold read coverage. This coverage and read depth allowed a confident examination of differential genomic methylation patterns (Figure 3.21). In mammals, 70% to 80% of CpG cytosines are methylated, however only very rare within CpG islands (Jabbari and Bernardi, 2004). Comparison of global CpG methylation levels within promoter, gene body and CpG islands between WT and Zeb2$^{\Delta/\Delta Mx1-Cre}$ HSCs showed no difference in the whole genome (Fig. 3.22).

Table 3.2 Statistics of RRBS analysis.

Sample	Total Read Number	Mapping Efficiency	Unique CpGs	Avg. CpG Coverage	Bisulfite Conversion Rate
Control 1	68,803,864	35%	6,012,631	16X	99%
Control 2	55,447,891	52%	6,263,200	18X	99%
Zeb2-KO 1	76,628,398	57%	6,567,027	28X	99%
Zeb2-KO 2	79,461,656	57%	6,383,905	30X	99%
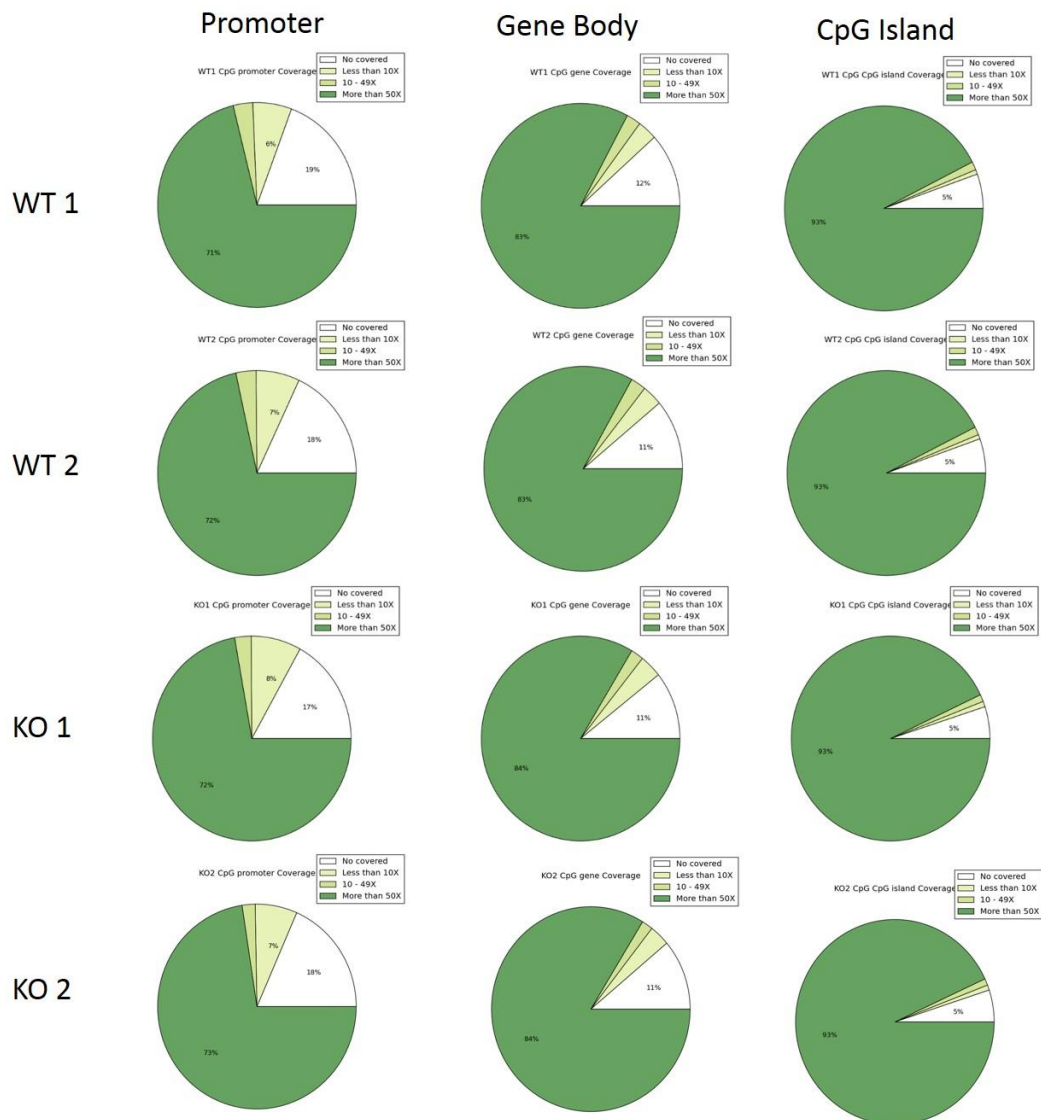
Figure 3.21 RRBS read coverage at the promoter, gene body and CpG islands.
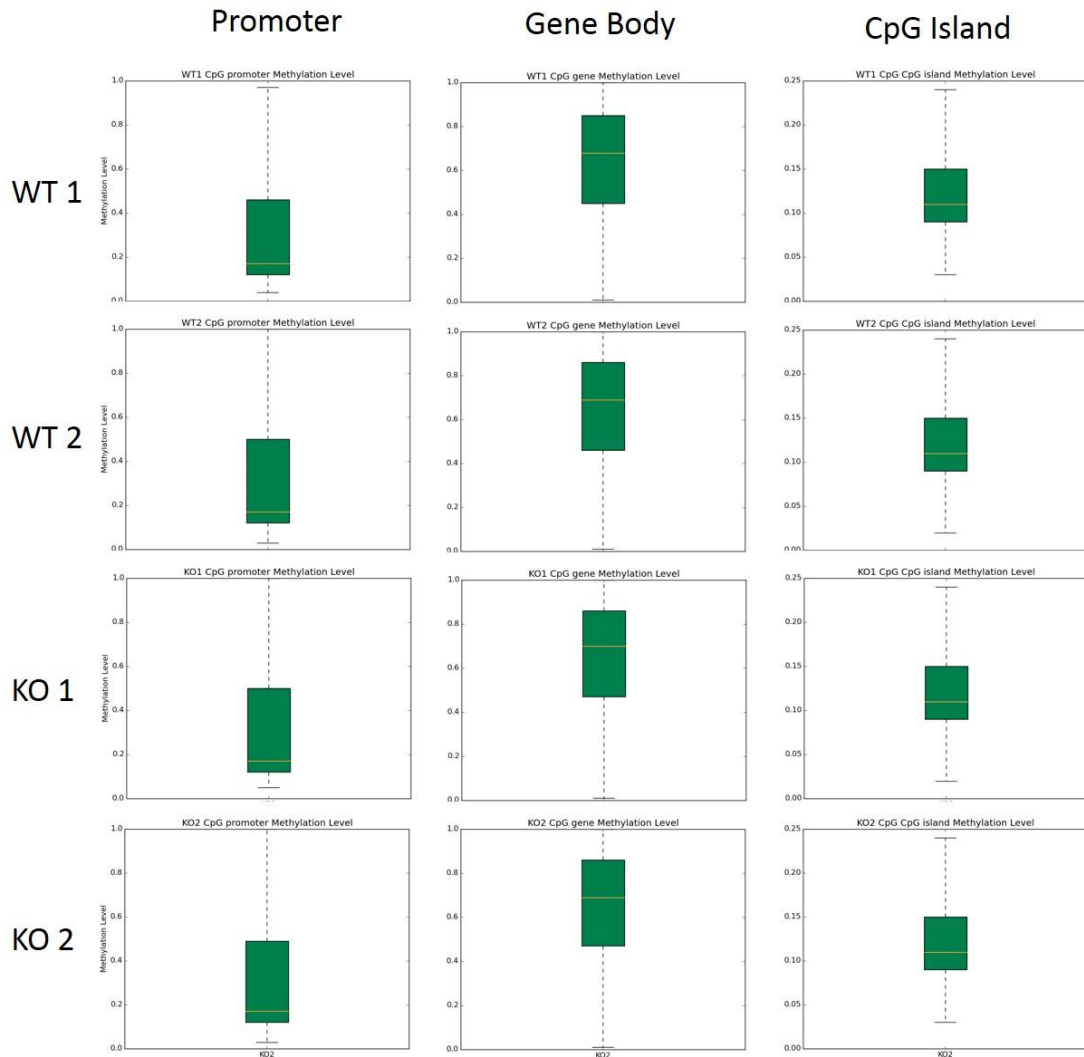
Figure 3.22 Comparison of global CpG methylation levels within promoter, gene body and CpG islands between Zeb2 WT and KO HSCs. The boxplot displays the mean DNA methylation levels as well as the distribution of methylation levels within a sample. The boxplots show the 2nd quartile (mean) as a yellow line, and 1st quartile and 3rd quartile as the bottom and upper bounds of the box, respectively.

