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The BTB/POZ Transcription Factor Miz-1 Is Required To Regulate The Commitment, Survival And Differentiation Of Early B And T Cell Lineages

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Résumé

Les lymphocytes B et T sont issus de cellules progénitrices lymphoïdes de la moelle osseuse qui se différencient grâce à l'action de facteurs de transcription, cytokines et voies de signalisation, dont l'interleukine-7 (IL-7)/IL-7 récepteur (IL-7R). Le facteur de transcription c-Myc est exprimé par les cellules lymphoïdes et contrôle leur croissance et leur différenciation. Cette régulation transcriptionnelle peut être coordonnée par le complexe c-Myc/Myc-Interacting Zinc finger protein-1 (Miz-1). Le but de ce projet était de comprendre les mécanismes qui impliquent Miz-1 et le complexe c-Myc/Miz-1 dans le développement des lymphocytes B et T. Pour réaliser ce projet, des souris déficientes pour le domaine de transactivation de Miz-1 (Miz-1$^{\Delta POZ}$) et des souris à allèles mutantes pour c-Myc^{V394D}, mutation qui empêche l'interaction avec Miz-1, ont été générées.

La caractérisation des souris Miz-1$^{\Delta POZ}$ a démontré que l'inactivation de Miz-1 perturbe le développement des lymphocytes B et T aux stades précoces de leur différenciation qui dépend de l'IL-7. L'analyse de la cascade de signalisation IL-7/IL-7R a montré que ces cellules surexpriment la protéine inhibitrice SOCS1 qui empêche la phosphorylation de STAT5 et perturbe la régulation à la hausse de la protéine de survie Bcl-2. De plus, Miz-1 se lie directement au promoteur de *SOCS1* et contrôle son activité. En plus de contrôler l'axe IL-7/IL-7R/STAT5/Bcl-2 spécifiquement aux stades précoces du développement afin d'assurer la survie des progéniteurs B et T, Miz-1 régule l'axe EBF/Pax-5/Rag-1/2 dans les cellules B afin de coordonner les signaux nécessaires pour la différenciation des cellules immatures. La caractérisation des souris c-Myc^{V394D} a montré, quant à elle, que les fonctions de Miz-1 dans les cellules B et T semblent indépendantes de c-Myc.

Les cellules T des souris Miz-1$^{\Delta POZ}$ ont un défaut de différenciation additionnel au niveau de la β-sélection, étape où les signaux initiés par le TCR remplacent ceux induits par IL-7 pour assurer la prolifération et la différenciation des thymocytes en stades plus

matures. À cette étape du développement, une forme fonctionnelle de Miz-1 semble être requise pour contrôler le niveau d'activation de la voie p53, induite lors du processus de réarrangement V(D)J du *TCR*. L'expression de gènes pro-apoptotiques *PUMA*, *NOXA*, *Bax* et du régulateur de cycle cellulaire $p21^{CIP1}$ est régulée à la hausse dans les cellules des souris Miz-$1^{\Delta POZ}$. Ceci provoque un débalancement pro-apoptotique qui empêche la progression du cycle cellulaire des cellules TCR-positives. La survie des cellules peut être rétablie à ce stade de différenciation en assurant une coordination adéquate entre les signaux initiés par l'introduction d'un TCR transgénique et d'un transgène codant pour la protéine Bcl-2.

En conclusion, ces études ont montré que Miz-1 intervient à deux niveaux du développement lymphoïde: l'un précoce en contrôlant la signalisation induite par l'IL-7 dans les cellules B et T, en plus de l'axe EBF/Pax-5/Rag-1/2 dans les cellules B; et l'autre tardif, en coordonnant les signaux de survie issus par le TCR et p53 dans les cellules T. Étant donné que les thymocytes et lymphocytes B immatures sont sujets à plusieurs rondes de prolifération, ces études serviront à mieux comprendre l'implication des régulateurs du cycle cellulaire comme c-Myc et Miz-1 dans la génération des signaux nécessaires à la différenciation non aberrante et à la survie des ces cellules. Enfin, les modèles expérimentaux, souris déficientes ou à allèles mutantes, utilisés pour ce travail permettront de mieux définir les bases moléculaires de la transformation maligne des lymphocytes B et T et de révéler les mécanismes conduisant au lymphome.

Mots-clés : Miz-1, c-Myc, IL-7R, TCR, différenciation, apoptose, Bcl-2, STAT5, SOCS1, p53.

Abstract

Signaling pathways control the differentiation and proliferation of blood cells, like B and T lymphocytes. They converge into regulating the activity of transcription factors that influence ultimately gene expression patterns. The transcription factor c-Myc is a central regulator of cellular proliferation and growth, and its deregulated expression has been demonstrated to be involved in many types of cancers, in particular lymphoma. Recent studies have shown that repression by c-Myc can be mediated by a complex formed with the BTB/POZ domain transcription factor Miz-1 (Myc Interacting Zinc finger protein-1). Given that both c-Myc and Miz-1 proteins are expressed in lymphoid precursors and since c-Myc has been shown to be important for B- and T-cell development, the aim of this thesis was to investigate the role of Miz-1 and the c-Myc/Miz-1 complex in regulating B and T cell survival, commitment and differentiation. To do so, mice expressing a non-functional Miz-1 protein lacking the BTB/POZ domain (Miz-$1^{\Delta POZ}$) and knock-in mice expressing a mutant c-Myc^{V394D} allele that no longer interacts with Miz-1 were generated.

B- and T-cell development requires the coordinated action of transcription factors and cytokines, in particular interleukin-7 (IL-7). The studies presented in this work demonstrated that mice deficient for the BTB/POZ domain of transcription factor Miz-1 almost entirely lack follicular B cells and T cells, since their progenitors fail to activate the JAK/STAT5 pathway and to up-regulate Bcl-2 upon IL-7 stimulation. Miz-1 exerts a dual role in the IL-7 receptor (IL-7R) pathway by directly repressing the JAK inhibitor *SOCS1* and by activating Bcl-2 expression. In B cells, a functional form of Miz-1 is also required for the proper expression of early B cell genes like E2A and EBF. These data suggest that Miz-1 represents a new regulatory element of early B- and T-cell differentiation required for the regulation of the IL-7/IL-7R/STAT5/Bcl-2 axis by monitoring SOCS1 for survival and by regulating the EBF/Pax-5/Rag-1/2 axis for the proper commitment and differentiation of the B-cell lineage. The regulation exerted by Miz-1 in B and T cells is

mostly likely independent of its interacting partner c-Myc, and seems specifically linked to the BTB/POZ domain of Miz-1.

Mice deficient for the BTB/POZ domain of Miz-1 have additionally a severe differentiation block at the pre-T cell "β-selection" checkpoint. Miz-1 deficient pre-T cells are highly apoptotic and do show cell cycle defects. This concurs with enhanced expression of p53-target genes such as $p21^{CIP1}$, *Bax*, *PUMA* and *Noxa*, most likely induced by the DNA double-strand breaks generated during the V(D)J recombination of the *TCR*. Only the co-expression of rearranged TCRαβ and Bcl-2 fully rescued *Miz-1*-deficient cell numbers and enabled them to differentiate into $TCR\beta^+$ cells. These data suggest that Miz-1 is required for both the regulation of the p53 response and proper expression of the pre-TCR to support the proliferative burst of pre-T cells.

In conclusion, the studies presented in this thesis revealed the so far unknown implication of Miz-1 in B- and T-cell development. More specifically, Miz-1 exerts early regulatory functions by monitoring the IL-7/IL-7R signaling in B and T cells. It regulates later stages of differentiation by controlling the EBF/Pax-5/Rag-1/2 in B cells and the TCR expression and the p53 response in T cells. These studies and the generated mice model (conditional knock-out and knock-in) will help characterize the implications of transcription factors that have been causally implicated in the altered genetic programming found in hematopoietic malignancies due to their capacities to regulate cell cycle. Ultimately the characterization of Miz-1 and c-Myc functions in B and T cells will help better understand the mechanisms responsible for the emergence of leukemia and lymphoma.

Keywords : Miz-1, c-Myc, IL-7R, TCR, differentiation, apoptosis, Bcl-2, STAT5, SOCS1, p53.

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List of abbreviations

AID: Activation-induced cytidine deaminase

ALP: All-lymphoid progenitor

Atm: Ataxia-telangiectasia-mutated

Atr: Atm-Rad3-related kinase

B-ALL: B-acute lymphoblastic leukemia

Bad: Bcl-2-antagonist of cell death

Bax: Bcl-2-associated X protein

Bcl-2: B-cell CLL/lymphoma-2

Bcl-X_L : Bcl-2-like 1

BCR: B cell receptor

BLP: B cell-biased lymphoid progenitor

BTB: Broad-complex, Tramtrack and Bric-à-brac

CCR9: CC-chemokine receptor 9

CDK: Cyclin-dependent kinase inhibitor

CFU-S: Colony Forming Units-Spleen

ChIP: Chromatin ImmunoPrecipitation

Chk1: Checkpoint kinase 1

CLP: Common lymphoid progenitor

CMP: Common myeloid progenitor

CSR: Class switch recombination

DC: Dendritic cell

DL: Delta-like ligand

DLBCL: Diffuse large B cell lymphoma

DN: $CD4^-CD8^-$ double negative

DP: $CD4^+CD8^+$ double positive

E2A: Transcription factor 3 (*Tcf3*)

Eμ: Immunoglobulin heavy chain enhancer

EBF: Early B cell factor

ELP: Early lymphoid progenitors

ERK: p44/42 Extracellular-Regulated Kinase

ETP: Early T-lineage progenitors

Fl: Floxed allele carrying loxP sites

Flt3: Fms-like tyrosine kinase 3

Flt3L: Flt3 ligand

Fo: Follicular

GM: Granulocyte/macrophage

GMP: Granulo-monocytic precursors

HLH-LZ: Helix-loop-helix and leucine-zipper motif

HSC: Hematopoietic stem cells

ICN: Intracellular domain of Notch

IEL: Intraepithelial lymphocytes

Ig: Immunoglobulin

IL-7 : Interleukin-7

IL-7Rα: Interleukin-7 receptor α chain

JAK: Janus kinase

JNK: c-Jun NH_2-terminal Kinase

Lin: Lineage marker

LMPP: Lymphoid-primed multipotent progenitors

LSK: $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$

LT-HSC: Long-term hematopoietic stem cells

MAML: Mastermind-like protein

MDM2: Double minute 2 protein or E3 ubiquitin-protein ligase

MAPK: Mitogen-activated protein kinase

Mcl-1: Myeloid cell leukemia sequence 1, Bcl-2-related

MegE: Megakaryocyte/erythroid

MHC: Major histocompatibility complex

Miz-1: Myc-Interacting Zinc finger protein-1 (*zbtb17*)

Miz-1$^{\Delta POZ}$: Miz-1 lacking the BTB/POZ domain

MPP: Multipotent precursors

Myc: Myelocytomatosis viral oncogene

Myc^{V394D}: c-Myc expressing an Aspartic Acid (D) instead of a Valine residue (V) at position 394 of its coding sequence

MZ: Marginal zone

NK: Natural killer

p53: Transformation related protein 53

Pax-5: Paired-homeodomain transcription factor

PI3K: Phosphatidylinositol 3-kinase

POZ: Pox virus Zinc finger domain

pTα: Pre-TCRα chain

RAG: Recombination activating gene

RSS: Recombination signal sequences

SCF: Stem cell factor

SHM: Somatic hypermutation

SOCS: Suppressor of cytokine signaling 1

SP: $CD4^+$ or $CD8^+$ single positive

STAT: Signal transducer and activator of transcription

ST-HSC: Long-term hematopoietic stem cells

T-ALL: T-cell Acute Lymphoblastic Leukemia

TCR: T cell receptor

Tdt: Terminal deoxynucleotidyl transferase

Tfh: T follicular helper cells

Tg: Transgenic

TGFβ: Transforming growth factor beta

Th: $CD4^+$ T helper cell

TopBP1: Topoisomerase II binding protein 1

TSLP: Thymic-stromal-derived lymphopoietin receptor

VCAM-1: Vascular cell adhesion molecule-1

V(D)J: Variable, diversity, joining recombination

WT: Wild-type

«Le plus grand ressort c'est l'espoir; quand
il est cassé tout mouvement s'arrête
en nous». Anonyme

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Overview

The immune system has evolved over the years to arm the body with many strategies of defense against pathogens and infections. The innate immune system possesses sensors that initiate innate responses after promiscuous recognition. On the contrary, the adaptive immunity integrates signals from specific antigen receptors to transcriptional regulation events that govern the acquired response. The shape and size of the antigen receptor repertoire are also tightly regulated by immuno-surveillance mechanisms such as tolerance to avoid cross-reactivity, inflammatory pathologies and autoimmune diseases.

All mature blood cells originate from hematopoietic stem cells that mature in the bone marrow or in primary lymphoid organs through coordinated proliferation and differentiation events. By acquiring lineage restrictions, the progenitors loose stemness, but gradually gain proliferative capacity that needs to be tightly regulated by transcription factors. These transcription factors ensure the maintenance of the genetic programming that regulates cell growth, commitment and survival. Lymphoid malignancies arise from different stages of development and are often linked to a deregulated signaling pathway or transcriptional regulatory functions in B and T cells.

The transcription factor c-Myc plays important roles in hematopoietic differentiation and in the emergence of lymphoma and other blood cancers. It can regulate the transcription of its target genes through two pathways, one E-box-dependent and the other E-box-independent. The second E-box-independent pathway, in which c-Myc regulates gene expression, is dependent on the association of c-Myc with Miz-1. Miz-1 itself can control the expression of genes involved in proliferation. The identity of most of these genes and their function in c-Myc lymphocyte biology are unknown.

This work aimed to define the role of Miz-1 and the c-Myc/Miz-1 complex in the regulation of T- and B-cell survival, commitment and differentiation. As c-Myc has been

shown to play important roles in the development of progenitors and T cells, this project hypothesis was that its interacting partner Miz-1 also had implications in precursor commitment and lymphoid development processes. The characterization of these two transcription factors was thought to enable the identification of the interplay between c-Myc and Miz-1 functions during critical developmental checkpoint of B- and T-cell development.

The results obtained for this project will be presented in three chapters. The first chapter will contain the published article in "Immunity" regarding the requirement for a functional form of Miz-1 to regulate IL-7R signaling at early commitment stages of B-cell differentiation. The second chapter will expose data regarding the role of Miz-1 in IL-7R-dependent survival and differentiation of early T-lineage progenitors that were published in "Blood". The third chapter will present data from a manuscript recently accepted for publication in "The Journal of Immunology" on the implication of Miz-1 later in T-cell development, where it is important to coordinate the expression of TCRβ and p53 effector genes at the pre-TCR ‘’β-selection’’ checkpoint.

These studies revealed that Miz-1 is a new regulator of normal lymphoid development and that it exerts this role independently of c-Myc. Since it is known that both c-Myc and Miz-1 can affect the process of malignant transformation, their function in normal cells needs to be properly monitored in order to avoid the emergence of leukemia and lymphoma. Hence, this study not only presents a new insight on the mechanisms underlying normal lymphoid development, but it also provides new knowledge that is very likely important to design future therapeutic strategies against different types of blood cancers.

Introduction

Hematopoietic stem cells (HSC) are responsible for the generation of mature blood cells through a series of well coordinated proliferation and differentiation events. As cells progress through the early stages of hematopoiesis, they give rise to precursors that are more restricted because they gradually loose their multilineage and self-renewal potential which characterized the originating HSC [1, 2]. By acquiring a certain lineage restriction, the progenitors gradually loose their ability for self-renewal but gain proliferative capacity. These processes are tightly regulated by transcription factors that ensure the maintenance of the genetic programming that regulates cell growth, lineage commitment and cell survival [3].

Throughout the lifespan of a mouse, the hematopoietic progenitors generate mature lymphoid and myeloid cells that continue to replenish the innate and adaptive or acquired immune systems. The innate system constitutes the first line of defense and recognizes a limited number of evolutionary conserved molecules expressed by pathogen-associated molecules such as lipopolysaccharides and peptidogylcans [4, 5]. The major difference between these two branches of the immune system is that the adaptive immunity provides specificity to antigen recognition by the B cell receptor (BCR) or the T cell receptor (TCR) and secretes antibodies [6, 7]. Moreover, the innate immune system responds to antigen with a fast kinetic that lacks memory capacity whereas the adaptive immune system responds with a delayed kinetic, but possesses effective memory responses [4, 6]. The specificity of antigen recognition is achieved through several developmental pathways and

selection steps orchestrated by the interplay between the TCR or BCR signaling and transcription factor regulation [8]. Another particularity of the adaptive immune response is the clonal selection, a process that enables each cell that expresses a single receptor to expand based on the affinity of that receptor for its ligand. Hence, a small number of specific naïve cells will expand following the recognition of a particular antigenic epitope. Consequently, the repertoire of the adaptive immune system is unique to each individual, whereas the receptors of the innate immune system, that are retricted to specific motifs, are not clonally distributed and may be shared within individuals [8].

Hematopoietic cells bear characteristics of both the innate and the adaptive immune system. Yet, some subsets of cells do not classify within the criteria of the innate or adaptive response, as they possess properties of both branches. Examples of such cells include natural killer T (NKT) cells, γδ T cells, CD8αα T cells, B-1 B cells, marginal zone B cells and subsets of NK cells [9]. T and B cells are a lymphocyte lineage that is critical for the host defense system against many types of pathogens. Their functions are well characterized in the periphery, but major questions still remain regarding their origin and their developmental processes.

1. Hematopoiesis and lineage progenitors

Most hematopoietic lineages, including B cells, develop in the bone marrow, while T cells complete their development in the specific environment of the thymus. The migration of

progenitors out of the bone marrow allows the circulation of cells in the bloodstream through which they finally reach the homing organ [10]. Many markers have been proposed to accurately identify the correct progenitor that generates a specific lineage. For instance, lineage markers (Lin) are cell surface antigens that define specific populations of mature blood cells. The lack of lineage marker expression is found in an enriched population of cells with primitive hematopoietic stem cell or early progenitor cell characteristics. A bone marrow fraction that lacks Lin expression and expresses c-Kit (CD117) and Sca-1 is referred to as $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ or "LSK" cells that contain long-term and short-term hematopoietic stem cells (HSC) [11]. Long-term (LT)-HSC cells are the true hematopoietic stem cells that are capable of self-renewal, have a multi-lineage potential and can repopulate a transplanted host. LT-HSC can also be identified by the absence or low expression levels of Thy-1.1 (CD90) and Flt3 (Fms-like tyrosine kinase 3, Flk2, CD135), *i.e.* as $Thy\text{-}1.1^{lo}$ $Flt3^{-}$ cells or as $CD34^{-}Flt3^{-}$ or $CD150^{+}CD244^{-}CD48^{-}$ cells [12, 13].

Self-renewing LT-HSC cells are the base of the hematopoietic system holding a very potent proliferative potential in order to produce differentiated mature blood cells. The differentiation steps required to generate blood cells are irreversible, as once a lineage potential is lost, it cannot be recovered [13, 14]. Many stages govern the differentiation and proliferation of hematopoietic precursors. LT-HSC give rise to short-term-HSC (ST-HSC) that can sustain hematopoiesis for only about 6 weeks in a mouse because of their restrictive self-renewal capacity [15]. ST-HSC can express Flt3 and are $Thy\text{-}1.1^{lo}Flt3^{+}$ or $CD34^{+}Flt3^{-}$ and $CD34^{+}Flt3^{+}$, functionally distinct subsets of short-term HSC [16], and

contribute to multi-lineage differentiation. The oldest technique for determining the capacity of progenitors to generate hematopoietic cell fates is an in vitro colony-forming cell assay where transplanted stem cells can form colonies in the spleen of irradiated mice [17-19]. ST-HSC are potent progenitors of the hematopoietic lineage and can generate colonies (CFU-S: Colony Forming Units-Spleen) that can be detected by this spleen colony technique after transplantation [15, 16]. ST-LSK will give rise to non-self renewing multipotent precursors (MPP) characterized by the expression of $Thy1.1^{-}Flt3^{+}$ or $CD34^{+}Flt3^{+}$. MPP have multilineage potential and mark the first step of lineage restriction during hematopoiesis [1, 2]. In addition, MPP have been identified as the progenitors of the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) [20, 21] (Figure 1).

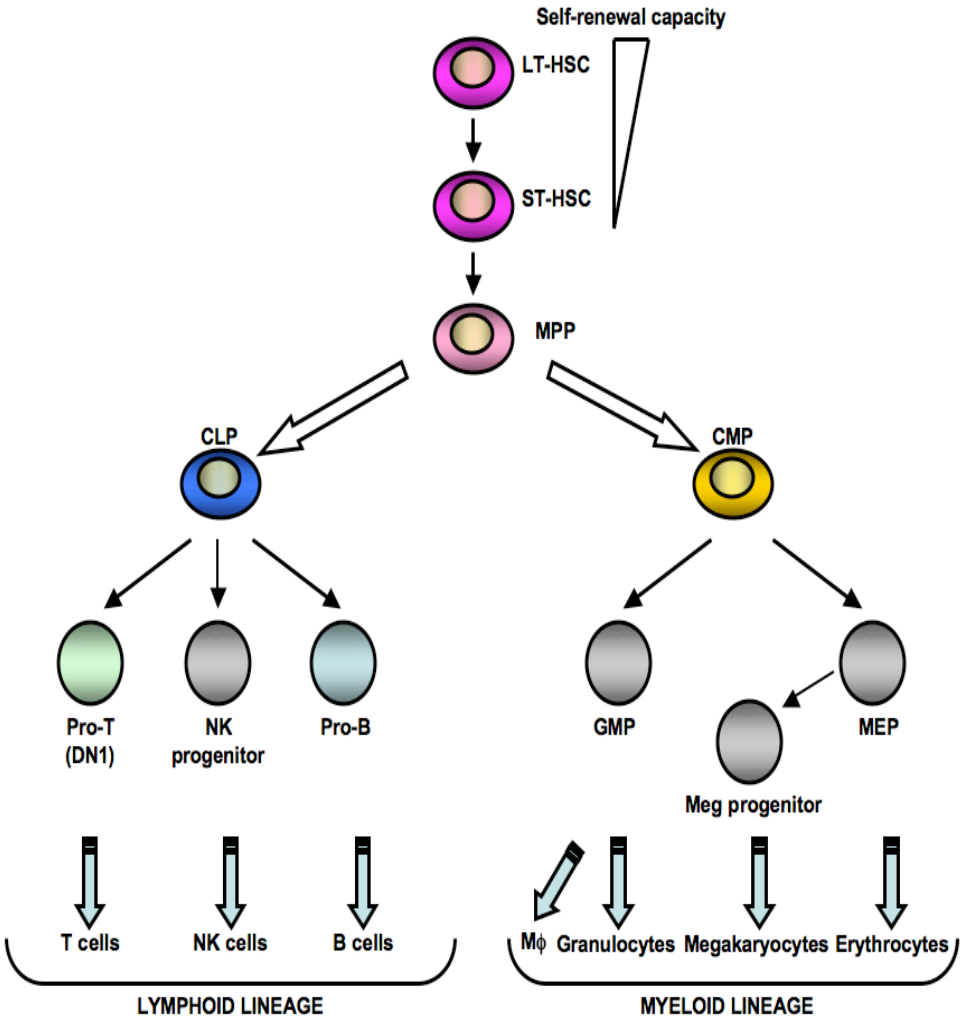

Figure 1. The classical model of hematopoiesis. Hematopoietic stem cells lose their self-renewal activity and their first lineage fate decision is to engage in lymphoid or myeloid differentiation. In this model, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) are symmetrically derived from the same multipotent precursors (MPP). Each progenitor gives rise to the indicated mature blood cells. GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythroid progenitor; Meg, megakaryocyte; NK, natural killer; Mϕ, macrophage (adapted from [22]).

1.1. Models for the hematopoietic cell differentiation

1.1.1. The classical model

Many groups have used the fractionation of hematopoietic precursors into subsets with different lineage specifications to further define hematopoietic differentiation. The first

dichotomy model was established in 1997 by the identification of the symmetrical division of MPP into CLP and CMP [20, 21]. CMP cells give rise to two sets of restricted bipotent progenitors: the megakaryocyte/erythroid (MegE) progenitors ($CD34^{-}FcγRIII^{-}Thy1.1^{-}IL\text{-}7Rα^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$) and the granulocyte/macrophage (GM) progenitors ($CD34^{+}FcγRIII^{+}Thy\text{-}1.1^{-}IL\text{-}7Rα^{-}Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$) [21]. CLP ($Lin^{-}IL\text{-}7Rα^{+}Sca\text{-}1^{low}c\text{-}Kit^{low}Thy\text{-}1.1^{-}$) are proposed to be the common lymphoid progenitor that gives rise to B and T cells. Refinements to this model were added by Adolfsson and his collaborators who reported that MPP could be divided into two groups based on Flt3 expression [23]. The highest Flt3 expressing subset had lost its MegE differentiation potential, but retained robust GM-, T- and B-cell differentiation potential [23]. Further analyses characterizing the expression of Flt3 expression allowed defining the branching point for most potent lymphoid progenitors to develop into the lymphoid lineage.

1.1.2. The revised classical model

By using Flt3 as a marker in combination with vascular cell adhesion molecule-1 (VCAM-1), Kondo's group could further subdivide the MPP population into three subsets: $Flt3^{lo}VCAM\text{-}1^{+}$, $Flt3^{hi}VCAM\text{-}1^{+}$, and $Flt3^{hi}VCAM\text{-}1^{-}$ MPP [24, 25]. The $Flt3^{lo}VCAM\text{-}1^{+}$ MPP population seems to contain the true multi-lineage progenitor able to differentiate into MegE, GM and lymphoid precursors [25]. In agreement with the findings from Adolfsson and collaborators, the $Flt3^{hi}VCAM\text{-}1^{+}$ MPP can no longer differentiate into MegE lineage, but can give rise to GM and T and B cells [25]. $Flt3^{hi}VCAM\text{-}1^{-}$ MPP cells still have GM differentiation potential, but preferentially give rise to lymphocytes [24, 25]. Only

Flt3^{lo}VCAM-1^{+} MPP can give rise to CMP, whether the more developmentally defined Flt3^{hi}VCAM-1^{-} MPP cell give rise to CLP [24]. Adolfsson and colleagues proposed an alternative lineage commitment step to the classical model where ST-HSC or MPP first diverge into the MegE lineage, and the loss of this MegE potential is a prerequisite for the differentiation into GM or lymphoid lineages [23]. This revised model suggests that CLP are not necessarily generated from the same MPP that commit to the CMP lineage (Figure 2). This refinement is particularly important to understand lymphoid lineage commitment and differentiation processes. These processes appear more complex compared to the myeloid-lineage commitment and differentiation program because of the multiple lineage restriction steps and the loss of the MegE and GM differentiating potentials prior to lymphoid lineage commitment.

1.1.2.1. Lymphoid versus myeloid lineage potential

One of the MPP subsets which expresses high levels of the Flt3 (Flt3^{+}LSK) has been termed 'lymphoid-primed multipotent progenitors' (LMPP). LMPP are the precursors of the common lymphoid progenitor (Lin^{-}IL-7Rα^{+}Sca-1^{low}CD117^{low}Thy-1.1^{-}) and early lymphoid progenitors (ELP, Lin^{-}IL-7Rα^{-}CD44^{+}Sca-1^{high}CD117^{high}) [20, 26-28] (Figure 2). ELP are lymphoid progenitors expressing the recombination activating gene-1 (RAG-1) [29, 30]. They are similar to Flt3^{hi}VCAM-1^{-} MPP which have been proposed to be lymphoid-biased progenitors that gradually down-modulate their myeloid potential. They also express CC-chemokine receptor 9 (CCR9), the chemokine receptor for CCL25 [31], which is only detected in the thymus [32], not in the bone marrow. Thus CCR9 enables

ELP to home to the thymus [20, 22, 29, 33]. CCR9 expression is not detected on CLP [34]. Consequently, their T cell potential is believed to occur prior to the silencing of the myeloid program and before the CLP stage [22]. Recently, the marker Ly6D has been used to identify the branch point where CLP divide into B cell-biased lymphoid progenitor (BLP) and all-lymphoid progenitor (ALP) that either give rise to the first stages of B-cell development or contribute to the T-cell development, respectively [35]. $Ly6D^-$ CLP possess B, T, natural killer (NK), dendritic cell (DC), and some degree of myeloid potential, whereas $Ly6D^+$ CLP are B cell lineage restricted progenitors [35]. $Ly6D^-$ CLP are thought to enter the thymus, since after intravenous transfer, they can generate thymocytes [35, 36] (Figure 2). Both the CLP that express *IL-7R* gene and the ELP progenitors relay on IL-7/IL-7R signaling or priming in order to contribute to the B- and T-cell development [37-39]. The notion of priming was demonstrated by visualizing the history of *IL-7R* gene expression in a study by Schelnner and collaborators. The experiments performed in this study showed that, although the vast majority of ELP lacked *IL-7R* mRNA expression, they were marked by a prior *IL-7R* expression. This indicated that they are primed by the *IL-7R* gene signature and that they descend from a progenitor that is IL-7R-positive [39].

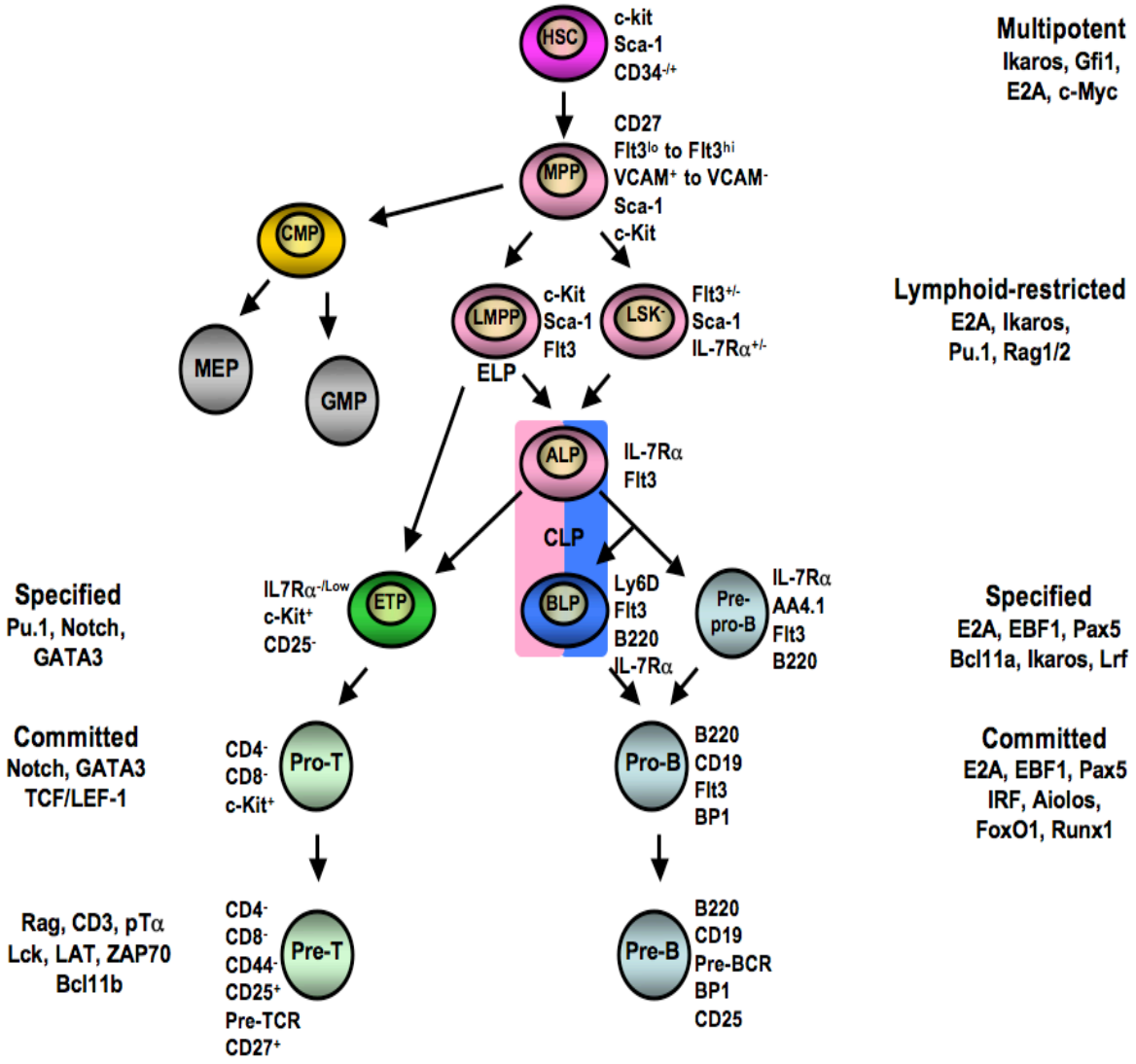

Figure 2. Schematic representation of the revised model of hematopoietic lineage differentiation, specification and commitment. Cells in pink can give rise to the myeloid lineage (gray) and the lymphoid lineages (blue-green). Common lymphoid progenitors (CLP) containing the ALP and BLP are in both pink and blue. ELP are also indicated under the LMPP cells as they represent a more immature stage compared to CLP. ELP and ALP can give rise to ETP, whereas BLP give rise to B cells. Specific surface markers for each cell types are indicated. Examples of key-player genes at the different differentiation stages are listed. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocytic/erythrocyte progenitor; GMP, granulocyte/macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; LSK, $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$; ALP, All lymphoid progenitor; ETP, early thymic progenitor; BLP, B cell-biased lymphoid progenitor; CLP, common lymphoid progenitor (adapted from [40]).

1.1.3. The myeloid-based model

The common lymphoid progenitors were described as the precursors that give rise to B, T and NK cells, but not to myeloid cells [20]. The prediction from this model was that CLP migrate to the thymus from the bone marrow to initiate T-cell development. However, some reports showed that the predominant thymus-seeding cells do not resemble CLP, but have characteristics of earlier hematopoietic progenitors [41]. Other evidence came from the characterization of Ikaros, member of the Ikaros family of transcription factors that contribute to multiple aspects of hematopoietic development and homeostasis [42, 43]. *Ikaros*-knockout mice lack B cells, but maintain their T cell pool despite the fact that they lack CLP because they retain the presence of an early T-lineage progenitor population in the thymus [44, 45]. These findings suggested that specific early T-lineage progenitors rather than CLP are the true T cell progenitors and that both can develop independently of each other. Therefore, an additional diversion at the CLP commitment step may be responsible for B- and T-cell development. Moreover, studies in mice showed differences in hematopoiesis between fetal and adult stages. In fetal mice, the myeloid potential persisted even after the lineage branch of hematopoiesis divided toward T and B cells [46-48]. Therefore, it was proposed that an additional 'myeloid-based' model of hematopoiesis exist, in which a hematopoietic stem cell initially generates common myelo-erythroid progenitors and common myelo-lymphoid progenitors. Recent studies validated this model for adult hematopoiesis, providing evidence that the early cell populations in the adult thymus contain progenitors that have lost the potential to generate B cells but retained substantial myeloid potential [49, 50]. These studies argue against the classical dichotomy

model in which T cells are derived from CLPs. It supports instead a myeloid-based model for both adult and fetal hematopoiesis.

Although compelling, the myeloid-based model of hematopoiesis is mainly supported by in vitro evidence. The proposed common progenitor for T and myeloid lineages has recently been challenged by an in vivo model able to map the fate of T- and myeloid cell development by visualizing the history of IL-7R expression. These elegant experiments provide evidence suggesting that lymphoid-restricted progenitors are the major source of T-cell differentiation and that the separation between lymphoid and myeloid progenitors is a fundamental hallmark of hematopoiesis [39]. In vitro and in vivo differentiation potentials must carefully be examined as both studies may generate artifactual results. On the one hand, non-physiological high concentrations of cytokines or growth factors may have been used in vitro that allow progenitors to differentiate into cell lineages that they are unable to generate under in vivo conditions. On the other hand, in vivo studies implicate isolation and manipulation of progenitors prior to their injection or transplantation which may force or alter the migration potential to distinct niches within the bone marrow or to the thymus [51] that only support the maturation of certain lineages.

Taken together, these studies show that the classical model of the CLP/CMP branching point of hematopoiesis is still valid, although some subdivisions in the subsets of MPP or ST-HSC that give rise to lymphoid or myeloid lineages must exist. Further analysis of bone marrow progenitors with T-cell differentiation potential will have to be done to clarify

whether only one or multiple subpopulations contribute to thymopoiesis. This clarification may have implications for improving bone marrow transplantation strategies to treat leukemia [52]. It takes months to fully reconstitute T cells after bone marrow transplantation [53]. During this period, patients are susceptible to infections. To improve the reconstitution of the peripheral T cell pool in a transplanted patient, it may become important to increase the number of true T-lineage committed progenitors in the transplant. In fact, while LMPP and CLP are able to generate $CD4^{+}CD8^{+}$ double positive (DP) cells in the thymus, HSC failed to do so after transfer. HSC, CLP and LMPP subsets can produce DP cells after intrathymic transfer, but HSC are unable to settle the thymus to generate DP cells. They can do so if the homing step is bypassed [54]. These experiments indicated that thymic settling is selective toward the circulating progenitors and that certain signals are required for thymic entry [54]. To improve stem cell transplantation efficiency, it was shown that the co-transplantation of CLP and hematopoietic stem cells in irradiated hosts can improve the recovery of the T-lineage [55]. Recently, Van den Brink's group successfully transferred precursors generated in vitro on OP9DL1 bone marrow stromal cell cultures that improved peripheral T cell numbers in irradiated mice [56]. This in vitro technique has the advantage of generating a large number of T-committed precursors in order to improve the peripheral T cell pool that is suitable for transfer and that expresses a normal T cell receptor repertoire. These precursor-derived T cells also had normal cytokine production and proliferated in response to antigenic stimulation [56]. The increase in donor T cells after transplantation was shown to improve the resistance to *Listeria* infection and enhanced graft-versus-tumor responses [56]. Finally, the generation of T cell precursors

from human $CD34^+$ cord blood cells using Notch signaling in vitro was also successful [57]. Therefore, whether T-lineage progenitors derived from bone marrow or from in vitro cultures are used, the co-transfer of true T cell progenitors and HSC may allow for an enhanced and accelerated T cell reconstitution after stem cell transfer.

2. Temporal and spatial regulation of transcription factors in stem cells

Lineage specification and commitment of HSC is dependent, for example, on the stem cell niche, the profile of chemokine expression, cytokine signaling and transcription factors that mediate their survival and development [58-60]. The molecular programming of hematopoietic progenitor subsets depend on gene regulation, which occurs based on transcription factors, co-factors, signaling molecules present in the cell and on the context of the regulatory elements of each gene. Moreover, the function of a transcription factor can change during developmental transitions, but the regulatory elements of each gene do not vary with each transitional step [61]. Stem cells and multilineage precursors co-express lymphoid, erythroid and myeloid genes [62, 63]. Some genes can be qualified as differentiation genes that are true markers of lineage commitment [64]. For example, GATA, Pu.1, Id factors, SCL, Ikaros, Gfi1 and Gfi1b are transcription factors required to enable precursors to make specific lineage fate decisions [65, 66].

2.1. GATA and Pu.1 during lineage commitment

Sequence-specific DNA-binding proteins are important regulators of chromatin configuration that either increase or inhibit gene expression [67]. For example, the zinc finger transcription factor GATA-1 directly occupy looped enhancers and target gene promoters at the β-globin locus [68]. GATA-1 is essential for the erythroid lineage differentiation [69] and mutations in the *GATA-1* gene are associated with megakaryoblastic leukemia and anemia [70]. GATA-1 is a transcriptional activator of many erythroid specific genes and can also function as a repressor of proto-oncogenes like *Myc* and *Kit* in order to terminate cell proliferation when erythroid maturation is achieved [71-73]. The zinc finger transcription factor GATA-2 is another GATA family member that is expressed in hematopoietic stem cells, multilineage progenitors and early committed erythroblasts. The erythroid differentiation is orchestrated by an exchange between the GATA factors as GATA-1 levels increase leading to the silencing of GATA-2 expression upon cell maturation [74, 75]. Furthermore, GATA2 exerts different functions regulating development, cell cycle and lineage commitment processes. It is expressed in hematopoietic stem cells during fetal and adult development [76, 77]. GATA2 deletion is lethal at mid-gestation due to severe defects in hematopoiesis [78]. GATA2 seems required to promote the proliferation and survival of early hematopoietic cells and mast cells, but it is not needed for terminal myeloid differentiation [79]. Mice deficient for one allele of GATA2 (*GATA2*$^{+/-}$) show decreased numbers of HSC that are unable to compete with normal cells in transplantation assays [80]. It has been suggested that the decrease in GATA2 expression increases apoptotic cell rates and ultimately provokes cell death [80].

The GATA/Pu.1 axis is important for the separation between erythro-megakaryocytic and myeloid lineages [81]. GATA-1 drives erythro-megakaryocyte cell differentiation in opposition to the Ets (E26 transformation specific) family factor Pu.1, which directs differentiation towards the myeloid fate [82-84]. The transcription factor Pu.1 is expressed at the highest level in macrophages. It induces myeloid genes such as Mac-1 (CD11b), F4/80, GM-CSF receptor (CD116) and M-CSF receptor (CD115) [85, 86]. The disruption of Pu.1 results in many hematopoietic abnormalities [87-89]. Up-regulation of Pu.1 drives the cells into the myeloid lineage, while low Pu.1 expression levels are necessary for B-cell development [90, 91]. At low levels of Pu.1 expression, the IL-7Rα is induced to promote both the survival and proliferation of pro-B and pro-T cells [91, 92]. Moreover, Pu.1 must be downregulated as cells differentiate toward the T cell lineage to avoid activation of myeloid genes and cell death in T cell precursors [89, 93, 94].

2.2. Id1 to Id4 during lineage commitment

Id1-4 proteins (inhibitor of differentiation) are members of the helix-loop-helix protein family of transcription factor that play important roles during embryogenesis, cell fate determination and cell cycle progression [95]. During myeloid development, *Id2* expression is up-regulated as the cells progress to terminally differentiated monocyte-macrophages, granulocytes and erythrocytes [96, 97]. Id proteins are also required for lymphopoiesis since $Id2^{-/-}$ mice display defects in NK cells that can emerge from the bone marrow or the thymus. Their development is regulated by the inhibition of basic helix-loop-helix E protein

functions which is mediated by high levels of Id protein expression [98, 99]. Id proteins lack the basic domain that allows helix-loop-helix proteins to bind to DNA. The heterodimerization of E- and Id-proteins abolishes the capacity of E-proteins, for example E2A (transcription factor 3), to bind to the DNA [100]. Id2 and Id3 are not expressed in early differentiating thymocytes as they interfere with their lineage commitment presumably by influencing the function of other factors such as E2A and HEB [101-103]. *Id2*-deficient precursors still give rise to T cells but not to NK cells, whereas forced expression of Id3 pushes thymocytes towards the NK fate [98, 103].

2.3. SCL/Tal-1 during lineage commitment

The helix-loop-helix stem cell leukemia (SCL/Tal-1) transcription factor is essential for the very early steps of hematopoiesis [104-107]. SCL/Tal-1 is also an erythroid differentiation cofactor along with the LIM-domain (for Lin11, Ist-1, Mec-3) protein LMO-2 [108, 109]. Maturation of T cells is associated with the downregulation of SCL/Tal-1 and LMO-1 and LMO-2, and a parallel up-regulation of E2A and HEB expressions. Enforced expression of SCL/Tal-1 and LMO-1 blocks T-cell development by inhibiting HEB target genes like the pre-TCRα chain (*pTα*) [110, 111]. Moreover, the deregulation of SCL/LMO-1 or LMO-2 complexes in T cells alters their normal growth pattern leading to the formation of leukemia [110, 112]. It is also involved in t(1;14) translocation observed in childhood T-cell acute lymphoblastic leukemia (T-ALL) [113, 114].

2.4. Ikaros during lineage commitment

Ikaros is the founding member of the Ikaros family of transcription factors that contributes to multiple aspects of hematopoietic development [115]. The Ikaros family members regulate, in particular, lymphocyte development and homeostasis [43, 44, 116, 117] and were originally described as transcription factors that recognize regulatory sequences of genes expressed in lymphoid cells [118, 119]. The *Ikaros* gene contains seven exons and can give rise to eight isoforms by alternative splicing [119]. The Ikaros proteins share a common C-terminal domain with two zinc fingers, whereas their N-terminal domains contain different combinations of one to four zinc finger motifs [119]. The N-terminal domains mediate DNA-binding and the C-terminal motif mediates self-dimerization or multimerization with other Ikaros family members such as Helios or Aiolos [120-122]. It takes three N-terminal zinc fingers for high affinity DNA interaction. Therefore only Ikaros-1, Ikaros-2 and Ikaros-3 are able to bind to sequences that share the core motif GGGA [119]. Ikaros-4 has two N-terminal zinc fingers and binds to tandem recognition sites that share the GGGA sequence [119]. On the contrary, Ikaros-5, Ikaros-6, Ikaros-7 and Ikaros-8, which have one or no N-terminal zinc fingers cannot bind to DNA [118]. The various isoforms of Ikaros act as activators or repressors of transcription [118]. For example, Ikaros-6 lacks a DNA-binding domain and acts as a dominant negative regulator of Ikaros function [118].

All Ikaros proteins are expressed in self-renewing populations of stem cells [115]. The role of the *Ikaros* gene was studied by deleting its last translated exon which is shared by all of

the Ikaros proteins. *Ikaros*-null mice lack B, NK and fetal T cells, but some T cell progenitors in the thymus and mature T cells in the periphery are found [44]. Although hematopoietic stem cell activity is defective in *Ikaros*-deficient mice, their myeloid differentiation is relatively normal [123, 124]. Mice expressing reduced amounts of Ikaros fail to undergo pro-B to pre-B cell differentiation and their bone marrow cells do not form colonies in response to IL-7 in vitro [125]. One explanation for these finding is provided by studies showing that Ikaros regulates the expression levels of several genes promoting lymphoid lineage differentiation such as terminal deoxynucleotidyl transferase (*Tdt*), *Rag-1/2*, *Flt3* and *IL-7R* [124, 125].

2.5. Gfi1, Gfi1b and their functions

Growth factor independent-1 (Gfi1) and its closely related paralogue Gfi1b are transcription factors with a SNAG domain at the N-terminus and six zinc fingers at their c-terminal end [126-129]. The region between these two domains is smaller in Gfi1b than Gfi1 and is not conserved between the two proteins. Gfi1 is a transcriptional repressor that also plays essential roles outside of the hematopoietic system. For example, Gfi1 is important for the inner ear development [130, 131] and for the development of subsets of intestinal cells [132, 133]. Moreover, the SNAG domain repressor Gfi1 can regulate cell cycle progression through its interaction with the zinc finger protein Miz-1 (Myc-interacting zinc finger protein-1) [134]. Miz-1 is a transcriptional transactivator or transrepressor depending on its interacting partner and the best documented activation potential of Miz-1 is exemplified by its regulation of the expression of two genes encoding

for the cyclin-dependent kinase inhibitors *Cdkn2b* ($p15^{INK4B}$) and *Cdkn1a* ($p21^{CIP1}$) [135-138]. It has been shown that Gfi1 can bind to the cell cycle inhibitor *Cdkn1a* promoter through the zinc finger protein Miz-1, thereby influencing the outcome of its transcriptional regulation [134]. In this study, the association of Gfi1 with Miz-1 seems to allow Gfi1 to control cell proliferation in response to TGFβ stimulation [134]. Another study showed that the Miz-1/Gfi1 complex was important to repress the transcription of another cell cycle inhibitor, *Cdkn2b* [139]. This mutual recruitment of Gfi1 and Miz-1 to target gene promoter represents an novel transcriptional regulation that require more work in order to elucidate the full potential of this mechanism.