To gain further insight into the similarities and differences in DNA methylation between WT and Zeb2 KO, we analyzed methylation changes at individual CpG site. We found that 5% of covered CpGs were differentially methylated between WT and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ HSC. Upon Zeb2 ablation, 72% of these

differentially methylated cites exhibited an increased methylation and 28% of

which exhibited increase (hyper) and decreased (hypo) in methylation at the

CpG cited compared to WT respectively (Table 3.3, Fig. 3.23).

Table 3.3 The counts of CpG sites in differential methylation

Classification of CpG sites	Count
insignificant	4195646
strongly hypermethylated	12633
hypermethylated	133578
strongly hypomethylated	5320
hypomethylated	52518

Hypermethylated: 0–33% more methylated than reference (p–value < 0.05); hypomethylated: 0–33% less methylated than reference (p–value < 0.05); strongly hypermethylated: 33–100% more methylated than reference (p–value < 0.05); strongly hypomethylated: 33–100% less methylated than reference (p–value < 0.05); insignificant: statistically insignificant difference in methylation.
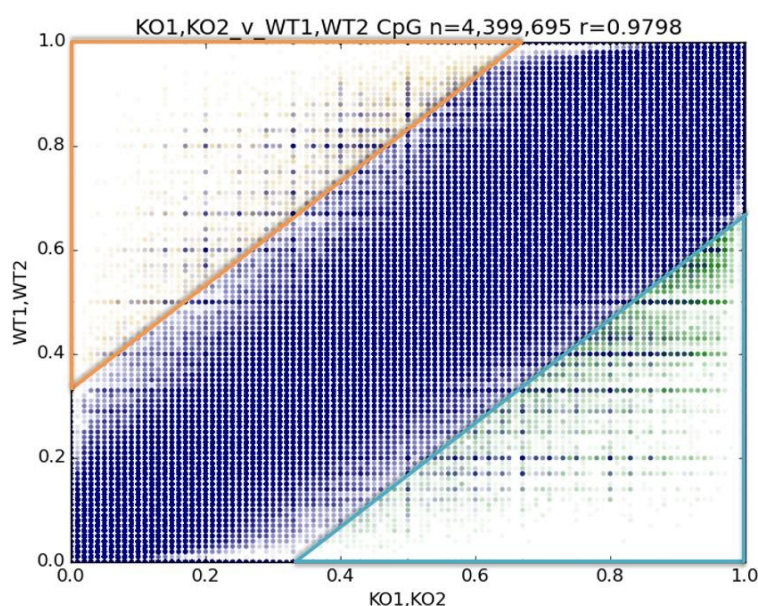

Figure 3.23 Scatter plots depict correlation between WT and Zeb2 KO. Blue dots show

sites with statistically insignificant differences between samples, significantly hypo- or

hyper-methylated sites are colored yellow or green respectively. The orange and blue triangle area indicates 33‐100% less and more methylated Zeb2 KO HSCs than control HSCs.

Although the overall DNA methylation changes did not differ greatly between Zeb2-deficient and control HSC, there may be specific sites where changes in methylation are biologically important. We therefore examined the CpGs of key HSC regulators, according to our previous qRT-PCR analysis, which were differentially expressed between WT and Zeb2$^{\Delta/\Delta Mx1-Cre}$ in LKS as well as Prepro-B population. Interestingly, some of those genes that we identified previously to be differentially expressed also showed changes in methylation, including PU.1, Foxo-1, Pax5 and ID-3. Moreover, additional genes with higher or lower methylation have been identified, which are also associated with stem cell fingerprint, such as Lmo2, Pbx1, Prdm16 and Runx3 (Fig. 3.24). The changes in methylation was also translated in changes in gene expression, as examined by real-time PCR (Fig. 3.25).
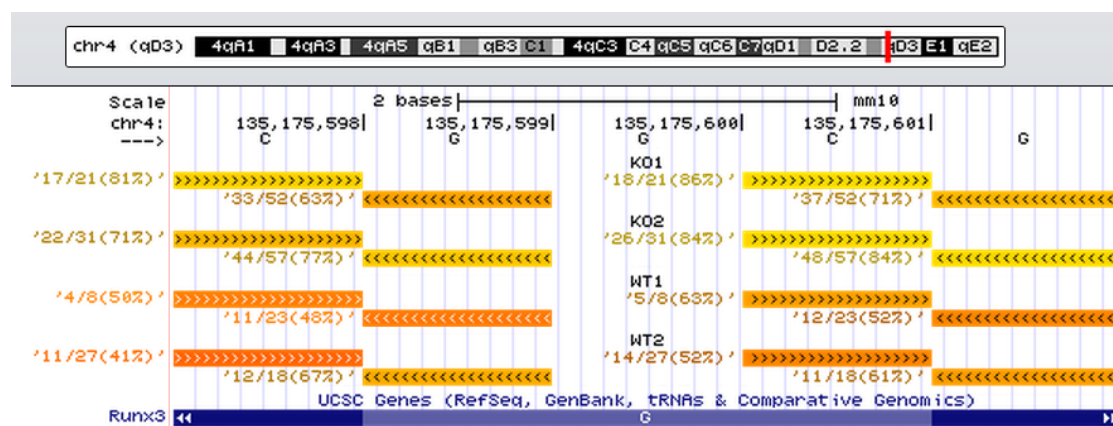
Figure 3.24 UCSC genome browser track of hypermethylated Runx3 fragment.

Chr4:135,175,598-135,175,602, 5 bp, in both WT and Zeb2 KO were displayed and the methylation ratios at each site were indicated. The label on the left of a CpG, say '17/21', indicates 17 reads were methylated in 21–fold sequencing read coverage and the DNA methlation ratio was 81%. Yellow corresponds to higher methylation levels, while red corresponds to lower methylation levels. Intermediate methylation levels are represented with orange.
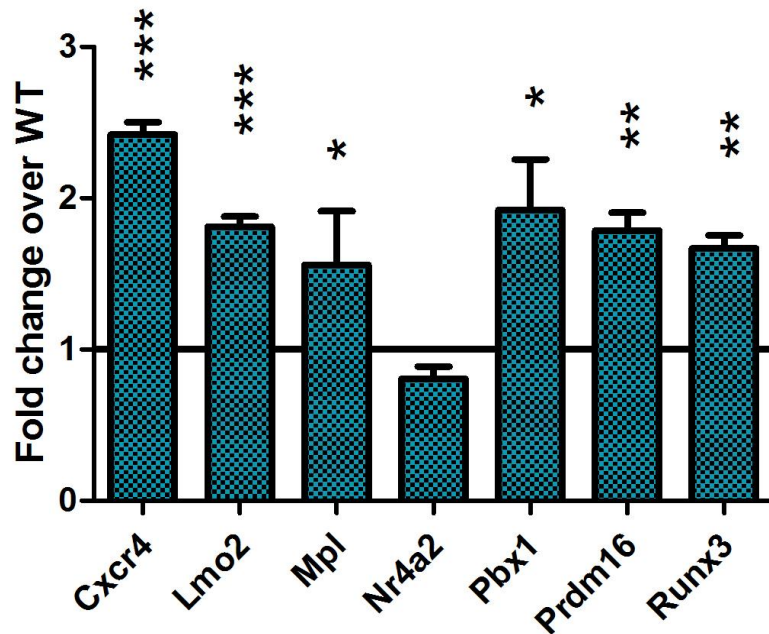
Figure 3.25 Relative expression levels of select HSC fingerprint genes containing hyper- or hypo-methylated CpG sites in LKS population. Values are in Zeb2$^{\Delta/\Delta Mx1-Cre}$ LKS cells normalized to the expression levels in controls. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

In conclusion, Zeb2 is essential for multilineage differentiation at different stages of hematopoiesis. The differentiation defects observed in Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice are at least in part due to changes in DNA methylation of key HSCs regulators.