During hematopoiesis, Gfi1 and Gfi1b are differentially expressed [140]. Myeloid cells, in particular granulocytes, express high levels of Gfi1 whereas Gfi1b is completely absent. Gfi1 is also expressed in activated macrophages, granulo-monocytic precursors (GMP), stem cells, thymocytes and developing B and T lymphocytes, but absent or expressed at low levels in mature resting B and T cells, respectively [126, 130, 141-144]. Loss of Gfi1 in mice affects early B- and T-cell development [145] and neutrophil development [142, 146]. In addition, the deletion of Gfi1 in mice affects the frequency and function of HSC and greatly affects CLP numbers in the bone marrow. Moreover, Gfi1 is important to restrains the proliferation of HSC in order to control their self-renewal capacity and long-term engraftment abilities [147]. It also protects HSC from stress-induced apoptosis [148]. As for the implication of Gfi1 in B-cell development, it was demonstrated that *Gfi1*-

deficient B cell precursors can no longer integrate the signals initiated by the IL-7/IL-7R cascade [149].

Conversely, Gfi1b is absent from granulocytes, activated macrophages that reside in the bone marrow and GMP. Gfi1b is not expressed in almost all stages of T-cell differentiation, but it is present in early steps of B-cell development [144]. Moreover, Gfi1b is highly expressed in hematopoietic stem cells and in erythroid precursors, megakaryocytes and their progenitor cells (MEP), where Gfi1 is mostly undetectable [144, 150, 151]. The deletion of Gfi1b is lethal at mid-gestation probably because of the disrupted erythroid and megakaryocyte development [144, 150]. To circumvent this lethality, conditional deletion of Gfi1b in adult mice was achieved and showed that the deficient HSC significantly expanded in the bone marrow and blood. This expansion correlates with increased levels of reactive oxygen species and disturbed expression of cell surface receptors that mediate stem cells niche localization [148]. The co-expression of Gfi1 and Gfi1b was observed in early stages of B cell and in a subset of early T cells, suggesting a tight regulation of both transcriptional factors in a cell-specific manner [143, 144].

2.6. Transcriptional regulation of B- versus T-lineage choice

The dichotomy between Notch signals and Paired-homeodomain transcription factor Pax-5 dictates the T- versus B-lineage commitment respectively. Notch signals activate RBPJ (Recombining binding protein suppressor of hairless) transcription factor, also known as CSL (CBF-1, Suppressor of Hairless, Lag-1) which triggers and sustains a T lymphocyte

program while blocking any B-cell differentiation potential. Conversely, Pax-5 directs the B cell program while blocking the T cell fate, in part by inhibiting Notch1 expression [152-154]. These transcription factors are key-players in the cell-type specific regulation of lineage fate. They can repress or inhibit a cascade of other factors important for alternative pathways in order to activate the cell-specific fate (Figure 3) [155].

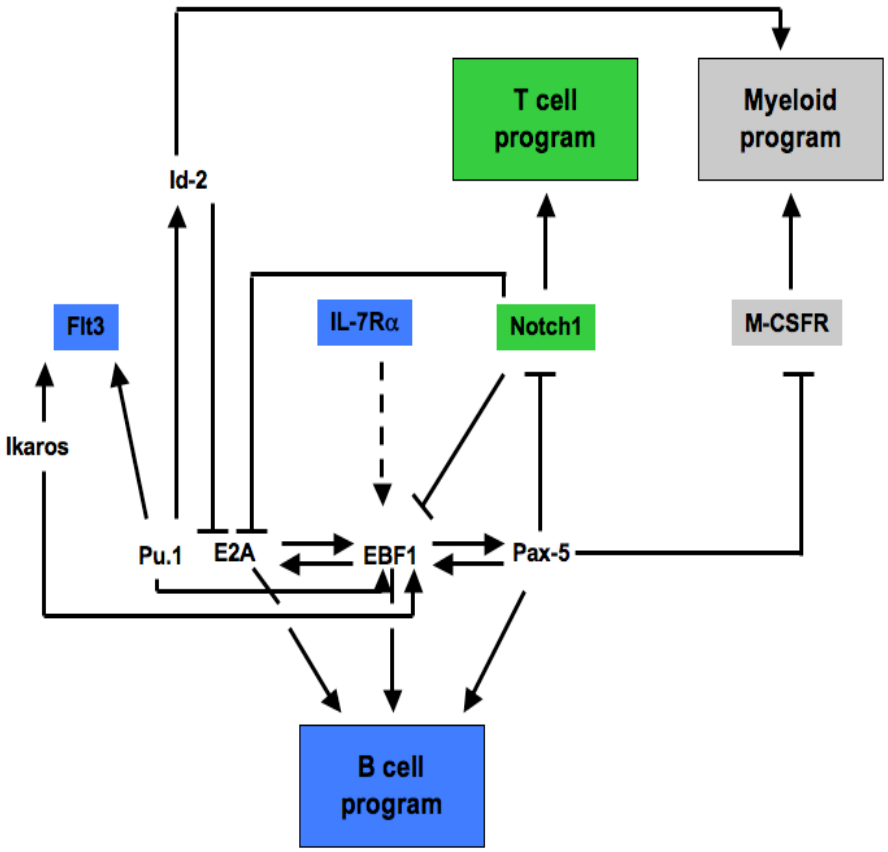

Figure 3. A simplified version of the transcriptional network governing B-, T- and myeloid lineage differentiation. Key surface receptors for B cells (blue), T cells (green) and myeloid cells (gray) are shown in rectangles. Transcription factors involved in different stages of hematopoiesis are in black text. Positive gene regulation is represented by arrows, while barred lines represent transcriptional repression. Indirect interactions are in dashed arrows (adapted from [40]).

3. B-cell development

B cells are typical representative members of the adaptive immune system because they provide both a specific response and a long-lasting protection against invading pathogens. B cell activation is initiated following the recognition of antigens by the BCR which results in cell proliferation and differentiation. Activated B cells can differentiate into either plasma cells that are responsible for antibody secretion or memory cells that provide protection during secondary responses against the same invading pathogen [156]. The proper functioning of B effector cells is coordinated during the immune response. Most importantly, it is tightly regulated earlier during development to assure that only functional mature B cells populate peripheral lymphoid organs to mount efficient responses against potential pathogens and infections.

3.1 Early stages of B-cell differentiation

Emergence of CLP from LMPP progenitors is marked by the up-regulation of IL-7Rα (CD127) expression that, together with the common γ chain (CD132), forms the IL-7R [157]. The IL-7Rα chain is also a component of the thymic-stromal-derived lymphopoietin (TSLP) receptor [158]. Given that IL-7Rα is required for two different receptors that function in B-cell development, it is to be noted that *IL-7Rα*-deficient mice have more pronounced B cell deficiencies compared to mice lacking the other chain of IL-7R, the common γ chain, since the *γ*-deficient mice have an intact TSLP-R [37, 159]. Mice lacking the IL-7 cytokine itself (IL-$7^{-/-}$) have a similar phenotype to *IL-7Rα*-deficient mice, but their B-cell development arrest at a later stage [38]. This difference is probably caused by other

cytokines that use the IL-7Rα, such as TSLP. IL-$7^{-/-}$ mice also showed similar lymphocyte abnormalities to γ-deficient mice [160], even if this receptor unit is shared by other common γ chain user cytokines. These studies indicate that the most severe lymphocyte developmental abnormalities observed in γ-deficient mice are mainly due to IL-7 and not the other cytokines that bind to this receptor.

Although CLP express IL-7Rα, this signaling cascade is not necessary for the generation of these progenitors, but is crucial for their ability to differentiate into pre-pro-B lymphocytes and to undergo cytokine-induced expansion [161, 162]. B-cell commitment can be analyzed by tracking the surface markers B220 (CD45R), HSA (CD24), CD43 and CD19 which follow a precise ordered expression pattern. The most immature B cells express B220 and CD43, whereas more mature stages start expressing HSA and CD19 [163]. The $B220^{+}$ subset in the CD19-negative cells is also referred to as fraction (Fr) A, which contain the uncommitted pre-pro-B cells ($B220^{+}CD19^{-}$) and are the source for committed $CD19^{+}$ pro-B cells ($B220^{+}CD19^{+}CD43^{+}IgM^{-}$). These cells can be further subdivided into Fr B and Fr C or pro-B cells, that differentiate into Fr C-prime or early/large pre-B cells and ultimately give rise to Fr D or late/small pre-B cells [164, 165] (Figure 4).

The transition from pro-B cells to pre-B cells is the first stage of B-cell development that is accompanied by the expression of the pre-B cell receptor. This pre-BCR is composed of the surrogate light chain, VpreB or Vλ5, that pairs with the immunoglobulin μ heavy chain (Igμ) in large pre-B cells [166].The diversity of the BCR repertoire is generated by the

variable (V), diversity (D), joining (J) recombination events that are sequentially coordinated during B-cell development [166]. The immunoglobulin heavy chain (*Igh*) locus undergoes rearrangements in the pro-B cells, and successful rearrangement leads to the expression of the Igμ heavy chain protein. Subsequently, the recombination of the Ig-light chain genes *Igk* or *Igl* takes place later in the small pre-B cells, which replaces the surrogate VpreB and Vλ5 chains resulting in the expression of the BCR [166] (Figure 4).

Diversity is generated during these early B-cell differentiation steps by random rearrangement of the immunoglobulin genes. This process mainly generates auto-reactive immature B cells that are eliminated from the repertoire by clonal deletion [167] or by the induction of anergy, which renders auto-reactive B cells non-responsive to a BCR-mediated simulation [167, 168]. Another mechanism to eliminate auto-reactive B cells is receptor editing, where a new *Ig* gene rearrangement occurs to generate a new light chain to pair with the existing Ig heavy chain [169-171]. The new combination, if successful, will generate non-self reactive BCR which prevents the cells from deletion by apoptosis [172]. Some auto-reactive B cells can also be generated later in the development, after mature B cells leave the bone marrow and enter the spleen where they undergo somatic mutations of the *Ig* variable region genes. Hence, tolerance mechanisms operate in the periphery such as receptor revision [173, 174] or by blocking activated B cells from becoming antibody-secreting plasma cells [175] (Figure 4).

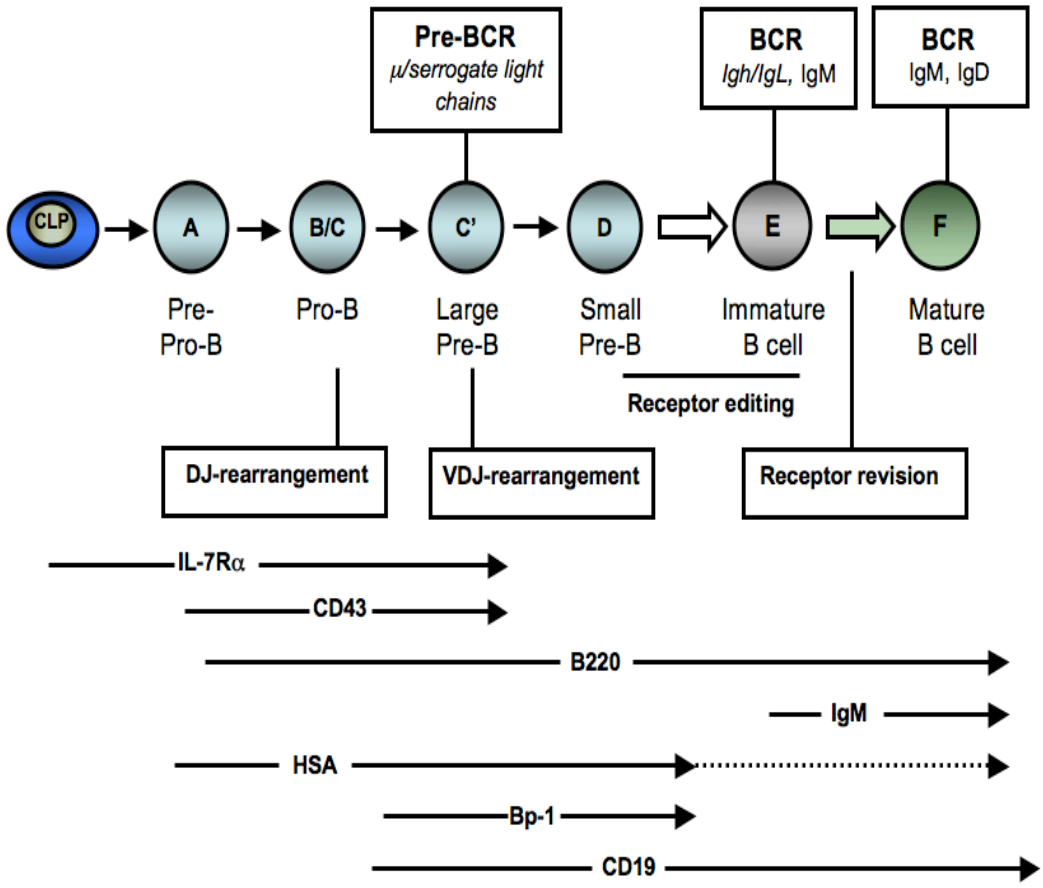

Figure 4. Illustration of B-cell development in the bone marrow. The B-cell lineage is initiated by a common lymphoid precursor (CLP) that give rise to cytokine-dependent pro-B cells. Pro-B cells start V(D)J recombination and the μ heavy chain is produced in the large pre-B cell stage. Cells that fail to produce this chain are eliminated by apoptosis. In large pre-B cells, the μ heavy chain pairs with two surrogate light chains, λ5 and VpreB, and form the pre-B cell receptor (BCR). The pre-BCR signaling mediates proliferation and differentiation of small pre-B cells. Subsequently, rearrangement of the kappa and lambda light chains takes place and a fully assembled BCR, mainly IgM molecules at this stage, is expressed on the surface of immature B cells. These immature B cells emigrate from the bone marrow to further maturate in the spleen and lymph nodes where they encounter antigen and go through the process of receptor editing. The different B cell maturation steps are defined by the presence of specific markers, some of which are indicated (adapted from [176-178]).

3.2. B-cell differentiation and cytokines

Adult B-cell development takes place in the bone marrow through well-defined steps involving cytokine signaling, V(D)J recombination and gene regulation [179, 180]. The differentiation, proliferation and survival of early B-cell progenitors are dependent on cytokine signaling, in particular on Flt3 and IL-7R [27, 181, 182]. Most of the relevant cytokines for B-cell development are produced in the bone marrow by stromal cells that reside in the medullary cavity. Stromal cells that support pre-pro-B cells are $CXCL12^{+}IL\text{-}7^{-}$, whereas those that support pro-B cell differentiation are $CXCL12^{-}IL\text{-}7^{+}$ [183].

IL-7 has been documented by many groups to be a necessary B lymphopoietic factor since B-cell differentiation does not occur in *IL-7R*-deficient mice [38]. The expression of the pre-BCR is dependent on stromal cell interactions and IL-7 receptor signaling. This signaling up-regulates the expressions of the IL-7Rα chain itself and the enzyme terminal deoxynucleotidyl transferase (Tdt), responsible for adding nucleotides at the joining region between V and D segments during recombination [184]. However, adult and fetal B lymphopoieses differ in their requirements for IL-7. For example, B-1 B cells, which are mainly produced during fetal development, may be independent of IL-7 since they are still produced in *IL-7*-deficient mice [185]. This may be attributable to the compensatory effect of thymic stromal lymphopoietin on fetal B cell progenitors [159, 186]. Contrary to *IL-7*-deficient mice, B-1 B cells are severely impaired in the absence of the IL-7 receptor. This indicates that the IL-7R is the key factor for adult B-cell development [187].

3.2.1. IL-7/IL-7R signaling in B cells

IL-7R signaling activates three major axes or pathways named after the Janus kinase- signal transducer and activator of transcription (JAK-STAT), the phosphatidylinositol 3-kinase (PI3K)-Akt and the RAS-mitogen-activated protein kinase (MAPK) [188]. The IL-7/IL-7R signaling cascade is induced when IL-7 binds to its receptor, which activates JAK3 that phosphorylates the IL-7Rα-associated JAK1 and the IL-7Rα chain itself. The phosphorylation of JAK proteins creates a docking site for STAT5, which is itself activated by phosphorylation following its recruitment to IL-7Rα chain [157]. The transcription factor STAT5 consists of two related isoforms, STAT5A and STAT5B, encoded by separate genes. In lymphocytes, STAT5A and STAT5B play redundant roles as the deletion of either one of them has only minor consequences on cellular functions [189]. Phosphorylated STAT5 proteins dimerize and translocate to the nucleus where they activate the transcription of IL-7-dependent target genes [157, 189] (Figure 5).

One outcome of IL-7R signaling is the maintenance of cell survival by promoting a positive balance of Bcl-2-family members. This is achieved by increasing the expression of anti-apoptotic Bcl-2 (B-cell CLL/lymphoma-2) and Mcl-1 proteins (Myeloid cell leukemia sequence 1, Bcl-2-related), and by redistributing the cell-death proteins Bax (Bcl-2-associated X protein) and Bad (Bcl-2-antagonist of cell death) [190]. On the one hand, pro-apoptotic Bax, Bad and Bak (Bcl-2-antagonist/killer) proteins form homo-oligomeric pores in the mitochondrial membrane and are critical for cytochrome *c* release. On the other hand, Bcl-2, Mcl-1 and Bcl-X_L (Bcl-2-like 1) are anti-apoptotic proteins that maintain

mitochondrial homeostasis, thus preventing mitochondrial outer membrane permeabilization [190]. A shift in this balance will induce cell death over cell survival. The expression of Bcl-2 is up-regulated after IL-7 stimulation and some studies have additionally shown that this up-regulation can be STAT5-dependent [191-194] or indirect, *i.e.* STAT5 induces another factor which expression can induce the transcription of Bcl-2 [195] (Figure 5).

The phosphorylation site on IL-7Rα chain that acts as a docking site for STAT5 also allows the recruitment of PI3K [194, 196, 197]. Some studies have shown that PI3K activation leads to the activation of AKT protein, which regulates the glucose metabolism of the cell and therefore maintains pro-survival and growth functions [157]. In some B cell lines, IL-7R signaling induced PI3K activity allowing the cells to enter cell cycle and to proliferate [198]. Mice deficient for the p85α subunit of PI3K or for the p85α together with splice variants p55α and p50α [199] have a severe B-cell differentiation block indicating that PI3K is a downstream mediator of IL-7 signaling during B-cell development. IL-7 can also activate Fyn and Lyn Src kinases in pre-B cell lines, but no member of this kinase family has been shown to be absolutely required for IL-7-induced proliferation [200] (Figure 5).

IL-7 induces a negative feedback loop on its own signaling by downregulating the activity of JAK1. Suppressor of cytokine signaling 1 (SOCS1) is known to inhibit phosphorylation

of STAT proteins by directly binding to JAK proteins. This competition for binding to JAK1 targets STAT proteins for proteasomal degradation. As a consequence, all further downstream signaling events are inhibited to ensure a return to steady state homeostasis after cytokine responses [201-203] (Figure 5).

3.2.1.1. IL-7/IL-7R regulation and B-cell differentiation

The expression of IL-7R is directly regulated by a subset of transcription factors, among them Ikaros, Pu.1 and E2A [204]. The signaling cascade initiated when IL-7 binds to its receptor not only activates developmental genes such as early B cell factor-1 (EBF1) and survival factors like Mcl-1 and Bcl-2; it also regulates cell proliferation [162, 205-207]. STAT5 was shown to bind to the *Pax-5* promoter at the same region where EBF also binds [208], which represent a critical signaling axis in the regulation of early B-cell development [209] (Figure 5).

The regulation of the downstream effectors of IL-7/IL-7R signaling pathway and their precise function during B-cell development is still a matter of debate. For instance, whether EBF1 is a direct downstream target of IL-7 is unclear since progenitors from *IL-7R* and *IL-7* knockout mice still express EBF1, albeit at very low levels [162]. Moreover, the ectopic expression of EBF1 can partially rescue a differentiation block at the pre-pro-B cell to pro-B cell transition in *IL-7R*-deficient mice. However, this rescue is transient suggesting that other factors than EBF, downstream of the IL-7/IL-7R signaling pathway, are equally required for early B-cell development [162]. It is also not clear whether IL-7/IL-7R signaling is required for the coordination of all B cells developmental processes. For

example, IL-7 was shown to be essential for CLP survival and development but not for their proliferative expansion [27]. Furthermore, a recent study by Malin and colleagues showed that the IL-7/STAT5 axis mainly controls B cell survival in pro-B cell rather than developmental processes [207] (Figure 5).

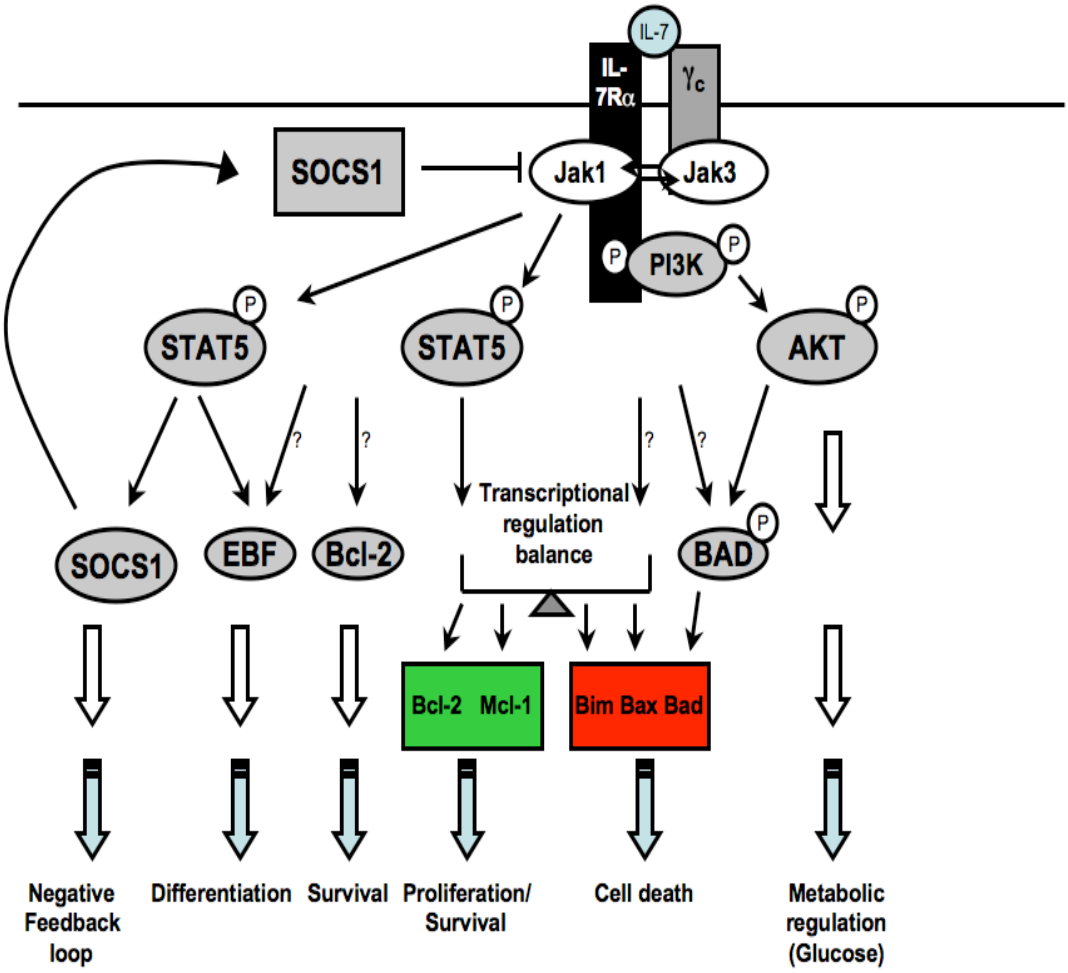

Figure 5. IL-7/IL-7R signaling cascade. IL-7 binds to the IL-7R and triggers a signaling cascade that phosphorylates PI3K, AKT, and STAT5. IL-7 cascade maintains a favorable balance between pro-apoptotic (Bcl-2, Mcl-1) and anti-apoptotic (Bim, Bax and Bad) proteins. The overall response after stimulation is indicated by big arrows ranging from differentiation or survival to cell death. SOCS1 inhibits the phosphorylation of STAT proteins to ensure a return to steady state homeostasis after IL-7/IL-7R responses. The involvement of other pathways in the activation of IL-7-downstream target genes is represented by a question mark (adapted from [157]).

3.2.2. Flt3L/Flt3 signaling in B cells

Flt3 ligand (Flt3L) is one of the cytokines that affects hematopoietic lineage development at multiple stages [210]. These effects are mediated when Flt3L binds to its receptor Flt3, expressed on hematopoietic stem and progenitor cells [211]. Flt3L is a membrane-bound protein that can be proteolytically cleaved to generate the soluble protein. Both soluble and membrane-bound forms are active [212]. Flt3L can synergize with other growth factors to promote hematopoietic cell proliferation and differentiation. For example, Flt3L in combination with IL-7, SCF, IL-11 and IL-3 will stimulate B-cell differentiation in vitro [213-216].

The administration of soluble Flt3L into mice generates an expansion of hematopoietic progenitors in the bone marrow and the spleen. Moreover, a potent mobilization of stem and progenitor cells into the peripheral blood occurs [217]. Expansion of B cells in the bone marrow and spleen was also noted [218]. On the contrary, mice lacking the receptor for Flt3L, *Flt3*$^{-/-}$, have hematological defects [219]. These mice lack B cell precursors in the bone marrow, but exhibit normal numbers of functional B cells in the periphery. The mild defects in B-cell differentiation in *Flt3*$^{-/-}$ mice can be attributable to other receptors, such as c-Kit, which may be able to susbstitute for Flt3 function in vivo [220]. Finally, mice lacking Flt3L have even more profound hematological defects compared to *Flt3*$^{-/-}$ mice. *Flt3L*$^{-/-}$ mice have a reduced cellularity of hematopoietic precursors in the bone marrow and of mature cells in the blood and the peripheral organs [221]. Whereas blood hematocrit, platelets and the thymic cellularity were not affected, both myeloid and B-cell progenitors

were reduced in the bone marrow of *Flt3L*$^{-/-}$ mice [221]. In addition NK and DC cell numbers were reduced in the spleen [221].

3.3. Mature B cells

Most immature B cells leave the bone marrow and become part of the mature, recirculating B cells in the spleen. These cells are referred to as follicular B cells (Fo) because of their localization to the follicular region in peripheral lymphoid organs [222]. The follicular B cell zone constitutes the last maturation site of B cells. Several transgenic models of B cell tolerance have shown that the self-reactive cells cannot enter the follicular zone and are therefore excluded from the recirculating B-cell repertoire [223, 224]. Fo B cells do not proliferate, but can persist in the resting state for months. Marginal zone B cells (MZ) are the second mature B lymphocytes. They are localized in the marginal zone of the spleen that represents the major antigen filtering area [225, 226]. MZ B cells respond very rapidly to antigens, likely in a T cell-independent manner, but also participate in the early phases of T cell-dependent responses [165]. These cells only produce IgM antibodies.

3.3.1 Somatic hypermutation and class switch recombination

B cells that leave the bone marrow and migrate to the spleen or lymph nodes encounter many types of antigens. If a naïve B cell-mediated antigenic activation occurs in a T cell-dependent manner, follicular structures called germinal centers are formed in the peripheral organ. Two maturation steps occur in germinal centers that greatly increase antibody affinity: somatic hypermutation (SHM) and class switch recombination (CSR) [227]. SHM can modify antibody affinities through the introduction of mutations in the variable region

of the *Ig* heavy and light genes [228]. CSR plays important roles in modulating antibody effector functions by replacing the antibody isotype. This occurs through DNA recombination events that join two switch regions [229].

Both SHM and CSR are triggered by activation-induced cytidine deaminase (AID) [230-232]. Specifically, AID induces the deamination of cytosines (dC) to uraciles (dU) in single-stranded DNA [228, 229, 233]. The resulting dU pairs with a Guanine (dG) and this dU:dG mismatch is processed to generate mutations in the variable region during SHM or DSBs in switch regions during CSR [228, 229, 233]. Mutations in AID are associated with hyper-IgM syndrome, which yields an increased susceptibility to infections and secondary antibody responses lacking characteristic class-switched, hypermutated antibodies [231]. CSR and SHM initiate DNA damage that is normally restricted to genes that encode antibodies [234]. The repair of the DSBs generally leads to diversification by SHM and CSR. However, aberrant damage of genes that do not encode for immunoglobulin genes and abnormal repair of AID-induced lesions most likely increase the tendency of B cells to undergo malignant transformation [234].

3.4. B-1 and B-2 cells

The differentiation of CLP into mature B cells mainly generates the conventional B cells termed B-2 cells [235]. Furthermore, a bone marrow bipotent progenitor that can generate B cells and macrophages has been described [236] and recognized as a special feature of fetal hematopoiesis [237-239]. Adult $Lin^{-}B220^{lo/-}CD19^{+}$ cells that are associated with

B/macrophage activities ultimately generate another B cell subset, the B-1 cells, but not B-2 cells [240, 241]. B-1 cells represent approximately 5 % of B cells in the mouse and they mainly participate in T cell-independent responses. They constitute a high proportion of B cells in the peritoneal and pleural cavities. In these sites, B-1 cells can be further subdivided into B-1a ($sIgM^{hi}$, $sIgD^{lo}$, $CD11b^{+}$, $CD5^{+}$) and B-1b ($sIgM^{hi}$, $sIgD^{lo}$, $CD11b^{+}$, $CD5^{-}$) [242, 243].

Two forms of circulating IgM antibodies exist: the natural and the antigen-induced-IgM. Natural IgM are mainly secreted by B-1 cells that can produce them in the absence of antigen stimulation [244-246]. In fact, natural antibodies are naturally occurring in individuals or 'antigen-free' mice independently of external antigens and non-specific mechanisms in response to environmental stimulations during an immune response [244]. They are encoded by germline genes with multireactivity but low affinity to a wide variety of antigens, such as cytoskeleton, nuclear proteins and DNA [244]. Although these preformed sIgM are used in their original germline configuration, they rapidly respond with more reactivity to both foreign antigens and self-components of the body, probably to provide a selective advantage to ensure the early survival of the host before the establishment of a more sophisticated immune response [245, 247]. Antigen-induced IgM are mostly produced by the conventional B-2 cells after antigen stimulation [246]. Both natural and induced IgM antibodies are polymeric. They can bind multimeric antigens, efficiently activate the complement cascade and be transported via the poly-Ig receptor onto mucosal surfaces to provide protection against invading pathogens [248, 249]. As IgM are

the first class of antibodies produced during an infection, and because of all their unique properties, they represent the first line of defense against mucosal and systemic pathogens [250, 251].

Even if B-1, MZ and Fo B cells develop from different precursors [239, 240, 252, 253], signaling at the immature B cell stage is critical for their development and can arise from crosslinking of the BCR and other microenvironmental sources. For example, Fo B-cell development is dependent on B cell-activating factor (BAFF) signaling, rather than BCR crosslinking, while B-1 development is dependent on BCR signaling and independent of BAFF [254]. MZ B-cell development uniquely requires Notch2 signaling [255, 256]. Most B cells in the periphery are quiescent and signaling from the BCR is the common mechanism that guides their transition to maturity after the appearance of surface IgM [257-262]. The mature B cell pool is determined by the proportion of immature B cells surviving transit to maturity and the longevity of the mature cells themselves [263]. Consequently, understanding the factors that control selection and the different B-cell development stages is critical to grasp how the size and composition of the B cell compartment is regulated in the periphery.

3.5. B-cell commitment is regulated by a transcription factor network

The differentiation, proliferation and survival of early B-cell progenitors are dependent on cytokine signaling, in particular on Flt3 and IL-7R. The expression of Flt3 and IL-7 receptors during B-cell development is directly regulated by a network of transcription

factors such as Ikaros, Pu.1 and E2A [204]. The helix-loop-helix proteins E2A and EBF1 specify the commitment of cells toward the B-cell lineage by activating B cell-specific genes such as *Pax-5*. A hierarchy seems to exist in the developmental regulation by these transcription factors whereby E2A effectors act upstream of EBF1, while terminal B-lineage commitment is regulated by Pax-5 [264]. In fact, Pax-5 restricts the potential of lymphoid progenitors to the B cell fate by repressing other lineage specific genes and by activating B cell specific genes [154]. It has been proposed that STAT5 regulates Pax-5 expression by interacting with its promoter region [208, 265]. STAT5 can also regulate EBF1 expression [162], although this regulation is still a matter of debate [207]. Both EBF and Pax-5 are required for the rearrangement of the immunoglobulin heavy chain locus Igh [266-268]. In contrast to adult B-cell development, EBF expression is independent of IL-7 signaling during fetal B-cell development [269], which may explain the presence of B cells in *IL-7*$^{-/-}$ mice [185]. Although some B cells can be detected in the peripheral organs of *IL-7*-deficient mice, it is believed that these B cells are mainly generated from fetal or neonatal origins [159, 185].

4. T-cell development

T and B cells are a lymphoid lineage that constitute the adaptive immune system. To generate T cells through the adult lifespan, the thymus needs to be continuously replenished with hematopoietic progenitors from the bone marrow via the bloodstream. Once homed to the thymus, these progenitor cells undergo extensive expansion and differentiation steps

that generate functionally competent, mature T cells that migrate to the peripheral lymphoid organs [51]. Several T cell subsets are involved in the host defense against invading pathogens. $CD8^+$ T cells are effector cells capable of lysing target cells by secreting perforin. $CD4^+$ cells orchestrate the immune response by secreting cytokines that control the functions of other immune cells. Among the best characterized $CD4^+$ subsets are the T helper 1 (Th1), Th2, Th17 and T regulatory cells [270].

4.1. The origin of T cells

The identification of the hematopoietic precursor best suited to home to the thymus has been a question of debate [41, 271]. The early T-lineage progenitors (ETP) represent the earliest known and most efficient intrathymic progenitors of T cells [272, 273], in comparison to other reported thymic precursors such as $CD4^{lo}$ precursors [274], common lymphoid precursors 2 (CLP-2) [275], L-selectin$^+$ precursors [276], double-negative $CD4^-$ $CD8^-$ (DN)1c, DN1d and DN1e precursors [277], thymic multipotent progenitors (TMP) [28], circulating T cell progenitors (CTP) [278] and $CCR9^+$ multipotent precursors ($CCR9^+$ MMP) [34]. Recently, $Ly6D^-$ CLP cells were proposed to enter the thymus and competently generate thymocytes even better than earlier LMPP precursors [35, 36]. Although $Ly6D^-$ CLP cells originate from LMPP, of both populations, only $Ly6D^-$ CLP rapidly reconstituted thymopoiesis after intravenous injections. This delayed kinetic was suggested to be attributable to the fact that LMPP need to reseed the bone marrow and regenerate CLP before migrating to the thymus [36].

4.2. Early T-lineage progenitors

The ability of bone marrow progenitors to settle the thymus has been attributed to different cytokines, adhesion molecules and chemoattractants. It has been suggested that the P-selectin/PSGL-1 axis plays a role in recruiting progenitors with T-cell potential to the thymus by mediating a step similar to the rolling step of the endothelial migration of mature leukocytes [279]. The chemokine receptors CCR9 and CCR7 support thymus settling [30, 34, 280-282] and act as migration signals. Finally, Flt3 signaling controls the expression of homing molecules such as CCR9 [30]. ETP in the thymus are believed to originate from LMPP/ELP in the bone marrow that express CCR9 [30, 273], or from the recently described Ly6D^{-} CLP that also originate from LMPP [35, 36]. ETP are phenotypically Lin$^{neg/low}$, CD117^{high}, CD25^{-} [272] and comprise less than 0,01% of the adult thymus. They have the potential to expand extensively and to repopulate the thymus after intrathymic transfer [272].

Experimental evidence indicate that ETP are functionally and phenotypically heterogeneous. Using CCR9-EGFP knock-in reporter mouse, Benz and Bleul found that ETP can be subdivided into CCR9-EGFP^{high} and CCR9-EGFP^{low} subsets [28]. Furthermore, Sambandam and colleagues fractionated ETP based on the expression of Flt3, which is expressed on the surface of bone marrow LSK cells, CLP and CLP-2 cells, but only 5%-20% of the thymic ETP population [33]. Flt3^{+}CCR9^{+} ETP are considered to be the most immature T-cell progenitors in the thymus that still have the potential to differentiate into the B cell lineage [28, 33]. Although immature thymocytes still retain the capacity to

differentiate into myeloid cells, it is strongly believed that thymus-seeding precursors are T/B bipotent progenitors that migrate from the bone marrow [35, 39].

4.3. $CD4^-CD8^-$ double negative cell differentiation

ETP cells undergo extensive expansion and differentiation steps that will generate functionally competent and mature T cells [41]. These steps are dependent on the stroma microenvironment for the expression of Notch ligands, mainly delta-like ligand 1 (DL1) and DL4 [153, 283, 284], in addition to other signaling pathway and cytokines such as IL-7 [285, 286]and the Kit ligand stem cell factor (SCF) [287]. The proper signaling initiated by these pathways is crucial for the maintenance of thymocyte survival and proliferation throughout the differentiating steps of the T cell program [288].

The most immature thymocytes do not express the CD4 or CD8 T cell co-receptors and are subdivided into four stages of double negative cells, DN1 to DN4. Development through the first differentiation stage, from ETP to DN3 cells, is dependent on cytokine signaling, independent of the T cell receptor and is coordinated by the migration of cells through distinct thymic microenvironments [51]. DN1 are defined by the expression of $CD44^+CD25^-$c-Kit^+IL-$7R\alpha^+$Thy-1.1^+. They can be subdivided into DN1a to DN1e according to the expression of the heat stable antigen (HSA) and c-Kit, where DN1a and DN1b have also been identified as the precursors that correspond to the ETP subset [277].

DN1 give rise to the $CD44^+$ $CD25^+$ DN2 subset, which is also fractionated into DN2a and DN2b based on c-Kit expression. Subsequently DN2 cells differentiate into DN3 cells expressing $CD44^-CD25^+$, which are classified as DN3a and DN3b based on their size [289] and CD27 expression [290]. DN3a cells, that successfully rearrange the *TCRβ* gene, express a TCRβ chain that associates with an invariant pTα chain to compose the pre-TCR. Pre-TCR-positive DN3 cells differentiate into DN3b cells that are selected by pre-TCR-dependent signals, referred to as the "β-selection" checkpoint [291]. This receptor-mediated step is the first critical checkpoint in T-cell development that resembles the one at the transition from pro-B cells to pre-B cells after the cytokine-dependent steps in B-cell development. Both pre-TCR and pre-BCR checkpoints are accompanied by the expression of a pre-B or a pre-T cell receptor composed of a surrogate or invariant chain (VpreB or Vλ5 for B cells and pTα for T cells) that pairs with the Igμ in pre-B cells or with the TCRβ in pre-T cells. DN3 cells that fail to undergo productive *TCRβ* V(D)J recombination can still be rescued by the expression of successful *TCRγ* and *TCRδ* rearrangements influencing the generation of γδ-T cells, also referred to as the "δ-selection" [292]. Cells that fail to rearrange their *TCRβ* or *TCRγ* and *TCRδ* or those that do not express in-frame rearrangements are eliminated at this point. On the contrary, successful rearrangements allow DN3 cells to reach the last subset of DN maturation, the $CD44^-CD25^-$ DN4 pre-T cells, or migrate to the periphery as γδ-T cells [291] (Figure 6).

4.4. $CD4^+CD8^+$ double positive cell differentiation

The newly developed DN4 cells up-regulate CD4 and CD8 co-receptors and become $CD4^+CD8^+$ DP cells by transiting through an intermediary stage referred to as "immature single positive (ISP)" cells [293]. DP cells are the largest thymic subpopulation that possesses a bipotent cell-fate potential, which needs to be coordinated throughout the T-cell differentiation program [291]. DP cells are also the first cells to express a fully assembled TCRαβ after the rearrangement of the *TCRα* gene that replaces the invariant pTα molecule [291, 294]. This recombination step resembles the one for the Ig-light chain genes *Igk* or *Igl* that takes place in the small pre-B cells, which replaces the surrogate VpreB and Vλ5 chains resulting in the expression of the BCR [166]. Most DP cells express TCRαβ that can not engage self major histocompatibility complex (MHC) molecules and are subjected to selection processes. They are mainly eliminated by "death by neglect" at this stage [294]. Cells bearing useful TCR are signalled to survive and continue their differentiation into functionally mature cells. These life versus death TCR-mediated signals in DP are referred to as "positive selection" [291, 294] (Figure 6). Because of this TCR-dependent restrictive step, DP cells do not depend on pro-survival cytokines like IL-7. They are also unique among the T cell lineage since they express both CD4 and CD8 co-receptors, do not express the IL-7R and express high amounts of SOCS1 [295, 296].

By expressing both co-receptors, DP cells get signalled by both MHC-class-I and MHC-class-II-restricted TCR molecules. DP signalled by MHC-class-II restricted TCR reach the

periphery as $CD4^+$ helper T cells, whereas cells signalled by MHC-class-I-restricted TCR differentiate into $CD8^+$ cytotoxic T cells [291, 294] (Figure 6). Many models have been proposed in order to explain the CD4/CD8 lineage choice in the thymus, all of each agreed on the importance of TCR-mediated positive selection signals [294]. The implications of CD4 and CD8 co-receptors and, more recently, of cytokine signals and co-receptor reversal [297, 298] added to the complexity of the lineage choice decision [294] and need to be addressed at the molecular level in order to clarify the complexity of this process. Moreover, TCR and co-receptor signaling can be integrated with transcriptional regulation involved in the CD4 versus CD8 lineage choice that explains the lineage commitment of DP cells [299]. For example, Th-POK zinc finger protein encoded by the *Zbtb7b* gene [300, 301] is very important for CD4 lineage choice [300-302]. Conversely, RUNX3, a member of the runt-domain family of transcription factors [303], can silence both *Zbtb7b* and *Cd4* gene transcription, leading to the termination of CD4 lineage potential and reinitiating thymocytes differentiation into mature $CD8^+$ cells [304, 305].

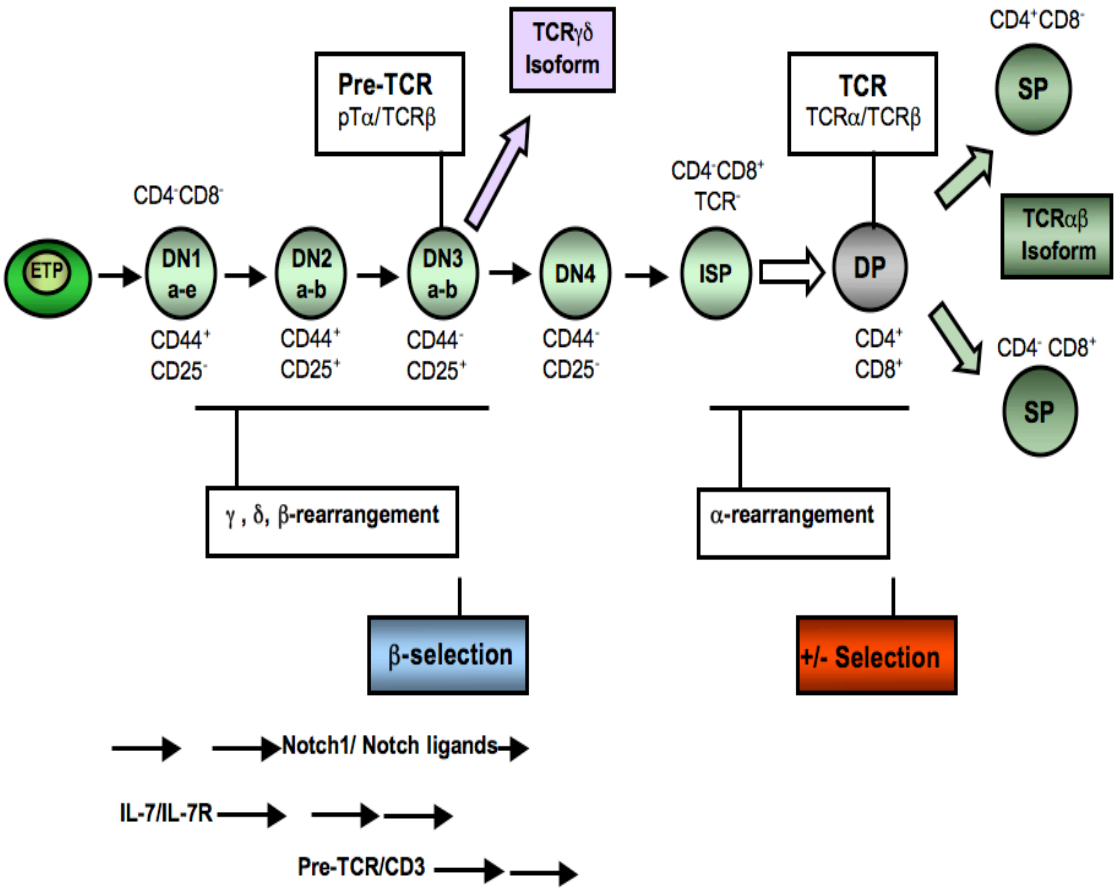

Figure 6. Illustration of T-cell development in the thymus. Early thymic progenitors (ETP) migrate from the bone marrow to the thymus, where they differentiate into four double negative $CD4^-CD8^-$ (DN) stages. DN1-DN4 subsets express CD44 and CD25 and undergo V(D)J rearrangements. The first critical selection checkpoint in this maturation process is at the DN3 stage. At this stage, a pre-T cell receptor (TCR) is expressed. If it is composed of the γδ chains, no further selection is required and the cells become γδ T cells. If the pre-TCR is composed of a TCRβ chain and the invariant pTα chain, the cells expressing this pre-TCR undergo the β-selection. Selected cells mediate proliferative signals which results in the expansion of the DN3 subset. Cells that fail to undergo productive V(D)J recombination at this point are eliminated. DN4 cells up regulate CD4 and CD8 co-receptors to become double positive (DP) cells that undergo the second checkpoint in T cell maturation: the positive and negative selection. Non-auto-reactive thymocytes are positively selected, downregulate CD4 or CD8 and reach the periphery as mature $CD4^+$ or $CD8^+$ αβ T cells. The main signaling pathways that govern DN differentiation are indicated (adapted from [306, 307]).