3.3 Myeloproliferative disorder upon Zeb2 loss is due to altered JAK/STAT signaling pathway

3.3.1 Zeb2 deficiency results in impaired JAK/STAT signaling pathway

To further characterize the main biological process targeted by Zeb2, gene ontology (GO) enrichment analysis was performed over 2,500 genes containing strongly hyper- or hypomethylated CpG sites. The results showed that genes involved in regulation of cell adhesion, transcription and protein kinase (including phosphorylation, serine/threonine protein kinase, tyrosine protein kinase) are the most enriched GO terms (Fig. 3.26). As an EMT regulator and transcriptional repressor, it is not surprising that altered cell adhesion and transcriptional regulation were altered after Zeb2 deletion. Interestingly, the GO term associated with protein kinase, in particular tyrosine kinase activity was highly enriched as well, which led us to investigate cell signaling activity in Zeb2 deficient cells.

Our previous work showed that upon induction of Zeb2 deficiency within the hematopoietic system we observed reduced erythrocytes and thrombocytes while granulocytes were increasing over time. In addition these mice developed a significant and progressive increase in spleen size. Histological analyses revealed a massive destruction of spleen architecture and a massive extramedullary hematopoiesis in Zeb2 depleted animals. Silver staining of

bone marrow sections showed a significant increase in the appearance of reticular fibers in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ animals. In addition, there was an increase in frequency of megakaryocytes with abnormal morphology in the bone marrow and spleen. All above features have been described as a hallmark of myeloproliferative diseases in humans (Lang et al., 2003).
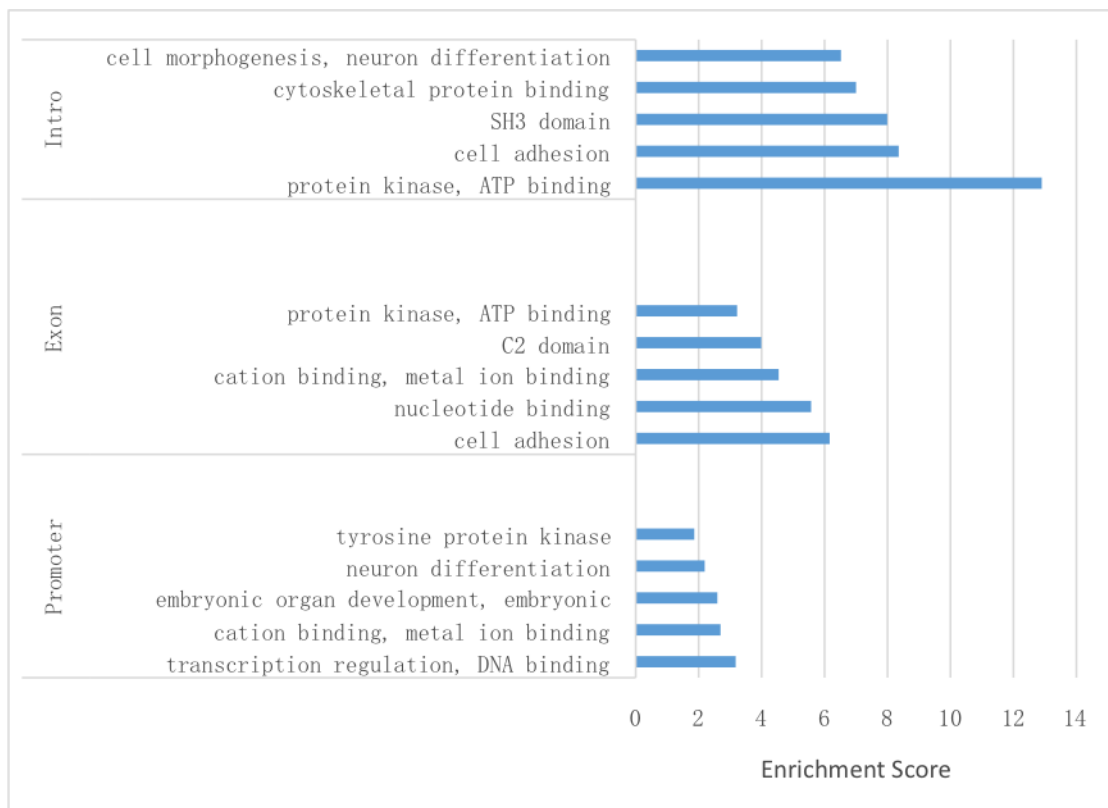
Figure 3.26. GO analysis of the differentially methylated genes.

In 2005, several groups identified a constitutively active JAK2V617F mutation in Janus kinase 2 (Jak2) gene in (Ph)-negative MPN bone marrow cells (Klarl et al., 2006). This mutation results in constitutively activation of Jak2 and subsequently activates signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase

(ERK) and phosphotidylinositide 3-kinase/protein kinase B-AKT (PI3K/AKT) signalling pathways resulting in expansion of the myeloid lineage within the bone marrow. More Jak2 mutations that contribute to activation of JAK signaling have been discovered in MNPs, which suggests a critical role of JAK kinase activity in myeloproliferative disorders (Bratosin et al., 2004). Since Zeb2 deficient mice exhibit features resembling MPN in humans, we examined the activity of those signaling pathways in the bone marrow from control and Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice by intracellular detection of phosphorylated signaling proteins utilizing flow cytometry. However, when we analyzed the signaling activity at freshly isolated bone marrow cells, we did not detect differences in the level of phosphorylated STAT3, STAT5, ERK1/2 between the genotypes at steady state (Fig. 3.27).

Next we sought to test, whether we would observe different sensitivity of the signaling pathways, when cells were treated with defined cytokines. To investigate the complex network of cytokine/ growth factor in control and Zeb2$^{\Delta/\Delta Mx1-Cre}$ hematopoietic system, we harvested the whole bone marrow cells and analyzed the activity of JAK/STAT, MAPK/ERK and PI3K/AKT signaling by western blot after stimulation with different cytokines. Interestingly, Zeb2 deficient bone marrow cells were more sensitive to the granulocyte colony- stimulating factor (G-CSF), resulting in striking increase in phosphorylation of STAT3, STAT5 and ERK compared to control cells (Fig. 3.28). In contrast, Zeb2$^{\Delta/\Delta Mx1-Cre}$ cells did not activate the JAK/STAT signaling

pathway neither by IL-3 nor IL-6 stimulation, as no change of phosphorylated STATs was detected (Fig. 3.28). However, analyses of p38 and AKT phosphorylation upon stimulation with G-CSF, IL3 and IL6 did not show difference in activation of these pathways between WT and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ bone marrow cells (Fig. 3.28).
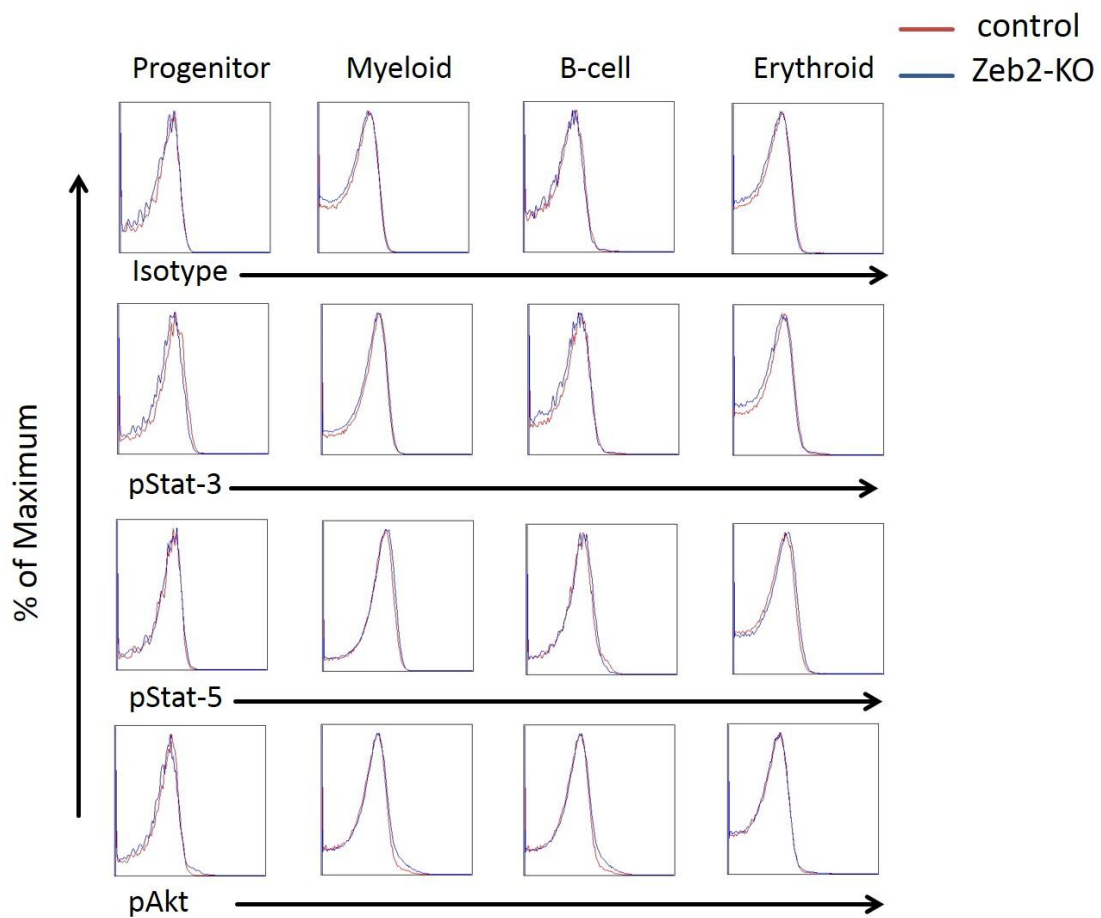
Figure 3.27 Representative flow cytometry analysis of intracellular phosphorylated STAT3, STAT5 and ERK in indicated populations.
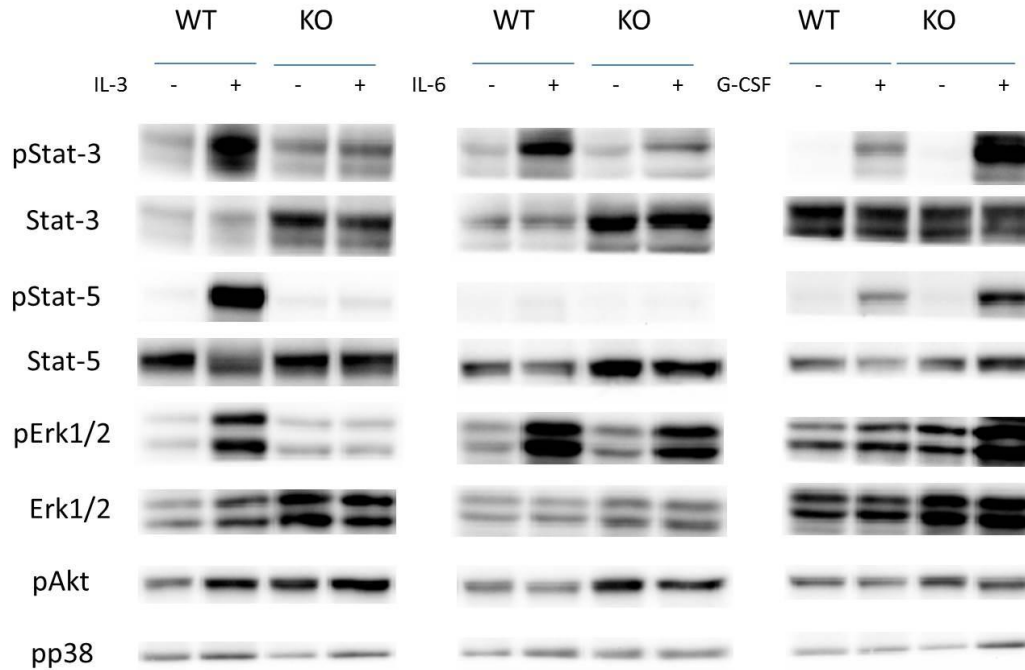
Figure 3.28 Representative western blot showing phosphorylation of STAT3, STAT5, ERK1/2, AKT and p38 in total lysates of bone marrow cells from control and Zeb2-KO mice in the presence of IL-3 (10ng/ul), IL-6 (10ng/ul) and G-CSF (10ug/ul) for 15 minutes. Non-phosphorylated antibodies were used as loading control.

Since bone marrow is containing various hematopoietic lineages, the increased activation of STATs in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ BM cells after G-CSF stimulation might be due to significant increase in the granulocytic lineages compared to control mice. Thus we performed western blot analyzes in selected Mac1 positive myeloid cells and Lineage negative cells, that contain mostly stem and progenitor cells. Consistent with our previous observation in total bone marrow cells we observed augmented STAT phosphorylation upon G-SCF exposure and diminished or absent signaling upon IL6 stimulation in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ cell subsets (Fig. 3.29, 3.30). In addition, intracellular phosphorylation levels in different hematopoietic lineages were detected by

flow cytometry, as this method allows the detection of signaling activity at the single cell level. As shown in Fig. 3.31 we not only confirmed our previous findings, but also demonstrated that attenuated STAT3 phosphorylation after IL-6 stimulation takes place in progenitor cells, which might be responsible for impaired differentiation in multiple hematopoietic lineages (Fig. 3.31).
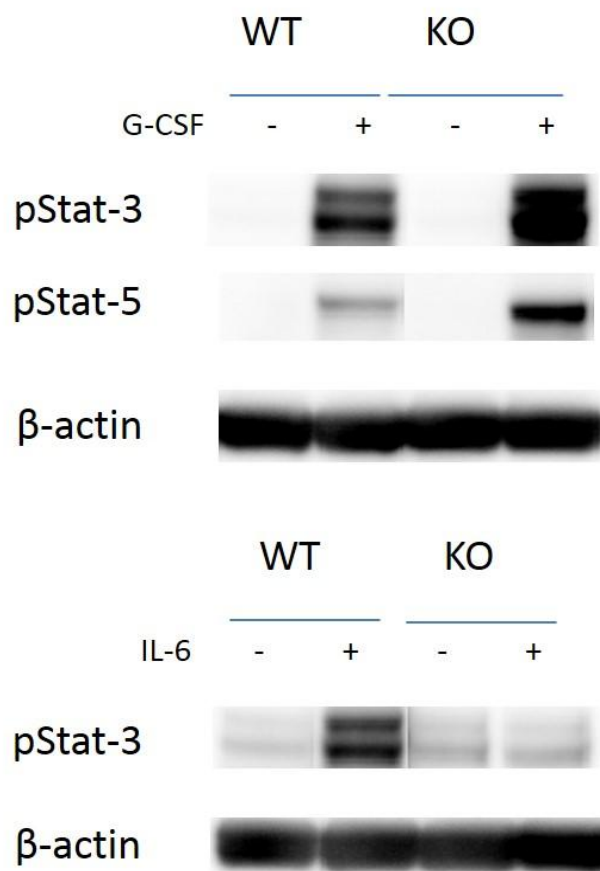
Figure 3.29 Representative western blot showing STATs phosphorylation MACS purified myeloid cells from control and Zeb2-KO mice in the presence of IL-6 (10ng/ul) and G-CSF (10ug/ul) for 15 minutes.
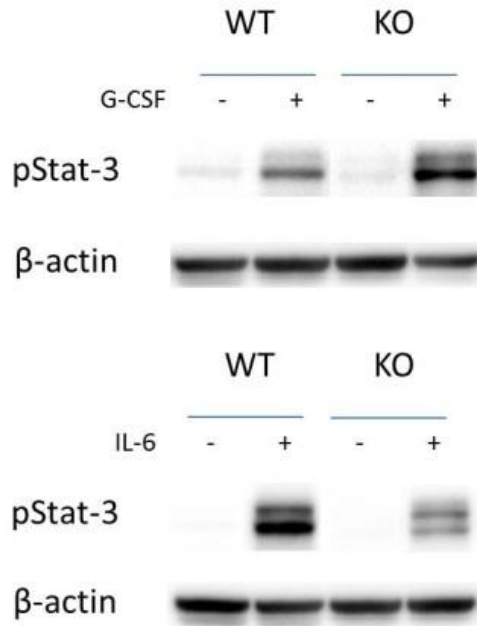
Figure 3.30 Representative western blot showing phosphorylation of Stat3 in MACS purified Lin- cells from control and Zeb2-KO mice in the presence of IL-6 (10ng/ul) and G-CSF for 15 minutes.
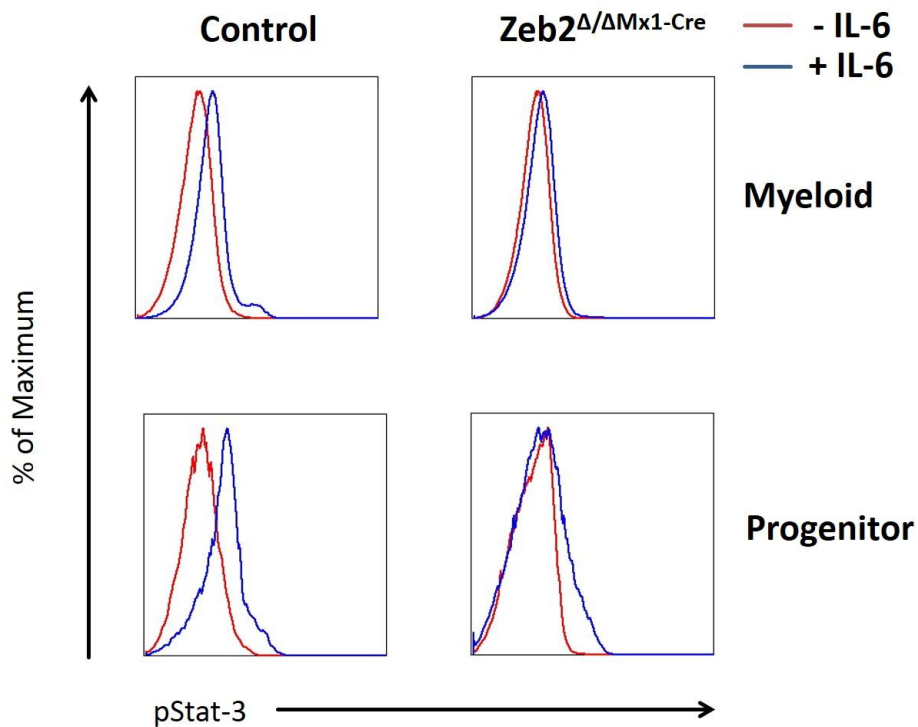
Figure 3.31 Representative flow cytometry analysis of intracellular phosphorylated STAT3 in control and Zeb2-KO myeloid and progenitor populations after IL-6 stimulation.