4.5. TCRγδ T cells

T cells bearing the TCRγδ receptor do not express precise surface markers in the periphery, and are therefore believed to have originated from $CD4^-CD8^-$ DN cells that have rearranged their TCRγ and TCRδ loci [308]. They do not proceed through a DP developmental stage in the thymus and do not express a pre-TCR or CD4/CD8 co-receptors [309, 310]. The potential of TCRγδ signaling is believed to be superior compared to the one initiated by TCRαβ [311]. Moreover, it is important to regulate the commitment of DN cells to the γδ-T cell fate and to assure their subsequent differentiation into mature TCRγδ cells [308, 312]. Although it is unclear how TCRγδ cells are selected in the thymus, some studies have shown that a least some γδ-T cells get positively selected when they express particular TCRγδ heterodimers that do not involve interaction between these TCR and polymorphic MHC molecules [313, 314]. This δ-selection favors in-frame *TCRγ* and *TCRδ* rearrangements positively influencing the generation of γδ-T cells [292]. γδ-T-cell-development also shows variations in the homing pattern of emigrating thymocytes which may be attributable to the TCR repertoire [313, 314]. The first γδ-T cells that emerge during fetal development express a restricted repertoire of $(V)_\gamma5V_\delta1$ TCR and preferentially migrate to the skin. A second wave generates γδ-T cells which express a $(V)_\gamma6V_\delta1$ TCR repertoire and populate the genital tract and tongue. Around or right after birth a third wave of γδ-T cells, mostly expressing $V_\gamma7^+$ or $V_\gamma1^+$ T cell receptors, emigrates to the small intestine and epithelium. As the mouse ages, more $V_\gamma4^+$ cells are produced and migrate to the peripheral blood and lymphoid tissues [307, 312].

In order to track γδ-T-cell development, mouse models have been generated such as the knock-in mutant for *TCRδ* tagged with enhanced green fluorescent protein (EGFP). This model has been used to visualize cells transcribing the TCRδ locus within the TCRγδ subset [315]. With this tool, it was observed that TCRγδ signaling in $EGFP^+$ DN3 cells up-regulated *TCRδ* transcription. The maturation of γδ-T cells occurs during the DN3/DN4 transitional stage and correlates with the up-regulation of the activation marker CD5. Another study has tracked CD27 and CD5 expressions to identify γδ-T cells and demonstrated that γδ-selected $CD27^+$ DN3b cells do not proliferate as much as the β-selected $CD27^+$ DN3b thymocytes [290], but did express higher CD5 on their surface correlating with the hypothesis that the strength of TCRγδ signaling is stronger compared to the one initiated by the TCRβ [316]. The use of fluorescent cells and new cell surface markers like CD27 helped to characterize the genes involved in the commitment of γδ- over αβ-T cell lineage. For example, γδ-T cell selection favors Bcl-2 rather than $Bcl\text{-}X_L$ prosurvival protein [290]. Moreover, γδ-T cells preferentially express Runx1, early growth response (Egr) proteins Egr2 and Egr3, and Id3 rather than HEB and Egr1 transcription factors in αβ-T cells [290].

4.6. Other unconventional T cells

In addition to the main αβ- and γδ- axes of T-cell development, other variations occur in the thymus. For instance, instead of the interaction between thymocytes and thymic epithelial cells that controls conventional T-cell development, some immature DP cells interact in a homotypic manner. This leads to the selection of NKT cells, a T cell subset with

characteristics typical for cells from the innate immune system. Such innate-like lymphocytes are subsets of T cells that express rearranged antigen receptors [317, 318] that are often invariant and recognize self-antigens or simple molecular structures on pathogens. Innate-like lymphocytes mainly reside in non-lymphoid tissues and express memory cell or activated memory cell markers. NKT cells can also colonize the spleen and, more abundantly, the mesenteric and pancreatic lymph nodes compared to the peripheral lymph nodes [319, 320]. In comparaison to B-1 B cells and marginal zone B cells, which can be considered as innate-like B cells [321], innate-like T lymphocytes include NKT, mucosal associated invariant T cells (MAIT), γδ-T cells and CD8αα cells [322].

Some αβ-T cells expressing a CD8αα homodimer instead of a CD8αβ heterodimer are differently selected and home directly to epithelial sites [323-326]. These cells seem to lack peptide-MHC restriction and may develop only by stroma support [327]. Cryptopatches (CP) along the intestinal wall were identified as primitive stroma that could support such unconventional T-cell development [328, 329]. These intraepithelial lymphocytes (IEL) express genes typically found in γδ-T cells and are functionally more similar to these T cells than to conventional $CD8^+$ αβ-T cells [330, 331]. Importantly, IEL populate the gut and contribute to the physiological integrity and the homeostasis of the intestinal epithelium in order to control immune response to commensal bacteria and the adaptive tolerance toward self-antigens [327]. When IEL functions are deregulated, inflammatory responses occur in the intestines emphasizing the importance of their proper regulation during unconventional T-cell development.

4.7. Extrathymic T-cell development

Some T cell progenitors have been detected in the spleen of wild-type (WT) C57/BL 6 mice, indicating that extrathymic T-cell development occurs [332]. Athymic mice or irradiated mice express abundant extrathymic T cell progenitors after bone marrow transplantation [333-336]. Although the developmental fate of these progenitors is unclear, they resemble DN3 cells expressing lower Notch1, Notch3 and Notch target genes [332-336]. Contrary to intrathymic T-cell development that requires Notch1, extrathymic T-cell development relies either on Notch1 or Notch2 for the generation of splenic T progenitors [333]. Splenic T-lineage progenitors have also been found to be dependent on IL-7R signaling [332].

4.8. Cytokine receptors and T-cell differentiation

Flt3 and IL-7 act together to support CLP and LMPP maintenance and to promote their development [2, 20, 25, 337]. Both cytokines also play important roles for the survival and proliferation of ETP. Given their pleiotrophic function, the regulation of their expression is important to understand their roles within the thymic environment.

4.8.1. Flt3L/Flt3 signaling in T cells

Both Flt3L and Flt3 play important roles in promoting the expansion of early hematopoietic progenitors and in the differentiation of mature cells. Flt3L increases the T-cell potential of multipotent bone marrow precursors in the presence of the thymic stromal microenvironment and IL-12 [338], or when cultured on bone marrow stromal cells in vitro

[339]. The T-cell potential of bone marrow precursors is also greatly augmented when they are cultured in the presence of Flt3L, IL-3, IL-6 and IL-7 [340]. In addition, T-lineage commitment is affected in mice deficient for the Flt3 receptor, shown by competitive repopulation experiments where stem cells from *Flt3*$^{-/-}$ mice transplanted into irradiated recipients did not effectively reconstitute the T cell compartment [219]. The reduced but not absent hematopoiesis in *Flt3*$^{-/-}$ mice has been attributed to the compensatory role of c-Kit. Mice with mutations in both c-Kit and Flt3 displayed an even further reduced hematopoiesis and life span [219, 341].

4.8.2. IL-7/IL-7R signaling in T cells

IL-7R signaling plays a central role in the development, survival and expansion of both B and T cell lineages [38, 182]. IL-7Rα and the common γ chain ensure proliferation signals and survival through most pro-T cell differentiation steps [342-346]. IL-7Rα is expressed in DN1, DN2 and DN3 stages and acts in concert with Flt3. ETP and their downstream subsets are significantly reduced in *IL-7R*-deficient and *Flt3L*-deficient mice [347]. Indeed, more severe defects in fetal and postnatal thymic progenitors are observed in *IL-7R*- and *Flt3L*-double deficient mice, which results in a loss of adult mice thymopoiesis. These results suggest that these two receptors have additive effects during early T-cell development [347, 348].

To return to a steady state of activation, IL-7 induces the expression of SOCS1 [349]. This induction needs to be carefully titrated as the overexpression of SOCS1 abrogates T-cell

development beyond the DN1 stage of differentiation [350]. Furthermore, SOCS1 transgenic mice showed inhibition of STAT5 phosphorylation further demonstrating the ability of SOCS1 to inhibit IL-7 signaling and its importance in regulating the αβ-T-cell development [351]. The deletion of *SOCS1* in mice leads to growth retardation and death by the third week after birth [203]. This lethality is in part due to excessive responses typical to the ones induced by IFNγ in mice, which suggests that SOCS1 regulates IFNγ action. It can be rescued by crossing *SOCS1*-deficient mice with *IFNγ*$^{-/-}$ mice [352]. The targeted deletion of SOCS1 in T cells did not show defects in DN differentiation, but revealed an exaggerated response of thymocytes to IL-7, inducing more CD8 differentiation. These CD8^{+} T cells also showed features of memory T cells [295].

IL-7/IL-7R pathway is also important for γδ-T-cell development by controlling the chromatin accessibility during V(D)J rearrangement and transcription of the *TCRγ* locus [345, 353]. Introducing STAT5 in *IL-7*-deficient mice partially rescued γδ-T-cell development suggesting that STAT5 is actively participating in the induction of V(D)J recombination and chromatin accessibility at the TCRγ locus [353].

4.9. Notch signaling

Notch signaling is required to ensure lineage commitment, survival and development of ETP into defined pro-T cell subsets [283]. Among the four Notch receptors, Noch1-4, Notch1 has been identified to play the key role in T-cell development [283, 354]. Two ligand families for the Notch receptors have been identified, Delta-like and Jagged [355].

The Notch signaling cascade is initiated by the cleavage of the Notch receptors when they encounter their ligands. The cleavage is generated sequentially by metalloproteases and γ-secretases, releasing the intracellular domain of Notch (ICN) [356-358]. The ICN part is translocated to the nucleus where it binds to the transcription factor CSL and recruits other co-activators such as mastermind-like proteins (MAML) [359]. This transcriptional complex activates and regulates the expression of Notch target genes.

Several line of evidence indicate that the biochemical events through which the cell detects and responds to Notch signaling depend on the signal strength. For example, sequence paired sites (SPS) have been identified for CSL which promote the assembly of transcriptionally active complexes of CSL, ICN and MAML1 [360] or favor the cooperation with basic helix-loop-helix E proteins binding at neighboring elements [361]. Moreover, it has been shown that different Notch cleavage sites may contribute to the gradation of the signal strength by generating two types of ICN, one more stable than the other, which results in different intensities of the transcriptional response [362]. Notch signaling is also modulated by antagonistic transcription factors such as LRF or Zbtb7a [363], or by antagonists of the assembly of the MAML1 complex like Msx2-interacting protein (MINT) [364]. Other feedback negative regulators such as Deltex1 and Nrarp [365, 366] also inhibit Notch target gene transcription (Figure 7).

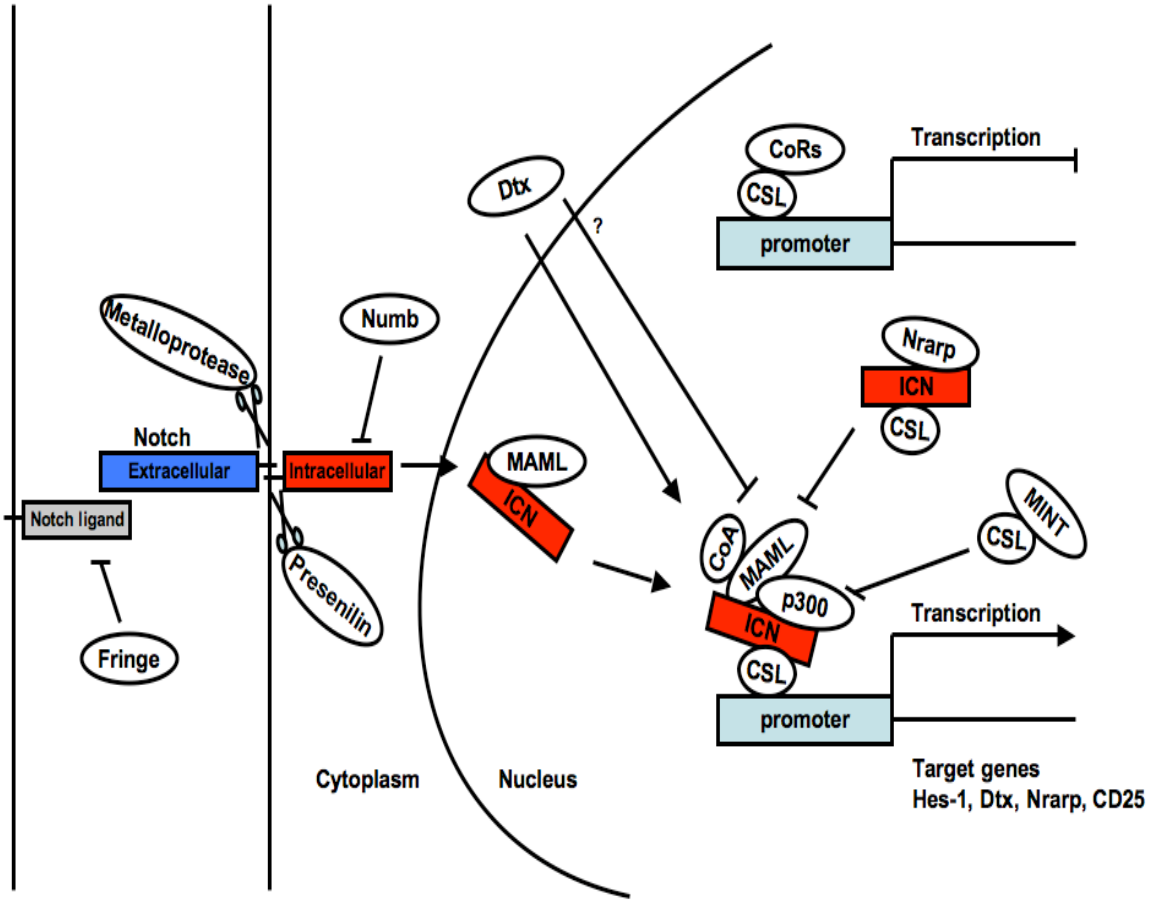

Figure 7. Notch signaling pathway. Notch is composed of an extracellular and intracellular domain. Once the intracellular domain of Notch (ICN) is cleaved by metalloprotease (extracellular site) and γ-secretase complex (transmembrane region), it translocates to the nucleus. ICN forms together with Mastermind and CSL a protein complex that recruits co-activators such as p300 to actively transcribe target genes. Notch activity is also inhibited by negative regulators like Deltex (Dtx), Numb, MINT, Nrarp, Fringe and others (adapted from [153]).

ETP are regulated by Notch signaling and have been shown to express high levels of Notch1, Hes1 (a Notch target gene) and Deltex1. Moreover, it has been proposed that these precursors express mostly lymphoid specific genes and other genes important for T-cell differentiation [34]. *Notch*-deficient bone marrow precursors adopt a B cell fate in the

thymus [283], whereas the overexpression of a constitutive active form of intracellular Notch1 in the same precursors leads to immature T cell differentiation in the bone marrow [354]. Sustained Notch signaling through DN1 and DN2 stages is required for both the differentiation of αβ- and γδ-T cells [367, 368]. At these stages, Notch promotes V(D)J rearrangement at the TCRγ, TCRδ and TCRβ loci [369, 370]. The synergy between Notch and TCR signaling determines the αβ versus γδ lineage choice during T-cell development [371]. According to this TCR-Notch synergy model, DN cells expressing the pre-TCR, require less Notch to progress to the DP stage and out-compete TCRγδ T cells for Notch ligands. The differentiation of γδ-T cells from the DN3 subset occurs in a Notch-independent way, although residual Notch signaling has been shown to be necessary in order to sustain their proliferation [290, 315]. In accordance with these observations, Notch1 expression and the expression of its target genes quickly diminish after the β-selection checkpoint [290], but several studies have demonstrated that Notch signaling is crucial for αβ-T-cell development at and after the β-selection stage [370-373]. Although the role of Notch at this first selection stage of T-cell development remains poorly understood, Ciofani and her collaborators showed that Notch signaling was required for the survival of pre-β selected cells [374]. Many in vitro studies that elucidated the implication of Notch signaling during T-cell development came from experiments with co-cultures of precursors or thymocyte subsets on modified OP9 bone marrow stromal cells [367] that mimic the thymic stromal microenvironment and express Notch ligands DL1 or DL4 [283, 284].

4.10. T cell gene expression network regulates the processes of differentiation

Many transcription factors play critical roles in T cell lineage commitment. These factors function in different stages of T-cell differentiation and control the T cell lineage fate. For instance, Bcl11b is a zinc finger transcription factor that plays an essential role during T-lineage commitment [375-377]. This role is comparable to the one Pax-5 plays in maintaining B cell identity of B cell progenitors. The expression of Bcl11b is up-regulated from ETP to DN2, and is required for the survival of DP thymocytes [378, 379]. Conditional deletion of Bcl11b arrests T-cell development at the expense of increased myeloid and NK fate [375, 376]. Pu.1 and GATA3 are other examples and are both required for early T-cell development. The ablation of their expression disturbs T-cell development, but their overexpression also inhibits the generation of T cells [93, 380]. Pu.1 regulates the expression of the cytokine receptors Flt3 and IL-7R in early prethymic stages and controls the commitment of progenitors toward the myelo-erythroid or lymphoid fate [381]. Once the cells reach the DN3 stage, enforced expression of Pu.1 dedifferentiates the committed T cells into cells with macrophage and DC phenotypes [382, 383]. GATA3 is a double zinc finger transcription factor that is expressed mainly in T cell progenitors. It is a direct Notch1 target, induced at the earliest stages of T cell precursors. It is expressed in ETP, DN2 and DN3 cells and its absence results in the complete loss of the T cell lineage [33, 384-386]. Studies have shown that GATA3 mainly regulates the differentiation, but not the survival or proliferation of early T-lineage progenitors [386].

T-lineage specific genes are responsible for the coordination of the main T-cell receptor initiated signaling pathways such as Ras/MAPK, PI3K and protein kinase C (PKC). This background fidelity is mainly coordinated by Notch1/CSL, GATA3, TCF-1, Ikaros, Runx factors, HEB, E2A and c-myb [387, 388]. However, other pathways such as bone morphogenetic proteins (BMP), Sonic hedgehog (SHH) and Wnt (wingless and integration site) are equally important and play crucial roles during early T-cell development [389-392]. Overall, in response to specific receptor stimulation, a complex network of transcriptional regulators mediates signaling pathways that control the stability of T-lineage commitment during the development and assures the production of T effector cells.

4.11. Pre-TCR rearrangement and double-stranded breaks

To produce a functional αβ TCR during T-cell development, thymocytes must rearrange the *TCR* genes. Each cell contains two copies of the *TCRβ* locus, one on each allele. The TCRβ chain is composed of V(D)J and constant (C) region exons. Although many exons encode for each segment, there are approximately 20 Vβ exons, and two exons of each Dβ, Jβ and Cβ [393, 394]. During the DN2/DN3 transition, V(D)J recombination occurs at the TCRβ locus mediated by enzymes encoded by the recombination activating genes *RAG-1* and *RAG-2* [395]. Mice deficient for either *RAG-1* or *RAG-2* lack mature B and T cells as both lineages are arrested during development since no V(D)J recombination occurs [396, 397]. The RAG proteins recognize recombination signal sequences (RSS) in the DNA that contain spacer sequences. These RSS are localized before and after the D and J regions and

at the 3' end of the V region coding exons. Only an RSS with a 12bp spacer sequence can pair with an RSS with a 23bp spacer, a rule known as the 12/23 rule [394, 395, 398, 399]. *Tdt* is another gene that takes part in the recombination process by adding random nucleotides to fill in the gap between the DJ and DJ to V regions. Finally, transcript processing through splicing removes internal introns producing a rearranged V(D)J-C *TCRβ* transcript [394, 400].

If errors occur during the recombination process leading to a non-productive allele, the rearrangement will continue on the second allele, using all available exons on the same chromosome [400, 401]. TCRβ chain rearrangement only takes place on one allele in order to express one polypeptide within a single cell. This process ensures the restriction of the rearrangement events and is referred to as the allelic exclusion [402]. Once a productive V(D)J recombination occurs, the TCRβ chain pairs with the pTα chain to form the pre-TCR that provides the proliferative and survival signals DN3 cells need for their further differentiation into DP cells [403].

V(D)J rearrangement at the DN3 stage generates DNA double-stranded breaks that activate the transformation related protein 53 (p53) in the course of a normal response to DNA damage. It has been proposed that p53 acts as a sensor for the β-selection checkpoint and evidence suggest that only thymocytes that express a functional pre-TCR are able to inactivate p53 in order to escape cell death as a consequence of DNA damage responses and further differentiate into DP cells [404]. In line with this hypothesis are data

demonstrating that ablation of p53 in *CD3γ*$^{-/-}$ [405], *RAG*$^{-/-}$ [406] or *SCID* mice [407-409], which all lack a functional pre-TCR, restores the generation of DN and DP cells. Most stress signals such as DNA damage response are regulated by checkpoint mechanisms [410, 411].

4.11.1. The p53 tumor suppressor protein

p53 (*Trp53*) is a tumor suppressor protein that orchestrate the transcriptional regulation of target genes in response to physiological or environmental stresses. The loss of p53 results in a strong predisposition to cancer in both humans and mice [412]. The activation of p53 usually leads to two outcomes: cell cycle arrest or apoptosis. In response to some stimuli, p53 will also induce DNA damage repair. This decision is mainly controlled by the differential activation or repression of p53 target genes [413]. On the one hand, a p53-dependent cell cycle arrest is induced by the up-regulation of the cyclin-dependent kinase (CDK) inhibitor *Cdkn1a*, coding for p21^{CIP1} protein, an inhibitor of the cell cyclin Ckd2 kinase. It is also mediated by the suppression of cell cycle regulatory genes such as *CDC25C*, coding for a phosphatase required for the activation of Cdk1 [414]. On the other hand, the apoptotic response mediated by p53 is coordinated by the up-regulation of pro-apoptotic genes such as *NOXA* (*Pmaip1*) and *PUMA* (*Bbc3*) (Figure 8). Moreover, the apoptotic activity of p53 involves other mechanisms that are not related to transcriptional regulation. These include the capacity of p53 to act as a cytoplasmic protein that can participate for example in the regulation of mitochondrial membrane permeabilisation [415]. Although the exact mechanism for this cytoplasmic function of p53 remains to be

fully elucidated, it has been shown that p53 can act as a pro-apoptotic BH3-domain expressing protein that leads to the release of cytochrome *c* from the mitochondria and to the induction of caspases and cell death [416].

4.11.2. DNA damage response

Replication of the DNA may lead to a stress signal that can activate checkpoint kinase 1 (Chk1), which promotes cell survival by blocking the start of the replication origins. This prevents the cell from entering mitosis, stabilizes stalled replication forks and facilitates DNA repair [417, 418]. Stalling of DNA replication, for example upon UV irradiation, leads to the accumulation of stretches of single-stranded DNA. The single-stranded DNA areas are coated with replication protein A (RPA) [419, 420], which in turn signals the recruitment of two checkpoint complexes, the ataxia-telangiectasia-mutated (Atm)-Rad3-related kinase-Atr-interacting protein (ATR-ATRIP) complex [421] and the Rad9-Hus1-Rad1 (9-1-1) complex [420]. Both complexes are essential for optimal ATR-mediated Chk1 phosphorylation and activation [422]. Rad9 does not participate in clamp formation, but binds to Topoisomerase II binding protein 1 (TopBP1). TopBP1 was found to be required for loading of Cdc45 to the origins of replication [423] and for the activation of Atr kinase in response to stalling of the replicative DNA polymerases δ and ε [424, 425] (Figure 8).

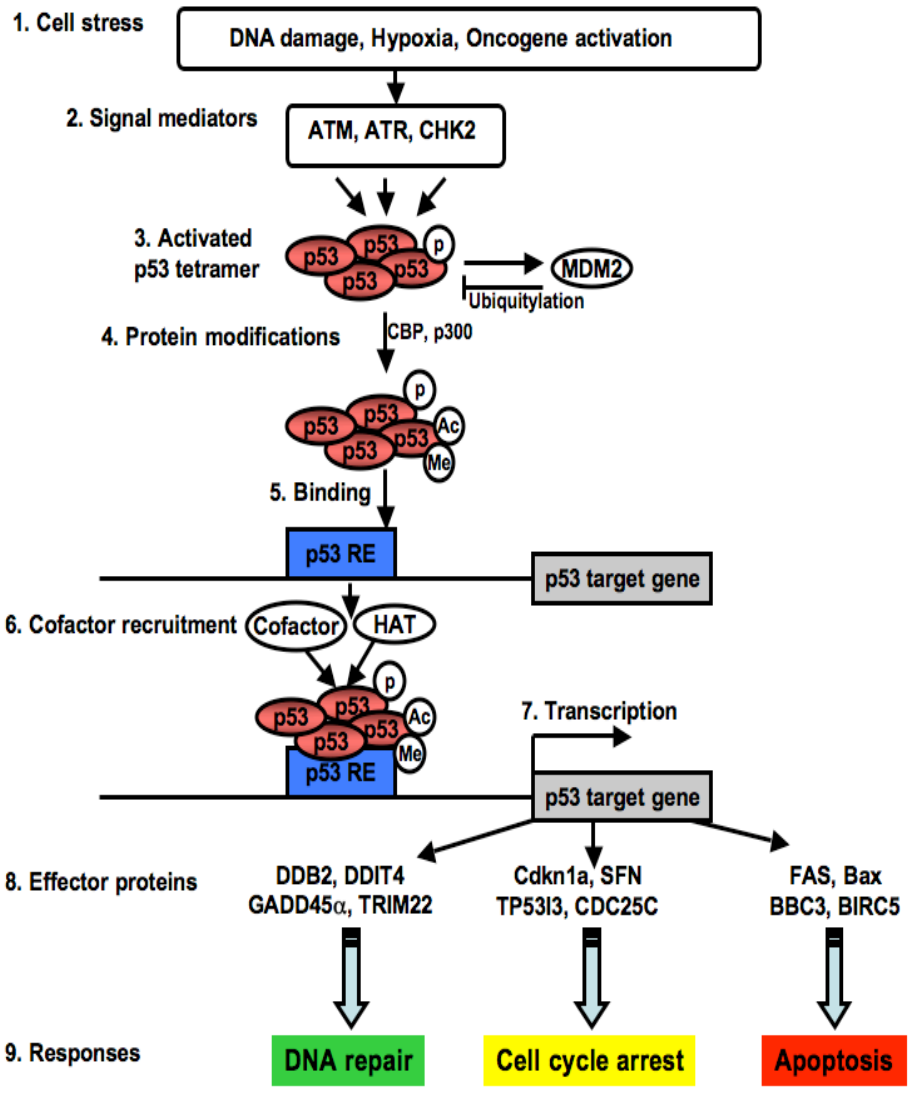

Figure 8. Schematic representation of p53 activation and regulation of its downstream target genes. Each signal in this cascade is represented by a number starting by the stimulus (1) to the response (9). p53 can bind to p53 response elements (RE) to regulate the transcription of nearby genes or to recruit co-factors such as histone acetyltransferases (HAT). p53 can mediate transactivation but also transcriptional repression. ATM, ataxia telangiectasia mutated; Bax, Bcl-2-associated X protein; BBC3, Bcl-2 binding component-3 (PUMA); BIRC5, survivin, Cdkn1a, cyclin-dependent kinase inhibitor-1A; CHK2, checkpoint kinase-2; DDB2, damage-specific DNA-binding protein-2; DDIT4, DNA-damage-inducible transcript-4; FAS, TNF receptor superfamily, member 6; GADD45α, growth arrest and DNA-damage inducible α; MDM2, Double minute 2 protein or E3 ubiquitin-protein ligase; SFN, stratifin; TP53I3, tumor protein p53-inducible protein-3; TRIM22, tripartite motif containing-22 (adapted from [426]).

4.11.3. Apoptosis versus cell cycle arrest

Several factors can influence the regulation of p53-induced target genes. In fact, p53 protein itself can integrate different signals which may favor apoptosis over cell cycle arrest or vice-versa [414]. For example, the phosphorylation of specific serine residues on p53 can contribute to the activation of pro-apoptotic genes and tumor suppression [427]. Other proteins may participate in the outcome of the p53 response. For instance, ASPP proteins, which bind to p53 and are homologous to 53BP2 (p53-binding protein 2), shift the transcriptional regulation toward induction of pro-apoptotic genes while inhibiting cell cycle regulators [428]. In addition, other transcription factors may also regulate specific p53 target genes. For example, p53 can cooperate with NF-κB to induce the expression of the death receptor DR5. DR5 is an apoptotic target that is under the regulation of a promoter containing both p53- and NF-κB-binding sites [429]. Similarly, the SLUG repressor protein, an inhibitor of PUMA, can be induced by p53 and in turn suppresses p53-dependent up-regulation of PUMA in hematopoietic cells [430]. Another example of the influence of p53 response to DNA damage can be demonstrated when c-Myc (myelocytomatosis viral oncogene) protein is deregulated. This deregulation represses p53-mediated induction of $p21^{CIP1}$ through binding to another transcription factor, Miz-1, thereby favoring apoptosis in response to p53 activation [137].

5. The Myc family of oncoproteins

The Myc protein family is composed of several nuclear phospho-proteins expressed in all proliferating cells. They play primordial roles in regulating cell growth, differentiation and proliferation [431]. These proteins are also involved in DNA damage response pathways and in malignant transformations [432-434]. The three main members of this family, c-, N-, and L-Myc, are paradigmatic examples of oncogenic transcription factors that exert their biological functions through the regulation of large sets of target genes in immune and hematopoietic cells. The structural composition of all Myc family members is conserved and contains a transactivating domain (TAD) at the amino terminal end and a basic region followed by a basic region and a helix-loop-helix and leucine-zipper motif (b-HLH-LZ) at the carboxy terminal end of the protein [431-434].

5.1 The role of c-Myc during lymphocyte development

In lymphoid cells, the expression of c-Myc is up-regulated by various mitogens such as concanavalin A (ConA) or lipopolysaccharide (LPS) [435-437] and by B- and T-cell receptors or by cytokine receptors like IL-2Rβ and the common γ chain. This latter chain is shared by the interleukin receptors IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [438, 439]. In response to these stimulations, c-Myc participates in the regulation of cell size [438, 440]. c-Myc also controls the development of CD8αα intestinal intraepithelial lymphocytes by regulating IL-15-dependent survival signals [441]. As for αβ-T-cell development, two different studies using *c-Myc*-deficient mice have demonstrated that c-Myc function is

required to ensure pre-T cell receptor-induced proliferation and expansion of both DN3 and DN4 thymic subpopulations [442, 443]. Although the block in the transition from DN to DP is observed in mice carrying a pre-T cell specific deletion of *c-Myc*, this effect does not cause a strong reduction in the overall thymic cellularity [442]. Finally, constitutive c-Myc expression is implicated in the induction of lymphomagenesis both in B- and T-cell compartments [444-452].

5.2. c-Myc and malignant transformation

The deregulation of c-Myc expression has been involved in many types of cancers, in particular lymphomas [444-453]. The process of malignant transformation is often linked to the activation of c-Myc expression either by gene amplification or chromosomal alterations or by the action of c-Myc in cooperation with other activated oncogenes, such as HA-RAS [432, 433].

c-Myc plays a particularly important role in the initiation of B- and T-cell lymphomas [434]. The paradigmatic example for the implication of c-Myc in a lymphoid malignancy is the B-cell Burkitt-type B-cell lymphoma, where c-Myc deregulation occurs as a consequence of the t(8;14) translocation juxtaposing the *c-Myc* gene to the vicinity of the immunoglobulin heavy chain enhancer (Eμ). This process can be mimicked in transgenic mice expressing a *c-Myc* gene under the control of the Eμ enhancer [444]. These mice are called *Eμ*-Myc and develop B-Acute Lymphoblastic Leukemia (B-ALL), which is similar to the Burkitt type lymphoma found in humans, characterized by immature B cells lacking

surface immunoglobulin [444, 454]. c-Myc also plays an important role in the development of T-cell lymphoma, in particular in T-cell Acute Lymphoblastic Leukemia. T-ALL develops as a consequence of hyperactivation of the Notch signaling or mutations in the *Notch* genes [455, 456], [457, 458] and c-Myc has been identified as a direct *Notch1* target gene [458]. Moreover, Notch and c-Myc expressions are induced in leukemia cells, as well as genes regulating cell cycle progression, protein biosynthesis and metabolism, that activate leukemic cell growth [459]. However, the precise implication of c-Myc in Notch-induced T-ALL has been recently debated, since Notch1 alone seems to rather be the dominant oncogene in T-ALL [460]. A t(8;14)(q24;q11) translocation also occurs in T cells where the gene encoding for the T cell receptor α chain becomes relocated to the vicinity of *c-Myc* gene [461, 462]. This translocation in T-cell leukemia is similar to the Burkitt lymphoma and, as a consequence, Myc does not respond normally and is inappropriately transcribed [463-465].

6. Miz-1 and c-Myc as transcriptional regulators

The protein c-Myc is a central regulator of cellular functions. The b-HLH-LZ domain of c-Myc is responsible for DNA binding and heterodimerization of c-Myc with its interacting partners such as the protein Max. Max, which also possesses a b-HLH-LZ domain, interacts with the b-HLH-LZ of c-Myc [433]. c-Myc can both transactivate and transrepress target genes. The molecular mechanism underlying c-Myc's transactivation abilities is well described and involves the c-Myc/Max complex, which binds to specific cognate sequences

called E-boxes “CACGTG” in upstream promoter regions of target genes [466]. For example, c-Myc can regulate factors involved in protein and ribosomal protein synthesis or cell cycle regulators such as the E2F family or Cyclins D and E [467]. However, the mechanisms by which c-Myc represses transcription are less well understood. Recent studies have shown that repression by c-Myc is mediated by a complex formed by the c-Myc/Max heterodimer with the transcription factor Miz-1(*Zbtb17*) [431, 468, 469].

6.1 Biochemical structure of Miz-1

Miz-1 is composed of a BTB/POZ domain in the N-terminal part of the protein and thirteen zinc-finger domains at its C-terminus. BTB/POZ is an abbreviation for Broad-complex, Tramtrack and Bric-à-brac/Pox virus zinc finger domain. It is a conserved evolutionary motif found in approximately 40 zinc-finger transcription factors, among them several POZ domain factors with oncogenic properties implicated in cancer [470, 471]. It has been shown that the POZ domain mediates protein-protein interactions via homo- or hetero-dimerization with other POZ domain-expressing proteins [470, 472, 473]. The recently characterized crystal structure of the Miz-1 POZ domain revealed two types of interfaces that mediate dimerization and tetramerization of this domain in solution [474]. In addition, proteins containing the BTB/POZ domain recruit histone-modifying enzymes, such as histone deacetylase or co-repressor complexes including N-CoR and SMRT to the promoter of target genes, thus enabling histone modifications. This results in the repression of transcription [471, 474-477]. The BTB/POZ domain is most likely not directly involved in any interaction of Miz-1 with DNA. Nevertheless, Miz-1 mutants that lack the BTB/POZ

domain are no longer able to stably bind the chromatin and lose their ability to regulate transcription of some target genes [478].

The zinc-finger domains contained in the C-terminus of Miz-1 are most likely implicated in the binding of the protein to DNA or for interaction with other co-factors [469]. For example, the interaction of Miz-1 with c-Myc has been located between the 12th and 13th zinc finger in the C-terminus of Miz-1, and the C-terminus helix II of the basic helix-loop-helix motif of c-Myc [135, 469] (Figure 9). A c-Myc mutant carrying an aspartic acid instead of a valine at position 394 in the helix II of its helix-loop-helix motif (c-Myc^{V394D}) leads to the abrogation of c-Myc binding to Miz-1 [135, 479-481]. This mutation also validates the mapped interacting domain on the c-Myc side to Miz-1 [469].

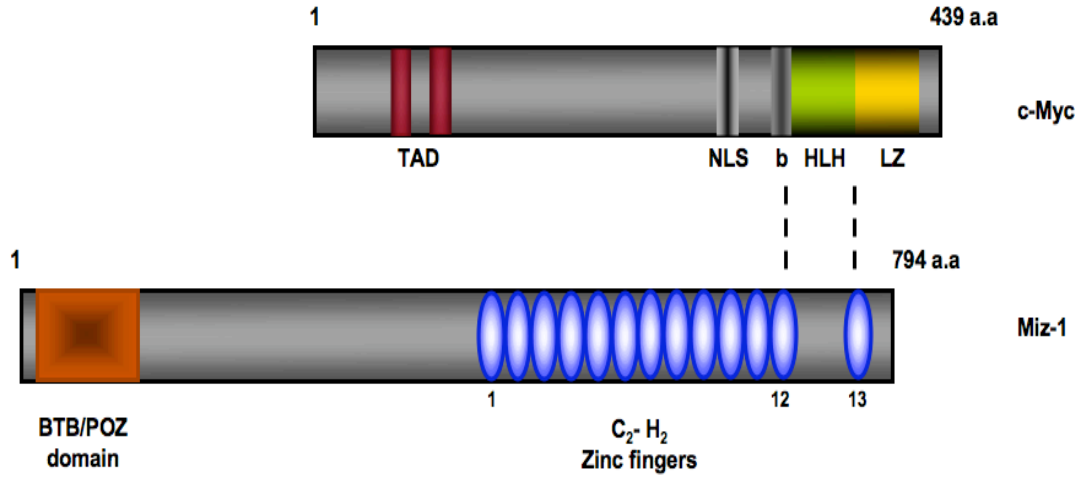

Figure 9. Schematic representation of c-Myc and Miz-1.
Upper part: c-Myc protein is composed of 439 amino acid (a.a). TAD, transactivating domain, b, basic region, NLS, nuclear localization signal, HLH, helix-loop-helix domain, LZ, leucine zipper domain. The dashed lines indicate the regions mediating the interaction between c-Myc and Miz-1.

Lower part: The Miz-1 protein is composed of 794 a.a. BTB/POZ, Broad-complex, Tramtrack and Bric-à-brac/Pox virus zinc finger domain, C_2-H_2, zinc fingers (adapted from [431, 434, 469, 471]).

6.2 Miz-1 and its functions

Miz-1 is a transcriptional transactivator or transrepressor and the best documented activation potential of Miz-1 is exemplified by its regulation of the expression of two genes encoding for the cyclin-dependent kinase inhibitors *Cdkn2b* ($p15^{INK4B}$) and *Cdkn1a* ($p21^{CIP1}$) [135-138]. Cyclin-dependent kinase inhibitors are negative regulators of cyclin and cyclin-dependent kinase (CDK) complexes which are grouped into two families: Ink4 and Cip/Kip proteins [482, 483]. The Ink4 ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p16^{INK4d}$) proteins block the G1 to S transition of the cell cycle, while the Cip/Kip proteins ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) bind to the entire cyclin/CDK holoenzymes, inhibiting transitions at all stages of the cell cycle.

Among the cyclin-dependent kinase inhibitors, $p21^{CIP1}$ plays a predominant role in both cell cycle and apoptosis by promoting cell cycle arrest at the G1 phase, inhibiting proliferation, influencing apoptotic players like p53 and contributing to cellular senescence [483-486]. Despite all these implications and the wide range of cells that express $p21^{CIP1}$ during their development and activation [483], *Cdkn1a*-deficient mice develop normally [487]. Nevertheless, these mice are defective in the G1 checkpoint control of their cell cycle [487]. $p21^{-/-}$ mice also exhibit enhanced primary antibody response, most likely caused by the accelerated proliferation of helper T cells [488]. Another study has reported that the homozygous deletion of *Cdkn1a* reduced cellular and histological diseases and increased survival of lupus-prone mice [488]. This resistance to autoimmunity seems related to an

increased susceptibility of activated/memory B and T cells to activation-induced cell death (AICD) [488]. The deletion of another cyclin-dependent kinase inhibitor locus, the *Cdkn2b-Cdkn2a* locus, is often associated with human cancers which underscore the importance of the proper regulation of these cell cycle inhibitors. This locus encodes for three cell cycle inhibitory proteins: *Cdkn2b*, *Cdkn2a* ($p16^{INK4a}$) and $p19^{ARF}$ another protein encoded by an alternative reading frame of *Cdkn2a* [489].

Miz-1 activates the transcription of its target genes by binding to the initiator (Inr) sites in the promoter and by recruiting activating co-factors such as the histone acetyl transferase p300/CBP or the ribosomal protein L23-nucleophosmin [135, 138, 469, 478]. The c-Myc/Max heterodimer is able to bind to Miz-1 and to silence $p15^{INK4B}$ and $p21^{CIP1}$ expression very likely by competing and displacing critical co-factors such as p300/CBP from Miz-1 [469]. This c-Myc mediated repression of gene expression is independent of E-boxes. As a result, cell fate decisions that depend on proliferative steps, for instance progression through the G1/S cell cycle, are altered by c-Myc. With this new discovery of Miz-1 it became clear that c-Myc can exert two pathways to regulate transcription, one E-box-dependent and another E-box-independent (Figure 10). In addition to its functions as a transcriptional regulator of cell cycle inhibitors, Miz-1 seems to be involved in other cell physiological processes than transcription. The initial discovery of Miz-1 as a c-Myc interacting partner showed that Miz-1 can be localized in both the cytoplasm and the

nucleus, indicating that the shuttling between the nucleus and the cytoplasm of Miz-1 is c-Myc-dependent [469].

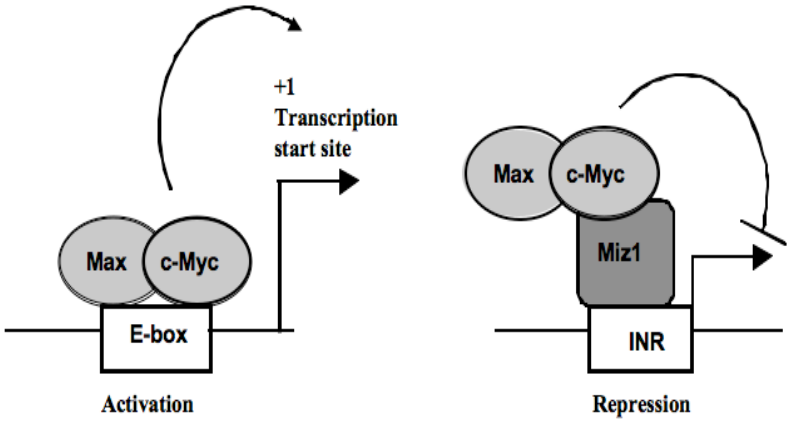

Figure 10. Schematic representation of the two c-Myc-dependent regulatory pathways.
Left: the Miz-1-independent pathway that involves the binding of the c-Myc/Max complex to E-box-dependent sites on target gene promoters, which leads to transcriptional activation by c-Myc.

Right: the Miz-1-dependent pathway: a trimeric c-Myc/Max/Miz-1 complex binds to initiator sequences (INR) located on target gene promoters. This E-box-independent binding leads to a repressory transcriptional regulation by c-Myc (adapted from [468, 469, 490]).

6.3 Miz-1 and its binding partners in cell regulation

The ability of Miz-1 to function as an activator or repressor of target gene transcription depends on its interacting partners. For example, in a complex with L-23 nucleophosmin or p300/CBP, Miz-1 stimulates the transcription of genes encoding for cell cycle regulators such as $p21^{CIP1}$ or $p15^{INK4B}$ or inhibitors of apoptosis such as Bcl-2 [135, 138, 469, 478, 480,

491]. In contrast, Miz-1 becomes a repressor of transcription when it binds to c-Myc or Bcl-6, which then enable the repression of $p21^{CIP1}$, $p15^{INK4B}$ or other target genes [469, 492].

6.3.1. The regulation of $p15^{INK4B}$ by Miz-1

Transforming growth factor beta (TGFβ) can arrest the division of epithelial cell by inhibiting c-Myc and turning on $p15^{INK4B}$ through a mechanism that involves Smad transcription factors. Studies suggested that Miz-1 is able to transactivate *Cdkn2b* which can be regulated by TGFβ. The Smad protein complexes containing Smad3 and Smad4 were shown to interact with Miz-1 and regulate its transcriptional activity [136]. The binding of Miz-1 to the transcriptional initiator of *Cdkn2b* prevents the recruitment of c-Myc which relieves the repression and enables the transcriptional activation of TGFβ downstream target genes [136]. Conversely, in mouse embryonic fibroblasts (MEF), c-Myc and Max can form a complex with Miz-1 at the initiator site of *Cdkn2b* promoter and inhibit its transcriptional activation by Miz-1. This activity of c-Myc inhibits the accumulation of $p15^{INK4B}$ that is associated with cellular senescence [138]. The SNAG domain repressor Gfi1 was also shown to associate with Miz-1 in a similar way as c-Myc. The Miz-1/Gfi1 complex seems to also repress *Cdkn2b* transcription [139], although additional experiments are needed to validate the formation of this complex in vivo. Another study presented finding that c-Myc, but not a mutant form of c-Myc ($c\text{-}Myc^{V394D}$) that no longer interacts with Miz-1 [135], could repress *Cdkn2b* in primary murine keratinocytes [479]. The conditional deletion of Miz-1 in keratinocytes also showed that Miz-1 is essential for controlling hair cell cycling and hair morphogenesis [493].

6.3.2. Regulation of $p21^{CIP1}$ expression by BTB/POZ domain transcription factors

Gfi1 was also shown to bind through Miz-1 to the *Cdkn1a* promoter, thereby influencing the outcome of its transcriptional regulation [134]. Although the formation of this complex needs to be more carefully studied, this work has elucidated a mechanism by which Gfi1 can control cell proliferation in response to TGFβ stimulation [134]. It has also been shown that the binding of Miz-1 to the proto-oncogene and BTB/POZ transcription factor Bcl-6 suppresses *Cdkn1a* transcription. This regulation was demonstrated to be important to suppress p53-independent cell cycle arrest in germinal center B cells and therefore favor the proliferative expansion of germinal centers during an immune response [492]. A deregulation of such a Miz-1-dependent mechanism may contribute to the aberrant expansion of B cell and eventually to B cell lymphoma. A different study demonstrated that Bcl-6 can suppress Bcl-2-induced activation via its interaction with Miz-1. This function of Bcl-6 is required to facilitate apoptosis in germinal center B cells via the suppression of Bcl-2. It also suggests that blocking this regulation may be critical for lymphomagenesis, in particular the pathogenesis of germinal center-derived diffuse large B cell lymphoma (DLBCL) and follicular lymphoma [494].

Miz-1 heterodimerizes with another BTB/POZ transcriptional repressor factor Zbtb4 to repress *Cdkn1a* transcription in neurons. This Miz-1/Zbtb4 complex recruits histone deacetylases to the promoter of *Cdkn1a* through the interaction with the Sin3 adaptor protein [495]. This repression of $p21^{CIP1}$ was shown to inhibit cell cycle arrest in response to p53 activation. These studies exemplify the many ways in which Miz-1 can exert its role

as a transcriptional regulator and also show that Miz-1 assumes different roles in response to a DNA damage signal ranging from the initiation of apoptosis to cell cycle arrest [137, 481] (Figure 11).

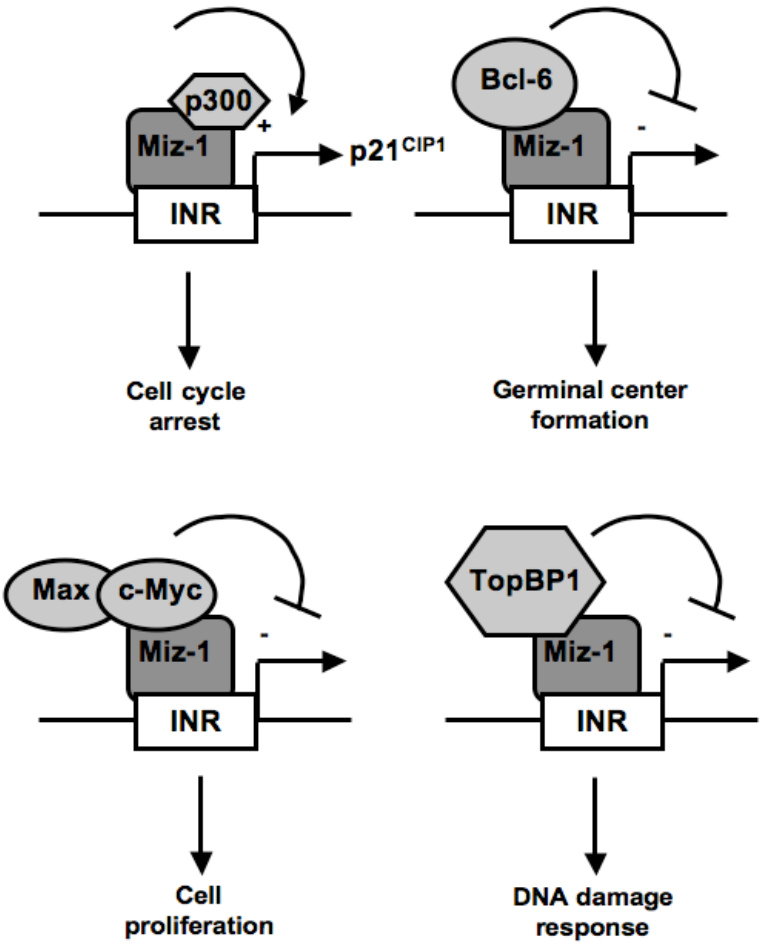

Figure 11. Examples of positive and negative regulation by Miz-1 on *p21^{CIP1}* transcription. Miz-1 associates with different partners at the initiator region (INR) of the *p21^{CIP1}* promoter. The Miz-1/p300 complex activates *p21^{CIP1}* transcription, whereas the association of Miz-1 with Bcl-6, c-Myc/Max or TopBP1 represses its transcription (adapted from [135, 468, 492, 496]).

6.3.3. Miz-1 is involved in regulating the DNA damage response

The association of TopBP1 with Miz-1 was shown to be critical for the regulation of proliferation in mammalian UV irradiated cells. This study showed that, through its binding to Miz-1, c-Myc negatively regulated the transcription of *Cdkn1a* upon UV irradiation to facilitate the recovery of cells from UV-induced cell cycle arrest [135]. Another study also demonstrated that a complex formed by the interaction of Miz-1 and E2F1 with TopBP1 led to transcriptional repression. The oligomerization of TopBP1 is induced by the PI3K-AKT signaling pathway that represses Miz-1 transcriptional activation. This interaction is important for the specific control of E2F1-induced apoptosis. [497]. These two studies illustrate that the interaction of TopBP1 with Miz-1 can induce or repress *Cdkn1a* transcription depending on the cell type, signaling pathway involved and the presence of interacting partners such as E2F1 or c-Myc.

The dissociation of TopBP1 from Miz-1 upon UV irradiation facilitates the induction of $p21^{CIP1}$ [135]. This is consistent with the fact that the deletion of TopBP1 led to p53-independent transcriptional activation of $p21^{CIP1}$ and to the inhibition of cyclin E/CDK2 [498]. To further characterize the implication of Miz-1 during UV irradiation response, Herold and colleagues demonstrated that TopBP1, an essential activator of the ATR kinase, requires Miz-1 for its recruitment to the chromatin [499]. The recruitment of Miz-1/TopBP1 complex protects TopBP1 from proteasomal degradation and is required for ATR-dependent signal transduction. TopBP1 that is not bound to the chromatin gets

degraded by ubiquitin ligase HectH9 [500, 501]. These observations are in accordance with earlier studies showing that Miz-1 inhibits HectH9-dependent ubiquitination, an important regulation for controlling tumor cell proliferation [500]. The activity of the ATR checkpoint was shown to be inhibited by c-Myc which antagonizes the binding of TopBP1 to Miz-1, resulting in the dissociation of TopBP1 from the chromatin and reducing the amount of TopBP1 available [499]. Taken together, these studies revealed that the levels of c-Myc and Miz-1 affect the function of the ATR-dependent DNA checkpoint through the regulation of TopBP1 stability.

6.3.4. Miz-1 controls cell survival

In addition to its function as a transcriptional activator or repressor of negative cell cycle regulators, Miz-1 can control cell survival by preventing apoptosis. Two independent studies have demonstrated that Miz-1 is required to up-regulate the anti-apoptotic protein Bcl-2. The c-Myc/Miz-1 complex is needed for the inhibition of this activation and the induction of apoptosis [480, 491]. Another study has established the implication of Miz-1 in controlling the p53-$p19^{ARF}$-MDM2 axis. Miz-1 can interact with both the cyclin-dependent kinase inhibitor $p19^{ARF}$ and p53. According to these findings, Miz-1 can directly interact with the DNA-binding domain of p53 and thus prevents it from activating pro-apoptotic target genes. If $p19^{ARF}$ is expressed in excess amounts, it binds to Miz-1 and therefore liberates p53 from the Miz-1/p53 complex. This enables p53 to transactivate its target genes and induce apoptotic responses [502].

All these studies helped establish Miz-1 as an important transcriptional regulator of cell cycle progression and apoptosis, but did not reveal many implications of Miz-1 during development. Miz-1 is ubiquitously expressed in all tissues and the first indication that Miz-1 is critical for regulating developmental processes was demonstrated by Adhikary and her colleagues. This study showed that the constitutive deletion of *Miz-1* led to an early block of gestation at day E7.5 [503].

7. Hypotheses and objectives

c-Myc plays important roles in hematopoietic differentiation and in the emergence of lymphoma and other blood cancers. It is therefore relevant to characterize the second E-box-independent pathway in which c-Myc regulates gene expression through Miz-1. Miz-1 itself can control the expression of genes involved in proliferation, and c-Myc represses at least a subset of these genes by binding to Miz-1. The identity of most of these genes and their function in c-Myc lymphocyte biology are unknown.

The thesis work presented here aimed to define the role of Miz-1 and the c-Myc/Miz-1 complex in the regulation of T- and B-cell survival, commitment and differentiation. As c-Myc has been shown to play important roles in the development of progenitor cells and T cells, we hypothesized that its interacting partner Miz-1 also had implications in precursor commitment and lymphoid development processes. The characterization of these two transcription factors also helped identify the interplay between Miz-1 and c-Myc at the major developmental checkpoint during B- and T-cell development, mainly at the cytokine receptor- and TCR- or BCR-dependent selection points. To reach these aims, different mouse models have been used for experimentation throughout this work.

8. Mouse models used for this project

The targeted disruption of *c-Myc* results in lethality before 10.5 days of gestation in homozygous mice [504]. Similarly, the complete deletion of the *Miz-1* gene is lethal at embryonic day E7.5 [503]. This early time point of lethality emphasized the importance of Myc-interacting zinc finger protein Miz-1 in embryonal development and demonstrated that Miz-1-deficiency arrested the development at the gastrulation stage [503]. These knock-out models represented limitations and could not support a full study of the implication of these transcription factors in hematopoiesis or lymphocyte development. Previous studies had shown that the implication of c-Myc in T-cell development could only be elucidated using conditional and cell type specific deletion of the gene [442, 443]. Therefore, we decided to address the importance of its interacting partner Miz-1 or the c-Myc/Miz-1 complex in lymphocyte development by conditional deletion of Miz-1 alleles.

Mice carrying loxP (floxed, fl) site flanking the coding region for the BTB/POZ domain of Miz-1 were generated in our laboratory. Using Cre-mediated recombination, it was possible to conditionally induce the deletion of Miz-1 BTB/POZ domain (Miz-$1^{\Delta POZ}$) in embryonic stem cells or in a cell specific manner in vivo. Since previous studies have shown that the deletion of the BTB/POZ domain of Miz-1 generated a truncated protein that was non-functional as a transcription factor [138, 478], the deletion of this domain was chosen to generate *Miz-1*-deficient mice. To study the role of Miz-1 in T- and B-cell development, conditional Miz-$1^{fl/fl}$ mice were crossed with several Cre-transgenic mice strains. A *Vav*-cre

transgene was used to enable the deletion of the BTB/POZ domain encoding exons in all hematopoietic cells [505]. *CD19*-cre [506] and *Lck*-cre [507] transgenic mice were used for the deletion of the domain in B and T cells, respectively.

To study the role of the c-Myc/Miz-1 complex in lymphocyte development, c-Myc^{V394D} knock-in mice were also generated. In these mice, a point mutation was introduced via homologous recombination into the c-Myc locus that altered the coding sequence in a way that the valine residue (V) at position 394 was replaced by an aspartic acid (D) (c-Myc^{V394D} knock-in mice). This mutation has been shown to inhibit the ability of c-Myc to bind to Miz-1 [135, 479-481]. Other mouse strains used in this study include the *VHT*-knock-in [508], *H2K-Bcl-2* transgenic (Tg) [206, 509], OTI transgenic [510] and *p21^{CIP1}*-deficient mice [487].

Results

Chapter I

Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation

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Running title: Miz-1 regulates IL-7 mediated B-cell development

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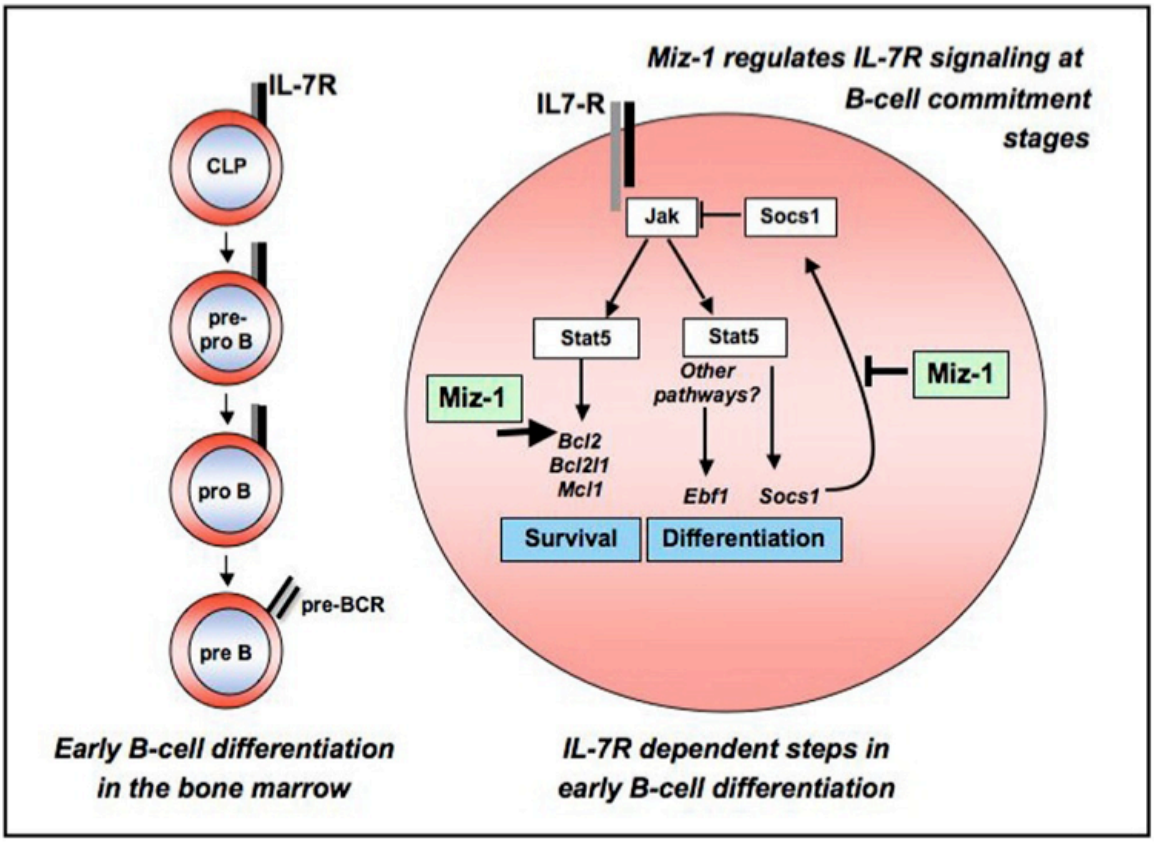
For this study, I actively participated in the characterization of Miz-1 requirement as a downstream effector of the IL-7/IL-7R signaling in B cells. I helped design the research and did the following experiments: i) flow cytometry analysis of phospho-Stat5 in Lin^- $CD19^-B220^+$ (Figure 3G) or in $IL\text{-}7R^+$ bone marrow cells (Figure 3H) from control or Vav-cre *Zbtb17*$^{fl/fl}$ mice, ii) chromatin immunoprecipitation (ChIP) showing binding of Miz-1 to the promoter region of *SOCS1* in MIG-*Zbtb17*-GFP transduced 70Z/3 cells, 70Z/3 cells and primary B cells ($B220^+$ cells) (Figure 4D), iii) immunoprecipitation of the Miz-1 protein in 70Z/3 cells (Figure S4), and iv) ChIP showing the binding of Miz-1 to the promoter region of *Bcl-2* in MIG-*Zbtb17*-GFP transduced 70Z/3 cells (Figure 5B). Christian Kosan designed the research, generated all the mice and cell lines, performed experiments, analyzed data, generated the figures, and wrote the manuscript. I also participated in writing the manuscript. Maren Godmann, Stefanie Herold and Barbara Herkert performed experiments. Tarik Möröy designed and supervised the research, wrote the manuscript, and provided funding.

Abstract

B-cell development requires the coordinated action of transcription factors and cytokines, in particular interleukin-7 (IL-7). We report that mice lacking the POZ (Poxvirus and zinc finger) domain of the transcription factor Miz-1 (*Zbtb17*$^{\Delta POZ/\Delta POZ}$) almost entirely lacked follicular B cells, as shown by the fact that their progenitors failed to activate the Jak-Stat5 pathway and to up-regulate the antiapoptotic gene *Bcl-2* upon IL-7 stimulation. We show that Miz-1 exerted a dual role in the interleukin-7 receptor (IL-7R) pathway by directly repressing the Janus kinase (Jak) inhibitor suppressor of cytokine signaling 1 (*Socs1*) and by activating *Bcl-2* expression. *Zbtb17*$^{\Delta POZ/\Delta POZ}$ (Miz-1-deficient) B cell progenitors had low expression of early B cell genes as transcription factor 3 (*Tcf3*) and early B cell factor 1 (*Ebf1*) and showed a propensity for apoptosis. Only the combined re-expression of *Bcl-2* and *Ebf1* could reconstitute the ability of Miz-1-deficient precursors to develop into CD19^{+} B cells.

Graphical Abstract



Highlights

► Miz-1 is required for early B-cell development

► Miz-1 directly represses the Jak (Janus kinase) inhibitor *Socs1*

► Miz-1 is required to up-regulate Bcl-2 upon IL-7 stimulation

Introduction

The development of mature functional B cells capable of producing specific antibodies is a highly regulated, multistep process that is initiated in the bone marrow, where early B cell differentiation takes place to yield immature immunoglobulin-M $(IgM)^+$ B cells. These immature cells can leave the bone marrow and migrate to the peripheral lymphoid organs, where they complete their maturation. The earliest step on the way to become a B cell is the generation of multipotent progenitors (MPPs) from hematopoietic stem cells (HSCs), which have the potential to develop into multiple hematopoietic lineages (Christensen and Weissman, 2001 and Morrison and Weissman, 1994). A subset of MPPs, which expresses the tyrosine kinase receptor Flt3, loses its ability to differentiate into the erythro-megakaryocytic lineage but retains myeloid and lymphoid potential. When these lymphoid-primed multipotent progenitors (LMPPs) up-regulate Flt3 (FMS-like tyrosine kinase 3) expression, they lose their myeloid potential and become restricted to the lymphoid lineage (Adolfsson *et al*., 2005 and Månsson *et al*., 2007). The LMPP population contains the early lymphoid progenitors (ELPs) (Igarashi *et al*., 2002), which give rise to the common lymphoid progenitors (CLPs) and, most probably, to the early T lineage progenitors (ETPs). CLPs can give rise to T cells, natural killer cells (NK cells), and dendritic cells, but in vivo are considered the sole progenitors of NK and B cells (Allman *et al*., 2003 and Kondo *et al*., 1997b). CLPs retain expression of Flt3 and express the receptor for IL-7 (IL-7R), which are both critical for B-cell development. Mice deficient for both receptors do not develop any B cells throughout fetal and adult life (Sitnicka *et al*., 2003).

Cytokine signaling and in particular IL-7 is indispensable for the adult B-cell development in the mouse bone marrow (Miller *et al*., 2002 and Namen *et al*., 1988). In CLPs, IL-7 signaling induces the expression of the early B cell factor 1 (*Ebf1*) (Kikuchi *et al*., 2005), which regulates the expression of the transcription factor *Pax5* (paired box gene 5) (O'Riordan and Grosschedl, 1999). In contrast, Malin and colleagues recently showed that Ebf1 regulation is independent of IL-7 signaling and suggest a role of IL-7 signaling solely in preventing apoptosis, as it has been shown for T cells (Malin *et al*., 2010). Consistent with this, it has been shown that the expression of Ebf1 is independent of IL-7 signaling during fetal B-cell development (Kikuchi and Kondo, 2006) and that $Il7^{-/-}$ mice still have a few B cells, which may however originate from a fetal or neonatal precursor (Carvalho *et al*., 2001). Also, overexpression of Ebf1 in $Il7r^{-/-}$ mice only partially rescues early B-cell development (Kikuchi *et al*., 2005), suggesting that other factors, induced by IL-7 signaling, must exist to support B-cell development such as E2A (*Tcf3*), Ebf1, and Pax5 that are required for expression recombination-activating genes 1 and 2 (*Rag1*, *Rag2*) and for a proper rearrangement of the immunoglobulin heavy chain locus (IgH) (Lin and Grosschedl, 1995, Nutt *et al*., 1997 and Urbánek *et al*., 1994).

The Miz-1 (*Zbtb17*) gene encodes a 87 kDa protein with 13 C_2H_2 zinc finger domains at the carboxy terminal half and a POZ domain at its N terminus (Peukert *et al*., 1997). Miz-1 belongs to the group of POZ domain zinc finger transcription factors that can act as both transcriptional activators and repressors. The POZ domain is required for multimerization and for the interaction with other cofactors that mediate their function (Staller *et al*., 2001).

Miz-1 binds to core promoters of RNA polymerase II-dependent target genes and recruits the histone acetyltransferase EP300 (E1A binding protein 300) and nucleophosmin (Herold *et al*., 2002, Staller *et al*., 2001 and Wanzel *et al*., 2008), which enables transcriptional activation of its targets such as the genes encoding the negative cell cycle regulators p15 (*Cdkn2b*) (Seoane *et al*., 2001 and Staller *et al*., 2001) or p21 (*Cdkn1a*) (Phan *et al*., 2005, Seoane *et al*., 2002 and Wu *et al*., 2003). Miz-1 can also form a complex with the bHLH (basic helix-loop-helix) leucine zipper transcription factor Myc, which displaces positive cofactors such as EP300 and nucleophosmin that leads to a repression of Miz-1 target genes (Herold *et al*., 2002, Seoane *et al*., 2001, Seoane *et al*., 2002, Staller *et al*., 2001 and Wanzel *et al*., 2008).

In this study we wished to elucidate the role of Miz-1 during hematopoiesis and have generated a loss-of-function mutant mouse by gene targeting introducing an allele that allowed the conditional deletion of the Miz-1 POZ domain. This strategy specifically disrupted the activity of Miz-1 as a transcriptional transregulator. Our studies demonstrated that Miz-1 is required for the very early steps of adult B-cell development in the bone marrow. Importantly, we found that the deletion of the Miz-1 POZ domain severely disrupted signaling events triggered by IL-7 that substantially affected B cell survival and differentiation through the regulation of early B cell genes and the antiapoptotic gene *Bcl-2*.

Results

Generation of Mice Carrying a Conditionally Deficient Allele of *Zbtb17*

The complete deletion of the *Zbtb17* gene leads to early embryonic lethality at E7.5 (Adhikary *et al*., 2003). To overcome this limitation, we generated mice carrying loxP (fl) sites flanking the region encoding the POZ domain to render it amenable to cre-mediated deletion. Analysis of protein extracts derived from $Zbtb17^{+/+}$, $Zbtb17^{+/\Delta POZ}$, or $Zbtb17^{\Delta POZ/\Delta POZ}$ embryonic fibroblasts (E12.5) confirmed that this targeting strategy yielded a truncated Miz-1 protein lacking the POZ domain (Figures S1A and S1B available online). Previous studies have shown that this truncated form is non-functional as a transcription factor (Staller *et al*., 2001 and Wanzel *et al*., 2008) and we could show that this form is unable to stably bind chromatin (Figure S1C). Adult $Zbtb17^{fl/fl}$ or $Zbtb17^{+/\Delta POZ}$ mice were indistinguishable from wild-type (WT) littermates and were used as control animals throughout this study, whereas animals with two deleted *Zbtb17* alleles ($Zbtb17^{\Delta POZ/\Delta POZ}$) arrested development at around E14 (Figure S1D). Because $Zbtb17^{+/\Delta POZ}$ mice are phenotypically indistinguishable from $Zbtb17^{fl/fl}$ or wild-type mice, a dominant-negative effect of the truncated Miz-1 protein could be excluded.

Miz-1 Is Required for Early B-cell development in Adult Mice

Zbtb17 is expressed in lymphoid cells notably in thymus, bone marrow, and spleen and in purified B and T cell populations (Figure S1E; data not shown). When $Zbtb17^{\Delta POZ/\Delta POZ}$ ES cells were used to generate chimeras, they did contribute to the formation of organs and

hematopoietic cells with the exception of T and B cells (Figure S1F), strongly suggesting an important role of Miz-1 in lymphoid development. Consistent with this, we observed a severe defect in B- and T-cell development when a *Vav-cre* transgene was present in the conditional *Zbtb17*$^{fl/fl}$ mice, which enabled the deletion of the POZ domain encoding exons in all hematopoietic cells as previously described (Figure S1G; de Boer *et al*., 2003).

In the spleen, *Vav-cre Zbtb17*$^{fl/fl}$ mice showed a reduction of the B cell population (B220^{+}) to 10% of control mice (Figure 1A). Within the residual B220^{+} cells of *Vav-cre Zbtb17*$^{fl/fl}$ mice, frequencies of mature B cells (IgM^{-}IgD^{+}) were reduced to half the values seen in wild-type mice (Figure 1B). In addition, follicular B cells (FO) were reduced in frequency (Figure 1B) and absolute numbers in *Vav-cre Zbtb17*$^{fl/fl}$ mice to about 4% of controls (Figure 1A), whereas marginal zone B cells (MZ) remained unaffected (Figures 1A and 1B). Similarly, in the bone marrow of *Vav-cre Zbtb17*$^{fl/fl}$ mice, cellular frequencies were strongly reduced and absolute numbers of B220^{+}CD19^{+} cells dropped to about 3% of controls (Figures 1C and 1D), indicating that Miz-1 deficiency leads to a severe reduction of the B cell compartment, particularly affecting follicular B cells.

B-cell development Is Arrested at the Pre-Pro-B to Pro-B Cell Transition in Miz-1-Deficient Animals

Hematopoietic progenitors including HSCs, MPPs, and LMPPs were present in *Vav-Zbtb17*$^{fl/fl}$ mice (Figure 2A). The frequencies and absolute numbers of CLPs (lin^{-} IL-7R^{+} AA4.1^{+}sca1^{med} c-kit^{med}) that are the precursors for the B cell lineage (Izon *et al*., 2001 and

Kondo *et al*., 1997b) as well as pre-pro-B cells ($lin^-B220^+CD43^+AA4.1^+CD19^-BP\text{-}1^-HSA^-$) that are contained within Fraction A ($B220^+CD43^+CD19^-BP\text{-}1^-HSA^-$) were not changed in *Vav-cre Zbtb17*$^{fl/fl}$ mice (Figures 2B–2F). However, the expression of AA4.1 on CLPs and pre-pro-B cells of *Vav-cre Zbtb17*$^{fl/fl}$ mice was severely reduced compared to control mice (Figures 2B and 2E), whereas IL-7R, c-kit, and Flt3 expression remained largely unchanged on pre-pro-B cells (Figure 2E). In addition, Fraction B ($B220^+CD43^+HSA^+BP\text{-}1^-CD19^+$), C, and C′ cells were almost absent in *Vav-cre Zbtb17*$^{fl/fl}$ mice (Figures 2C and 2F) and the proportion of $CD19^+$ cells in Fraction B was strongly reduced from 92.8% to 31.1% (Figure 2C). This suggested that Miz-1 is required for the generation of committed $CD19^+$ B cells; a phenotype that could be confirmed by adoptive transfer experiments of Miz-1-deficient bone marrow cells into syngeneic recipients (Figures 3A and 3B; Figures S2A–S2C). These experiments also showed that hematopoietic stem cells from *Vav-cre Zbtb17*$^{fl/fl}$ mice are able to home to the bone marrow and still have T lymphoid and myeloid potential (Figures S2A–S2C). However, although T-cell development was severely perturbed, myeloid development was not affected in the absence of functional Miz-1 (data not shown; Figures S2A and S2B). Conversely, transfer of control bone marrow cells into *Vav-cre Zbtb17*$^{fl/fl}$ mice resulted in a normal lymphoid and myeloid reconstitution, indicating that the observed disruption of B-cell development is a cell-autonomous phenotype (Figures S2D–S2F). Taken together, Miz-1 is required for the development of $CD19^+$ B cells and T cells but does not affect progenitor function and myeloid development.

Adult Miz-1-Deficient B Cell Progenitors Fail to Develop In Vitro in Response to IL-7

LSKs and CLPs from adult *Vav-cre Zbtb17*$^{fl/fl}$ were purified and cultured with IL-7 and Flt3L on OP9 cells. Under these conditions, Miz-1-deficient cells did not develop into B220^{+}CD19^{+} B cells and were lost from the cultures reproducibly, whereas progenitors from control animals developed efficiently into B cells (Figures 3C and 3E). Under myeloid conditions and in the absence of IL-7, both wild-type and Miz-1-deficient LSK cells developed into Mac-1^{+} cells (Figure 3D). To further analyze the response to IL-7, we cultured CLPs under stroma-free conditions, which have been shown to provide a survival and differentiation signal, but no proliferative stimulus (Miller *et al*., 2002). CLPs from control animals differentiated in the presence of IL-7 into B220^{+}CD19^{+} B cells, whereas Miz-1-deficient CLPs did not develop and were again lost from the culture (Figure 3F). Because DNA content analysis and BrdU (bromodeoxyuridine)-labeling experiments demonstrated that progression through the cell division cycle is not disturbed in Miz-1-deficient CLPs or pre-pro-B cells in vivo (Figure S3), these findings suggested that loss of Miz-1 function affects those elements of IL-7 signaling that control differentiation and survival but not proliferation.

A key event in IL-7 signaling is the phosphorylation of Stat5, which is mediated by Janus kinases (Jaks) upon IL-7 stimulation (Lin *et al*., 1995). We found that in Miz-1-deficient lin^{-}CD19^{-}B220^{+} or IL-7R^{+} bone marrow cells, Stat5 phosphorylation was reduced upon IL-7 stimulation compared to similarly treated control cells (Figures 3G and 3H). To confirm the defect in IL-7 responsiveness, we stimulated CLPs with IL-7 and found that

several IL-7-responsive genes such as *Socs1*, *Socs3*, and *Bcl-2* were no longer IL-7-inducible in Miz-1-deficient cells, whereas a normal up-regulation was seen in WT control cells (Figure 4A). We also observed that the expression of *Ebf1*, *Mcl1*, or *Bcl-2l1* was not dependent on IL-7 in CLP cells and their expression level in the presence of IL-7 were not affected by Miz-1 deficiency (Figure 4A). In freshly isolated CLPs from *Vav-cre Zbtb17*$^{fl/fl}$ mice, *Socs1* was (35-fold) increased in comparison to CLPs from control mice (Figure 4B). In contrast, the Miz-1-overexpressing 70Z/3 pre-B cell line showed a strong reduction in *Socs1* expression (Figure 4B). To identify potential binding sites of Miz-1 in the *Socs1* promoter, we performed chromatin immunoprecipitation (ChIP) with extracts from 70Z/3 pre-B cells transduced with a Miz-1-expressing retrovirus, 70Z/3 pre-B cells, or primary B cells (Figures 4C and 4D; Figures S4A and S4B). Of note, Miz-1 overexpression in 70Z/3 cells did not influence their cell cycle progression (Figure S4C). We analyzed a genomic region of 10 kb and found that Miz-1 bound to promoter sequences of the *Socs1* gene that are close to the transcription initiator site (Figures 4C and 4D; data not shown). We could not find a similar binding site in the *Socs3* promoter region (data not shown). This suggested that Miz-1 functions as a direct transcriptional repressor of the *Socs1* gene and that *Socs3* regulation by Miz-1 must be indirect.

Apoptosis in *Zbtb17*-Deficient B Cells Can Be Partially Rescued by *Bcl-2* or Inhibition of *Socs1*

CLPs or Fraction A cells from *Vav-cre Zbtb17*$^{fl/fl}$ mice did not indicate a deregulation of survival genes such as *Bcl-2* and *Mcl1* or proapoptotic genes like *Bad* and *Bax* (Figure 5A),

which is also consistent with findings reported for the *IL7r*$^{-/-}$ mice (Kikuchi *et al*., 2005). One exception is *Bcl-2l1*, which we found slightly downregulated in CLPs from *Vav-cre Zbtb17*$^{fl/fl}$ mice (Figure 5A). ChIP on the promoter regions of *Bcl-2l1* or *Ebf1* did not show Miz-1 binding (data not shown); however, scanning over a 10 kb region of the *Bcl-2* promoter revealed a Miz-1 binding site about 1.4 kb upstream of the initiator site (Figure 5B; Figure S5A). Together with the fact that *Bcl-2* can no longer be induced by IL-7, these findings suggest that Miz-1 may be required for the up-regulation of *Bcl-2* upon IL-7 and acts by directly binding to the *Bcl-2* promoter at a site different from the initiator.

To test whether absence of a functional Miz-1 affected the survival of B lineage cells in vivo, we stained CLPs and pre-pro-B cells (lin$^-$B220$^+$CD43$^+$CD19$^-$) of Miz-1-deficient mice or controls with AnnexinV, but we did not find different rates of cell death (Figure 5C). In contrast, 5- to 10-fold increased frequencies of apoptotic cells (AnnexinV$^+$) were observed in lin$^-$B220$^+$CD43$^+$CD19$^+$ cells (Fraction B–C′) and in B220$^+$CD43$^-$ cells, respectively, in the absence of a functional Miz-1 (Figure 5C), which may explain the loss of committed CD19$^+$ B cells (described in Figure 2).

In an attempt to counteract this enhanced rate of cell death, we crossed an *H2-K1-Bcl-2* transgene, which directs a high constitutive expression of Bcl-2 throughout hematopoiesis, including HSCs and early lymphoid progenitors (Figure S5B; Domen *et al*., 1998 and Kondo *et al*., 1997a) into *Vav-cre Zbtb17*$^{fl/fl}$ mice. In the resulting combinatorial mutant animals, B220$^+$CD19$^+$ B cells were now detected in the bone marrow, reaching about 40% of control frequencies (Figure 6A). Importantly, lin$^-$CD19$^+$B220$^+$CD43$^+$ pro-B cells, which

were almost undetectable in Miz-1-deficient mice, now reappeared in the presence of the *H2-K1-Bcl-2* transgene (Figure 6A). We also found that in contrast to CLPs from *Vav-cre Zbtb17$^{fl/fl}$,* CLPs from *Vav-cre Zbtb17$^{fl/fl}$ H2-K1-Bcl-2* mice now survived and expanded in vitro on OP9 cells (Figure 6B), but gave rise to only a very small number of CD19^{+} cells, possibly reflecting the partial rescue seen in vivo (Figure 6A). This indicated that *Bcl-2* alone is not sufficient to overcome the developmental block imposed by Miz-1 deficiency.

Socs1 is a negative regulator of the IL-7 signaling pathway and we did not only observe high *Socs1* expression in Miz-1-deficient CLPs, but our ChIP experiments also suggested that Miz-1 directly binds to *Socs1* promoter, possibly modulating its expression. Hence, we reasoned that inhibition of *Socs1* could restore IL-7 responsiveness in Miz-1-deficient cells and allow the development of CD19-positive cells. To test this, we performed a silencing experiment with gene-specific "Morpholino" oligonucleotides that are able to interfere with Socs1 protein expression (Figure 6C). LSKs from *Vav-cre Zbtb17$^{fl/fl}$* transfected with *Socs1*-specific Morpholinos showed a higher percentage of survival compared to *Vav-cre Zbtb17$^{fl/fl}$* transfected with a control Morpholino. These cells were able to expand on OP9 cells in the presence of IL-7 but did not up-regulate CD19 (Figure 6D). This suggested that high *Socs1* expression was at least partially responsible for the lack of survival of Miz-1-deficient B lineage precursors on OP9 cells in vitro.

Ebf1 and Bcl-2 Restore the Ability of Miz-1-Deficient Precursors to Generate CD19^{+} B Cells

The inability to fully rescue B cell differentiation in Miz-1-deficient progenitors by restoration of the IL-7-Stat5-Bcl-2 pathway either by providing excess Bcl-2 or by inhibiting *Socs1* suggested that an additional defect exists (Domen *et al*., 1998 and Kondo *et al*., 1997a). Comparative expression analysis showed that lymphoid-specific genes such as *Tcf3*, *Ebf1*, *Dntt*, *Pax5*, *Rag1*, and *Rag2* were decreased in Miz-1-deficient CLPs versus controls (Figure 7A). Ebf1 is essential for B-cell development and responsible for the expression of Pax5 and eventually also Rag1 and Rag2. Therefore, we expected to be able to rescue B cell differentiation in Miz-1-deficient cells by overexpressing Ebf1. We restored Ebf1 expression in Miz-1-deficient progenitors via a murine stem cell virus (MSCV) containing the *Ebf1* gene and GFP as a marker (Figure S6A). However, LSK cells from *Vav-cre Zbtb17*$^{fl/fl}$ mice transduced with the *Ebf1*-expressing virus did not develop into CD19^{+} cells and almost all cells were lost when cultured on OP9-stroma cells, whereas control LSK cells showed an accelerated differentiation into CD19^{+} B cells (Figure 7B). Similar to this, forced expression of a rearranged transgenic V(D)J segment at the IgH locus (Cascalho *et al*., 1996) did not rescue adult B-cell development in Miz-1-deficient mice (Figure S6B).

We hypothesized that because the IL-7-Stat5-Bcl-2 signaling pathway that regulates cell survival and the Ebf1-Pax5-Rag1-Rag2 axis that regulates differentiation are both affected by Miz-1 deficiency, a full rescue of B lineage differentiation might be achieved only if both pathways are restored. To test this, we sorted LSK cells from *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl-2* mice and transduced them with a retroviral vector expressing *Ebf1* or a virus

made with an empty vector control. When GFP^+ cells obtained after infection were cultured on OP9 cells, those that had received the *Ebf1*-expressing retrovirus were indeed able to fully differentiate into $CD19^+$ cells, but LSKs from *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl-2* infected with the control retrovirus alone did not (Figure 7C). This indicated that re-expression of both Bcl-2 and Ebf1 is needed to restore B cell differentiation from Miz-1-deficient progenitors and confirms that the transcription factor Miz-1 is required to regulate survival and developmental networks during early B-cell development.

Discussion

The development of B cells in the adult bone marrow is a well-defined process, in which cytokine signaling, V(D)J recombination, and the regulation of gene expression by transcription factors play a central role (Medina *et al*., 2004 and Singh *et al*., 2007). The signal transduction process initiated by IL-7 and its direct and indirect downstream effectors and also the transcription factors Ebf1 and Pax5 are essential for the early commitment and differentiation stages of B-cell development. In this study, we present evidence that the POZ-domain protein Miz-1 (*Zbtb17*) is essential for B-cell development. Our data suggest that Miz-1 regulates IL-7 signaling by monitoring on one hand the expression of *Socs1*, a negative regulator, and on the other hand *Bcl-2*, a positive effector of IL-7 signaling. In addition, Miz-1 is required for the proper expression of *Tcf3* and *Ebf1* and thus assures the functioning of the Ebf1-Pax5-Rag1-Rag2 axis.

Cre-mediated *Zbtb17* deletion initiated in hematopoietic stem cells by the *Vav* promoter confirmed this and suggested a role of Miz-1 at a stage of early uncommitted B cell progenitors. Flow cytometry data further supported the view that Miz-1 deficiency affects mainly follicular B cells and their differentiation, but not the formation of marginal zone B cells. This is similar to the phenotype reported in *Il7*- or *IL7r*-deficient mice (Carvalho *et al*., 2001 and Hesslein *et al*., 2006), where most residual peripheral B cells are marginal zone (MZ) B cells. MZ B cells are long-lived cells and are less affected by the absence of IL-7 signaling (Hesslein *et al*., 2006 and Lu and Cyster, 2002), which might explain their accumulation in Miz-1-deficient mice. Hence, the fact that we see a loss of follicular B

cells but not MZ B cells could point to a possible role of Miz-1 in IL-7 signaling. Along this line, the severe drop in absolute $CD19^+$ pro-B cell numbers and the fact that CLP or pre-pro-B cell numbers were unaffected in Miz-1-deficient mice suggested a block precisely at the pre-pro-B to pro-B cell transition, which is very similar to the reported observations in mice deficient for *IL7* or *IL7r* (Dias *et al*., 2005 and Kikuchi *et al*., 2005).

Our data clearly show that activation of Stat5 by Jak-mediated phosphorylation in response to IL-7 is almost undetectable in Miz-1-deficient cells. This is probably due to the fact that *Vav-cre Zbtb17*$^{fl/fl}$ CLPs highly express the Jak inhibitor *Socs1* and are thus unable to up-regulate IL-7-responsive target genes. Moreover, our ChIP and mRNA expression data very strongly suggest that the normal function of Miz-1 during IL-7-dependent early B-cell development is to dampen the expression of *Socs1* through direct binding to its transcription initiator site and to allow the activation of Stat5 and the subsequent up-regulation of IL-7 target genes. Because the inhibition of *Socs1* allows Miz-1-deficient cells to survive on OP9 and in the presence of IL-7, it is conceivable that Miz-1 plays an important role in regulating IL-7-mediated survival signals.

In a close perspective to our findings, it has been shown that during early B-cell development, *Bcl-2* expression is highly up-regulated in uncommitted "Fraction A" B cells that contain pre-pro-B cells to provide a survival signal (Li *et al*., 1993). It has also been reported that under stroma-free conditions, IL-7 alone enables CLPs to both survive and differentiate into $B220^+CD19^+$ pro-B cells (Kikuchi *et al*., 2005 and Miller *et al*., 2002). Our expression analysis showed that in the absence of Miz-1, *Bcl-2* expression cannot be

up-regulated upon IL-7 stimulation in CLPs, and ChIP experiments demonstrated a direct binding of Miz-1 to the *Bcl-2* promoter. This suggests that Miz-1 exerts a second function in the IL-7R pathway, which is different from the repression of *Socs1* and acts as a direct transcriptional activator of *Bcl-2*, probably in response to IL-7. Such a role would be consistent with two different reports that have previously demonstrated that *Bcl-2* is a direct effector gene of Miz-1 (Patel and McMahon, 2007 and Saito *et al*., 2009).

Miz-1 has been discovered as a Myc binding protein and its function as a repressor has been described in a complex with Myc on the promoters of cell cycle regulator genes such as *Cdkn1a* and *Cdkn2b* (Phan *et al*., 2005, Seoane *et al*., 2001, Seoane *et al*., 2002, Staller *et al*., 2001 and Wu *et al*., 2003). None of these Miz-1 targets that regulate cell cycle progression were altered in Miz-1-deficient cells (data not shown). Consistent with this, we could not find evidence for disturbed cell cycle progression in Miz-1-deficient B cell progenitors, suggesting that the regulatory function of Miz-1 that we describe here is independent of Myc. In addition, the few remaining peripheral follicular B cells did not show a proliferation defect (data not shown) in spite of the fact that they emerged from a disturbed early B-cell development. To obtain further insights in the role of Miz-1 in peripheral follicular B cells, other Cre deleter strains will have to be used.

The knockout of either *Ebf1* or *Tcf3* leads to a complete block at the pre-pro-B to pro-B transition (Bain *et al*., 1994 and Lin and Grosschedl, 1995), which resembles the phenotype seen in *Miz-1*-deficient mice. However, *Tcf3* is necessary for CLP formation and E2A-deficient mice have a marked reduction in their CLP numbers (Bain *et al*., 1997 and

Borghesi *et al*., 2005), which is different from our observations in the Miz-1-deficient mice. In addition, E2A is necessary to up-regulate *Ebf1* for allowing progenitors to develop into B cells, and ectopic expression of Ebf1 in *Tcf3*-deficient progenitors rescued B lymphocyte differentiation (Bain *et al*., 1994 and Seet *et al*., 2004). In Miz-1-deficient CLPs, *Tcf3* expression is reduced to about 50% and this residual expression might be sufficient to maintain CLP numbers in Miz-1-deficient mice but may be the cause of the reduced Ebf1 amounts. It is unlikely that Miz-1 regulates *Ebf1* expression directly because ChIP experiments did not provide evidence for Miz-1 binding on the *Ebf1* promoter. It is, however, possible that Miz-1 acts further upstream and interferes with the expression of *Tcf3* via a mechanism that remains to be elucidated.

If regulation of the E2A-Ebf1-Pax5-Rag1-Rag2 axis would fully describe the biochemical function of Miz-1, the retrovirally re-expression of *Ebf1* or a rearranged variable heavy chain gene should have rescued B cell differentiation in Miz-1-deficient progenitors. However, this is not the case, suggesting that another defect outside the Ebf1-Pax5-Rag1-Rag2 signaling pathway must exist in cells lacking Miz-1. A very likely solution to this is the role of Miz-1 in the part of IL-7 signaling that provides a survival signal to B cell progenitors described above. Ectopic expression of Ebf1 in *IL7r*$^{-/-}$ progenitor cells leads to an initial development of B220^{+}CD19^{+}IgM^{+} B cells but cannot overcome a defect in cell survival (Kikuchi *et al*., 2005). Our data are consistent with this and corroborate recent reports indicating that regulation of Ebf1 is independent of IL-7 (Malin *et al*., 2010). Our own findings that *Ebf1* cannot be induced by IL-7 and that re-expression of *Ebf1* alone was

not sufficient to rescue B-cell development in Miz-1-deficient mice confirm this notion further.

When we crossed Miz-1-deficient mice with transgenic mice overexpressing *Bcl-2*, a substantial but still partial rescue of B cell differentiation was seen in vivo, but differentiation of Miz-1-deficient B cell precursors expressing *Bcl-2* did not occur when cultured on OP9 stroma in vitro. The introduction of both Bcl-2 and Ebf1, which has been shown to promote B-cell development by up-regulating B cell genes like *Pax5*, *Rag1* and *Rag2*, and *Cd19* (Medina *et al*., 2004, O'Riordan and Grosschedl, 1999 and Pongubala *et al*., 2008) finally enabled us to fully reconstitute B cell commitment in Miz-1-deficient precursors when cultured in vitro. This was evident because only with *Bcl-2* and *Ebf1*, Miz-1-deficient progenitors were able to significantly up-regulate CD19 expression, which indicates that B cell lineage commitment has taken place. We found that *Cd19-Cre Zbtb17*$^{fl/fl}$ mice, which excise later than *Vav-Cre Zbtb17*$^{fl/fl}$ at pro-B and pre-B stages (Rickert *et al*., 1997), have normal B-cell development (data not shown), so we conclude that Miz-1 acts precisely at the pre-pro-B to pro-B transition, where Ebf1 is critical for commitment. This experiment supports a comprehensive model, in which Miz-1 exerts two functions in early B lineage progenitors: one in the regulation of the IL-7-independent E2A-Ebf1-Pax5-Rag1-Rag2 axis responsible for B cell differentiation and another role in the IL-7-dependent up-regulation of *Bcl-2* that ensures survival of B cell progenitors. In summary, the evidence that we present here establishes the POZ transcription factor Miz-1 as a regulatory element required at a critical point where signals from the IL-7 receptor

have to be relayed to effector genes that mediate survival (such as *Bcl-2*) and to be coordinated with signals that enable differentiation (such as *Ebf1*) to allow full lineage commitment and differentiation along the B cell lineage.

Experimental Procedures

Mice

All experiments performed on mice were approved by the IRCM animal care committee and done in accordance with the regulation of the Canadian Council of Animal Care.

For additional information see Supplemental Experimental Procedures.

Flow Cytometry, Cell Surface Staining, Stimulation, and Cell Sorting

Single-cell suspensions were prepared at the time of autopsy from thymus, bone marrow, or spleen in PBS supplemented with 1% FCS (staining solution). Antibody incubation was performed at 4°C for 15 min in 1% FCS in PBS (for more detail see Supplemental Information). For the intracellular flow cytometry analysis of pStat5, cells were incubated for 1.5 hr at 37°C to shut off endogenous signaling prior to stimulation. Cells were harvested, washed with PBS, and incubated with or without 10 ng/ml IL-7 (Peprotech 217-17) for 15 min at 37°C. After stimulation, cells were fixed with formaldehyde (BD cytofix) and additionally permeabilized with methanol (BD Phosflow Perm III). Samples were then stained with anti-phospho-Stat5(Y694)-Alexa488 (BD Phosflow 612598) or Alexa488 Mouse IgG1κ isotype control (BD Phosflow). AnnexinV staining was performed with the AnnexinV-FITC Detection Kit I (Becton Dickinson) and by following the manufacturer's instructions.

Chromatin Immunoprecipitation Assay

ChIP assays were performed with ChIP-IT Express (Active Motif) according to the manufacturer's instructions. Cells were fixed with 1% paraformaldehyde (PFA) neutralized with glycine, washed, and lysed with IGEPAL lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% IGEPAL, 1 mM PMSF). The lysate was sonicated (Branson Digital Sonifier). One percent of the soluble fraction (input) was kept and the rest was precleared with salmon sperm DNA-Protein G-agarose (Upstate). This was then divided into two tubes and incubated with 15 μg/ml of rabbit anti-Miz-1 polyclonal IgG (H190, Santa Cruz) or 15 μg/ml rabbit control IgG-ChIP grade antibodies (Abcam). The immune complexes were then precipitated with Protein G-agarose and eluted according to the manufacturer's instruction. DNA was purified with a polymerase chain reaction (PCR) purification kit (QIAGEN). Quantitative PCR was performed with the SYBR Green system on the Invitrogen Mx3005. Primers used for experiments are listed in Table S1.