Differential activation of JAK/STAT in control and Zeb2-deficient cells stimulated by IL-3, IL-6 and G-CSF might be ascribed to differently expressed receptors on those cells. Therefore, we examined the expression levels of receptors for different cytokines in different populations. Interestingly, IL-6Rα was significantly downregulated (0.5-fold) in both progenitors and myeloid cells, while the mRNA of G-CSFR and IL-3Rα only decreased in progenitors but not in myeloid populations (Fig 3.32). This suggested that the inability of Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ cells facilitating JAK/STAT pathway by IL-6 may due to the reduced abundance of its receptors at the cell surface. In contrast, differential activation of JAK/STAT to IL-3 and G-CSF in control and Zeb-KO cells might be due to other receptor-independent mechanisms.
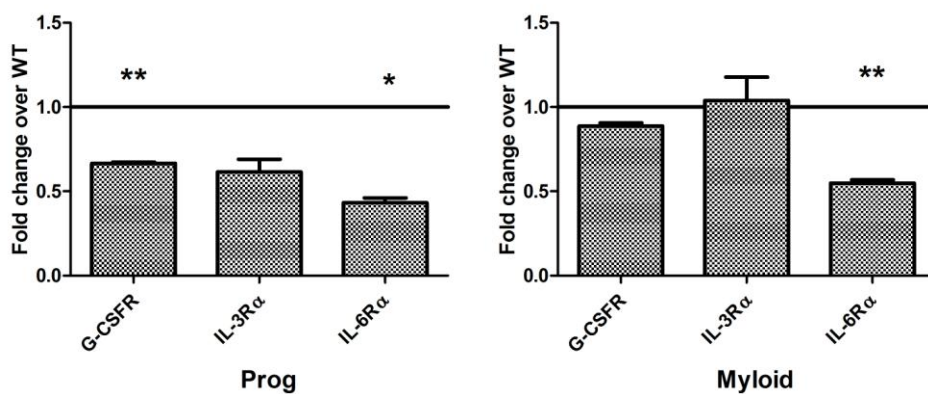
Figure 3.32 Relative expression levels of receptors to G-CSF, IL-3 and IL-6 measured by real-time PCR analysis. Values are in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ progenitor and myeloid cells normalized to the expression levels in controls. Data are shown as mean ± SEM. *, P< 0.05, **, P < 0.01; ***, P < 0.001.

3.3.2 JAK inhibition by Ruxolitinib ameliorates some myeloproliferative features

Ruxolitinib is a drug, which is used for the treatment of MPN, related symptoms in patients with primary or secondary myelofibrosis. Ruxolitinib is a small molecule that selectively inhibits JAK1 and JAK2 kinases (Quintas-Cardama et al., 2010). Because Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice exihibit MPN phenotype and hematopoietic cells are hypersensitive to G-CSF, we next evaluated the effect of ruxolitinib in Zeb2-deficient mice. Firstly, western blot analyses confirmed that ruxolitinib was able to abrogate G-CSF-induced activation of JAK/STAT pathway in vitro (Fig. 3.33).
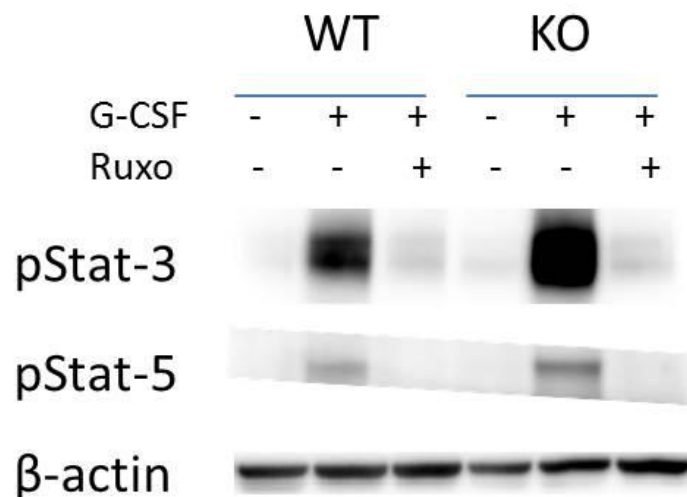
Figure 3.33 Representative western blot showing phosphorylation of STAT3 and STAT5 in total lysates of bone marrow cells from control and Zeb2-KO mice in the presence of only G-CSF (10ug/ul) or pretreated cells with ruxolitinib followed by G-CSF stimulation for 15 minutes.

Ruxolitinib was then evaluated in in vivo assays by administration to control and Zeb2-KO mice twice a day with either ruxolitinib or vehicle via oral gavage. Mice were sacrificed and analyzed one week later. As we expected, Zeb2-KO mice, which receiving ruxolitinib, exhibited a significant shrinkage of the spleen, but not control mice (Fig. 3.34). However, we did not observe any rescue in differentiation of other lineages in Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice (data not shown).
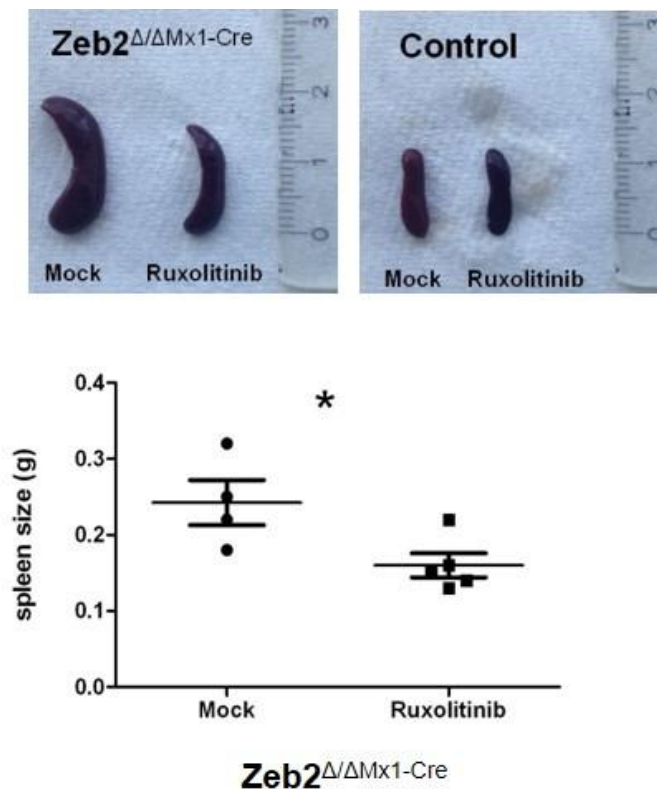
Figure 3.34 Representative spleen images of Ctrl and Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice at 1w (top) after ruxolitinib or mock administration via oral gavage. The statistical data (bottom) in KO mice are shown as mean ± SEM. *, P< 0.05.