Retroviral Transduction

Retroviruses were generated with 293-GPG cells. GP+E cells were infected with retroviruses from the 293-GPG cells for coculture and virus production. Hematopoietic cells were transduced spin-infection. For spin infection, sorted LSK cells were resuspended in viral supernatant in the presence of polybrene (12.5 μg/ml) and were centrifuged by 1400 × g for 2 hr. LSK cells were washed with Opti-MEM and cocultured on OP9 cells (Opti-MEM [10% (vol/vol) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol] containing 10 ng/ml SCF, 10 ng/ml Flt3L, and 1 ng/ml IL-7). Four to five days after infection, transduced cells were cell sorted by flow cytometry on the basis of

GFP expression. Coculture transduced cells were plated on OP9 cells. After 4 days of coculture, cells were transferred onto new stroma cells supplied with new cytokine containing media and cultured for the indicated time periods.

Morpholino Silencing

LSK cells sorted by flow cytometry were cultured in the presence of SCF, Flt3L, and IL-7 for 1 hr under stroma-free conditions. Morpholinos were added to the culture in the presence of EndoPorter followed the manufacturer's instructions (Gene Tools, USA). After 4 hr progenitor cells were transferred on OP9 cells. Every 4 days, cells were transferred on new OP9 cells and new cytokines were added (see Supplemental Information).

Statistics

Two-tailed Student's t tests were used to calculate p values where indicated. A p value $\leq$ 0.05 was indicated as statistically significant.

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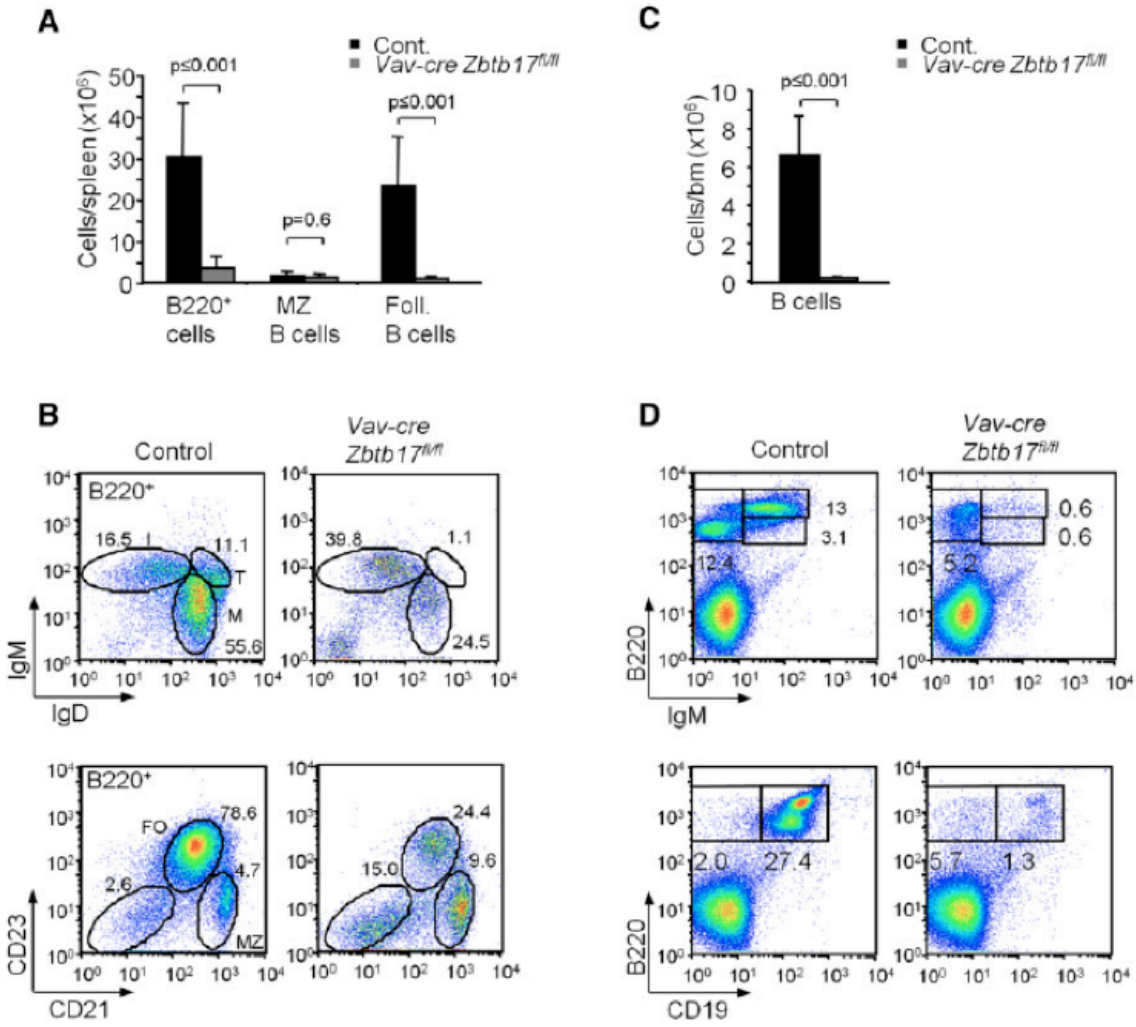
A
Cont.
Vav-cre Zbtb17fl/fl
Cells/spleen (x10^6)
p≤0.001
p=0.6
p≤0.001
B220+ cells
MZ B cells
Foll. B cells
C
Cont.
Vav-cre Zbtb17fl/fl
Cells/bm (x10^6)
p≤0.001
B cells
B
Control
Vav-cre Zbtb17fl/fl
B220+
16.5
11.1
T
M
55.6
39.8
1.1
24.5
IgM
IgD
B220+
FO
78.6
4.7
2.6
MZ
24.4
9.6
15.0
CD23
CD21
D
Control
Vav-cre Zbtb17fl/fl
13
3.1
12.4
0.6
0.6
5.2
B220
IgM
2.0
27.4
5.7
1.3
B220
CD19

Figure 1

Figure 1. POZ Domain Transcription Factor Miz-1 Is Essential for B-cell development

(A) Absolute numbers of splenic B cells ($B220^+$), marginal zone B cells (MZ; $B220^+CD21^+CD23^-$), and follicular B cells (Foll.; $B220^+CD21^+CD23^+$) of control mice (black) and *Vav-cre Zbtb17*$^{fl/fl}$ (gray). A minimum of five mice was analyzed for each group. Error bars indicate standard deviation (±SD); p values are indicated in the figure.

(B) Flow cytometric analysis of splenic B cells from control mice and *Vav-cre Zbtb17*$^{fl/fl}$ mice. $B220^+$ cells were analyzed for surface expression of IgM and IgD or CD21 and CD23. Immature (I), transitional (T), mature (M), follicular (FO), and marginal zone (MZ) B cells. Numbers in plots indicate percentages relative to gated $B220^+$ cells.

(C) Absolute numbers of bone marrow B cells ($B220^+CD19^+$; femur and tibia) were corrected to living cells (based on forward scatter [FSC] and side scatter [SSC] gating) of control mice (black) and *Vav-cre Zbtb17*$^{fl/fl}$ (gray). A minimum of five mice was analyzed for each group. Error bars indicate standard deviation (±SD); a p value is given in the figure.

(D) Flow cytometric analysis of bone marrow B cells from control mice and *Vav-cre Zbtb17*$^{fl/fl}$ mice. Cells were analyzed via antibodies for B220, IgM, and CD19; numbers in dot plots indicate percentages of each gate. (B and D) Data represent at least three independent experiments. Numbers in plots indicate percentages calculated on live cells (based on FSC and SSC gating). Controls were either WT, *Vav-cre Zbtb17*$^{fl/+}$, or Zbtb17$^{fl/fl}$ mice. See also Figure S1.

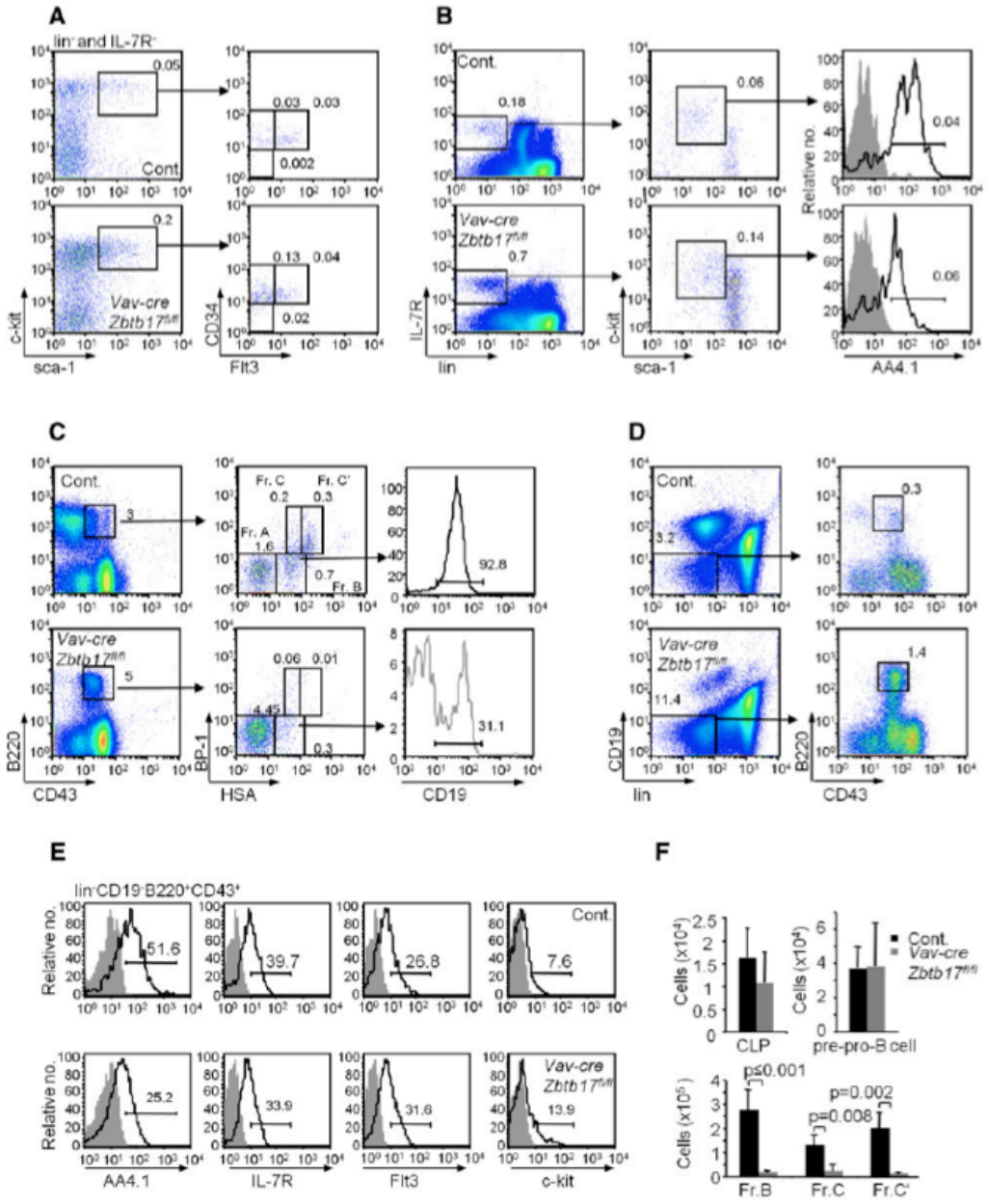
A
lin- and IL-7R-
Cont.
Vav-cre Zbtb17fl/fl
c-kit
sca-1
CD34
Flt3
B
Cont.
Vav-cre Zbtb17fl/fl
IL-7R
lin
c-kit
sca-1
Relative no.
AA4.1
C
Cont.
Vav-cre Zbtb17fl/fl
B220
CD43
BP-1
HSA
CD19
Fr. A
Fr. B
Fr. C
Fr. C'
D
Cont.
Vav-cre Zbtb17fl/fl
CD19
lin
B220
CD43
E
lin-CD19-B220+CD43+
Relative no.
AA4.1
IL-7R
Flt3
c-kit
Cont.
Vav-cre Zbtb17fl/fl
F
Cells (x10⁴)
CLP
pre-pro-B cell
Cont.
Vav-cre Zbtb17fl/fl
Cells (x10⁵)
p≤0.001
p=0.002
p=0.008
Fr.B
Fr.C
Fr.C'

Figure 2

Figure 2. Miz-1 Function Is Required to Generate Pro-B Cells

Bone marrow cells from control and *Vav-cre Zbtb17*$^{fl/fl}$ mice were analyzed via flow cytometry.

(A) Lineage-negative and IL-7R-negative cells (lin$^-$ and IL-7R$^-$) were analyzed for c-kit, sca-1, CD34, and Flt3 expression.

(B) Surface expression of lineage marker (lin), IL-7R, sca-1, c-kit, and AA4.1 on bone marrow cells.

(C) Flow cytometric analysis of B220, CD43, HSA, BP-1, and CD19 surface expression on bone marrow cells. B220$^+$CD43$^+$ cells were electronically gated and analyzed for HSA and BP-1 expression. Fraction B (Fr. B) (B220$^+$CD43$^+$HSA$^+$BP1$^-$) cells were analyzed for CD19 expression. Numbers on gates represent percentages of living cells. Numbers in histograms represent percentages of cells in indicated gates. Fraction (Fr.) A–C′ are indicated on the gates.

(D) Expression of lin, CD19, B220, and CD43 on bone marrow cells. Lineage panel for pre-pro-B cells contains CD11b, Gr-1, Ter119, IgM, TCR-β, TCR-γδ, CD8, CD4, CD3, NK1.1, Ly-6c, and CD5. Lin$^-$CD19$^-$ cells were analyzed for the expression of B220 and CD43. Numbers in plots indicate percentages calculated on total cells (based on FSC and SSC gating).

(E) The expression of AA4.1, IL-7R, Flt3, and c-kit on pre-pro-B cells ($lin^{-}CD19^{-}B220^{+}CD43^{+}$). Open histograms represent the expression of each surface marker and filled histograms represent isotype-matched irrelevant antibodies, respectively. Numbers in histograms represent percentages of ($lin^{-}CD19^{-}B220^{+}CD43^{+}$).

(F) Absolute numbers of CLPs ($lin^{-}IL\text{-}7R^{+}AA4.1^{+}sca\text{-}1^{med}c\text{-}kit^{med}$), pre-pro-B cells ($lin^{-}CD19^{-}B220^{+}CD43^{+}AA4.1^{+}$), Fr. B cells ($B220^{+}CD43^{+}HSA^{-}BP1^{-}CD19^{+}$), Fr. C cells ($B220^{+}CD43^{+}HSA^{+}BP1^{+}CD19^{+}$), and Fr. C′ cells ($B220^{+}CD43^{+}HSA^{hi}BP1^{+}$ $CD19^{+}$). Absolute cells numbers (femur and tibia) were corrected to living cells (based on FSC and SSC gating) from control mice (black) and *Vav-cre Zbtb17*$^{fl/fl}$ (gray). A minimum of five mice was analyzed for each group. Error bars indicate the standard deviation (±SD); a p value is given in the figure. See also Figure S2.

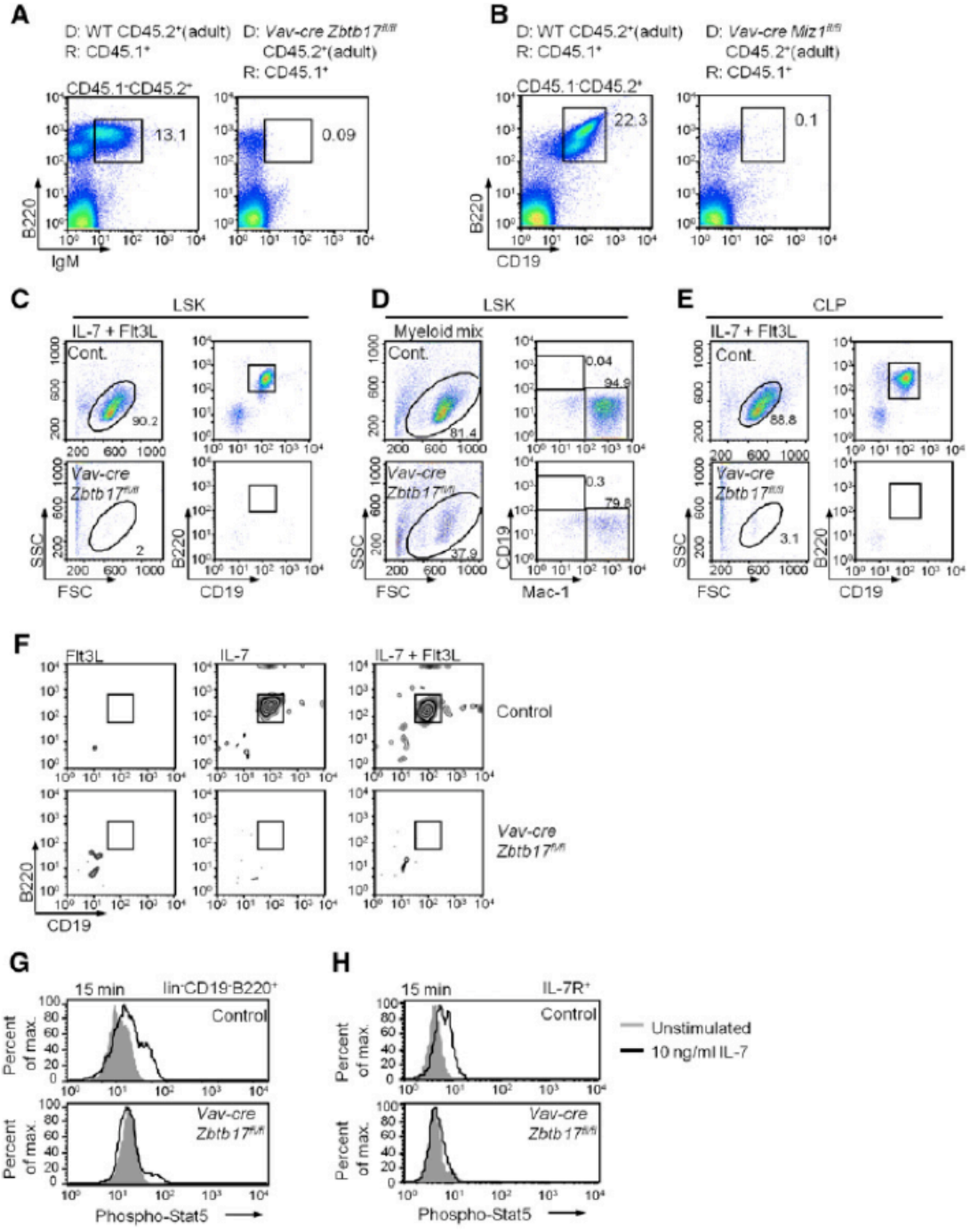
A
D: WT CD45.2+(adult)
R: CD45.1+
D: Vav-cre Zbtb17fl/fl
CD45.2+(adult)
R: CD45.1+
CD45.1-CD45.2+
13.1
0.09
B220
IgM
B
D: WT CD45.2+(adult)
R: CD45.1+
D: Vav-cre Miz1fl/fl
CD45.2+(adult)
R: CD45.1+
CD45.1-CD45.2+
22.3
0.1
B220
CD19
C
LSK
IL-7 + Flt3L
Cont.
90.2
Vav-cre Zbtb17fl/fl
2
SSC
FSC
B220
CD19
D
LSK
Myeloid mix
Cont.
81.4
0.04
94.9
Vav-cre Zbtb17fl/fl
37.9
0.3
79.8
SSC
FSC
CD19
Mac-1
E
CLP
IL-7 + Flt3L
Cont.
88.8
Vav-cre Zbtb17fl/fl
3.1
SSC
FSC
B220
CD19
F
Flt3L
IL-7
IL-7 + Flt3L
Control
Vav-cre Zbtb17fl/fl
B220
CD19
G
15 min
lin-CD19-B220+
Control
Vav-cre Zbtb17fl/fl
Percent of max.
Phospho-Stat5
H
15 min
IL-7R+
Control
Vav-cre Zbtb17fl/fl
Unstimulated
10 ng/ml IL-7
Percent of max.
Phospho-Stat5

Figure 3

Figure 3. Miz-1 Is Indispensable for Progenitor Differentiation and Survival upon Cytokine Stimulation

(A and B) Lethally irradiated recipients (CD45.1) were reconstituted with bone marrow cells (Terr119$^-$B220$^-$CD19$^-$) from WT control mice or *Vav-cre Zbtb17$^{fl/fl}$* mice (both CD45.2$^+$). Bone marrow cells were analyzed 3–4 months after transplantation by flow cytometry with antibodies for CD45.1, CD45.2, B220, and IgM (A) and with antibodies for CD45.1, CD45.2, B220, and CD19 (B). Data represent three independent experiments. Numbers on gates indicate percentages. (D, donor; R, recipient.)

(C) LSK (lin$^-$sca-1$^+$c-kit$^+$) cells from control mice or *Vav-cre Zbtb17$^{fl/fl}$* mice were flow cytometrically sorted and cocultured on OP9 cells in the presence of Flt3L and IL-7 for 10–12 days. Cells were analyzed for forward (FSC) and side (SSC) scatter and the surface expression of B220 and CD19 by flow cytometry.

(D) LSK (lin$^-$sca-1$^+$c-kit$^+$) cells from control mice or *Vav-cre Zbtb17$^{fl/fl}$* mice were flow cytometrically sorted and cocultured on OP9 cells in the presence of a myeloid cytokine cocktail for 6–8 days. Cells were analyzed for FSC and SSC and the surface expression of Mac1 and CD19 by flow cytometry.

(E) CLP (lin$^-$IL-7R$^+$sca-1^{med}c-kit^{med}) cells from control mice or *Vav-cre Zbtb17$^{fl/fl}$* mice were flow cytometrically sorted and cocultured on OP9 cells in the presence of Flt3L and IL-7 for 10–12 days. Cells were analyzed for FSC and SSC as well as the surface expression of B220 and CD19 by flow cytometry.

(F) CLP were sorted and cultured under stroma-free conditions in the presence of Flt3L, IL-7 or Flt3L, and IL-7 for 6 days. Cells were analyzed for the surface expression of B220 and CD19 by flow cytometry. Data represent at least four (C, E, F) or two (D) independent experiments.

(G and H) Flow cytometry of Stat5 phosphorylation in $lin^{-}CD19^{-}B220^{+}$ (lin: Mac1, Terr119) (G) or in IL-$7R^{+}$ bone marrow cells (H) from control mice or *Vav-cre Zbtb17*$^{fl/fl}$ mice. Open histograms represent the expression of phospho-Stat5 after 15 min IL-7 stimulation and filled histograms represent phospho-Stat5 in unstimulated cells, respectively. Data represent three (G) or two (H) independent experiments. See also Figure S3.

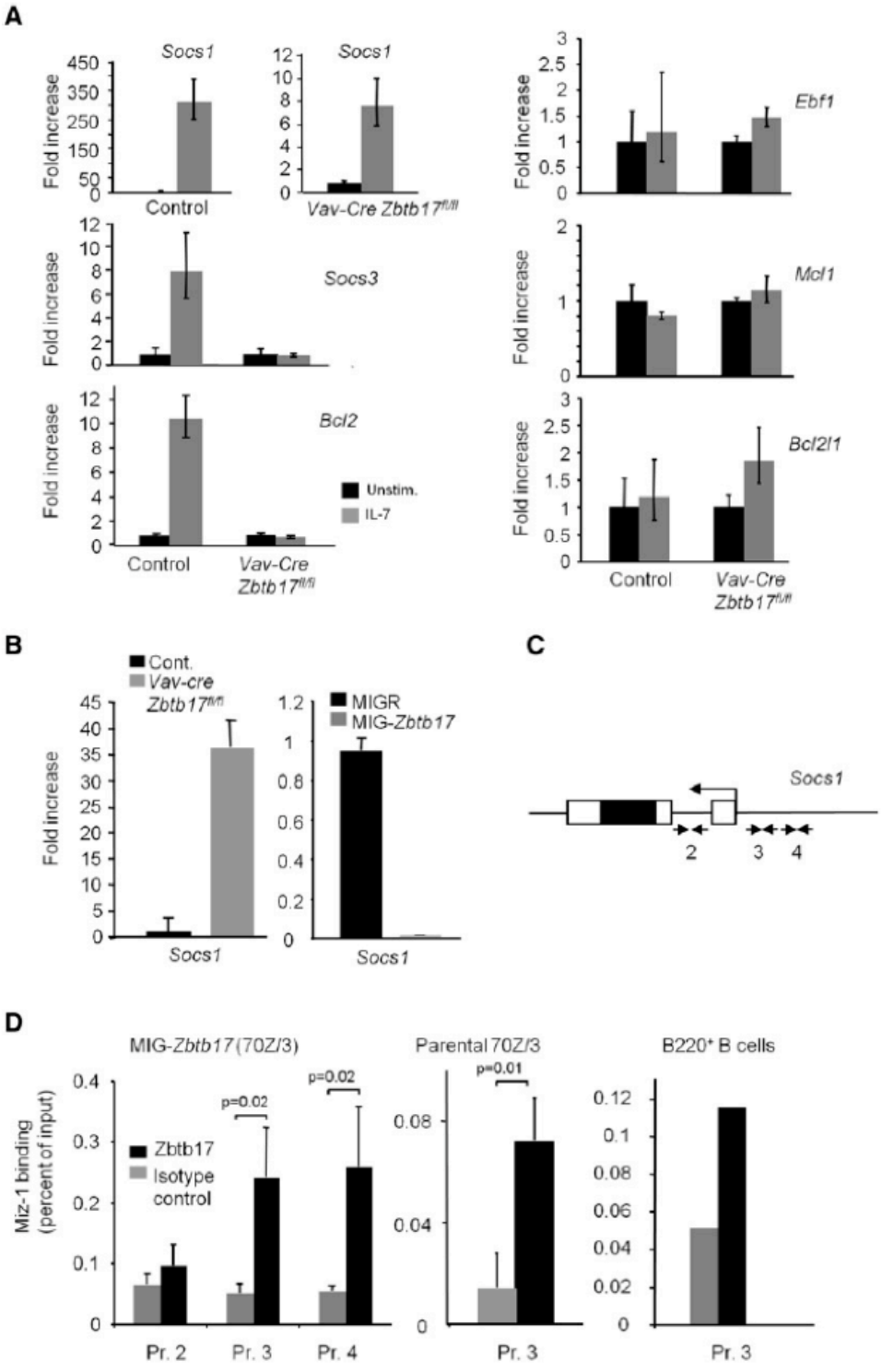
A
Socs1
Fold increase
Control
Vav-Cre Zbtb17fl/fl
Socs3
Bcl2
Unstim.
IL-7
Ebf1
Mcl1
Bcl2l1
B
Cont.
Vav-cre Zbtb17fl/fl
MIGR
MIG-Zbtb17
C
Socs1
2
3
4
D
MIG-Zbtb17 (70Z/3)
Parental 70Z/3
B220+ B cells
Miz-1 binding (percent of input)
Zbtb17
Isotype control
p=0.02
p=0.02
p=0.01
Pr. 2
Pr. 3
Pr. 4
Pr. 3
Pr. 3

Figure 4

Figure 4. Miz-1 Is Required to Regulate the Expression of IL-7-Responsive Genes in CLPs

(A) CLPs from the bone marrow of control and *Vav-cre Zbtb17*$^{fl/fl}$ mice were separated by flow cytometric cell sorting and cultured for 120 min in the absence and presence of IL-7. RNA was extracted and reverse-transcribed and used for quantitative PCR. Expression of the indicated genes was measured and normalized to the expression of the *Gapdh* gene and is presented as the fold increase relative to cDNA from control mice or unstimulated cells (set as 1-fold). Data represent three independent experiments each done in triplicate. Error bars indicate the standard deviation (±SD).

(B) Expression of *Socs1* determined by qRT-PCR in CLPs from control and and *Vav-cre Zbtb17*$^{fl/fl}$ mice (left), and in 70Z/3 cells transduced with empty vector (MIGR1) or with a MIG-*Zbtb17*-GFP virus (right). Error bars indicate the standard deviation (±SD).

(C) *Socs1* locus, showing coding regions (black boxes) and non-coding regions (open boxes) and location of primers (black triangles).

(D) Chromatin immunoprecipitation (ChIP) showing binding of Miz-1 to the promoter region of *Socs1*. Used were α-Miz-1 antibodies (H190) and α-isotype control antibodies on extracts of MIG-*Zbtb17*-GFP transduced 70Z/3 cells (left), 70Z/3 cells (middle), or primary B cells (B220^{+} cells) (right), followed by a quantitative RT-PCR via primer pairs of indicated regions. Data represent two independent experiments each done in triplicate (PCR on primary B cells was done in duplicates). Error bars indicate the standard deviation (±SD). See also Figure S4 and Tables S1 and S2.

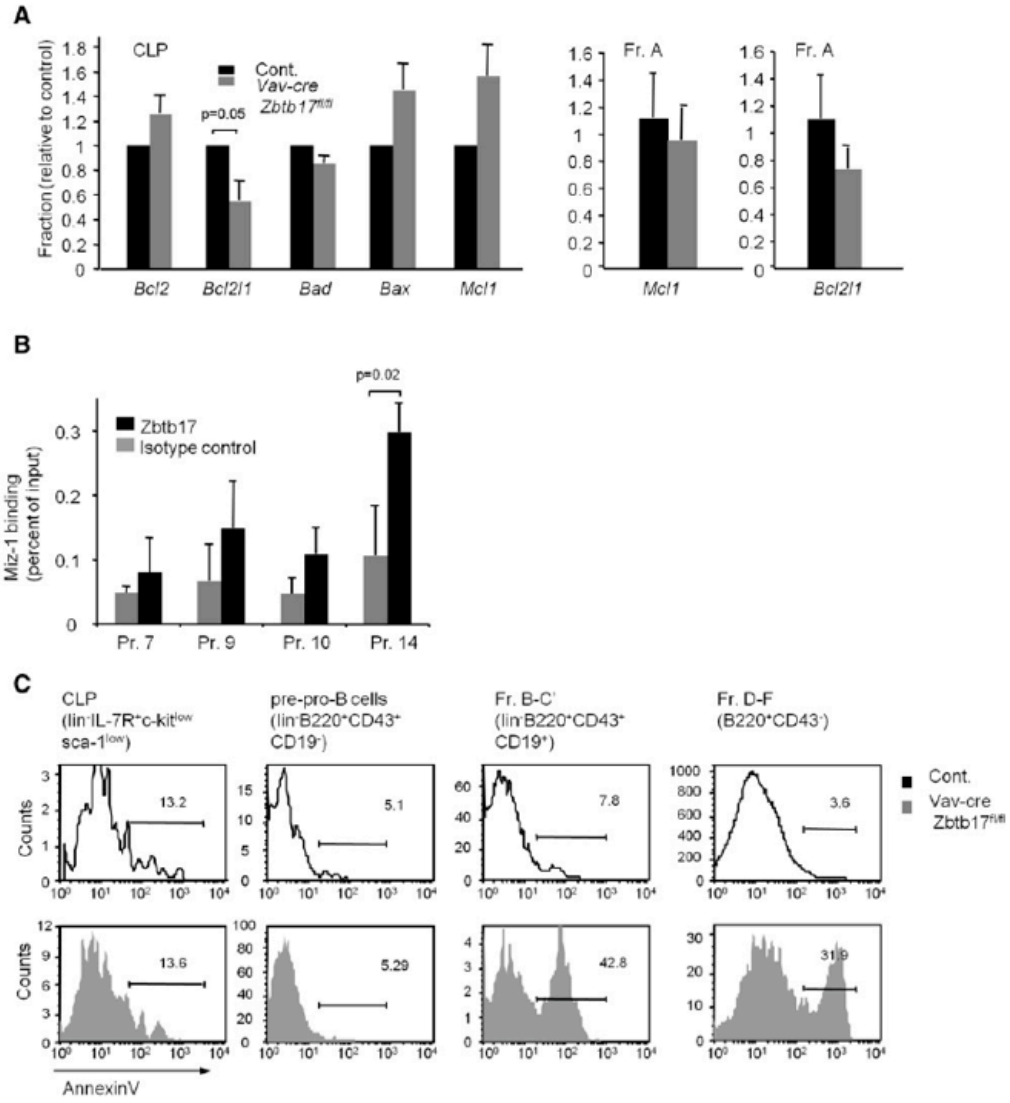
A
CLP
Cont.
Vav-cre
Zbtb17fl/fl
p=0.05
Fraction (relative to control)
Bcl2
Bcl2l1
Bad
Bax
Mcl1
Fr. A
Mcl1
Fr. A
Bcl2l1
B
p=0.02
Zbtb17
Isotype control
Miz-1 binding (percent of input)
Pr. 7
Pr. 9
Pr. 10
Pr. 14
C
CLP (lin-IL-7R+c-kitlow sca-1low)
pre-pro-B cells (lin-B220+CD43+ CD19-)
Fr. B-C' (lin-B220+CD43+ CD19+)
Fr. D-F (B220+CD43-)
Cont.
Vav-cre Zbtb17fl/fl
Counts
13.2
5.1
7.8
3.6
13.6
5.29
42.8
31.9
AnnexinV

Figure 5

Figure 5. Apoptosis during B-cell development in Miz-1-Deficient Mice

(A) CLPs or Fraction A ($CD19^{-}B220^{+}CD43^{+}$) cells from control and *Vav-cre Zbtb17*$^{fl/fl}$ mice were separated by flow cytometric cell sorting. RNA was extracted and reverse transcribed and used for quantitative PCR with indicated primer. All values were normalized to the expression of the *Gapdh* gene and are presented as the fraction relative to cDNA from control mice (set as 1-fold). Data show three independent experiments, each done in triplicates. Error bars indicate the standard error of the mean (±SEM).

(B) Chromatin immunoprecipitation (ChIP) of the binding of Miz-1 to the promoter region of *Bcl-2*. Used were a Miz-1 antibody (H190) and an isotype control antibody and extracts of MIG-*Zbtb17*-GFP transduced 70Z/3 cells, followed by a qRT-PCR of the indicated genomic regions. Data represent two independent experiments, each done in triplicate. Error bars indicate the standard deviation (±SD). A p value is indicated.

(C) CLPs ($lin^{-}IL\text{-}7R\alpha^{+}c\text{-}kit^{med}sca\text{-}1^{med}$), pre-pro-B ($lin^{-}B220^{+}CD43^{+}CD19^{-}$), pro-B ($lin^{-}B220^{+}CD43^{+}CD19^{+}$), and Fr. D–F B ($B220^{+}CD43^{-}$) cells were electronically gated and analyzed for apoptotic cells via AnnexinV staining. Numbers in histograms indicate percentages of each indicated gate. Data represent three independent experiments. See also Figure S5 and Tables S1 and S2.

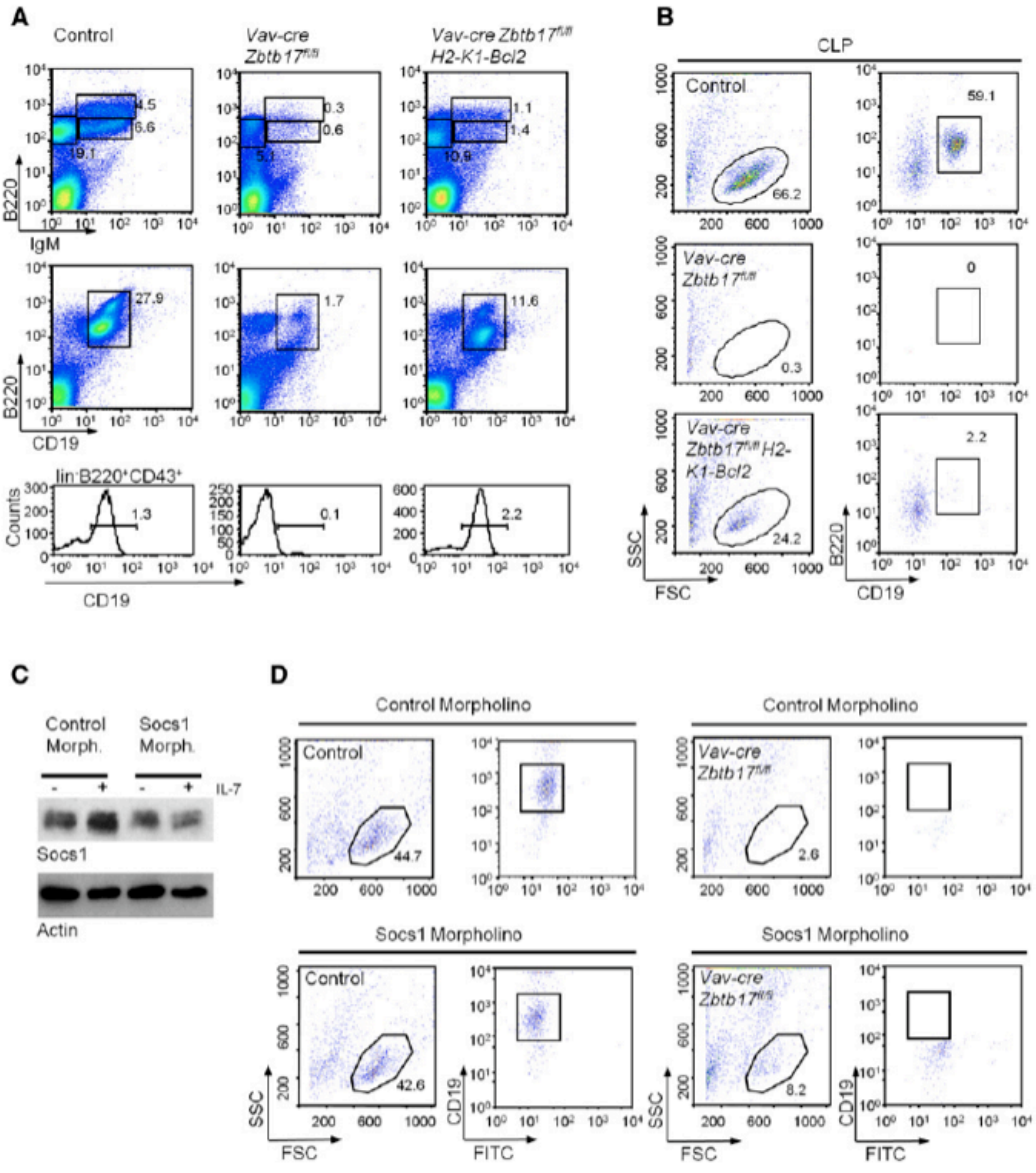
A
Control
Vav-cre Zbtb17fl/fl
Vav-cre Zbtb17fl/fl H2-K1-Bcl2
B220
IgM
CD19
lin-B220+CD43+
Counts
B
CLP
Control
Vav-cre Zbtb17fl/fl
Vav-cre Zbtb17fl/fl H2-K1-Bcl2
SSC
FSC
B220
CD19
C
Control Morph.
Socs1 Morph.
IL-7
Socs1
Actin
D
Control Morpholino
Socs1 Morpholino
Control
Vav-cre Zbtb17fl/fl
SSC
FSC
CD19
FITC

Figure 6

Figure 6. *Bcl-2* Expression or *Socs1* Inhibition Enables Miz-1-Deficient Precursors to Survive with IL-7

(A) Flow cytometry analysis of bone marrow from control, *Vav-cre Zbtb17*$^{fl/fl}$, and *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl-2* mice with the indicated surface markers. Numbers in dot plots indicate percentages of each gate. Bone marrow cells were analyzed with the pre-pro-B lineage markers (CD11b, Gr-1, Ter119, IgM, TCR-β, TCR-γδ, CD8, CD4, CD3, NK1.1, Ly-6c, and CD5) and B220, CD43 CD19, and IgM to define the pro-B cell population. Numbers in histograms indicate percentages of pro-B cells for the indicated mice. Data are representative of three independent experiments.

(B) CLP (lin^{-}IL-7R^{+}sca-1^{med}c-kit^{med}) cells from control, *Vav-cre Zbtb17*$^{fl/fl}$, and *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl-2* were flow cytometrically sorted and cocultured on OP9 stroma cells in the presence of Flt3L and IL-7 for 7–8 days. Cells were analyzed for FSC and SSC as well as the surface expression of B220 and CD19 by flow cytometry. Data are representative of two independent experiments.

(C) Expression of Socs1 protein in 70Z/3 cells stimulated or not with IL-7 in the presence or absence of an anti-Socs1 Morpholino demonstrates its effect to knockdown Socs1 protein expression in the presence of IL-7.

(D) Sorted LSKs from control or *Vav-cre Zbtb17*$^{fl/fl}$ mice were incubated with Morpholino against *Socs1* mRNA or a control Morpholino. Shown is the fluorescein isothyocyanate fluorescence after incubation, indicative of an almost complete and efficient transfer of morpholino. Cells from control or *Vav-cre Zbtb17*$^{fl/fl}$ treated either with control or anti-

Socs1 Morpholinos were analyzed after 8 days of OP9 coculture (representative of at least three independent experiments).

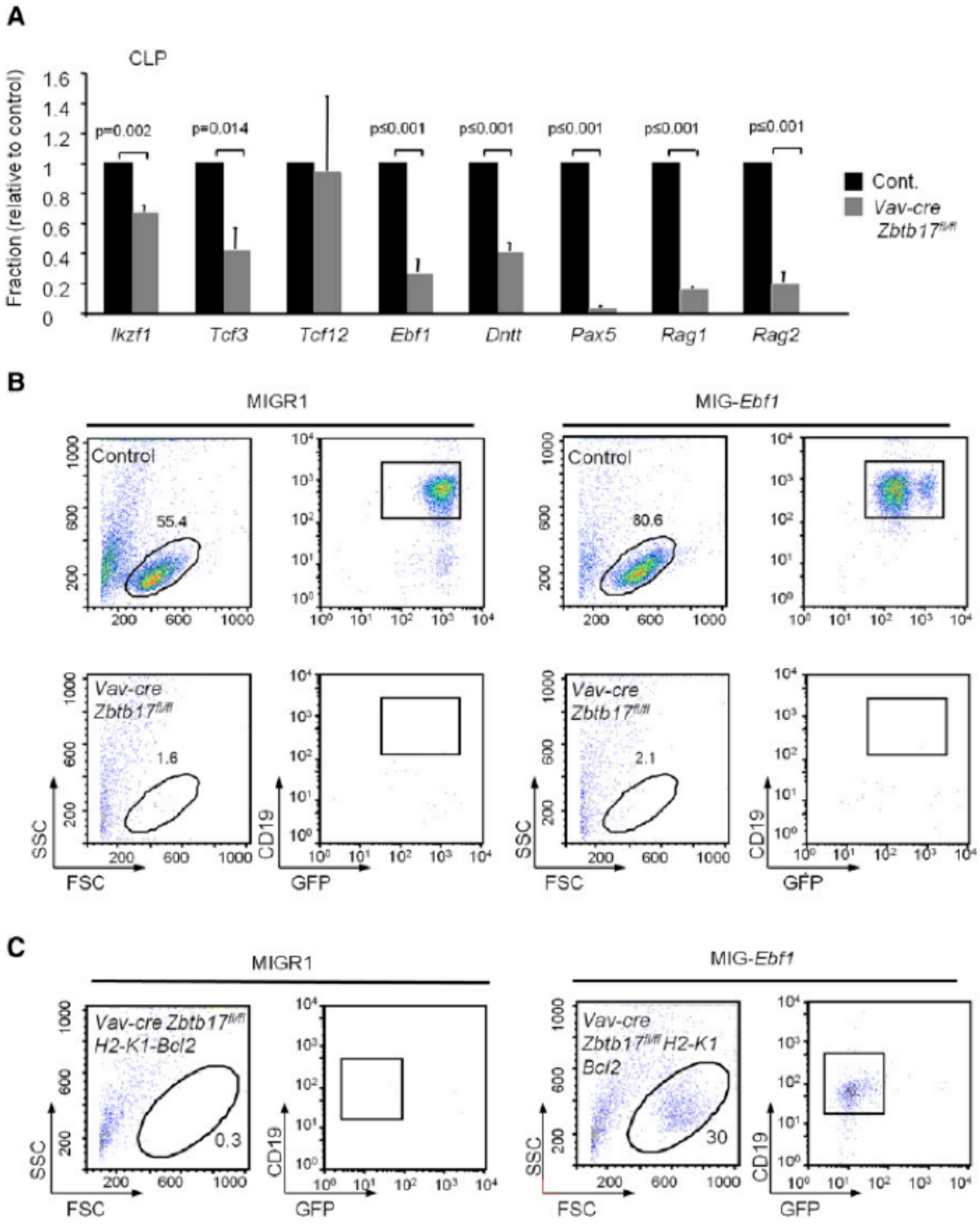
A
CLP
Fraction (relative to control)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p=0.002
p=0.014
p≤0.001
p≤0.001
p≤0.001
p≤0.001
p≤0.001
Cont.
Vav-cre Zbtb17fl/fl
Ikzf1
Tcf3
Tcf12
Ebf1
Dntt
Pax5
Rag1
Rag2
B
MIGR1
MIG-Ebf1
Control
55.4
80.6
Vav-cre Zbtb17fl/fl
1.6
2.1
SSC
FSC
CD19
GFP
C
MIGR1
MIG-Ebf1
Vav-cre Zbtb17fl/fl H2-K1-Bcl2
0.3
30

Figure 7

Figure 7. Reconstitution of B-cell development in Miz-1-Deficient Precursors by Ebf1 and Bcl-2

(A) CLPs (lin^{-}IL-$7R^{+}$c-kit^{med}sca-1^{med}) were isolated by flow cytometric cell sorting from the bone marrow of control mice and *Vav-cre Zbtb17*$^{fl/fl}$ mice. RNA was extracted and reverse-transcribed and used for quantitative PCR. All values are normalized to the expression of the *Gapdh* gene and are presented as the fraction relative to cDNA from control mice (set as 1-fold). Data show three independent experiments each done in triplicates. Error bars indicate the standard error of the mean (±SEM).

(B) LSK cells sorted from bone marrow were infected with MIGR1-GFP or MIG-*Ebf1*-GFP retroviruses. Transduced cells (GFP^{+}) were FACS sorted (Figure S6) and equal cell numbers were directly cultured on OP9 cells in the presence of SCF, Flt3L, and IL-7. Cells were analyzed for CD19 and GFP expression by flow cytometry 10–14 days after infection. Numbers on gates indicate percentages. Data are representative of three independent experiments.

(C) LSK cells were sorted from *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl-2* mice and were infected with MIGR1-GFP or MIG-*Ebf1*-GFP retroviruses. After infection, LSK cells were cultured in the presence of SCF, Flt3L, and IL-7 on OP9 cells and the GFP^{+} fraction was isolated by sorting and further cultured under the same conditions. Cells were analyzed for CD19 and GFP expression by flow cytometry 16 days after infection, Numbers on gates indicate percentages. Data are representative of two independent experiments. See also Figure S6 and Table S2.

Supplementary materials

Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor signaling at Early Commitment Stages of B Cell Differentiation

Christian Kosan, Ingrid Saba, Maren Godmann, Stefanie Herold, Barbara Herkert, Martin Eilers, and Tarik Möröy

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Supplemental Figures:

Supplemental Tables:

Supplemental Experimental Procedures:

Mice

Antibodies and lineage panel

Cell cycle analysis

Generation of conditional Zbtb17 deficient mice

RNA isolation, cell sorting and real-time PCR

Cell culture

RNA isolation and RT PCR

PCR analysis

Morpholinos

Immunoprecipitation

Adoptive transfer

Western blotting

Antibodies

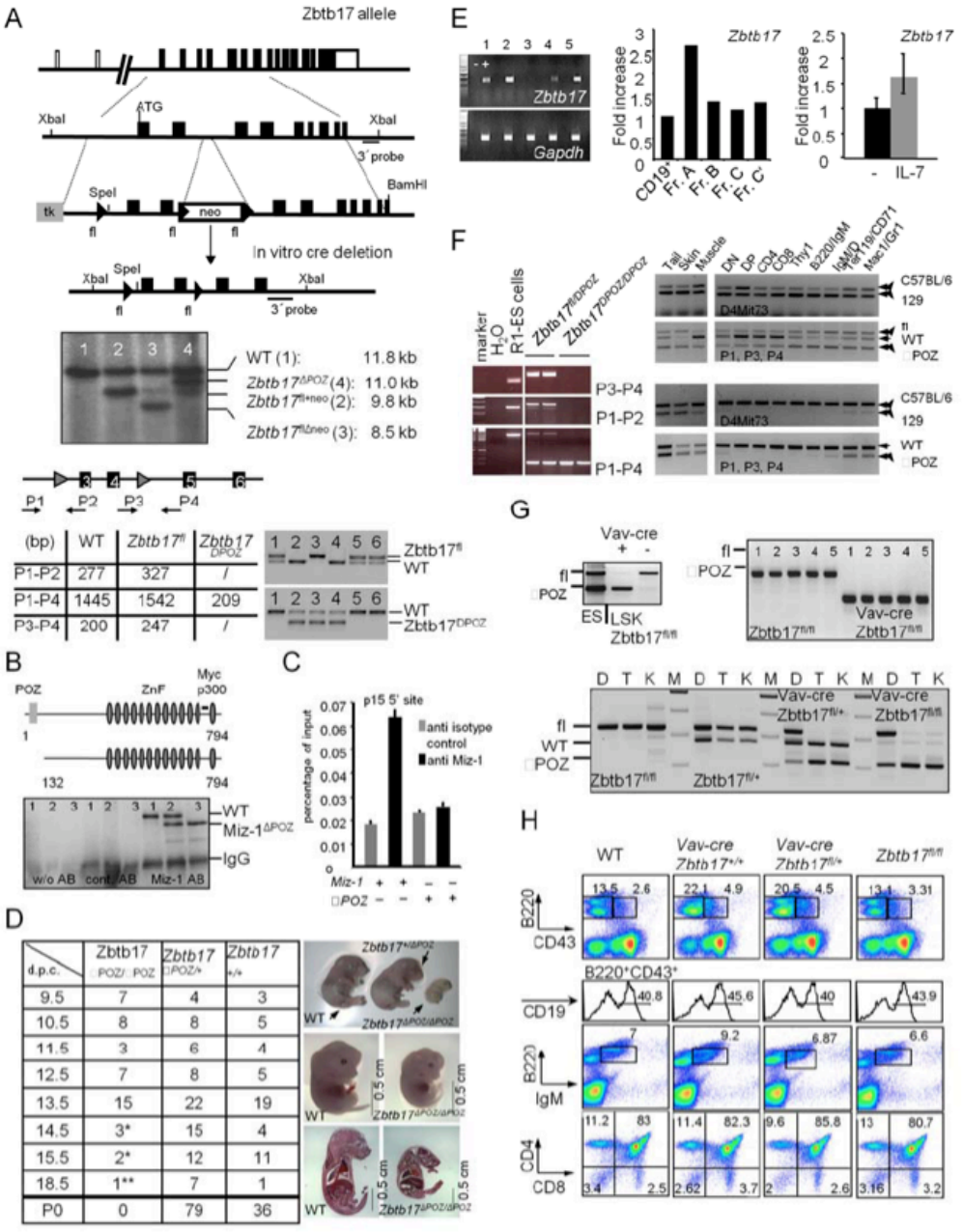
A
Zbtb17 allele
XbaI
ATG
XbaI
3´probe
SpeI
BamHI
tk
neo
fl
In vitro cre deletion
WT (1): 11.8 kb
Zbtb17ΔPOZ (4): 11.0 kb
Zbtb17fl+neo (2): 9.8 kb
Zbtb17flΔneo (3): 8.5 kb
P1
P2
P3
P4
(bp) WT Zbtb17fl Zbtb17 ΔPOZ
P1-P2 277 327 /
P1-P4 1445 1542 209
P3-P4 200 247 /
Zbtb17fl
WT
Zbtb17ΔPOZ
B
POZ
ZnF
Myc
p300
1
794
132
794
WT
Miz-1ΔPOZ
IgG
w/o AB
cont. AB
Miz-1 AB
C
p15 5' site
anti isotype control
anti Miz-1
percentage of input
Miz-1
POZ
D
d.p.c.
Zbtb17 POZ/ POZ
Zbtb17 ΔPOZ/+
Zbtb17 +/+
9.5 7 4 3
10.5 8 8 5
11.5 3 6 4
12.5 7 8 5
13.5 15 22 19
14.5 3* 15 4
15.5 2* 12 11
18.5 1** 7 1
P0 0 79 36
Zbtb17+/ΔPOZ
WT
Zbtb17ΔPOZ/ΔPOZ
0.5 cm
E
Zbtb17
Gapdh
Fold increase
CD19+
Fr. A
Fr. B
Fr. C
Fr. C'
- IL-7
F
marker
H2O
R1-ES cells
Zbtb17fl/ΔPOZ
Zbtb17ΔPOZ/ΔPOZ
Tail
Skin
Muscle
DN
DP
CD4
CD8
Thy1
B220/IgM
IgM/D
IgM/19/CD71
Mac1/Gr1
C57BL/6
129
D4Mit73
P1, P3, P4
P3-P4
P1-P2
P1-P4
G
Vav-cre
fl
POZ
ES
LSK
Zbtb17fl/fl
Zbtb17fl/+
D T K M D T K M D T K M D T K
H
WT
Vav-cre Zbtb17+/+
Vav-cre Zbtb17fl/+
Zbtb17fl/fl
B220
CD43
B220+CD43+
CD19
IgM
CD4
CD8

Figure S1

Figure S1. Deletion of the Miz-1 POZ domain leads to a loss of T and B cells in chimeric mice

(A) Generation of a conditional Zbtb17 allele and Miz-1 deficient mice. Schematic representation of the targeting strategy at the Zbtb17 locus. Shown is the genomic locus of the murine *Zbtb17* gene (white boxes untranslated exons; black boxes translated exons), the targeting construct, the targeted genomic locus after homologous recombination and the resulting conditional $Zbtb17^{fl}$ alleles. The size of different *Zbtb17*

alleles before and after homologous recombination and after in vitro cre recombination is given in kilobases (kb) after *Xba*I/*Spe*I digestion. Southern blot analysis of embryonic stem (ES) cell lines with the indicated genotypes that were obtained after homologous recombination and in vitro cre recombination. Genomic DNA was digested with *Spe*I/*Xba*I and probed with the 3´-probe, indicated in panel 2. Orientation and localization of primers P1, P2, P3 and P4 used for genotyping on the genomic Zbtb17 locus. The different sizes of fragments obtained with these primers and DNA containing the WT-, $Zbtb17^{fl}$ and $Zbtb17^{\Delta POZ}$ alleles in a PCR reaction are indicated (1-6, different embryonic DNAs). (B) Scheme of the protein structure of the full-length Miz-1 protein and the truncated form lacking the POZ domain. Immunoprecipitation of the full length and the truncated Miz-1 protein lacking the POZ domain from primary embryonic fibroblasts (MEFs) derived from day E12.5 embryos with the indicated genotypes (1) WT, (2) $Zbtb17^{+/\Delta POZ}$ and (3) $Zbtb17^{\Delta POZ/\Delta POZ}$. Precipitates were generated without antibody, with a control antibody or an anti-Miz-1 antibody (H190), separated by SDS-PAGE and transferred to a solid support. Miz-1 proteins were revealed with the Miz-1 monoclonal antibody 10E2. (C) Chromatin

immuno-precipitation (ChIP) analysis showing the binding of Miz-1 and Miz-1$^{\Delta POZ}$ to the p15 promoter region in Hela cells. ChIP was performed using α-Miz-1 antibodies and isotype control antibodies followed by a QRTPCR of indicated regions. Data represent two independent experiments each done in triplicates. Error bars indicate the standard deviation (±S.D.). (D) Genotypes and numbers of offspring produced by matings between *Zbtb17*$^{+/\Delta POZ}$ mice. Wildtype *Zbtb17*$^{+/+}$, *Zbtb17*$^{+/\Delta POZ}$, *Zbtb17*$^{\Delta POZ/\Delta POZ}$. (*), pale and much smaller size, (**), severe defects in development. Embryos at E18.5; a wild type and a *Zbtb17*$^{+/\Delta POZ}$ animal are shown side by side with a *Zbtb17*$^{\Delta POZ/\Delta POZ}$ animal, which is significantly smaller and displays a noticeable delay in development. Representative photomicrograph of wild type (WT) and *Zbtb17*$^{\Delta POZ/\Delta POZ}$ embryos at day E13.5. Sections of control and *Zbtb17*$^{\Delta POZ/\Delta POZ}$ embryos at day E15.5 stained with Haematoxylin and Eosin (HE). (E) RT-PCR analysis with RNA from different mouse tissues ((1) spleen, (2) liver, (3) thymus, (4) kidney and (5) lymph node). The PCR reaction was performed with 30 cycles. (-) -RT: without reverse transcriptase; ($^{+}$) $^{+}$RT: with reverse transcriptase. Real-time PCR with cDNA from FACS sorted cells. All values are normalized to the expression of the *Gapdh* gene and are presented as the fold induction relative CD19^{+} respectively (set as 1-fold). Data represent two experiments, each done in duplicates. CD19^{+}: splenic B cells; Fr.A (B220^{+}CD43^{+}HSA^{-}BP-1^{-}), Fr. B (B220^{+}CD43^{+}HSA^{+}BP-1^{-}), Fr.C (B220^{+}CD43^{+}HSA^{+}BP-1^{+}) and Fr.C' (B220^{+}CD43^{+}HSA^{high}BP-1^{+}). CLPs (lin^{-}IL7Rα^{+}c-kit^{med}sca-1^{med}) from the bone marrow of control and *Vav-cre Zbtb17*$^{fl/fl}$ mice were separated by flow cytometric cell sorting and cultured in the presence and absence of IL-7. RNA was

extracted and reverse-transcribed and used for quantitative-PCR. Expression of *Zbtb17* was measured and normalized to the expression of the *Gapdh* gene and is presented as the fold induction relative to cDNA from control mice or unstimulated cells (set as 1-fold). Data represent two independent experiments each done in triplicates. Error bars indicate the standard deviation (±S.D.). (F) Genotyping of embryonic stem (ES) cell lines that were obtained after targeting both Zbtb17 alleles and after in vitro cre recombination. ES cells with one deleted and one targeted, but functional (floxed) allele (*Zbtb17*$^{fl/\Delta POZ}$) were injected into C57BL/6 blastocysts. ES-cells with two deleted alleles (*Zbtb17*$^{\Delta POZ/\Delta POZ}$) were injected into C57BL/6 blastocysts. Depiction of PCR fragments generated with DNA from tail, skin and muscle as control and with DNA from FACS sorted cells from different hematopoietic lineages (DN, DP, CD4$^+$, CD8$^+$, Thy1$^+$, B220$^+$/IgM$^+$, IgM$^+$/IgD$^+$, Ter119$^+$/CD71$^+$ and Mac1$^+$/Gr1$^+$ cells). The PCR reactions were performed with *Zbtb17* specific primers P1, P2 and P4 to distinguish between wt and targeted alleles or with primers D4Mit73 that amplify minisatellite sequences to distinguish between C57Bl/6 (blastocysts) and 129J specific alleles (ES cells). Data represent the analysis of three (*Zbtb17*$^{fl/\Delta POZ}$) or five (*Zbtb17*$^{\Delta POZ/\Delta POZ}$) chimeras. (G) Detection of the efficiency of cre recombination in different cell types and FACS analysis from control mice. PCR reactions with genomic DNA from *Zbtb17*$^{fl/\Delta POZ}$ ES cells and FACS sorted LSK cells (lin$^-$Sca1$^+$c-kit$^+$), which contain hematopoietic stem cells, from *Vav-cre Zbtb17*$^{fl/fl}$ and from *Zbtb17*$^{fl/fl}$ mice. *Zbtb17*$^{fl/\Delta POZ}$ ES cells were used as a control. PCR reactions with genomic DNA from FACS sorted different hematopoietic cell populations derived from *Zbtb17*$^{fl/fl}$ and from

Vav-cre Zbtb17$^{fl/fl}$ mice. Splenic T cells ((1)Thy1.2^{+}), splenic B cells ((2) B220^{+}), bone marrow derived (3) Mac1^{+}/Gr1^{lo} cells (monocytes), (4) Mac1^{+}/Gr1^{+} cells (granulocytes) and (5) Ter119^{+}/CD71^{+} cells (erythrocytes). All cells were derived from *Zbtb17*$^{fl/fl}$ and from *Vav-cre Zbtb17*$^{fl/fl}$ mice. PCR reactions with genomic DNA from tail (D) and FACS sorted fetal liver Ter119^{+}ckit^{-} (T) and Ter119^{-}c-kit^{+} (K) cells from mice with the indicated different genotypes (*Zbtb17*$^{fl/fl}$, *Zbtb17*$^{fl/+}$, *Vav-cre Zbtb17*$^{fl/fl}$, *Zbtb17*$^{fl/fl}$, *Vav-cre*). (H) Flow cytometry analysis of bone marrow a thymocytes from wildtype mice (WT), *Vav-cre Zbtb17*$^{+/+}$, *Vav-cre Zbtb17*$^{+/fl}$ and *Zbtb17*$^{fl/fl}$ mice using the indicated surface markers. Numbers in dot plots indicate percentages of each gate.

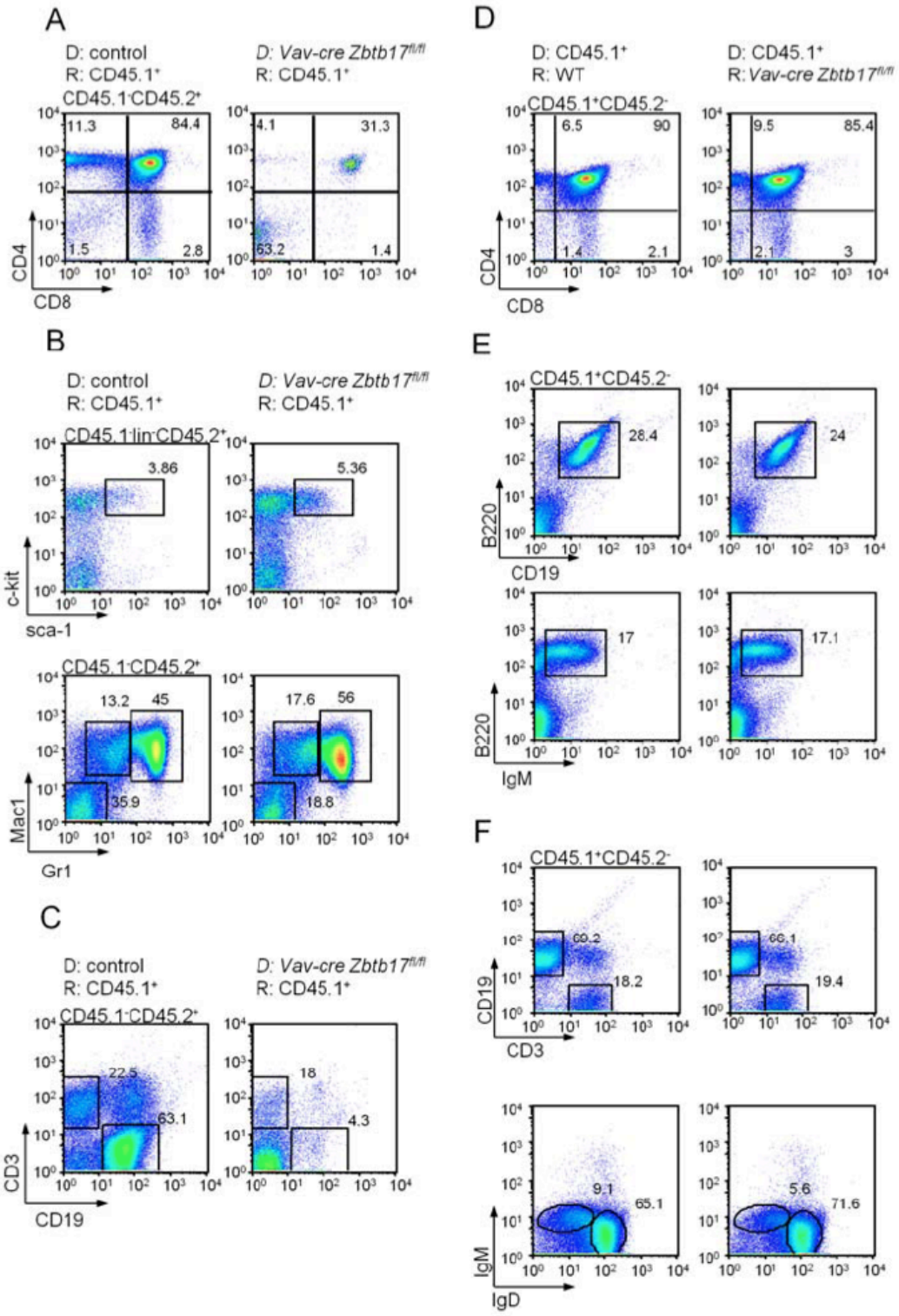
A
D: control
R: CD45.1+
CD45.1-CD45.2+
11.3
84.4
1.5
2.8
D: Vav-cre Zbtb17fl/fl
R: CD45.1+
4.1
31.3
63.2
1.4
CD4
CD8
B
D: control
R: CD45.1+
CD45.1-lin-CD45.2+
3.86
c-kit
sca-1
D: Vav-cre Zbtb17fl/fl
R: CD45.1+
5.36
CD45.1-CD45.2+
13.2
45
35.9
Mac1
Gr1
17.6
56
18.8
C
D: control
R: CD45.1+
CD45.1-CD45.2+
22.5
63.1
CD3
CD19
D: Vav-cre Zbtb17fl/fl
R: CD45.1+
18
4.3
D
D: CD45.1+
R: WT
CD45.1+CD45.2-
6.5
90
1.4
2.1
CD4
CD8
D: CD45.1+
R: Vav-cre Zbtb17fl/fl
9.5
85.4
2.1
3
E
CD45.1+CD45.2-
28.4
B220
CD19
24
17
B220
IgM
17.1
F
CD45.1+CD45.2-
69.2
18.2
CD19
CD3
66.1
19.4
9.1
65.1
IgM
IgD
5.6
71.6

Figure S2

Figure S2. Progenitors from Miz-1 deficient mice have homing capacity and reconstitute the immune system and developmental defects in Miz-1 deficient mice are cell intrinsic

Lethally irradiated recipient mice ($CD45.1^+$) were reconstituted with lineage depleted progenitor cells ($B220^-CD19^-Ter119^-$) from either control ($CD45.2^+$) or *Vav-cre Zbtb17*$^{fl/fl}$ ($CD45.2^+$) mice. Flow cytometric analysis was performed 3-4 month after transplantation on $CD45.1^-CD45.2^+$ cells. (A) Thymocytes were analyzed by flow cytometry using antibodies against CD45.1, CD45.2, CD4 and CD8. (B) Bone marrow cells were analyzed by flow cytometry using antibodies against CD45.1, CD45.2, lin, ckit, sca-1, Mac-1 and Gr-1. (C) Flow cytometric analysis of spleen cells stained with antibodies CD45.1, CD45.2, CD3 and CD19. All cells were pre-gated as indicated. Numbers on gates indicate percentages. Data represent three independent experiments. D: donor, R: recipient.

Lethally irradiated control ($CD45.2^+$) mice or *Vav-cre Zbtb17*$^{fl/fl}$ ($CD45.2^+$) were reconstituted with lineage depleted progenitor cells ($B220^-CD19^-Ter119$) from wild type mice ($CD45.1^+$). Flow cytometric analysis was performed 3 months after transplantation on $CD45.1^+CD45.2^-$ cells (D) Thymocytes were analyzed by flow cytometry using antibodies against CD45.1, CD45.2, CD4 and CD8. (E) Bone marrow cells were analyzed by flow cytometry using antibodies against CD45.1, CD45.2, B220, IgM, CD19. (F) Flow cytometric analysis of splenic B cells with antibodies against CD45.1, CD45.2, CD19, CD3, IgM and IgD. All cells were pre-gated as indicated. Numbers on gates indicate percentages. Data represent three independent experiments. D: donor, R: recipient.

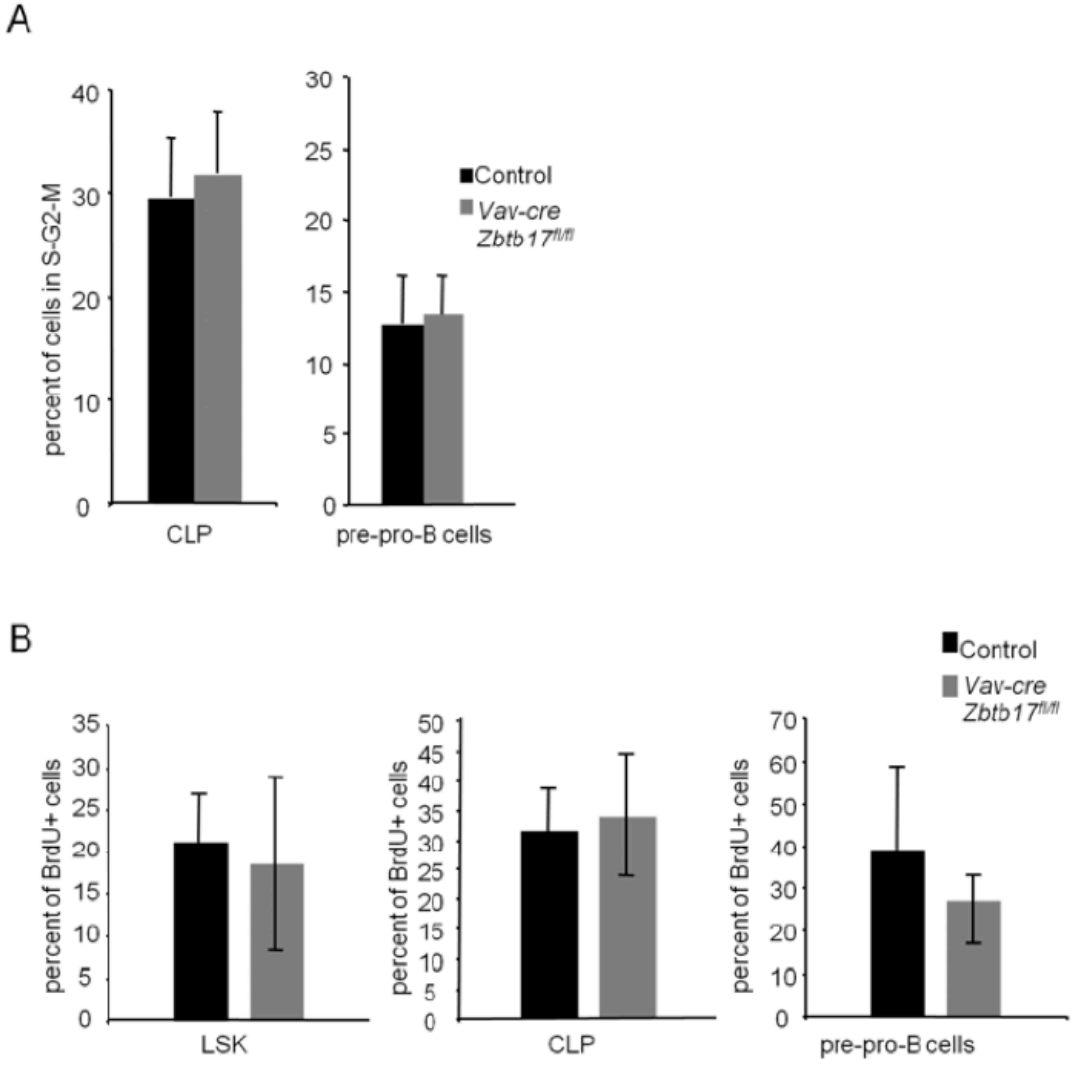
A
percent of cells in S-G2-M
40
30
20
10
0
CLP
30
25
20
15
10
5
0
pre-pro-B cells
Control
Vav-cre
Zbtb17fl/fl
B
percent of BrdU+ cells
35
30
25
20
15
10
5
0
LSK
50
45
40
35
30
25
20
15
10
5
0
CLP
70
60
50
40
30
20
10
0
pre-pro-B cells
Control
Vav-cre
Zbtb17fl/fl

Figure S3

Figure S3. Cell cycle analysis of B cell progenitors from Miz-1 deficient mice

(A) Cell cycle analysis using Propidium iodide staining on sorted and permeabilized CLPs (lin^-IL-$7R^+$sca-1^{med}c-kit^{med}) and pre-pro-B cells ($lin^-CD19^-B220^+CD43^+$). Shown are the percent of S-G2-M (Synthese-Mitose-Gap2 phase) cells from control mice (black) and *Vav-cre Zbtb17*$^{fl/fl}$ (gray). Data are representing three independent experiments. (B) BrdU (Bromodeoxyuridine) labeling experiments were performed on control and *Vav-cre Zbtb17*$^{fl/fl}$ mice. LSK (lin^-IL-$7R^-$sca-1^+c-kit^+), CLP (lin^-IL-$7R^+$sca-1^{med}c-kit^{med}) and prepro-B cells ($lin^-CD19^-B220^+CD43^+$). Bone marrow cells were analyzed for BrdU positive cells. Data represent four mice analyzed in two experiments for each genotype.

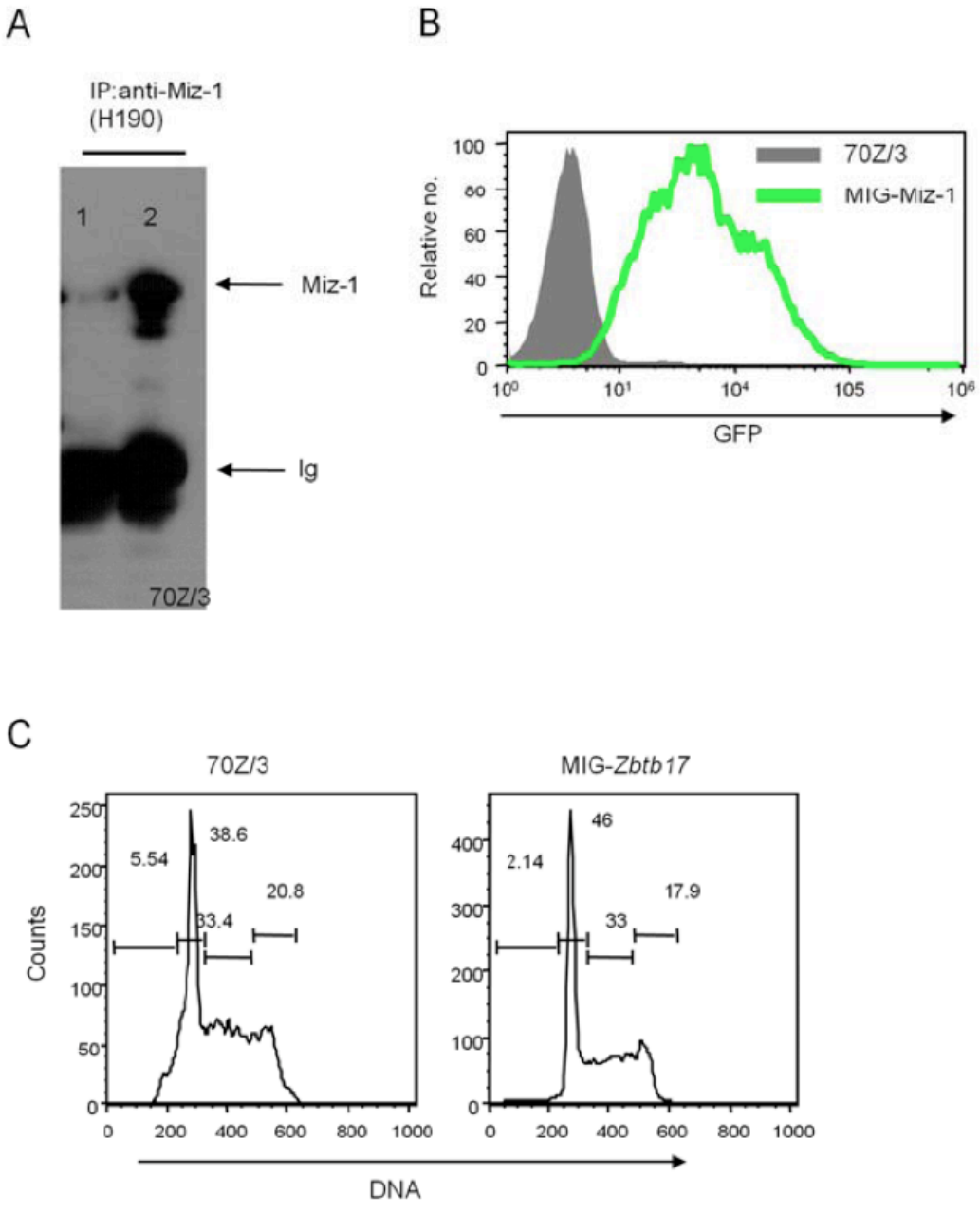
A
IP:anti-Miz-1
(H190)
1
2
Miz-1
Ig
70Z/3
B
Relative no.
70Z/3
MIG-Miz-1
GFP
C
70Z/3
MIG-Zbtb17
Counts
5.54
38.6
20.8
33.4
2.14
46
17.9
33
DNA

Figure S4

Figure S4. Expression of Miz-1 in 70Z/3 and in 70Z/3 cells retrovirally transduced to express Miz-1

70Z/3 cells were tranduced with MIG-*Zbtb17* and 5 days after transduction GFP^+ cells were sorted by FACS. (A) Immuno-precipitation of the Miz-1 protein in 70Z/3 (1) cells and 70Z/3 cells transduced with MIG-*Zbtb17* (2). Precipitates were generated with an anti-Miz-1 antibody (H190), separated by SDS-PAGE and transferred to a solid support. Miz-1 proteins were revealed with the Miz-1 monoclonal antibody 10E2. (B) Flow cytometry of the expression of GFP in 70Z/3 cells (gray line) and MIG-*Zbtb17* expressing 70Z/3 cells (green line). (C) Cell cycle analysis using Propidium iodide staining on permeabilized 70Z/3cells and MIG-*Zbtb17* expressing 70Z/3 cells.

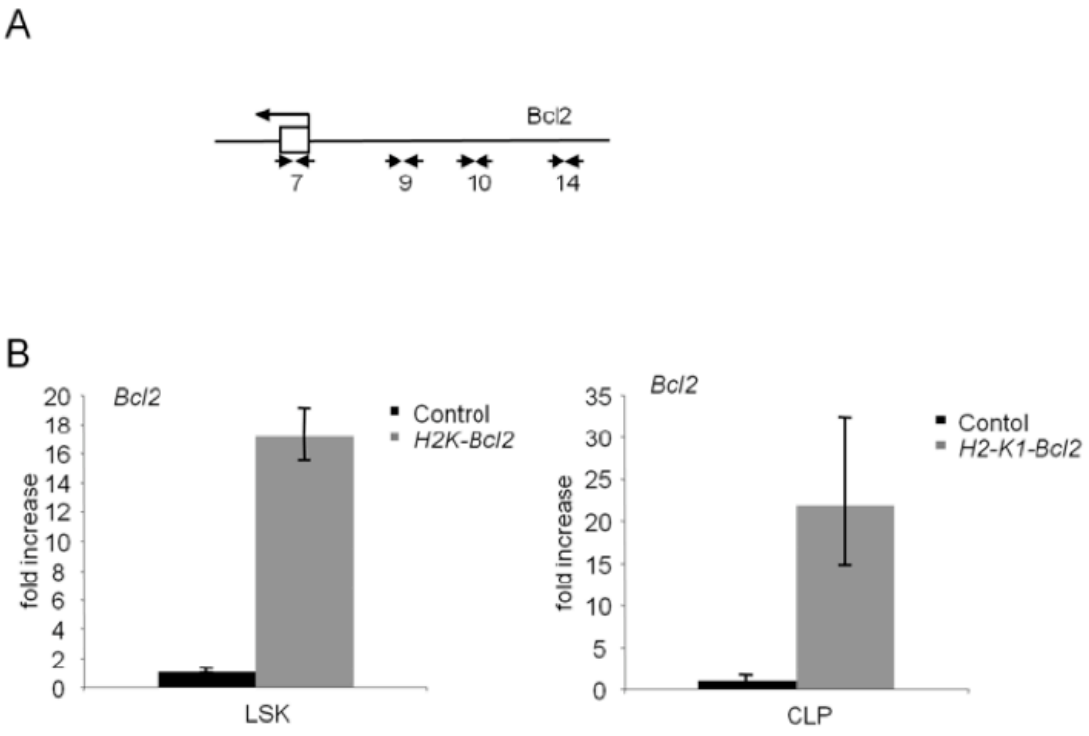
A
Bcl2
7
9
10
14
B
Bcl2
fold increase
20
18
16
14
12
10
8
6
4
2
0
Control
H2K-Bcl2
LSK
Bcl2
fold increase
35
30
25
20
15
10
5
0
Contol
H2-K1-Bcl2
CLP

Figure S5

Figure S5. Schema of the *Bcl-2* promoter region and *Bcl-2* expression in progenitor cells from *H2-K1-Bcl-2-tg* mice

(A) Bcl-2 locus, showing the most 5' non-coding exon (open box) and locations of primers (black triangles). (B) LSK cells ($lin^-sca\text{-}1^+$ kit^+) or CLP ($lin^-sca\text{-}1^+c\text{-}kit^{med}IL\text{-}7R^{med}$) cells from the bone marrow of control or *H2-K1-Bcl-2* transgenic mice were isolated by FACS sorting. RNA was extracted, reverse-transcribed and used for quantitative-PCR. Expression of *Bcl-2* was measured in cells from *H2-K1-Bcl-2* transgenic mice and was normalized to the expression of the *Gapdh* gene and is presented as the fold induction relative to cDNA from wt control mice (set as 1-fold). The data indicate a significantly elevated *Bcl-2* expression in the analyzed cells from the transgenic mice compared to control cells. The data set represent one experiments for each cell type, done in triplicates. Error bars indicate the standard deviation (±S.D.).

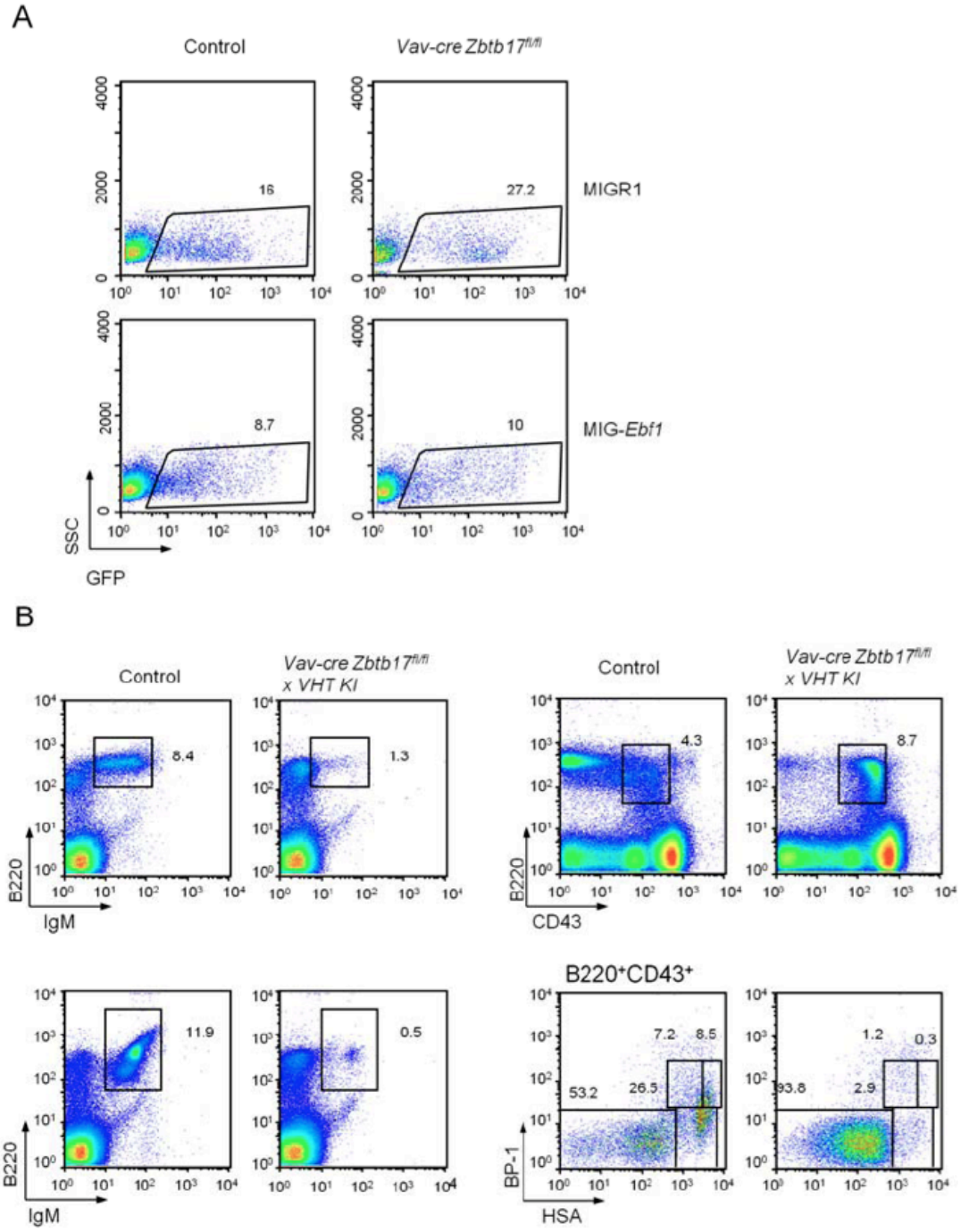
A
Control
Vav-cre Zbtb17fl/fl
MIGR1
MIG-Ebf1
16
27.2
8.7
10
SSC
GFP
B
Control
Vav-cre Zbtb17fl/fl x VHT KI
B220
IgM
CD43
8.4
1.3
4.3
8.7
11.9
0.5
B220+CD43+
BP-1
HSA
53.2
26.5
7.2
8.5
93.8
2.9
1.2
0.3

Figure S6

Figure S6. Transduction of progenitors with different retroviruses (MIGR1 and MIG-*Ebf1*) and developmental defects in Miz-1 deficient mice cannot be rescued by a constitutively expressed immunoglobulin heavy-chain

(A) Progenitor cells were transduced with control (MIGR1) or Ebf containing (MIG*Ebf1*) retrovirus. After transduction, cells were analyzed for GFP expression before cell sorting. Numbers indicate percentages of transduced (GFP^+) cells. (B) Flow cytometry analyzes of bone marrow from control mice or *Vav-cre Zbtb17*$^{fl/fl}$ *VHT-KI* mice were analyzed by FACS cells with antibodies against B220, IgM, CD43, CD19, HSA and BP-1. $B220^+/CD43^+$ cells were electronically gated and analyzed for HSA and BP-1 expression. Numbers on gates indicate percentages. Data represent three independent experiments.

Table S1: Primer for ChIP

Primer (Pr.)	Sequence	Distance to Inr (bp)
bcl_2_7f	GACCGGGACCGGAATCC	+ 349
bcl_2_7r	AGCTGCTTTTTTTCCCGTTTC	+ 424
bcl_2_9f	GCCCGGCCTCTTACTTCATT	- 50
bcl_2_9r	GGAGGGCTTTCTTTCTTCTTTTTT	- 144
bcl_2_10f	GCTGGCTGGACGTGCCTATA	- 363
bcl_2_10r	TTGTGCCCATCCTGTTAGAAAA	- 438
bcl_2_14f	CCATGACCCTAACACGGCATA	- 1352
bcl_2_14r	GAAGCCGTCTCAGGTTGCAT	- 1428
Socs1_2F	GAATCCGCCCGGAGCTT	+ 459
Socs1_2R	TCTGGAAGGGTTCCGCATAC	+ 534
Socs1_3F	GAGACGGTTCCTCTAACTTGGTTTC	- 125
Socs1_3R	GAACACAAGATTCCGGTTGGA	- 204
Socs1_4F	CGGCTCCAACCGGAATCT	- 194
Socs1_4R	GGGCGAAGGTAGAGCAAAAGA	- 267
Socs3_8F	CCTTCCCTTTCTTTTGATCC	- 447
Socs3_8R	GCTTGGAACTGTACATGAGGA	- 532
Socs3_9F	TCCAGAAGTGGAGGAGACAC	- 656
Socs3_9R	GTCTGTCACCGAAGAACCAG	- 740
Socs3_10F	GGACCCTCTCTTGCCTCTAC	- 1153
Socs3_10R	CCTTTCACCTCAATCACCTG	- 1250
P15_chip_F	AGGAAAAGCCCGGAGCTAAC	- 152
P15_chip_R	CCTGGGCTCAGCTTCATTAC	- 246

Primer names, sequences and distance to the Initiator (Inr) used in this study for Chromatin-immuno-precipitation (ChIP)

Table S2: Real-time assays on demand (Applied Biosystems)

Assay ID	Gene symbol	Gene name
Mm00456421_m1	Ikzf1	IKAROS family zinc finger 1
Mm00395519_m1	Ebf1	early B-cell factor 1
Mm00493500_m1	Dntt	deoxynucleotidyltransferase, terminal
Mm00441699_m1	Tcf12	transcription factor 12
Mm01270936_m1	Rag1	recombination activating gene 1
Mm00501300_m1	Rag2	recombination activating gene 2
Mm00477635_m1	Bcl6	B-cell leukemia/lymphoma 6
Mm01175597_m1	Tcff3	transcription factor E2a
Mm00435501_m1	Pax5	paired box gene 5
Mm99999915_g1	Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Mm00477631_m1	Bcl2	Apoptosis regulator Bcl-2
Mm00437783_m1	Bcl2l1	BCL2-like 1
Mm00782550_s1	Socs1	suppressor of cytokine signaling 1
Mm00545913_s1	Socs3	suppressor of cytokine signaling 3
Mm00494286_m1	Zbtb17	zinc finger protein 100 (Miz-1)
Mm00432050_m1	Bax	BCL2-associated X protein
Mm00432042_m1	Bad	BCL2-associated agonist of cell death

All Real-time assays used were obtained from Applied Biosystems (“assays on demand”). Shown are the assay IDs, gene symbols and gene names.

Supplemental Experimental Procedures

Mice.

Mice were housed at the animal facility of the "Institut für Zellbiologie", University of Essen Medical School or at the animal facility at the "Institut de recherche clinique de Montréal" (IRCM) in single ventilated cages and under specific pathogen free conditions. Unless specified otherwise, mice were used for analysis at 4 to 12 weeks of age. The following mice were used in this study and maintained on the C57BL/6 background: *CD19-cre* (Rickert *et al*., 1997), *Vav-cre* (de Boer *et al*., 2003), *VHT-KI* (Cascalho *et al*., 1996) and *H2K-Bcl-2* (Domen *et al*., 1998; Kondo *et al*., 1997).

Antibodies and lineage panel.

Monoclonal antibodies were from BD Bioscience (clone identified in parentheses): CD3 (145-2C11), Thy1.2 (53-21), CD4 (129.19 and RM 4-5), CD8 (53- 6.7), CD25 (PC61), CD44 (IM7), TCRβ (H57-597), TCRγδ (GL3), CD43 (S7), CD19 (1D3), B220 (RA3-6B2), pre-BCR (SL156), BP-1 (6C3) Gr-1 (RB6-8C5), Mac-1 (M1/70), Ter-119 (Ter-119), Pan-NK (DX5) NK1.1 (PK136), HSA (M1/69), CD21 (7G6), CD23 (B3B4), IgD 11-26.c2a) and IgM (R6-60.2). To analyze the bone marrow lineage negative population, lineage marker negative cells (Lin) were selected by staining cells with the biotinylated-antibodies against CD3, CD8, B220, Gr-1, Mac-1, Ter-119, CD5, IgM and NK1.1, followed by incubation with streptavidin-coupled antibody. To analyze the pre-pro-B cell population the

lineage marker (pre-B lin) CD11b, Gr-1, Ter119, IgM, TCRβ, TCRγδ, CD8, CD4, CD3, NK1.1, Ly-6c and CD5 were used.

Cell cycle analysis.

Propidium iodide (Pi) staining: Cell cycle analysis was performed using Propidium iodide (Pi) staining on FACS sorted and permeabilized cell. Cells were sorted in modified 'Krishan buffer'(Dressler et al., 1988; Krishan, 1975) (0.1% sodium citrate; 0.3% NP-40; 0.05 mg/ml propidium iodide; 0.02 mg/ml RNaseA) and incubated on ice for 30 min. BrdU labeling: Mice were injected with 100 μg/g body weight. After 18h cells were collected from hematopoietic organs and stained with indicated antibodies. After staining with surface antibodies, cells were washed with Perm/wash (BD, biosciences), fixed and permeabilized with Cytofix/Cytoperm buffer for 20 min at 4°C. Cells were washed with Perm/Wash buffer and incubated with DNaseI (300 μg/ml) for 1hr at 37°C. After an additional washing step (Perm/wash buffer) cells were stained with anti-BrDU for 30 min at room temperature.

Generation of conditional Zbtb17 deficient mice.