In summary, Zeb2 ablation in the hematopoietic compartment led to a phenotype with several features reminiscent of myeloproliferative disorders in

humans, such as bone marrow fibrosis, splenomegaly and extramedullary hematopoiesis. These features may partly be attributed to hypersensitivity of the JAK/STAT signaling pathway to selected cytokines, such as G-CSF. While retarded activation of JAK/STAT by IL-3 and IL-6 stimulation might be responsible for distinct differentiation defects in other hematopoietic lineages in Zeb2-deficient mice.

4. Discussion

4.1 The role of Zeb2 in terminal differentiation

It has been described previously that deletion of Zeb2 within hematopoietic compartment results in early embryonic lethality due to differentiation and migration defects of fetal liver hematopoietic cells (Goossens et al., 2011). In this study, we used the interferon sensitive Mx1-Cre based inducible knockout model to generate Zeb2 deficient mice, in which Zeb2 was deleted in adult hematopoietic cells upon Poly (I:C) administration. In the first part of the study we showed that the transcriptional repressor Zeb2 is important for adult erythropoiesis. After Zeb2 allele deletion, mice exhibited progressive anemia over time. If the same amount of BM and spleen cells were plated in methylcellulose medium, we were not able to detect abnormal number of colonies derived from BFU-E or CFU-E in BM, but accumulative CFU-E from spleen. This is most likely the result of a higher proportion of erythroid precursors in the spleen of Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice. Flow cytometry analysis demonstrated a noticeable accumulation of proerythroblasts (CD71^{+}Ter119^{-}) and erythroblast population (CD71^{+}Ter119^{+}) in both BM and spleen upon ablation of Zeb2. As a result of erythroid terminal differentiation defect, Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice display anemia and partially compensate this by extramedullary erythropoiesis.

The hemoglobin-oxidizingtoxin PHZ induced hemolytic anemia is mechanically

due to increased reactive oxygen species (ROS). ROS production triggers cation channels in the erythrocyte membrane. This allows Ca^{2+} entering the cell and translocate the phosphatidylserine (PS) from the inner to the outer of the plasma membrane. This translocation can be recognized and further engulfed by macrophages (Foller et al., 2008; Lang et al., 2003). Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice displayed a pronounced anemia upon PHZ induced oxidative stress which implies that Zeb2-deficient mice display impaired (increased) ROS levels in response to stress. It has been reported that p53 inhibits stress erythropoiesis and the p53-/- mice showed a faster stress response to oxidative challenge (Ganguli et al., 2002). The activation of p53 regulates cellular ROS generation and mediates apoptosis, and higher ROS levels can stabilize p53 and trigger its activation (Liu et al., 2008). Significantly increased p53 mRNA level was observed in Zeb2-deficient CD71^{high}TER119$^{+/-}$ population compared with controls in the steady-state (data not shown). This might account for the increased sensitivity of Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice to stress erythropoiesis.

We also found that upon Zeb2 ablation, several erythropoiesis promoting genes, including Gata1, Fli1 and Klf1 were significantly down-regulated in Zeb2$^{\Delta/\Delta Mx1-Cre}$ CD71^{+}Ter119^{+} population, where the maturation block occurs. Interestingly, in addition to the critical roles in erythropoiesis, all of those genes are essential for megakaryopoiesis as well. Our previous data showed more MEP cells but massively diminished thrombocytes in Zeb2 deficient animals.

The histological analyzes of the bone marrow cavity and spleens revealed the number of megakaryocytes was significantly increased, but the megakaryocytes displayed a rather immature morphology with reduced amount of cytoplasm and less condensed nucleus in Zeb2$^{\Delta/\Delta Mx1-Cre}$ animals. In concordance with this observation ex vivo culture of megakaryocytes using CFU-Mk assay revealed a lack of mature, acetylcholinesterase positive megakaryocytes in absence of Zeb2, which represents the final maturation step of megakaryopoiesis (data from concurrent project, not shown here). These data indicate that more megakaryocyte-erythroid progenitors were generated in absence of Zeb2, but both erythrocytes and megakaryocytes show maturation impairment at the stage of terminal differentiation. Mx1-Cre mediated Gata1 deletion in adult hematopoiesis leads to differentiation arrest at the proerythroblast stage, severe thrombocytopenia, as well as excessive megakaryocytosis in the spleen (Gutierrez et al., 2008), a phenotype similar to what we observed in Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice. Klf1 and Fli1 are both Gata1 binding partners which play cross-antagonistic roles in the regulation of erythroid versus megakaryocytic development (Starck et al., 2003). We have investigated the expression level of Klf1 and Fli1 in MEP populations, and both genes are up-regulated in Zeb2$^{\Delta/\Delta Mx1-Cre}$ MEPs compared with controls. This might explain the excessive erythropoiesis and megakaryopoiesis after Zeb2 deletion.

In conclusion, Zeb2 is essential for terminal erythroid differentiation under

steady state conditions and maybe dispensable for stress erythropoiesis. The maturation arrest upon Zeb2 ablation is mostly due to impaired networks of transcription factors, rather than altered cell cycle.

An important finding of this study is that Zeb2 is not necessary for the specification but rather for the differentiation of B-cells. A slightly increased number of LMPP was observed in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ BM compartment, and this may be result from increased LKS population. There were no changes in the absolute numbers of CLP cells. However, the transition from Prepro-B to Pro-B cells is hindered strikingly in Zeb2 deficient mice. Mechanistically, we demonstrated that ablation of Zeb2 leads to inhibited IL-7R expression and impaired IL-7 receptor signaling cannot be effectively activated in prepro-B cell subset. It is well known that IL-7R signaling is indispensable in orchestrating B lymphopoiesis (Clark et al., 2014). A recent study has shown that gain-of-function Zeb2 leads to constitutive activation of IL-7/STAT5 signaling via direct binding of Zeb2 to the IL-7R promoter element (Goossens et al., 2015). This finding strongly supports our current findings, as we see in absence of Zeb2 the opposite effect on IL-7 signaling to the overexpression of Zeb2. In IL-7 or IL-7R-deficient mice, Ebf1 and Pax5 are absent, while E2A is normally expressed (Dias et al., 2005; Kikuchi et al., 2005). Consistently, expression of Pax5 was almost depleted in Zeb2-deficient Prepro-B population and accompanied with mild down-regulated Ebf1. However, in our study E2A expression was controversially increased in this cells. This indicates that there

might be some other mechanisms responsible for its over-expression. Inhibitor of DNA binding 2 and 3 (ID2 and ID3) function to repress E2A and play negative role in B-lineage specification. Enforced expression of Ebf1 or E2A in IL-7R$^{-/-}$ Prepro-B cells leads to dramatic reduction and induction of both ID2 and ID3 respectively (Thal et al., 2009). High levels of ID2 were shown in Zeb2-deficient Prepro-B population and may be due to inhibited IL-7R and E2A. In turn, up-regulated ID2 can inhibit activities of E2A proteins posttranslationally and further results in insufficient activation of E2A to rescue expression of target genes. Interestingly, ID3 was potently down-regulated and might promote B-lymphopoiesis in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ Prepro-B cells. However, it has been shown before that loss of ID3 in IL-7R-/- mice was not sufficient to rescue impaired B-cell development (Thal et al., 2009). This suggest that Zeb2 is necessary for the transcriptional regulatory network of B-lymphocyte differentiation. However, no direct evidence can show the link of Zeb2 to other transcription factors, including Gata-3, PU.1 and Runx-1.

4.2 Role of Zeb2 in epigenetic regulation

Zeb2 has been reported to be a transcriptional repressor by interacting with multiple subunits of nucleosome remodeling and histone deacetylation (NuRD) complex (Verstappen et al., 2008). Our qRT-PCR analysis revealed that in addition to NuRD complex, genes associated with polycomb repressive complexes 2 (PRC2) (Ezh2 and Suz12), DNA methylation (Dnmt1, 3a and 3b) and tet methylcytosine dioxgenases (Tet1, 2, 3) displayed altered expression levels in Zeb2-deficient Prepro-B cells as well. A set of important H3 modifications was dysregulated upon Zeb2 deletion, including H3K4me1, H3K4me3 and H3K27me3, which are able to regulate specific B-cell fate transcriptomes directly or interplay with transcription factors to establish correct genomic landscape of B-lymphopoiesis. These findings implicate the important role of Zeb2 in epigenetic regulation of B-cells.