The Zbtb17 targeting construct was generated from a 5.9-kb *Bam*HI fragment containing exons 3 to 11 subcloned from 129Sv mouse genomic phage library. The distal loxP site was introduced in a *Hind*III site (insert an additional *Xba*I and *Spe*I site) 5' of exon 3 and the neo-cassette was integrated in a NheI site 3' of exon 4 (Supplementary Fig. S1). The

targeting construct was linearized with *Not*I and electroporated in R1 ES cells, which were screened by Southern blot analysis after neomycin/ganciclovir selection using an external 700 bp fragment (*Sph*I/*Bgl*II) from the 5' region as well as an external 300 bp fragment (*Eco*RI/*Bam*HI) from the 3' region on XbaI/SpeI digested genomic DNA. Cre recombinase was transiently transfected and ES cell clones were analyzed with primers P1-P4 for recombination. Two independently derived embryonic stem (ES) clones were injected into C57BL/6 blastocysts. The resulting chimeric mice produced the expected heterozygous offspring (*Zbtb17*$^{wt/\Delta POZ}$ and *Zbtb17*$^{wt/fl}$, Supplementary Figure 1), which were phenotypically indistinguishable from wt littermates (data not shown). Chimeric mice were mated with C57BL/6 mice for more than 8 generations. To generate *Zbtb17*$^{\Delta POZ\ /\Delta POZ}$ and *Zbtb17*$^{fl\ /\Delta POZ}$ ES cell lines, *Zbtb17*$^{wt/\Delta POZ}$ cells were targeted for a second time with same targeting construct. After cre recombination, the resulting ES cells were injected into C57BL/6 blastocysts.

RNA isolation, cell sorting and real-time PCR.

RNA was extracted using TRIZOL reagent (Invitrogen) and reverse transcribed using Superscript II (Invitrogen) following the manufacturer's protocol. Real time PCR was performed in a 20µl reaction volume containing 900 nM of each primer, 250 nM TaqMan probe and 1x TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Reactions were monitored on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and Mx 3005 (Stratagene). Assays on Demand

(Applied Biosystems) were used. To correct for the amount of cDNA added to any individual reaction, PCR was performed in duplicates or triplicates. The expression of the gene of interest was calculated relative to the Gapdh mRNA (Δct). Assays used for experiments are listed in Supplementary Table S2.

Cell culture.

OP9 stroma cells were cultured as in αMEM containing 20% FCS (vol/vol); 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2.2 g/l Sodium bicarbonate. Bone marrow progenitor cells were cell sorted and cultured on OP9 stroma cells or stroma-free conditions in Opti-MEM (10% (vol/vol) 100 U/ml Penicillin, 100 µg/ml Streptomycin 50µM β-mercaptoethanol) supplemented with 10 ng/ml SCF, and/or 5 ng/ml Ft3L and/or 5 ng/ml IL-7 for co-culture and with 5 ng/ml Ft3L and/or 10 ng/ml IL-7 in stroma-free conditions. OP9-culture for myeloid differentiation was performed in Opti-MEM (see above) supplemented with SCF, IL-3, IL-6, Flt3L, M-CSF, GM-CSF, G-CSF (10 ng/ml; only Flt3L 5ng /ml).

PCR analysis.

Genomic DNA from mouse tissues or from FACS sorted cells was analyzed using primers for the Zbtb17 genomic region: P1: 5'- GTATTCTGCTGTGGGGCTATC-3', P2: 5'-GGCTGTGCTGGGGGAAATC-3', P3: 5'- TGCCTGCCTCTGGGTCTCC-3' and P4: 5'-GGCAGTTACAGGCTCAGGTG-3'. D3Mit73 forward: 5'-

CCACATTCTGATAAGAATTGAGAA-3' reverse: 5'-GACACTGTATTCCAGAAAAACACA-3'. Amplified PCR products were separated by gel electrophoresis followed by ethidium bromide staining.

Morpholinos.

anti-Socs1 Morpholino: CTGCCACCTGGTTGCGTGCTACCAT; control-Morpholino: CCTCTTACCTCAGTTACAATTTATA

Immunoprecipitation.

Embryonic fibroblasts (MEFs) were lysed and extracted on ice (30 min) in TNN-Lysis buffer (50 mM Tris-HCl; pH7,5; 120 mM NaCl; 5 mM EDTA; 0.5% NP-40; 10 mM Na4P2O7; 2 mM Na3VO4; 100 mM NaF). 500 µg of protein lysate was incubated with an antibody against Miz-1 (H-190; Santa Cruz) over night at 4°C. Proteins were separated on a 10% SDS-PAGE and were then transferred to Immobilon-P Transfer membrane (Millipore). Protein blots were probed with the monoclonal anti-Miz-1 antibody (10E2).

Adoptive transfer.

Recipient mice were lethally irradiated with 9.6 Gy (CD45.1 or CD45.2). Irradiated mice were reconstituted with fetal liver cells or lineage depleted bone marrow cells ($Ter119^-$ $B220^-CD19^-$).

Western blotting.

Cells were lysed and extracted on ice (30 min). Lysis buffer contains 20 mM Tris-HCl (pH 7.5); 420 mM NaCl; 2 mM EDTA; 1% NP-40 and protease inhibitors (Roche). Proteins were separated on a SDS-PAGE and were then transferred to Hybond-C membranes (Amersham) and probed with indicated antibodies.

Antibodies.

The following antibodies were used in this study: anti-Socs-1 (4H1) (Millipore), antiactin (I-19) (Santa-Cruz), anti-Miz-1 (H-190) (Santa-Cruz). anti-Miz-1 (10E2) (Prof. Martin Eilers lab, Wuerzburg, Germany).

RNA isolation and RT PCR.

Tissue RNA was isolated by preparing single cell suspension and using TRIZOL Reagent (Invitrogen). Cells from different developmental stages were FACS sorted and TRIZOL Reagent (Invitrogen) was applied. RT-PCR was performed using Superscript II (Invitrogen) following manufacturer's protocol.

Primer:

Zbtb17-1F: 5'-CCTCTACGCTTGTGATTCCT-3'

Zbtb17-1R: 5'-GGAGCACCTTCTGAGTCCTA-3'

Mcl1-1F: 5'-TCAAAGATGGCGTAACAAACTGG-3' (Malin et al., 2010)

Mcl1-1R: 5'-CCCGTTTCGTCCTTACAAGAAC-3' (Malin et al., 2010)

GAPDH F: 5’-TGTCTTCACCACCATGGAGA-3’

GAPDH R: 5’-CGGCCATCACGCCACAGCTT-3’

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Chapter II

IL-7R-dependent survival and differentiation of early T-lineage progenitors is regulated by the BTB/POZ domain transcription factor Miz-1

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Running title: Miz-1 in pro-T cell survival and differentiation

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For this study, I generated all the figures and supplementary figures in the paper. Tarik Möröy, Christian Kosan and I designed the research and analyzed the results. Christian Kosan and myself performed all the experiments and Lothar Vassen helped with providing *SOCS1* promoter construct. Tarik Möröy and I wrote the paper. Tarik Möröy also provided funding.

Abstract

T cells originate from early T lineage precursors that have entered the thymus and differentiate through well-defined steps. Mice deficient for the BTB/POZ domain of zinc finger protein-1 (Miz-1) almost entirely lack early T lineage precursors and have a $CD4^{-}CD8^{-}$ to $CD4^{+}CD8^{+}$ block causing a strong reduction in thymic cellularity. Miz-$1^{\Delta POZ}$ pro-T cells cannot differentiate in vitro and are unable to relay signals from the interleukin-7R (IL-7R). Both STAT5 phosphorylation and Bcl-2 up-regulation are perturbed. The high expression levels of *SOCS1* found in Miz-$1^{\Delta POZ}$ cells probably cause these alterations. Moreover, Miz-1 can bind to the *SOCS1* promoter, suggesting that Miz-1 deficiency causes a deregulation of SOCS1. Transgenic overexpression of Bcl-2 or inhibition of *SOCS1* restored pro-T cell numbers and their ability to differentiate, supporting the hypothesis that Miz-1 is required for the regulation of the IL-7/IL-7R/STAT5/Bcl-2 signaling pathway by monitoring the expression levels of SOCS1.

Introduction

Hematopoietic precursors differentiate into mature blood cell lineages through a series of well-coordinated steps. T cells are generated in the thymus, which is continuously replenished with lymphoid progenitors from the bone marrow via the bloodstream.[1] Early lymphoid progenitors (ELPs) enter the thymus and become early T lineage precursors (ETPs), defined as $Lin^{-/low}$, $CD117^{high}$, and $CD25^{-}$.[2] The capacity of ELPs to migrate to the thymus has been attributed to their expression of CCR9.[3,4] In addition to $CCR9^{+}$ ELPs, other progenitors, such as CLPs, may home to the thymus and generate T cells. Recently, Ly6D has been used to identify the branch point of CLPs that gives rise to the first stages of B-cell development, B cell-biased lymphoid progenitor (BLP), and all-lymphoid progenitor (ALP), which contribute to the T-cell development.[5]

The subsequent development of ETPs starts with $CD4^{-}CD8^{-}$ double-negative 1 (DN1) cells. DN1s are subdivided into DN1a-e according to the expression of CD24 and CD117, DN1a/b corresponding to the ETP subset.[6] DN1s give rise to DN2a-b cells, which differentiate into DN3s, subdivided into DN3a-b based on their size and CD27 expression.[7] DN3a cells that have productively rearranged the T-cell receptor β-gene (*TCR*-β) become activated by TCR-dependent signals (β-selection), differentiate into DN3b, and become DN4 pre-T cells. The newly developed DN4s become $CD4^{+}CD8^{+}$ double-positive (DP) cells and undergo positive/negative selection before reaching the periphery as mature $CD4^{+}$ or $CD8^{+}$ T cells.[8]

Pro-T-cell differentiation steps depend on the expression of Notch ligands, mainly δ-like ligand 1 (DL1) and DL4 on thymic stroma,[9] and on cytokines, such as interleukin-7 (IL-7).[10] Notch signaling assures lineage commitment, survival, and development of ETPs into further DN subsets.[11] The IL-7/IL-7R pathway drives proliferation, survival, and progression of pro-T cells,[12] and also induces the rearrangement and transcription of the *TCR*-γ locus.[13] The IL-7R signaling activates Janus kinase 1/3 (Jak1/3), which phosphorylate signal transducer and activator of transcription 5 (STAT5). Phosphorylated STAT5 then activates the transcription of IL-7–dependent target genes.[14]

A key player in IL-7R cascade is the maintenance of cell survival by promoting a favorable balance of B-cell lymphoma-2 (Bcl-2) family members.[15] The expression of the antiapoptotic protein Bcl-2 is up-regulated after IL-7 stimulation. Some studies have shown that the up-regulation of Bcl-2 can be STAT5-dependent.[16–18] Other studies have shown that STAT5-mediated activation of AKT protein regulates the glucose metabolism of the cell and maintains prosurvival and growth functions.[19] Suppressor of cytokine signaling 1 (SOCS1) is known to inhibit phosphorylation of STAT proteins by directly binding to the Jak proteins and therefore inhibiting all further downstream signaling events to ensure a return to steady-state homeostasis after cytokine responses.[20]

Miz-1 (*Zbtb17*) is a transcription factor of 87 kDa that is composed of 13 zinc finger domains at its carboxy-terminal end and of a BTB/POZ domain at its N-terminus.[21] It has originally been identified as an interacting partner of the c-Myc proto-oncogene.[21] The BTB/POZ domain of Miz-1 is essential for its trans-activating functions and for its capacity

to bind to the DNA.[22] Miz-1 can activate or repress the transcription of its target genes depending on its interacting partner. For example, Miz-1 acts as a transcriptional trans-activator by binding to core elements of RNA Pol II–dependent target gene promoters and by recruiting coactivators, such as the histone deacetylase p300/CBP.[22–24] Miz-1 can also be a transcriptional trans-repressor, for example, by recruiting c-Myc to an E-box-independent site around the initiator of its target gene promoters. Genes that encode the negative cell cycle regulators CDKN2b[22,25] and CDKN1a[26,27] have been validated as direct Miz-1 targets that are repressed by Miz-1/c-Myc complex.

Because Miz-1 deletion is lethal,[28] we have used conditional Miz-1–deficient mice, in which the exons coding for the BTB/POZ domain[29] are deleted via Cre recombinase.[30] This deletion generates a truncated form of Miz-1 that lacks the BTB/POZ domain and thus eliminates specifically its activity as a transcriptional regulator in all hematopoietic cells.[22,31] In this report, we describe findings that identify Miz-1 as a new regulator of early T-cell differentiation, at stages where the IL-7/IL-7R interaction assures survival and lineage commitment. Our data suggest that Miz-1 regulates the expression of SOCS1 and thus controls the activation of STAT5 phosphorylation in response to IL-7 to gauge the level of Bcl-2 expression required for the survival and development of ETP/DN1 and DN2 cells.

Methods

Mice

All mice used in this study are described in the supplemental Data (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Mice have been bred on C57BL/6 background for at least 10 generations and were maintained in Specific-Pathogen-Free Plus^{+}. The Institutional Review Board approved all animal protocols, and experimental procedures were performed in compliance with the Institut de recherches cliniques de Montréal guidelines.

Antibodies and FACS analysis

Antibodies were from BD Biosciences, except when indicated. DN1-DN4, ETP, ELP, ALP, and BLP were analyzed using lineage marker-negative cells (Lin^{-}) selected by staining with biotinylated antibodies described in the supplemental Data, followed by streptavidin-peridinin-chlorophyll protein-Cy5.5 or phycoerythrin-Cy5. Cells were analyzed with a FACSCalibur, FACSScan, or LSR (BD Biosciences). Cell sorting was performed using a MoFlo cell sorter (Cytomation).

Intracellular staining and cell activation

After 1 hour of incubation at 37°C to shut off endogenous signaling, cells were activated with or without 10 ng/mL IL-7 (PeproTech 217-17) for 18 hours to detect Bcl-2 or 15 minutes for pSTAT5. To verify Bcl-2, cells were fixed with Cyto Fixation/Permeabilization kit (BD 554714). For pSTAT5, cells were fixed with formaldehyde (BD cytofix 554655) and permeabilized with methanol (BD phosflow Perm III 558050). For gene expression

profiling, DN1-DN2 cells were sorted, activated, and lysed in TRIZOL Reagent (Invitrogen). RNA was extracted as described in the supplemental Data.

OP9DL1 cocultures

OP9 stromal cells expressing DL1[32] were plated at 2.5×10^4 cells/well and cocultured with sorted DN1-4 and ETPs/ELPs. The cells were incubated in Opti-α-modified Eagle medium supplemented with 1 to 5 ng/mL IL-7 and 5 ng/mL Fms-like tyrosine kinase 3 ligand (PeproTech) and charcoal-stripped fetal bovine serum.

ChIP assay

Assays were performed using ChIP-IT Express (Active Motif) on purified primary $CD4^-CD8^-$ DN cells (preparation purity > 90% by Auto-MACS), which rested at 37°C 1 hour in phosphate-buffered saline, or on SCID.adh murine thymic lymphoma (clone P6D4).[33] Variation in the cell lysis was as follow: first lysis with 5mM piperazine-N, N-bis[2-ethanesulfonic acid], pH 8, 85mM KCl, 0.5% NP-40, 1mM phenylmethylsulfonyl fluoride, protease inhibitors cocktail (complete Mini; Roche Diagnostics), and second with 50mM Tris-HCl, 10mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulfate, protease inhibitor cocktail, and 1mM phenylmethylsulfonyl fluoride. After sonication (Branson Digital Sonifier), immunoprecipitation was performed using salmon sperm DNA/Protein G-agarose (Upstate Biotechnology) and 10 μg of rabbit anti–Miz-1 (H190; Santa Cruz Biotechnology) or rabbit control IgG antibodies (Abcam).

SOCS1 knockdown

Sorted DN1 or $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) were cultured in Opti-α–modified Eagle medium supplemented with 1 ng/mL IL-7, 5 ng/mL Fms-like tyrosine kinase 3 ligand, and 10 ng/mL stem cell factor for 1 hour. Cells were then incubated with Endo-Porter reagent (Gene Tools) for delivering fluorescein isothiocyanate-morpholino oligo against *SOCS1* mRNA or a control morpholino oligo in vitro. After 4 hours, the cells were transferred on OP9DL1 stroma layer and cultured for 6 to 20 days.

Statistical analysis

Two-tailed Student *t* tests were used to calculate *P* values. A *P* value < .05 was considered statistically significant.

Results

Miz-1$^{\Delta POZ}$ mice have severe defects in T-cell development

To investigate the role of Miz-1 during T-cell development, we used *Vav*-Cre Miz-1$^{flox/flox}$ mice (described in the supplemental Data, and hereafter named Miz-1$^{\Delta POZ}$). We observed that Miz-1$^{\Delta POZ}$ mice have a block at the transition from DN to DP cells (Figure 1A), which results in a 100-fold reduced thymic cellularity compared with a wild-type (WT) littermate (Figure 1B). The deletion of the POZ domain of Miz-1 also caused a 1000-fold reduction of αβ-T cells compared with a 7-fold reduction of γδ-T cells (Figure 1B), and a significant reduction of all DN subpopulations compared with controls (Figure 1C).

One of the most striking phenotypes of Miz-1$^{\Delta POZ}$ mice was the reduction of the DN1 population. DN1a-e subsets were reduced by 70- to 130-fold; and the ETP subset (DN1a/b), which is the most affected, showed a reduction of 230-fold compared with WT (Figure 1D-F). Similarly, DN2a and DN2b cells were reduced by 100- and 40-fold (Figure 1G), respectively, whereas DN3a and DN3b cell numbers were only reduced by 3- and 10-fold, respectively, in Miz-1$^{\Delta POZ}$ mice (not shown), suggesting that Miz-1 has an important function in ETP/DN1 and DN2 cells.

Lack of early T-cell precursors in the thymus of Miz-1 mutant mice

The few thymic ETPs that are present in Miz-1$^{\Delta POZ}$ mice were phenotypically normal according to the expression of CD117, CD44, CCR9, and CD135 (supplemental Figure 1A). Compared with ETPs, the frequencies of bone marrow ELPs or the ALP and BLP subsets of CLPs were not altered, and even present at higher frequencies compared with

WT (Figure 2A-C). Miz-1$^{\Delta POZ}$ ELPs showed normal expression levels of CD117 and CCR9, with a small reduction in CD135 expression (Figure 2A), and ALPs and BLPs showed normal expression of Ly6D (Figure 2B). Moreover, Miz-1$^{\Delta POZ}$ ELPs sorted from the bone marrow expressed T-lineage specific genes, such as *GATA3, Notch1, Rag1, Tdt*, and *E2A, Ikaros*, *c-Myb*, and *PU.1* at WT levels (Figure 2D), suggesting that Miz-1 deficiency does not alter the expression program related to T-lineage specification.

Similar to ETPs, ELPs were reduced in the blood of Miz-1$^{\Delta POZ}$ mice compared with WT controls (supplemental Figure 1B). To evaluate whether a homing problem was responsible for the observed lack of thymic ETPs, LSK progenitor cells from WT mice were sorted and transplanted into Miz-1$^{\Delta POZ}$ irradiated mice. These cells successfully reconstituted the thymus of Miz-1$^{\Delta POZ}$ mice. Sorted Miz-1$^{\Delta POZ}$ LSKs transplanted into WT mice showed the same phenotype as Miz-1$^{\Delta POZ}$ mice, generating a hypocellular thymus (not shown). These data indicate that the effect is cell intrinsic and not caused by a defect in the thymic stroma. Furthermore, the absence of early T-cell differentiation in the thymus does not seem to be related to aberrant Notch1 signaling in Miz-1$^{\Delta POZ}$ mice. Indeed, the intracellular expression of Notch1 and Notch1 target genes, such as *Notch1* itself and *Hes1*, were not reduced in Miz-1$^{\Delta POZ}$ cells (supplemental Figure 1C-D). In addition, no aberrant T-cell development in the bone marrow was noticeable, and no B-cell development was detected in the thymus of Miz-1$^{\Delta POZ}$ mice (not shown). Moreover, the expression of chemokine receptors CXCR4, CCR7, and CCR9 on DN subsets from Miz-1$^{\Delta POZ}$ mice was intact or even higher compared

with WT cells (supplemental Figure 2). Therefore, the observed T-cell development defects are most probably cell autonomous.

Nevertheless, sorted bone marrow or blood (not shown) ELPs and thymic ETPs from Miz-$1^{\Delta POZ}$ mice were unable to differentiate into mature T-cell stages in the presence of IL-7 (Figure 2E-F). It is thus probable that Miz-1 affects cytokine-dependent survival or proliferation signals needed for the intrathymic differentiation of ETP/DN1 cells.

ETP and DN1 from Miz-$1^{\Delta POZ}$ mice do not differentiate in vitro because of increased apoptosis

The developmental defect of ETPs and ELPs in vitro was also seen when sorted thymic DN1 cells from Miz-$1^{\Delta POZ}$ mice were cultured on OP9DL1. Sorted DN2 or DN3 cells from Miz-$1^{\Delta POZ}$ thymus survived better in vitro compared with DN1 cells but were still less efficient at generating DP cells compared with WT (Figure 3A). The development of γδ-T cells from Miz-$1^{\Delta POZ}$ sorted DN2 and DN3 was comparable with WT levels on OP9DL1 (not shown). This suggests that Miz-1 is important for ELP and ETP/DN1 survival and/or differentiation, but this requirement seems to decrease as cells reach the DN2 or DN3 stage. Indeed, the deletion of Miz-1 after the DN3 transition does not influence T-cell differentiation. The overall thymic cellularity (not shown) and development are normal in *Lck*-cre Miz-$1^{\Delta POZ}$ mice (supplemental Figure 3A-B).

To explain the decrease in DN differentiation, we sorted cells from Miz-$1^{\Delta POZ}$ mice and verified their expression of T-cell genes. *Rag-1*, *Rag-2*, and *Tdt* and genes encoding for *HEB*, *Idb2*, *E2A*, and *Egr1-3* were at WT levels, whereas *Idb1*, *Tgfb1*, *Notch1*, and *IL-7R* at

levels slightly higher than WT (supplemental Figure 3C). This confirms again that Miz-1 does not regulate the expression of genes specifying the T cell lineage. The negative cell cycle regulators *CDKN2b* and *CDKN1a* are direct Miz-1 target genes. Whereas *CDKN2b* could not be detected in thymocytes, the expression of *CDKN1a* was elevated in sorted DN1 and DN2 subsets (supplemental Figure 3D), where T-cell differentiation in Miz-1$^{\Delta POZ}$ mice is affected the most. Because *CDKN1a* regulates cell cycle, we next examined whether the thymic atrophy in Miz-1$^{\Delta POZ}$ mice and the absence of ETP/DN1 differentiation in vitro were the result of a reduction in cell division, cell proliferation, or an increase in apoptosis. In vivo bromodeoxyuridine (BrdU) labeling or propidium iodide staining did not show significant defects in cell cycle progression of Miz-1$^{\Delta POZ}$ pro-T cells (Figure 3B-C). Moreover, crossing *Vav*-cre Miz-1$^{\Delta POZ}$ mice with CDKN1a-deficient mice did not restore the DN to DP transition, confirming that the block seen in Miz-1$^{\Delta POZ}$ mice cannot be explained by a cell cycle defect (supplemental Figure 3E). By contrast, annexin V staining revealed increased apoptosis in DN1 (37%), DN2 (60%), and DN3 (26%) subpopulations of Miz-1$^{\Delta POZ}$ thymocytes compared with WT controls, but not in Miz-1$^{\Delta POZ}$ DN4 cells (8%; Figure 3D). These results indicated that Miz-1$^{\Delta POZ}$ pro-T cells exhibit an excessive cell death, particularly at the critical cytokine-dependent step (DN1 and DN2) of pro-T-cell differentiation.

Miz-1$^{\Delta POZ}$ pro-T cells lack an IL-7–dependent survival signal because of a deregulated *Bcl-2* expression

IL-7 signaling assures survival and proliferation in DN1-3 subsets mainly by controlling the increased expression of antiapoptotic Bcl-2 and Mcl-1, and by redistributing the cell-death proteins Bax and Bad.[15] In Miz-1$^{\Delta POZ}$ thymocytes, both CD127 (IL-7Rα) and CD132 (common γ-chain) are expressed at WT levels on all DN subsets (supplemental Figure 4). However, compared with the respective WT, mRNA level of *Bax* was elevated in particular in the DN2 subsets of Miz-1$^{\Delta POZ}$ mice, and *Bcl-2* expression was either at the WT level (DN1) or increased (DN2; Figure 4A). *Bcl-xL*, *Mcl-1*, *Bad*, and *Pim-1* expression levels were similar between WT and Miz-1$^{\Delta POZ}$ cells (Figure 4A). *Miz-1* expression was also at comparable levels in DN1 and DN2 cells isolated from WT thymus, with a decrease in its expression in DN4 pre-T cells (supplemental Figure 5A). *Miz-1* was also expressed in DP cells (supplemental Figure 5B), with a noticeable reduction in mature splenic CD3^{+} cells, consistent with a predominant role of Miz-1 in early pro-T-cell development. Because Miz-1 has been described to interact with *c-Myc*, we have evaluated *c-Myc* expression, which was at WT levels in all DN subsets isolated from Miz-1$^{\Delta POZ}$ mice (supplemental Figure 5C). Moreover, it has been shown that a mutated c-Myc allele, in which the valine residue (V) at position 394 is substituted by an aspartic acid (D), can no longer interact with Miz-1.[24] We have generated homozygous c-Myc^{V394D} knockin mice (described in supplemental Data). These mice did not show any defects in thymic development or in the overall cellularity of the thymus, indicating that the phenotype we observe in Miz-1$^{\Delta POZ}$ mice is probably c-Myc-independent (supplemental Figure 5D).

Expression levels of the Jak inhibitor *SOCS1* and, to a smaller extent, *SOCS3* were elevated in DN, but not in DP subsets of Miz-1$^{\Delta POZ}$ mice compared with controls (Figure 4A; supplemental Figure 5E). The already increased expression of *SOCS1* was still further inducible by IL-7 in DN1-2 from Miz-1$^{\Delta POZ}$ mice, albeit at a much lower extent than in WT cells (Figure 4B-C), contributing to *SOCS1* overexpression in the absence of a functional Miz-1. The induction of *Bcl-2* expression by IL-7 was almost completely abrogated in all Miz-1$^{\Delta POZ}$ pro-T cell subsets compared with WT cells (Figure 4B-C). In contrast, *Miz-1* mRNA expression was not induced by IL-7, making it doubtful that Miz-1 itself is an IL-7 effector gene (supplemental Figure 5F). These data suggest that the part of IL-7R signaling that is involved in protecting cells from apoptosis and promoting survival in early T-cell subsets is defective in Miz-1$^{\Delta POZ}$ cells. The elevated expression levels of *SOCS1* could be the responsible factor that prevents IL-7 from initiating signal transduction in Miz-1$^{\Delta POZ}$ DNs.

Miz-1 controls the IL-7/IL-7R signaling pathway by regulating SOCS1

IL-7 stimulation did not activate STAT5 phosphorylation in Miz-1$^{\Delta POZ}$ DN1 cells and was also less efficient in DN2, DN3 (not shown), and TCR-γδ cells compared with the respective WT control cells, despite similar STAT5A/B protein expression levels (Figure 5A-B). Similarly, even if DN2 cells expressed higher *Bcl-2* levels than DN1s, IL-7-mediated induction of Bcl-2 expression was completely blocked in all Miz-1$^{\Delta POZ}$ DN subsets at the protein level (Figure 5C). Given that Bcl-2 is a target of STAT5 and thus a downstream effector of IL-7R signaling, and that SOCS1 binds to the Jak proteins and

thereby blocks IL-7R signaling, we reasoned that one mechanism that could explain the lack of signaling observed in Miz-1$^{\Delta POZ}$ thymocytes is the regulation of SOCS1 by Miz-1 itself.

To determine whether Miz-1 can bind to the *SOCS1* promoter, we performed chromatin immunoprecipitation (ChIP) on purified primary DN cells from WT thymi. Quantitative polymerase chain reaction (PCR) analysis, using primers located around the initiator start site of *SOCS1* (Figure 5D), revealed a significant 1.8-fold enrichment using anti–Miz-1 antibodies compared with control IgG (Figure 5E). DN1 and DN2 cells, where Miz-1 is actually promoting survival, represent a small percentage of the total DN purified, probably causing the little enrichment obtained. We therefore confirmed this result in SCID.adh murine thymic lymphoma clone P6D4 (Figure 5F; $P \leq .01$), which highly expresses endogenous Miz-1 (supplemental Figure 6A-B) and validated the specific binding around the initiator using control primers upstream of *SOCS1* where Miz-1 did not bind (Figure 5E-F). As an additional control, we did not detect any enrichment with an anti–Miz-1 ChIP using primers designed to detect binding sites near or upstream of the initiator of *SOCS3* promoter (supplemental Figure 6C). These data indicate that Miz-1 is specifically binding to the initiator site of *SOCS1* but not *SOCS3* promoter in primary DN and P6D4 cells. To further validate the regulation of SOCS1 by Miz-1, we transduced P6D4 cells with a retroviral vector expressing Miz-1–IRES-GFP or a control empty vector (MIGR1-GFP; supplemental Figure 6D). Protein band intensities were quantified and, when GFP$^+$ sorted cells were stimulated with IL-7, P6D4 cells overexpressing 5 times more Miz-1 were less

efficient in up-regulating SOCS1 compared with control cells (supplemental Figure 6E). This experiment provided additional evidence that increased levels of Miz-1 repress SOCS1 expression.

Inhibition of SOCS1 or overexpression of Bcl-2 can restore the differentiation block of Miz-1$^{\Delta POZ}$ pro-T cells in vitro

To further test our hypothesis, we treated sorted Miz-1$^{\Delta POZ}$ LSK or DN1 cells with either a fluorescein isothiocyanate-labeled morpholino-oligonucleotide against *SOCS1* mRNA or a control morpholino and cocultured them on OP9DL1. After 6 to 20 days, Miz-1$^{\Delta POZ}$ LSK or DN1 cells treated with the morpholino against *SOCS1* mRNA developed into DN3 cells, whereas the control morpholino-treated cells did not differentiate (Figure 6A; supplemental Figure 7A-B). The efficiency of the transfection of fluorescein isothiocyanate-labeled morpholinos was monitored by flow cytometry, which was diluted out as cells proliferated (supplemental Figure 7C). Miz-1$^{\Delta POZ}$ LSK or DN1 went through similar cycles of cell division after 19 days on OP9DL1 as WT cells, although their mean fluorescence intensities were slightly higher. The reason for that higher fluorescence may be attributable to fewer cells in culture, which are apoptotic. The cells did not survive past the DN3 stage and did not generate DP cells compared with WT (Figure 6A). This suggested that high SOCS1 levels might indeed be responsible for the observed block of differentiation or lack of survival seen in Miz-1$^{\Delta POZ}$ ETP/DN1 cells by blocking IL-7R signaling.

It has previously been shown that overexpression of Bcl-2 restores defective T-cell development in mice lacking IL-7 or IL-7R.[34,35] Hence, to further investigate whether Miz-

1 controls the part of the IL7-R signaling that assures survival of early T-cell subsets through the induction of Bcl-2, we crossed Miz-1$^{\Delta POZ}$ mice with *H2K-Bcl-2* transgenic mice, which express high constitutive Bcl-2 levels throughout hematopoiesis.[36] In contrast to the ETPs from Miz-1$^{\Delta POZ}$ mice, ETPs from Miz-1$^{\Delta POZ}$ x Bcl-2Tg animals now survived and expanded in vitro on OP9DL1 stroma cells, differentiated into DN3-DN4 cells, and gave rise to a small number of DP cells (Figure 6B). Of note, ETPs from Miz-1$^{\Delta POZ}$ x Bcl-2Tg (Figure 6B), like sorted DN2 and DN3 cells from Miz-1$^{\Delta POZ}$ thymus (Figure 3A), survived better in vitro but were less efficient at generating DP cells compared with WT or Bcl-2Tg cells on OP9DL1. This indicates that a second differentiation block at the DN3/DN4 transition exists in Miz-1$^{\Delta POZ}$ thymus (Figure 1A-C) that can only partially be rescued by Bcl-2 overexpression.

Overexpression of Bcl-2 restores T-cell differentiation in Miz-1$^{\Delta POZ}$ mice

The introduction of the *H2K-Bcl-2* transgene into Miz-1$^{\Delta POZ}$ mice reduced the DN to DP block observed in Miz-1$^{\Delta POZ}$ thymus, as demonstrated by CD4/CD8 fluorescence-activated cell sorter (FACS) analysis (Figure 7A), and restored the numbers of both αβ-T cells and TCR-γδ cells (Figure 7B). As αβ-T cells, DP cells were also significantly increased in Miz-1$^{\Delta POZ}$x Bcl-2Tg, but their cell numbers remained 20-fold lower than WT or Bcl-2Tg mice, consistent with the few DP generated in vitro when Miz-1$^{\Delta POZ}$ x Bcl-2Tg ETPs were cocultured on OP9DL1. The second block observed at the DN3/DN4 transition in Miz-1$^{\Delta POZ}$ thymus was not rescued by Bcl-2 overexpression in vivo, as demonstrated by CD44/CD25 FACS analysis (Figure 7A-B). Nonetheless, the ETP and blood ELP subsets,

which were almost undetectable in Miz-1–deficient mice, were completely restored in Miz-$1^{\Delta POZ}$ x Bcl-2Tg animals, reaching more than WT levels (Figure 7A-C). The number of cells in DN1 and DN2 subsets, as well as their subpopulations, was also completely restored (supplemental Figure 7D). Therefore, increased levels of Bcl-2 are sufficient to rescue Miz-$1^{\Delta POZ}$ early ETP/DN1/DN2 defects, allowing their survival and differentiation both in vitro and in vivo until the DN3 stage.

Discussion

ELPs initiate pro-T-cell differentiation in the thymus in response to the appropriate signaling pathways, such as Notch or IL-7. ELPs from the bone marrow are similar to thymic ETPs, exhibiting comparable gene expression patterns.[37] Both Miz-1$^{\Delta POZ}$ ELPs and ETPs express normal critical markers and T-cell genes, suggesting that Miz-1 deficiency does not alter the expression program related to their T-lineage specification. Nevertheless, Miz-1$^{\Delta POZ}$ ELPs/ETPs and DN1s failed to differentiate into more mature pre-T cells. In this study, we present evidence that Miz-1 controls an IL-7–dependent survival step in pro-T cells, particularly at the ETP/DN1/DN2 stages, by regulating Bcl-2 induction through the control of SOCS1.

It is still not clear whether ETPs, contained in the Lin^{-}CD117^{+}IL-7R$\alpha^{-/low}$ fraction of DN1s, depend on IL-7R signaling for survival, but early T-cell expansion is severely reduced in IL-7$^{-/-}$ and IL-7R$^{-/-}$ mice,[38,39] and more recent data showed that ETPs or their progenitors have encountered IL-7/IL-7R priming throughout their development.[40] This history of *IL-7R* signaling makes it plausible to hypothesize that progenitor cells require IL-7 and benefit from IL-7 accessibility in the bone marrow.[41] Miz-1 may be needed at or before thymic settling of T-cell progenitors to regulate *Bcl-2* and help the cells benefit from IL-7 availability to survive (Figure 7D). This hypothesis would explain the lack of survival of blood ELPs and thymic ETPs in Miz-1$^{\Delta POZ}$ mice that can be rescued by Bcl-2 overexpression.

One outcome of IL-7R signaling is the maintenance of cell survival by promoting a positive balance of Bcl-2-family members.[15] Our findings indicate that the balance between proapoptotic and antiapoptotic factors downstream of the IL-7R signaling is altered in Miz-$1^{\Delta POZ}$ pro-T cells. Miz-1–deficient DN2s express elevated levels of *Bcl-2* mRNA but show higher levels of apoptosis. This may be the result of a selection of Miz-$1^{\Delta POZ}$ DN2 cells that express enough antiapoptotic *Bcl-2*, possibly through an IL-7-independent signal, to escape the lack of survival signals caused by Miz-1 deficiency at the ETP/DN1 stage. It is also possible that a residual and very inefficient IL-7–dependent signal could be responsible for the high *Bcl-2* expression despite elevated *SOCS1* expression levels because DN2 cells from Miz-$1^{\Delta POZ}$ still had a low level of STAT5 phosphorylation in response to IL-7 stimulation.

The *Bax/Bcl-2* ratio in Miz-$1^{\Delta POZ}$ DN1 and DN2 cells seems to be in favor of apoptosis rather than survival. Because *Bax* is a target gene of the Gfi1 transcriptional repressor and Miz-1 can recruit Gfi1 to target gene promoters, it is possible that up-regulation of *Bax* in Miz-$1^{\Delta POZ}$ cells is the result of a disruption of the Miz-1–Gfi1 complex,[42,43] and future work would be required to clarify such regulation. Collectively, our data led us to conclude that Miz-1 deficiency probably interrupts the IL-7/IL-7R/STAT5/Bcl-2 axis, which assures cell survival.

Consistent with these observations, IL-7R deficiency causes a block in early T-cell differentiation that can be reversed by overexpressing *Bcl-2*[34,35] or by deleting the *Bax* gene.[44] We could observe that transgenic overexpression of Bcl-2 restored most of the early

αβ-T-cell deficiencies in Miz-1$^{\Delta POZ}$ mice. Previous reports proposed that *Bcl-2* could be an effector gene of Miz-1.[45,46] Our data show that Miz-1 deficiency inhibits Bcl-2 up-regulation on IL-7 treatment. However, a role of Miz-1 in Bcl-2 regulation in T cells is probably indirect because we did not detect binding of Miz-1 to the *Bcl-2* promoter in primary sorted DN thymocytes (data not shown). It is however possible that such a direct regulation happens in T-cell progenitors that settle in the thymus because ELP survival can be rescued by Bcl-2 overexpression in Miz-1$^{\Delta POZ}$ mice.

In the IL-7–dependent DN1 and DN2 subsets, it is more likely that Miz-1 regulates *SOCS1*, which inhibits IL-7R signaling and is highly expressed in Miz-1$^{\Delta POZ}$ DNs. ChIP experiments suggest that Miz-1 binds to the *SOCS1* promoter at the initiator site. Although additional experimental evidence would be required to definitively prove that Miz-1 directly represses *SOCS1* transcription, our data show that Miz-1 overexpression inhibited SOCS1 up-regulation in response to IL-7. Moreover, Miz-1$^{\Delta POZ}$ LSKs and DN1 cells treated with a morpholino oligo against *SOCS1* mRNA regain their ability to differentiate in vitro. Finally, high *SOCS1* levels in Miz-1$^{\Delta POZ}$ cells correlated with an interruption of IL-7 signaling. We could also show that *Miz-1* is highly expressed in WT DN thymocytes, where *SOCS1* levels are normally low. Although DP thymocytes express the highest *SOCS1* levels, we did not observe an inverse correlation between *SOCS1* and *Miz-1* expression in this subset, which is consistent with data from *Lck*-cre Miz-1$^{\Delta POZ}$ mice that argue against a role of Miz-1 in DP cells.

Because *SOCS1* overexpression was only observed in Miz-1$^{\Delta POZ}$ DN not DP, and Miz-1 is not involved in T-cell development beyond the DN stage, our data support a model in which Miz-1 monitors SOCS1 expression levels to ensure a proper IL-7 response in ETP/DN1/DN2 cells (Figure 7D). It would have been conceivable that *Miz-1* expression would be down-regulated in response to IL-7 to derepress *SOCS1* transcription. However, *Miz-1* expression level is not regulated after IL-7 stimulation, suggesting that Miz-1 itself is doubtful a direct IL-7–dependent effector gene.

Whether Miz-1 regulates IL-7R signaling in a c-Myc–dependent manner in T cells is an intriguing question because Miz-1 was originally identified as a c-Myc binding protein.[21] It has been reported that a pre-T cell–specific deletion of c-Myc leads to a block at the transition between DN to DP cells.[47,48] However, c-Myc–deficient mice do not show a strong reduction in their thymic cellularity.[47] Moreover, the reported DN3 and DN4 cell frequencies in c-Myc–deficient mice are at WT levels, and the DN4 cells have high levels of cytoplasmic TCR-β chain, contrary to what we observed in Miz-1$^{\Delta POZ}$ mice (I.S., T.M., unpublished data, June 2010).

Moreover, the c-Myc^{V394D} knockin mice do not phenocopy Miz-1$^{\Delta POZ}$ mice and have a normal T-cell development. Although these data suggest a c-Myc-independent function of Miz-1, it could be argued that, in the absence of a functional Miz-1, there is an increase in c-Myc levels that do not form a complex with Miz-1, which would explain the developmental defects seen in Miz-1$^{\Delta POZ}$ mice. This also seems doubtful because: (1) constitutive c-Myc expression in T cells leads to T-cell lymphomas rather than to T-cell

depletion[49]; and (2) higher c-Myc activity would lead to a higher proliferation of pre-T cells,[50] which is not the case in Miz-1$^{\Delta POZ}$ mice.

In conclusion, we show that the BTB/POZ domain transcription factor Miz-1 regulates the part of IL-7R signaling that is involved in protecting cells from apoptosis and promoting differentiation of early pro-T cell subsets. Miz-1 deficiency causes a deregulation in *SOCS1* expression levels, an interruption of Jak/STAT5 signaling, an unbalanced ratio of *Bcl-2* to *Bax*, and a high rate of apoptosis. Our data indicate that Miz-1 is required for the regulation of the IL-7/IL-7R/STAT5/Bcl-2 signaling pathway in ETP/DN1/DN2 cells by monitoring the expression levels of *SOCS1* in a c-Myc-independent manner. Our study not only establishes Miz-1 as a new factor in early T-lymphoid differentiation but also implies that these functions are linked to the BTB/POZ domain of Miz-1.

Authorship

Contribution: I.S., C.K., and T.M. designed the research and analyzed the results; I.S., C.K., and L.V. performed experiments; C.K. generated the mice; I.S. and T.M. wrote the paper; and T.M. provided funding.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Footnotes

* I.S. and C.K. contributed equally to this study.

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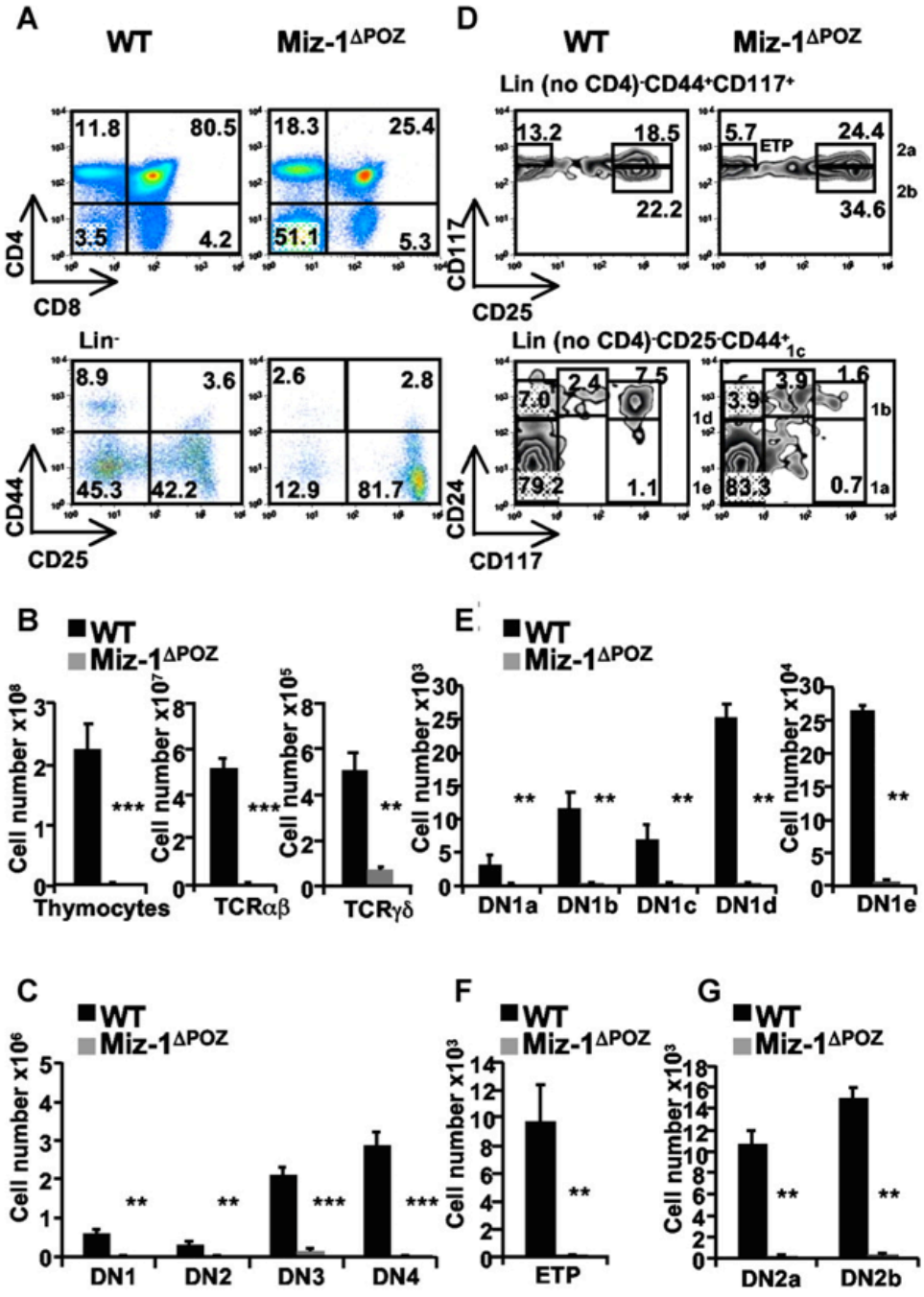
A
WT
Miz-1ΔPOZ
11.8
80.5
3.5
4.2
18.3
25.4
51.1
5.3
CD4
CD8
Lin-
8.9
3.6
45.3
42.2
2.6
2.8
12.9
81.7
CD44
CD25
D
WT
Miz-1ΔPOZ
Lin (no CD4)-CD44+CD117+
13.2
18.5
22.2
5.7
ETP
24.4
34.6
2a
2b
CD117
CD25
Lin (no CD4)-CD25-CD44+1c
7.0
2.4
7.5
79.2
1.1
3.9
1.6
3.9
83.3
0.7
1d
1b
1e
1a
CD24
CD117
B
WT
Miz-1ΔPOZ
Cell number x10^8
Thymocytes
Cell number x10^7
TCRαβ
Cell number x10^5
TCRγδ
E
WT
Miz-1ΔPOZ
Cell number x10^3
DN1a
DN1b
DN1c
DN1d
Cell number x10^4
DN1e
C
WT
Miz-1ΔPOZ
Cell number x10^6
DN1
DN2
DN3
DN4
F
WT
Miz-1ΔPOZ
Cell number x10^3
ETP
G
WT
Miz-1ΔPOZ
Cell number x10^3
DN2a
DN2b

Figure 1

Figure 1. The deletion of the POZ domain of Miz-1 disrupts T-cell development at the transition from DN to DP and at the ETP/DN1 stage. Flow cytometric (FACS) analysis (A,D) and total cell numbers (B,C,E-G) of thymic populations of wild-type (WT) and Miz-$1^{\Delta POZ}$ mice. (A) CD4 and CD8 surface staining (top panel) and lineage-negative (Lin^-) cells (bottom panel), described in supplemental Methods are analyzed for the surface expression of CD44 and CD25 to assess DN1 ($Lin^-CD44^+CD25^-$), DN2 ($Lin^-CD44^+CD25^+$), DN3 ($Lin^-CD44^-CD25^+$), and DN4 ($Lin^-CD44^-CD25^-$). Numbers in quadrants indicate the percentage of cells. (B-C) Absolute numbers of thymocytes, TCR-$\alpha\beta^+$, TCR-$\gamma\delta^+$, and DN1 to DN4 cells are calculated relative to the live cells gated according to the FSC/SSC profile and expressed as absolute cell count. (D) FACS analysis of ETP (Lin^- no CD4, $CD25^-$ $CD44^+CD117^+$), DN1a-e (Lin^- no CD4, $CD25^-$, $CD44^+$, and $CD24/CD117^{medium/high}$) and DN2a-b (Lin^- no CD4, $CD44^+CD117^+CD25^{medium/high}$) populations. (E-G) Percentages of positive cells in panel D are calculated relative to the total live cells and expressed as absolute cell count. Average counts of at least 8 mice and error bars representing the SD are shown. $^{**}P \leq .01$. $^{***}P \leq .001$.