In recent years, investigators put much effort to clarify the epigenetic machinery involved in B-cell development and reprogramming. PU.1 is required for lymphoid commitment, and mice lacing PU.1 fail to generate B, T, NK and myelomonocytic cells (McKercher et al., 1996; Scott et al., 1994). PU.1 was reported to promote IL-7R transcription by binding to its Ets sites of the promoter (DeKoter et al., 2007). The activated PU.1 in Zeb2-deletion Prepro-B cells may be due to the genetic compensation induced by insufficient IL-7R expression. A genome-wide study revealed that collaborative interactions

between PU.1 and small sets of lineage type specific transcription factors lead to nucleosome remodeling and the deposition of H3K4me1, result in activation of cistromes of lineage-restricted factors (Heinz et al., 2010).

A hall mark of B cell lymphopoiesis is the B cell receptor (BCR) immunoglobulin rearrangement that consists of heavy chain (Igμ) and light chain (Igκ and Igλ). The correct expression of BCR subunits mb-1, which encodes the Igα component of Pre-BCR as well, VpreB and λ5, and initiation of D-J rearrangements lead to B cell development. The promoter of mb-1 is hypermethylated at CpG dinucleotides in HSCs and progressively demethylated during B cell development. Synergistic effects of EBF with Runx-1 and its partner CBFβ, as well as E2A, results in modified epigenetic events of mb-1 promoter, including chromatin structure modulation and CpG demethylation and further cause mb-1 activation by Pax5. (Maier et al., 2004). Knock-down of Mi-2β, the Mi-2/NuRD complex subunit, in cultured B cells results in the demethylation of the mb-1 promoter that indicated restrained function of Mi-2/NuRD for EBF and Pax5 induced mb-1 reprogramming (Gao et al., 2009). In addition, Mi-2/NuRD complex interacts with Ikaros and Blimp-1 in B cell development (Dege and Hagman, 2014). A genome-wide analysis has been performed to explore the DNA-binding of E2A, EBF1 and Foxo1 by Chip-seq. During the transition from Prepro-B to Pro-B cell stage, E2A occupied genes were mostly with cistromes were monomethylated at H3K4 (H3K4me1), characterized as enhancer elements (Lin et al., 2010). In Pro-B

cells, gain- and loss- of Ebf1 studies demonstrated that H3K4me3 and H3 acetylation marks were enriched while repressive H3K27me3 were hampered in activated genes (Treiber et al., 2010). The abnormal levels of H3 modifications in Zeb2-deficiency Prepro-B cells, as shown in our current study, might cause broad chromatin changes of B-cell specifying genes, due to uncontrolled transcription network during B cell development.

Interestingly, two members of the PRC2 complex, Eed and Ezh2 and all three Dnmts showed significantly increased expression levels in HSC enriched LKS population. Aberrant epigenetic regulators are associated with perturbed stem cell function (Cullen et al., 2014). Overexpression of Ezh2 is associated with a variety of human cancers, including breast and prostate carcinoma (Simon and Lange, 2008). In hematopoietic system, gain-of-function mutation of Ezh2 Y641 results in B-cell lymphomas through selectively increased H3K27me3 (Sneeringer et al., 2010; Yap et al., 2011). A recent study points to a pivotal role of Ezh2 in myeloproliferative diseases. Conditional Ezh2 knock-in mice develop MPN features, like excessive myelopoiesis, leukocytosis and splenomegaly (Herrera-Merchan et al., 2012), similar to what we observed in Zeb2$^{\Delta/\Delta Mx1-Cre}$ mice. As a homolog of Ezh2, Ezh1 could build an alternative PRC2 complex with Suz12 and Eed when Ezh2 is insufficient, that could explain the limited effects on HSC maintenance upon Ezh2 deletion alone, while ablation of the core subunit Eed is likely abolishes complete canonical PRC2 function (Xie et al., 2014). Although PRC2 was recognized as a

transcriptional repressor via tri-methylation of H3K27, some studies indicated that Ezh1 and Ezh2 could function in a histone methyltransferase independent way (Mousavi et al., 2012; Xu et al., 2012). However, very little stem cell number impedes further mechanistical investigation.

Numerous studies have pointed out that histone modifications result in and are influenced by DNA methylation (Cedar and Bergman, 2009). Fortunately, the rapid development of sequencing methods is helping to draw a picture of genome-wide methylation profiles from control and Zeb2-deficient HSCs. Herein we use reduced representation bisulfite sequencing (RRBS) which enables to quantify global DNA methylation in as little as 20ng of DNA. The analysis revealed that 72% of all differently methylated CpG sites showed higher methylation level in Zeb2-KO HSC. That might be due to the up-regulation of both maintenance methyltransferase Dnmt1 and the de novo methyltransferases Dnmt3a and Dnmt3b in LKS cells lacking Zeb2. Interestingly, 70% of those hyper-methylated CpG sites are located in the gene bodies (exons and introns). DNA methylation in promoter regions is relatively well characterized as a repressive epigenetic mark. However, the relationship between DNA methylation of gene bodies and gene transcription is still unclear. However, some studies have shown that methylation in the gene body stimulates transcription elongation and splicing, and is positively correlated with gene expression (Jones, 2012). Here we show a set of HSC fingerprint genes, including Pbx1, Prdm16 and Runx3, harboring differentially methylated

CpG sites with higher expression levels in Zeb-KO HSCs. These findings could be result from hyper-methylated bodies of those genes. A more recent study has explored that 5-Aza-2′-deoxycytidine, the DNA methylation inhibitor, down-regulates genes through triggering gene bodies' demethylation. This suggests that high levels of gene body methylation in some overexpressed oncogenes could be the potential therapeutic target by methylation inhibitors in various cancer (Yang et al., 2014).

All three DNA methyltransferases are essential for hematopoietic maintenance and development. Two studies investigated the impact of Dnmt1 in hematopoiesis and were published almost at the same time. Induced loss of Dnmt1 in adult hematopoietic cells resulted in rapid mice death due to profound anemia and cytopenia caused by absence of HSCs and progenitors, and depletion of all hematopoietic lineage cells. Dnmt1$^{-/chip}$ mice, which express low Dnmt1 levels, further help to reveal that distinct threshold levels of methylation are required for commitment to myeloerythroid and lymphoid lineages (Broske et al., 2009b). Another group showed much milder phenotype upon ablation of Dnmt1, this may due to insufficient amount of poly (I:C) used in this study and thus no efficient deletion of the floxed allele (Trowbridge et al., 2009). Dnmt3a and Dnmt3b were considered to be important for self-renewal of HSCs but not for their differentiation (Tadokoro et al., 2007). A later in vivo study showed that Dnmt3a deficiency impairs HSC differentiation over serial transplantation while HSC numbers were accumulated. Deletion of Dnmt3a

alters DNA methylation status and selectively increases the expression of HSC multipotency genes while downregulate differentiation factors, including increased Runx1, Gata3, Nr4a2 and decreased Pu.1, E2A, whose expression also changed in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ HSCs in our current study (Challen et al., 2012). However, the understanding of the mechanism, in which Zeb2 orchestrates epigenetic changes remains to be defined in further studies.

Moreover, all three Dnmts were reported to be substantially overexpressed in acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (Mizuno et al., 2001). Dnmt3a mutations, including missense, nonsense, frame-shift and splice-site mutations have been reported highly recurrent in de novo AML patients (22.1%) (Hou et al., 2012; Ley et al., 2010). Moreover, both Dnmt3a and Dnmt3b are downregulated in CLL samples. Mice lacking Dnmt3a alone or simultaneously with Dnmt3b can develop chronic lymphoid leukemia (CLL) rather than AML (Peters et al., 2014). These findings suggested that not all of the Dnmt3a mutations in AML completely or partially lose the methyltransferase activity, some may result in gain of function as well, and lymphocytes might be more sensitive to the expression level of Dnmt3a and Dnmt3b. Dnmt1 is highly expressed in leukemia stem cells (LSCs), its conditional deletion is able to block transformation and development of MLL-AF9-induced leukemia. Haploinsufficiency of Dnmt1 is also sufficient to delay leukemic progression (Trowbridge et al., 2012). This indicates that Dnmt1 and Dnmt3a/3b might play different roles during leukemogenesis. In

this study, we have not observed any bone marrow malignancies in Zeb2-deficient mice, in which all three Dnmts were overexpressed in LKS population. That could be due to the substraction effect of three Dnmts, or additional mutations are likely to be needed, like Flt3 and Npm1.

4.3. Role of Zeb2 in cytokine signaling

Hematopoiesis is tightly regulated by a variety of external stimulating factors (e.g cytokines), of which all can transmit signals and activate different signaling pathways via their cognate receptors. Ligand binding induces receptor dimerization and can rapidly result in the recruitment and phosphorylation of downstream kinases like JAKs, which subsequently phosphorylate STATs. The activated and dimerized STATs translocate to the nucleus and activate or repress target genes. Similarly, AKT and ERK signaling pathways can be activated as well (Baker et al., 2007).

In this study, we observed accelerated activation of the JAK/STAT pathway upon G-CSF stimulation, but attenuated activation of the same pathway in the presence of IL-3 and IL-6 in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice compared with controls. However, we did not detect altered abundance those cytokine levels in the serum of Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ mice (data not shown). It is known that a particular cytokine is not lineage restricted and able to function on different cells, while several different cytokines can play similar or overlapping roles in the same cell type (Rane and Reddy, 2002). IL-6 signaling is broadly required for monocytes, megakaryocytes, hepatocytes and adipocytes, and its physiological role could be compensated by leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) since they shared the same signal transducer gp130 (Kishimoto, 1989; Yoshida et al., 1996). IL-6 contributes to

the survival and self-renewal of HSCs and early progenitors. Despite the fact that IL-6-/- mice can develop normally and grow into healthy adults IL-6 deficiency leads to abnormal differentiation and commitment of granulocytic-monocytic, megakaryocytic and erythroid lineages upon serial transplantation (Bernad et al., 1994). This indicated that reduced expression of the IL-6Rα in Zeb2-deleted progenitor and myeloid cells compared with control is not sufficient to maintain normal differentiation leading to the sever differentiation impairment of different lineages in Zeb2-deficient mice. In vitro and in vivo studies highlight the critical trombopoietic role of IL-6 in megakaryocyte terminal differentiation and maturation (Clarke et al., 1996; Ishibashi et al., 1989; Kimura et al., 1990; Stahl et al., 1991). It has been shown that IL-6 is able to augment the size and increase the DNA content of megakaryocytes, but has no effect on cell cycle. Combining treatment with IL-3 and IL-6 can considerably increase the megakaryocytic colony formation compared with IL-6 alone, suggesting that synergistic effect of IL-3 and IL-6 is important for megakaryopoiesis (Kimura et al., 1990). The simultaneous attenuated STATs activation response to IL-3 and IL-6 upon Zeb2 deletion might account for the severe thrombocytopenia in Zeb2-KO mice.

It is known that IL-3 stimulates a wide spectrum of hematopoietic progenitors and committed lineages, including myeloid cells, megakaryocytes, lymphocytes, erythroid cells, and mast cells (Reddy et al., 2000). IL-3 has been described as a necessary stimulus in culture medium for proliferation and

maturation of HSCs in vitro (Mangi and Newland, 1999). The presence of IL-3 induces the dimerization of the unique α and the common β chain of the IL-3 receptor and trigger multiple intracellular signaling transduction cascades. IL-5 and GM-CSF share the common β (β_c) signal transducing subunit as well. We analyzed the expression level of IL-3Rα in myeloid cells and progenitors, but did not find significant changes. It has been reported that β_c does not bind IL-3 by itself, but associates with IL-3Rα and forms high affinity receptors (Nicola et al., 1996). So it is necessary to compare the β_c expression in Zeb2-deficient and control cells. Moreover, similar to IL-6, either IL-3 or β_c–deficient mice exhibit no obvious hematopoietic abnormalities, suggesting the compensation effect in hematopoiesis by other cytokines (Nicola et al., 1996).

G-CSF is widely used to induce the mobilization of HSCs from the bone marrow to the bloodstream for transplantation (To et al., 1997). In the steady state, G-CSF is known to be the major cytokine producing neutrophilic granulocytes (Panopoulos and Watowich, 2008). Recombinant G-CSF is clinically used for treatment of neutropenia resulting from chemotherapy in cancer patients (Morstyn et al., 1988). In hematopoietic system, the G-CSF receptor (G-CSFR) is necessary for signal transduction. Its expression has been detected in myeloid lineage and correlates with G-CSF sensitivity (Panopoulos and Watowich, 2008). Here we detected higher G-CSFR expression in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ myeloid cells. It has been reported that deletion of either G-CSF or G-CSFR result in severe neutropenia phenotype in mice, but

a residual level of neutrophil could still be detected (Lieschke et al., 1994; Liu et al., 1996). This suggested that in addition to G-CSF/G-CSFR signaling pathway, other mechanisms could support granulopoiesis in vivo as well. However, when comparing the members of the JAK family that needed for G-CSF and IL-3 signaling, we found that G-CSF induces the activation of STATs via tyrosine phosphorylation of JAK1 and JAK2, while the activation of JAK1 by IL-3 is very limited (Kouro et al., 1996; Tian et al., 1996). It has been shown that in the absence of JAK1, G-CSF failed to activate STATs, indication that JAK1 is more critical for G-CSF-induced STAT activation (Shimoda et al., 1997). Interestingly, several CpG sites in exon of Jak1 gene are hyper-methylated in Zeb2-KO HSCs, suggesting an increased Jak1 expession level. This might account for the more enhanced STATs activation in Zeb2$^{\Delta/\Delta Mx1-Cre}$ cells in the presence of G-CSF.

Our result indicated that increased proportion of granulocytes and progressive myeloproliferative disorders in Zeb2-deficiency mice may be due to abnormal STATs activation in the presence of G-CSF. In contrast, cells lacking Zeb2 are not able to respond to IL-3 and IL-6 stimulation, partly due to reduced receptor expression, which might be in part attributing to impaired differentiation of multiple hematopoietic lineages.

5. Summary

The differentiation of hematopoietic stem cells (HSC) into specialized blood cells is tightly controlled by a complex network of transcription factors (TF). The zinc finger E-Box binding TF Zeb2 is known to govern the epithelial to mesenchymal transition (EMT) during embryonic development and tumor progression and metastasis in an adult organism. Our previous work showed that deletion of Zeb2 within hematopoietic compartment resulted in early embryonic lethality due to differentiation and migration defects. In order to identify the role of Zeb2 in adult hematopoiesis, the Mx1-Cre based inducible Zeb2 knockout model was used. We found drastic reduction of B-lymphocytes, monocytes, platelets and erythrocytes in peripheral blood while accumulation of hematopoietic stem and progenitor cells in the bone marrow after Zeb2 deletion.

In this study, we firstly demonstrated that impaired terminal differentiation of the erythroid lineage takes place at the transition of $CD71^{high}TER119^{+}$ to $CD71^{med}TER119^{+}$ population upon Zeb2 ablation. This may be caused by the dysregulation of several transcription regulators, including Klf1 and Fli1 and Gata1. In addition, the most prominent differentiation block was observed within B lymphopoiesis at the Prepro-B to Pro-B cell transition. The early B-cell receptor IL-7R as well as fingerprint transcription factors such as Runx1, E2A, EBF1 and Pax5 were markedly changed in $Zeb2^{\Delta/\Delta Mx1-Cre}$ Prepro-B cells.

Moreover, the expression level of DNA methylation related genes and histone modifications were significantly changed in Zeb-deficient early B-cells, suggesting the B-cell differentiation block due to altered epigenetic regulation.

A set of higher or lower methylated key HSC genes were discovered by reduced representation bisulfite sequencing (RRBS) analysis between Zeb2 WT and Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ HSCs. Gene ontology (GO) enrichment analysis highlights altered protein kinases (including phosphorylation, Serine/threonine protein kinase, tyrosine protein kinase) after Zeb2 deletion. This was further confirmed by abnormal JAK/STAT activity in Zeb2$^{\Delta/\Delta Mx1\text{-}Cre}$ cells.

In summary, Zeb2 is essential for multilineage differentiation at different stages of hematopoiesis. Ablation of Zeb2 in adult hematopoiesis results in defective epigenetic regulation, a causative event in impaired transcriptional network and JAK/STAT activation that account for multilineage defects.

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- The Third prize Scholarship, Chongqing University, China, 09. 2007
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- Investigate the effect of *c-Myc* in hair follicle development process.
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