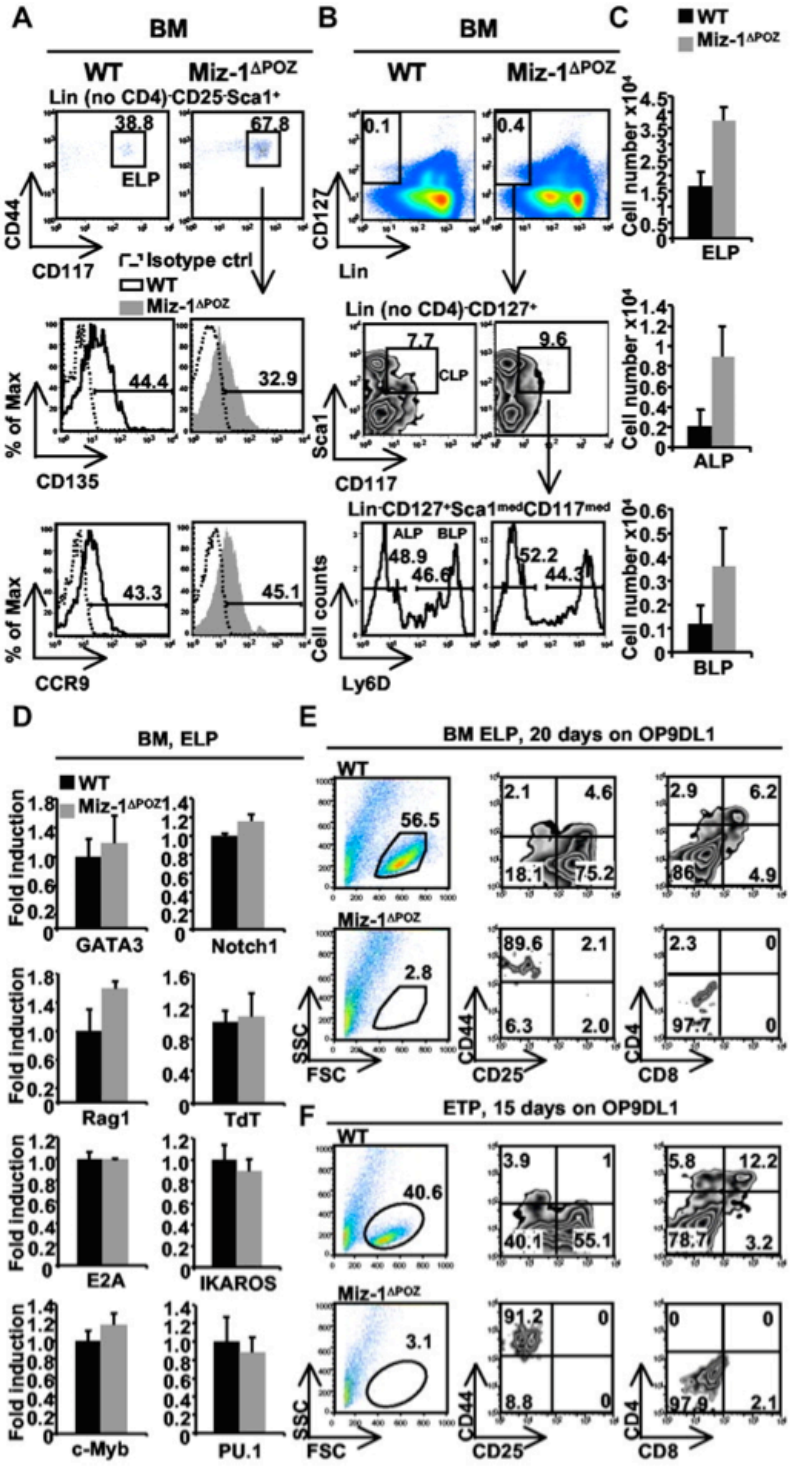
A
BM
WT
Miz-1ΔPOZ
Lin (no CD4)CD25Sca1+
38.8
67.8
ELP
CD44
CD117
Isotype ctrl
WT
Miz-1ΔPOZ
44.4
32.9
% of Max
CD135
B
BM
WT
Miz-1ΔPOZ
0.1
0.4
CD127
Lin
Lin (no CD4)CD127+
7.7
9.6
CLP
Sca1
CD117
LinCD127+Sca1medCD117med
ALP
BLP
48.9
46.6
52.2
44.3
43.3
45.1
% of Max
CCR9
Cell counts
Ly6D
C
WT
Miz-1ΔPOZ
Cell number x10⁴
ELP
ALP
BLP
D
BM, ELP
WT
Miz-1ΔPOZ
Fold induction
GATA3
Notch1
Rag1
TdT
E2A
IKAROS
c-Myb
PU.1
E
BM ELP, 20 days on OP9DL1
WT
56.5
2.1
4.6
18.1
75.2
2.9
6.2
86
4.9
Miz-1ΔPOZ
2.8
89.6
2.1
6.3
2.0
2.3
0
97.7
0
SSC
FSC
CD44
CD25
CD4
CD8
F
ETP, 15 days on OP9DL1
WT
40.6
3.9
1
40.1
55.1
5.8
12.2
78.7
3.2
Miz-1ΔPOZ
3.1
91.2
0
8.8
0
0
0
97.9
2.1

Figure 2

Figure 2. Loss of Miz-1 POZ domain affects frequencies of bone marrow ELPs, ALPs, and BLPs and in vitro differentiation of ETPs and ELPs. (A) ELPs were gated on Lin^- no CD4, $CD25^-$ $Sca1^+CD44^+CD117^+$ (top panel) and further analyzed for CD135 and CCR9 expression (bottom panel). The plots are composed of an overlay of the CD135 or CCR9 staining in black (WT) or gray (Miz-$1^{\Delta POZ}$) with the matching isotype control antibodies staining (dotted black; n = 4). (B) Bone marrow $Lin^-CD127^+Sca1^{med}CD117^{med}$ CLPs were examined for the expression of Ly6D dividing the progenitor population into $Ly6D^-$ ALPs and $Ly6D^+$ BLPs. (C) Percentages of positive cells in panels A and B are calculated relative to the total live cells and expressed as absolute cell count (n = 4 for ELPs and n = 2 for ALPs and BLPs). (D) Quantitative real-time PCR analysis of target genes involved in ELP development. RNA was extracted from 5000 sorted bone marrow ELPs from WT and Miz-$1^{\Delta POZ}$ mice. All values are presented as fold induction relative to values obtained with the respective wild-type control. Average of triplicate values and SD are shown (n = 3). FACS analysis indicating the development of sorted Lin^- no CD4, $CD25^-$ $Sca1^+CD44^+CD117^+$ ELPs (E) and ETPs (F) from the bone marrow and thymus of WT or Miz-$1^{\Delta POZ}$ mice. Fifty sorted ETPs or ELPs were cocultured on OP9DL1 stroma cells for 15 or 20 days in T-cell media. The developmental progression of the cells is evaluated by flow cytometry using T-cell markers CD44, CD25, CD4, and CD8 (n = 4).

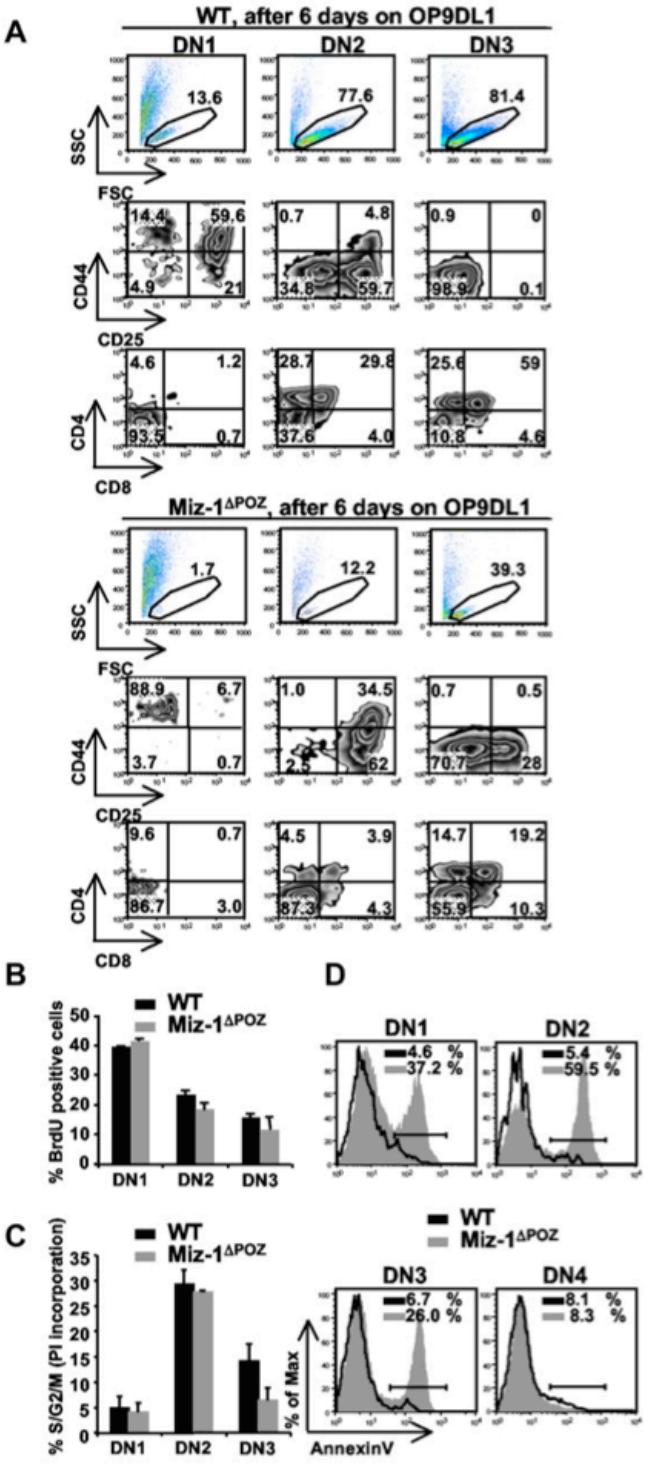
A
WT, after 6 days on OP9DL1
DN1
DN2
DN3
SSC
FSC
13.6
77.6
81.4
CD44
CD25
14.4
59.6
4.9
21
0.7
4.8
34.8
59.7
0.9
0
98.9
0.1
CD4
CD8
4.6
1.2
93.5
0.7
28.7
29.8
37.6
4.0
25.6
59
10.8
4.6
Miz-1ΔPOZ, after 6 days on OP9DL1
1.7
12.2
39.3
88.9
6.7
3.7
0.7
1.0
34.5
2.5
62
0.7
0.5
70.7
28
9.6
0.7
86.7
3.0
4.5
3.9
87.3
4.3
14.7
19.2
55.9
10.3
B
WT
Miz-1ΔPOZ
% BrdU positive cells
50
40
30
20
10
0
DN1
DN2
DN3
C
WT
Miz-1ΔPOZ
% S/G2/M (PI incorporation)
35
30
25
20
15
10
5
0
DN1
DN2
DN3
D
DN1
DN2
4.6 %
37.2 %
5.4 %
59.5 %
WT
Miz-1ΔPOZ
DN3
DN4
6.7 %
26.0 %
8.1 %
8.3 %
% of Max
AnnexinV

Figure 3

Figure 3. DN1s from Miz-1$^{\Delta POZ}$ mice lack in vitro differentiation and/or survival signals on OP9DL1 cocultures. (A) Comparative differentiation kinetics of 500 cells sorted from DN1, DN2, and DN3 subsets after 6 days of culture on stromal OP9DL1 cells. Gated live cells (top panel) were further gated on $CD4^{-}CD8^{-}$TCR-$\gamma\delta^{-}$ and analyzed for CD44 and CD25 surface expression to assess DN stages of differentiation (middle panel). CD4 and CD8 surface expression shows developmental progression of more mature T cell (bottom panel). The numbers in dot plots are percentages of cells (n = 6). (B) Cell cycle analysis after in vivo BrdU labeling. Cells were stained for surface markers and anti-BrdU and gated on DN1, DN2, and DN3. Data show percentage of BrdU-positive cells (n = 2). (C) Cell cycle analysis using propidium iodide staining was performed on permeabilized, sorted DN1, DN2, and DN3 cells. Data show percentage of cells in S/G2/M phase and are representative of 4 independent experiments. (D) Single-cell suspensions of thymocytes were stained with antibodies against lineage markers, CD44 and CD25 followed by annexin V staining. Percentages of annexin V-positive cells are indicated (n = 4).

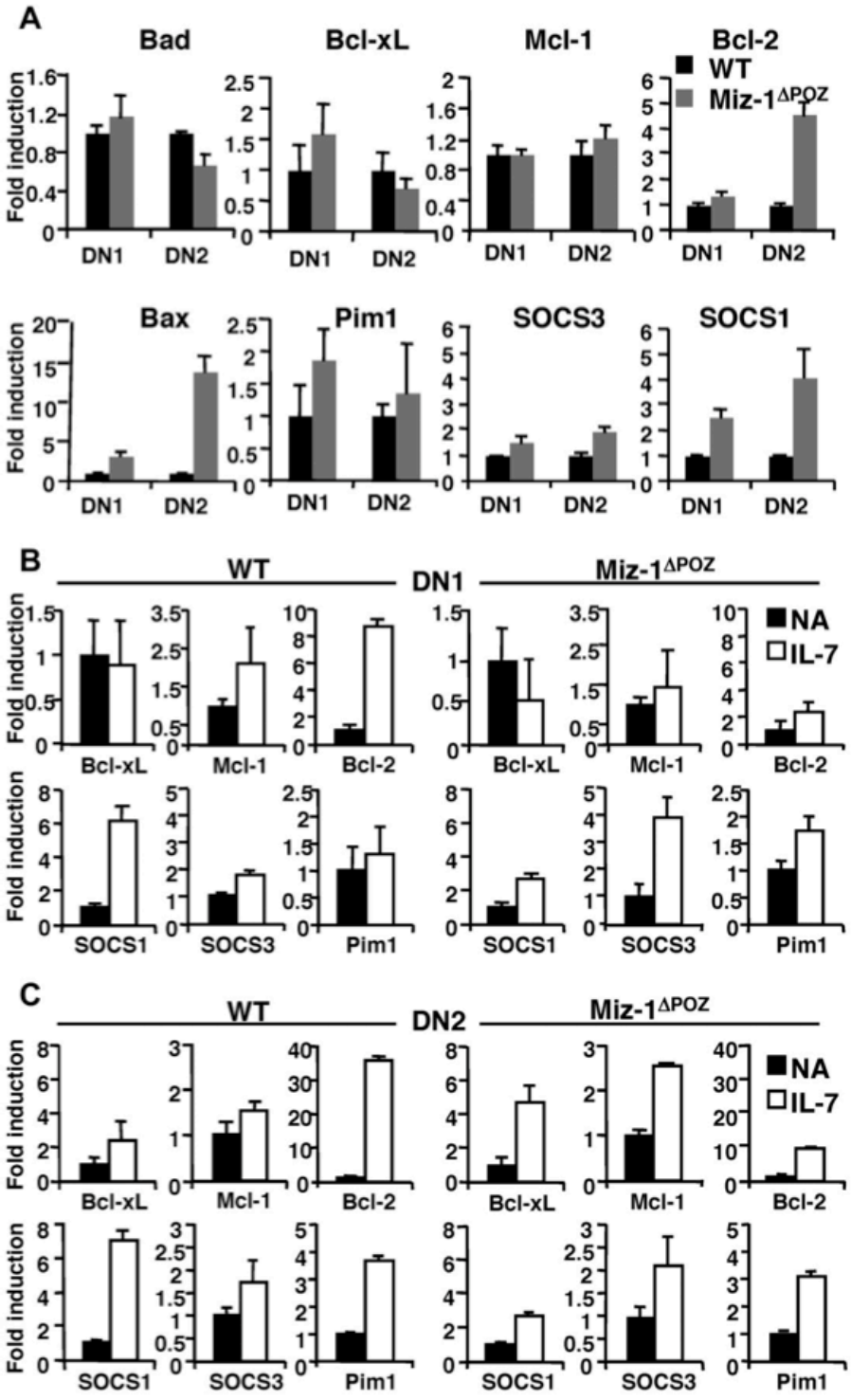
A
Bad
Bcl-xL
Mcl-1
Bcl-2
WT
Miz-1ΔPOZ
Fold induction
DN1
DN2
Bax
Pim1
SOCS3
SOCS1
B
WT
DN1
Miz-1ΔPOZ
NA
IL-7
Bcl-xL
Mcl-1
Bcl-2
SOCS1
SOCS3
Pim1
C
WT
DN2
Miz-1ΔPOZ
NA
IL-7
Bcl-xL
Mcl-1
Bcl-2
SOCS1
SOCS3
Pim1

Figure 4

Figure 4. Miz-1$^{\Delta POZ}$ DN thymocytes show aberrant IL-7-related gene expression profile. (A) RNA was extracted from 5 to 10 × 10^3 sorted DN1 and DN2 cells. All values are normalized to the expression of *GAPDH* gene and are presented as fold induction relative to values obtained with the respective wild-type control (set as 1-fold). Average of triplicate values ± SD are shown (n = 5). (B-C) Quantitative real-time PCR analyses of indicated genes in sorted DN1 and DN2 cells non-activated (NA) or activated with IL-7 for 4 hours. Data are presented as fold induction relative to values obtained with the respective NA sample for wild-type control (left panel) and for Miz-1$^{\Delta POZ}$ (right panel). Average of triplicate values ± SD are shown (n = 3).

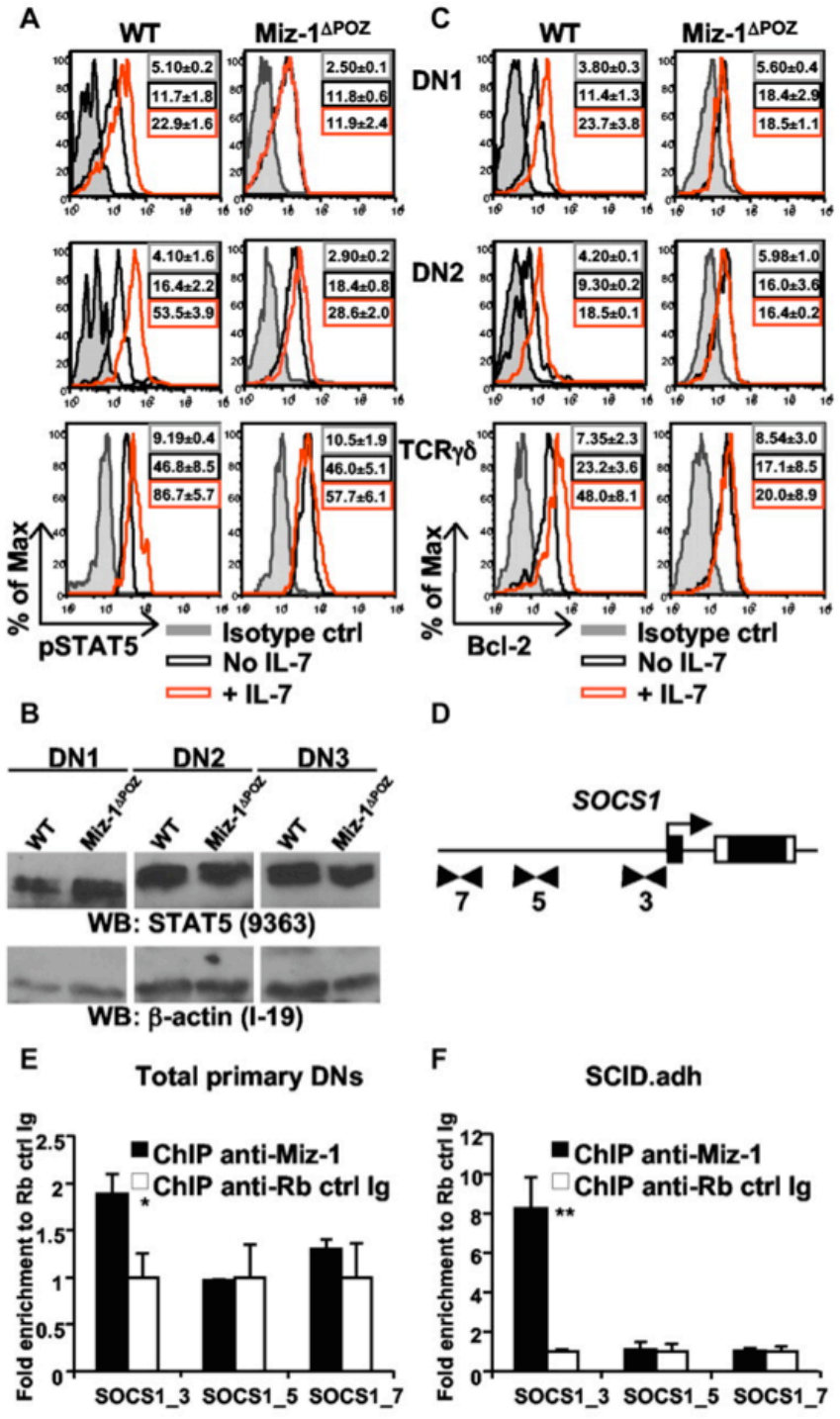
A
WT
Miz-1ΔPOZ
5.10±0.2
11.7±1.8
22.9±1.6
2.50±0.1
11.8±0.6
11.9±2.4
4.10±1.6
16.4±2.2
53.5±3.9
2.90±0.2
18.4±0.8
28.6±2.0
9.19±0.4
46.8±8.5
86.7±5.7
10.5±1.9
46.0±5.1
57.7±6.1
% of Max
pSTAT5
Isotype ctrl
No IL-7
+ IL-7
C
WT
Miz-1ΔPOZ
DN1
3.80±0.3
11.4±1.3
23.7±3.8
5.60±0.4
18.4±2.9
18.5±1.1
DN2
4.20±0.1
9.30±0.2
18.5±0.1
5.98±1.0
16.0±3.6
16.4±0.2
TCRγδ
7.35±2.3
23.2±3.6
48.0±8.1
8.54±3.0
17.1±8.5
20.0±8.9
% of Max
Bcl-2
Isotype ctrl
No IL-7
+ IL-7
B
DN1
DN2
DN3
WT
Miz-1ΔPOZ
WB: STAT5 (9363)
WB: β-actin (I-19)
D
SOCS1
7
5
3
E
Total primary DNs
ChIP anti-Miz-1
ChIP anti-Rb ctrl Ig
Fold enrichment to Rb ctrl Ig
SOCS1_3
SOCS1_5
SOCS1_7
F
SCID.adh
ChIP anti-Miz-1
ChIP anti-Rb ctrl Ig
Fold enrichment to Rb ctrl Ig
SOCS1_3
SOCS1_5
SOCS1_7

Figure 5

Figure 5. Miz-1 is required for proper IL-7/IL-7R signaling and binds to the *SOCS1* promoter. (A) Intracellular pSTAT5 detection in WT and Miz-$1^{\Delta POZ}$ thymocytes after ex vivo stimulation with IL-7. Histograms show isotype control (ctrl) antibodies staining in gray, and pSTAT5 antibody stainings in unstimulated (No IL-7) and stimulated with IL-7 (+ IL-7) cells. Mean fluorescence intensities ± SD are indicated; n = 4 for DN1 and DN2 and n = 3 for $CD4^{-}CD8^{-}$TCR-$\gamma\delta^{+}$. (B) Total STAT5 proteins in DN1, DN2, and DN3 cells. Cells were sorted and whole protein extracts were evaluated by Western blot for STAT5 (top blot) and β-actin loading control (bottom blot; n = 2). (C) Bcl-2 detection in WT and Miz-$1^{\Delta POZ}$ thymocytes after ex vivo stimulation with IL-7. Mean fluorescence intensities ± SD are indicated. (D) ChIP analysis to identify Miz-1 binding to potential sites within *SOCS1* promoter. Cells were rested at 37°C in phosphate-buffered saline for 1 hour, and ChIP was performed on primary DN cells (E) or on SCID.adh murine thymic lymphoma cells (F). Quantitative real-time PCR was performed using primers flanking the initiator region (SOCS1_3) or upstream (SOCS1_5 and _7) of *SOCS1* promoter (indicated as arrows in panel D and described in supplemental Table 3). Data are fold enrichment of specific anti–Miz-1 ChIP over rabbit control Ig ChIP (set as 1-fold) from triplicates ± SD (n = 4). *$P \leq .05$. **$P \leq .01$.

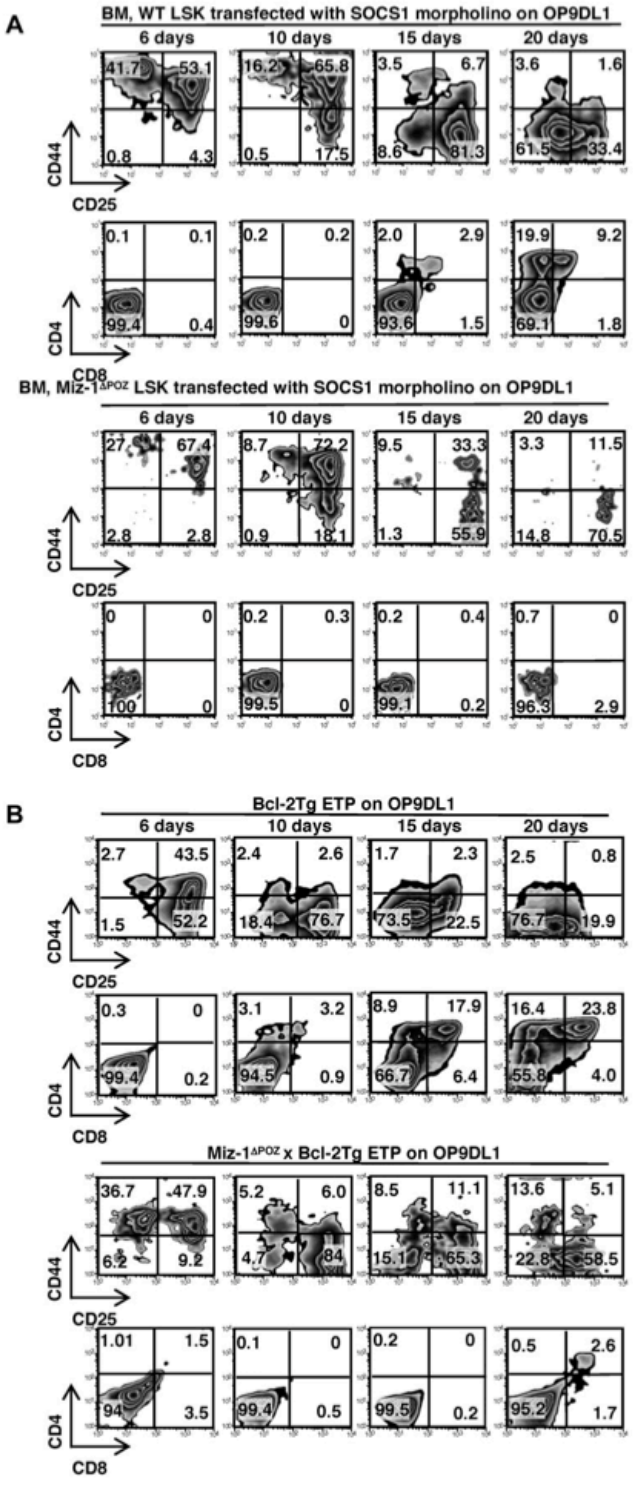
A
BM, WT LSK transfected with SOCS1 morpholino on OP9DL1
6 days
10 days
15 days
20 days
41.7
53.1
0.8
4.3
16.2
65.8
0.5
17.5
3.5
6.7
8.6
81.3
3.6
1.6
61.5
33.4
CD44
CD25
0.1
0.1
99.4
0.4
0.2
0.2
99.6
0
2.0
2.9
93.6
1.5
19.9
9.2
69.1
1.8
CD4
CD8
BM, Miz-1ΔPOZ LSK transfected with SOCS1 morpholino on OP9DL1
6 days
10 days
15 days
20 days
27.4
67.4
2.8
2.8
8.7
72.2
0.9
18.1
9.5
33.3
1.3
55.9
3.3
11.5
14.8
70.5
CD44
CD25
0
0
100
0
0.2
0.3
99.5
0
0.2
0.4
99.1
0.2
0.7
0
96.3
2.9
CD4
CD8
B
Bcl-2Tg ETP on OP9DL1
6 days
10 days
15 days
20 days
2.7
43.5
1.5
52.2
2.4
2.6
18.4
76.7
1.7
2.3
73.5
22.5
2.5
0.8
76.7
19.9
CD44
CD25
0.3
0
99.4
0.2
3.1
3.2
94.5
0.9
8.9
17.9
66.7
6.4
16.4
23.8
55.8
4.0
CD4
CD8
Miz-1ΔPOZ x Bcl-2Tg ETP on OP9DL1
36.7
47.9
6.2
9.2
5.2
6.0
4.7
84
8.5
11.1
15.1
65.3
13.6
5.1
22.8
58.5
CD44
CD25
1.01
1.5
94
3.5
0.1
0
99.4
0.5
0.2
0
99.5
0.2
0.5
2.6
95.2
1.7
CD4
CD8

Figure 6

Figure 6. Early T-cell development is restored in Miz-1$^{\Delta POZ}$ cells by *SOCS1* knockdown or Bcl-2 overexpression in vitro. (A) A total of 1000 sorted LSK cells from WT or Miz-1$^{\Delta POZ}$ were incubated with morpholino against *SOCS1* mRNA. Comparative in vitro differentiation kinetics of the cells was monitored after 6 to 20 days of coculture on OP9DL1. (B) A total of 500 sorted ETP cells from Bcl-2Tg or Miz-1$^{\Delta POZ}$ x Bcl-2Tg were analyzed by FACS after 6 to 20 days of culture. Gated live cells were further gated on $CD4^{-}CD8^{-}$TCR-$\gamma\delta^{-}$ and analyzed for CD44 and CD25 surface expression to assess DN stages of differentiation. CD4 and CD8 surface expression shows development progression of more mature T cells. Data are representative of 4 independent experiments for panel A and 5 experiments for panel B.

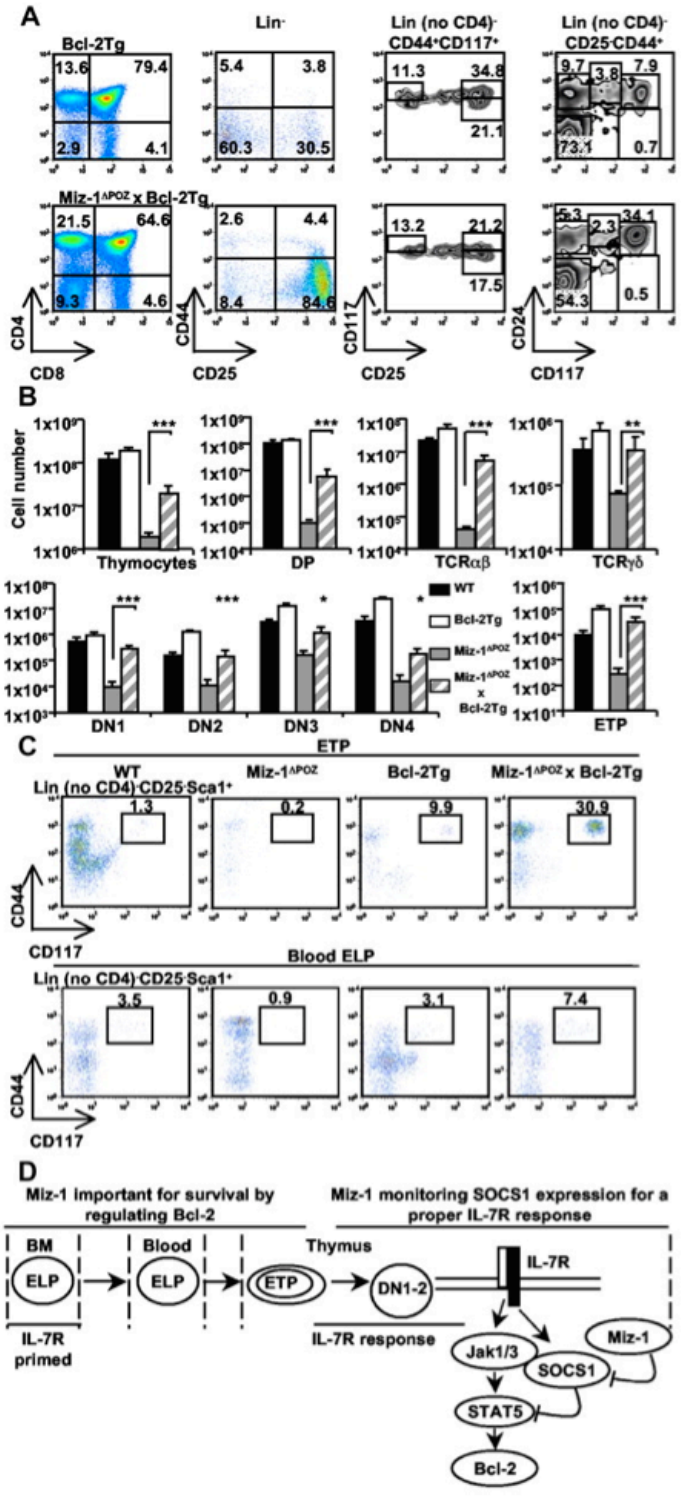
A
Bcl-2Tg
Lin-
Lin (no CD4)- CD44+CD117+
Lin (no CD4)- CD25-CD44+
13.6
79.4
2.9
4.1
5.4
3.8
60.3
30.5
11.3
34.8
21.1
9.7
3.8
7.9
73.1
0.7
Miz-1ΔPOZ x Bcl-2Tg
21.5
64.6
9.3
4.6
2.6
4.4
8.4
84.6
13.2
21.2
17.5
5.3
2.3
34.1
54.3
0.5
CD4
CD8
CD44
CD25
CD117
CD25
CD24
CD117
B
Cell number
Thymocytes
DP
TCRαβ
TCRγδ
DN1
DN2
DN3
DN4
ETP
WT
Bcl-2Tg
Miz-1ΔPOZ
Miz-1ΔPOZ x Bcl-2Tg
C
ETP
WT
Miz-1ΔPOZ
Bcl-2Tg
Miz-1ΔPOZ x Bcl-2Tg
Lin (no CD4)-CD25-Sca1+
1.3
0.2
9.9
30.9
CD44
CD117
Blood ELP
Lin (no CD4)-CD25-Sca1+
3.5
0.9
3.1
7.4
CD44
CD117
D
Miz-1 important for survival by regulating Bcl-2
Miz-1 monitoring SOCS1 expression for a proper IL-7R response
BM
ELP
Blood
ELP
ETP
Thymus
DN1-2
IL-7R
IL-7R primed
IL-7R response
Jak1/3
Miz-1
SOCS1
STAT5
Bcl-2

Figure 7

Figure 7. The early T-cell differentiation block in Miz-1$^{\Delta POZ}$ mice is overcome by Bcl-2 overexpression in vivo. FACS analysis (A,C) and total cell numbers (B) of thymic and blood lymphoid populations from Bcl-2Tg or Miz-1$^{\Delta POZ}$ x Bcl-2Tg mice (n = 7). (A) CD4 and CD8 surface staining is shown (left panel). CD4$^-$CD8$^-$ DN cells gated on lineage-negative cells were further analyzed for the surface expression of CD44 and CD25 to assess the 4 DN populations (middle left panel). Within the DN populations, DN2s (Lin$^-$ no CD4, CD44$^+$CD117$^+$CD25$^{medium/high}$), ETPs (Lin$^-$ no CD4, CD25$^-$ CD44$^+$CD117$^+$; middle right panel), and DN1s (Lin$^-$ no CD4, CD25$^-$, CD44$^+$, and CD24/CD117$^{medium/high}$; right panel) were also characterized. (B) Numbers in rectangular gates or quadrants indicate the percentage of cells. Total cell numbers of the thymocyte subsets and of gated TCR-αβ$^+$ and TCR-γδ$^+$ cells are shown. (C) FACS analysis of Lin$^-$ no CD4, CD25$^-$ Sca1$^+$CD44$^+$CD117$^+$ thymic ETPs (top panel) and peripheral blood ELPs (bottom panel; n = 5). (D) Schematic representation of Miz-1 implication throughout T-lineage progenitor and pro-T-cell development. *P ≤ .05. **P ≤ .01. ***P ≤ .001.

Supplementary materials

IL-7R–dependent survival and differentiation of early T-lineage progenitors is regulated by the BTB/POZ domain transcription factor Miz-1

Ingrid Saba, Christian Kosan, Lothar Vassen, and Tarik Möröy

Inventory:

Supplemental material and methods

Mice

Generation of Miz-1$^{\Delta POZ}$ mice.

We followed a triple loxP gene targeting strategy (flox) to conditionally delete exon 3 and 4 coding for the POZ domain of Miz-1, as well as the selectable neomycin marker gene.[29] Mice carrying one deleted allele and one conditional allele (Miz$^{\Delta POZ/flox}$) were crossed with Vav-Cre mice, which express the Cre recombinase in all hematopoietic cells. All hematopoietic stem cells, erythroid cells and myeloid cells had efficient deletion of the POZ domain of Miz-1, referred to as Miz-1$^{\Delta POZ}$ mice. Mice carrying the conditional alleles (Miz-1$^{flox/flox}$) were also crossed with *Lck*-Cre mice, where the Cre recombinase starts to be expressed at the DN2/DN3 transition of T-cell development. This model provided mice with a T cell-specific deletion of the POZ domain of Miz-1. Finally, Vav- cre Miz-1$^{\Delta POZ}$ mice were crossed with the cyclin kinase inhibitor CDKN1a-deficient mice (CDKN1a$^{-/-}$) to generate *Vav*-cre Miz-1$^{\Delta POZ}$x CDKN1a$^{-/-}$ mice.

c-Myc V394D knock-in (KI) mice.

Homozygous c-Myc^{V394D} KI mice were also generated and are viable. The targeting strategy for this model will be described elsewhere (Kosan *et al.* in preparation). Briefly, a c-Myc mutant has been generated where a Valine residue (V) at position 394 has been exchanged for an Aspartic Acid (D). This mutation has been shown to inhibit the ability of c-Myc to bind to Miz-1.[24]

Antibodies

CD3ε (145-2C11), CD4 (RM 4-5), CD8α (53-6.7), CD25 (PC61.5 from eBioscience), CD44 (IM7), TCRβ (H57-597), TCRγδ (GL3), CD45R/B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (Mac-1, M1/70), Ter-119, Pan-NK (DX5), NK1.1 (PK136 from e-Bioscience), CD19 (1D3), CD5 (53-7.3 from e-Bioscience), IgM (R6-60.2), CD24 (I, M1/69), CD135 (Flt-3, A2F10.1), CD117 (cKit, 2B8), Sca-1 (D7), Ly6D (49-H4), CCR7 (4B12 from e-Bioscience), CXCR4 (2B11 from e-Bioscience), CCR9 (R&D systems), Notch1 (mN1A), CD127 (IL-7Rα, A7R34 from e-Bioscience) and CD132 (γc, TUGm2), Bcl-2 (BD, 556537), pSTAT5 (BD pY694, 612567), STAT5 (Cell Signaling, 9363), SOCS1 (Millipore, 4H1), β-actin (Santa Cruz, I-19). Thymic DN lineage cocktail contained biotinylated antibodies against CD3ε, CD4, CD8α, CD45/B220, Gr-1, Mac-1, Ter-119, NK1.1, DX5, TCRγδ. ETPs were analyzed using DNs Lin^- cocktail, without CD4 but with biotin anti- CD25. ELPs were gated on the same ETP Lin^- with the addition of biotin anti-CD19 and anti-TCRβ. CLP lineage contained CD3ε, CD8α, CD45/B220, Gr-1, Mac-1, Ter-119, NK1.1, CD5, IgM antibodies, without CD4. ALP and BLP populations were distinguished by gating on Lin^- CLP cocktail, $Sca1^{med}$, $CD117^{med}$ $CD127^+$, and Ly6D-negative (ALP) or Ly6D-positive (BLP) cells.

Retroviral transduction

Retroviruses were generated using 293-GPG cells. GP+E cells were infected with retroviruses from the 293-GPG cells for co-culture and virus production. Cells were transduced by spin-infection. P6D4 cells were resuspended in viral supernatant (from

GP+E- 86 cells in DMEM containing 10% (vol/vol) FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin), in the presence of polybrene (12.5 μg/ml) and were centrifuged by 1400g for 2h. The cells were then washed with RPMI and cultured in RPMI 10% (vol/vol) FBS, 2% Penicillin/Streptomycin, 1% Non Essential Amino Acids, 1% Na-pyruvate and 100 μM β- mercaptoethanol. Four to five days after infection transduced cells were sorted by flow cytometry on the basis of GFP expression.

Cell cycle and cell death analysis

Cell cycle analyses were performed using a FITC bromodeoxyuridine (BrdU) flow kit (BD 559619) according to the manufacturer's instructions. Mice were injected intraperitoneally with BrdU (100 μg/g body-weight) and the thymi were collected 4h after injection. Propidium Iodide (PI) analyses were carried out on sorted DN1-DN4. Cells were directly sorted in modified krishan buffer (0.1% sodium citrate, 0.3% NP-40) containing 0.05 mg/ml PI and 0.02 mg/ml Rnase and analyzed after 30 min of incubation. Apoptosis rates were measured by AnnexinV apoptosis detection kit (BD 556547).

RNA isolation and real-time PCR

Cells were FACS-sorted directly in TRIZOL Reagent (Invitrogen). RT-PCR was performed using Superscript II (Invitrogen) and oligo (dT) 12-18 primers following manufacturer's protocol. Real time PCR was performed on the Invitrogen Mx3005, in triplicates, in 20μl using TaqMan Universal PCR Master Mix (Applied Biosystems) or in 10μl using PerfeCTa SYBR Green PCR SuperMixUNG (Quanta Biosciences). The expression of the gene of

interest was calculated relative to the GAPDH mRNA (Δct) and is presented as fold induction relative to values obtained with the respective control (set as 1-fold). Primers used are listed in Tables S1, S2, and S3.

Table S1: Real-time assays used from Applied Biosystems

Taqman Assay ID	Gene symbol	Gene name
Mm00456421_m1	Ikzf1	IKAROS family zinc finger 1
Mm00493500_m1	Dntt/Tdt	Deoxynucleotidyltransferase, terminal
Mm00441699_m1	HEB/Tcf12	Transcription factor 12
Mm01270936_m1	Rag1	Recombination activating gene 1
Mm00501300_m1	Rag2	Recombination activating gene 2
Mm01175597_m1	Tcfe2a	Transcription factor E2a
Mm99999915_g1	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Mm00782550_s1	SOCS1	Suppressor of cytokine signaling 1
Mm00545913_s1	SOCS3	Suppressor of cytokine signaling 3
Mm00477631_m1	Bcl-2	B-cell lymphoma-2
Mm00432050_m1	Bax	Bcl-2-associated X protein
Mm00432042_m1	Bad	Bcl-2-associated agonist of cell death
Mm01342018_m1	Zbtb17	Miz-1
Mm00775963_g1	Idb1	Inhibitor of DNA binding 1
Mm00711781_m1	Idb2	Inhibitor of DNA binding 2
Mm00487803_m1	c-Myc	Myelocytomatosis oncogene
Mm00441724_m1	Tgfb1	Transforming growth factor, beta 1
Mm00435249_m1	Notch1	Notch gene homolog 1 (Drosophila)
Mm00432939_m1	E2f1	E2F transcription factor 1
Mm00434295_m1	IL7R	Interleukin-7 receptor
Mm00501741_m1	c-Myb	Myeloblastosis viral oncogene homolog
Mm00656724_m1	Egr1	Early growthresponse 1
Mm00456650_m1	Egr2	Early growthresponse 2
Mm00516979_m1	Egr3	Early growthresponse 3
Mm01342805_m1	Hes1	Hairy and enhancerof split 1, (Drosophila)
Mm00484683_m1	Gata3	GATA binding protein 3
Mm00488140_m1	Sfpi1	Spi-B transcription factor (Spi-1/PU.1 related)
Mm00435712_m1	Pim1	Pim-1 oncogene (proviral integration site 1)

Table S2: Primer sequence for Sybrgreen assays

Sybrgreen Primers	Gene symbol	Gene name	Reference
Mcl-1	Forward: TCAAAGATGGCGTAACAAACTGG	Myeloid Cell Leukemia sequence 1	*Nature Immunol*., 2010, p.171
Mcl-1	Reverse: CCCGTTTCGTCCTTACAAGAAC	Myeloid Cell Leukemia sequence 1	*Nature Immunol*., 2010, p.171
Bcl-xL	Forward: CTGGCTAGGAGCCCTTCAG	BCL2-like 1	-
Bcl-xL	Reverse: AGGGATGAGACAGGCCAAG	BCL2-like 1	-
GAPDH	Forward: ACTCCACTCACGGCAAATTCA	Glyceraldehyde-3-phosphate dehydrogenase	*Dev Biol*, 2009, p.444
GAPDH	Reverse: GCCTCACCCCATTTGATGAT GTT	Glyceraldehyde-3-phosphate dehydrogenase	*Dev Biol*, 2009, p.444

REFERENCES

- Malin S, McManus S, Cobaleda C, Novatchkova M, Delogu A, Bouillet P, Strasser A, Busslinger M. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B-cell development. Nat Immunol. 2010;11(2):171–179.

- David-Fung ES, Butler R, Buzi G, Yui MA, Diamond RA, Anderson MK, Rowen L, Rothenberg EV. Transcription factor expression dynamics of early T-lymphocyte specification and commitment. Dev Biol. 2009; 325(2): 444–467.

Table S3: Primer sequences and their relative location for ChIP experiments

ChIP primers	Sequence	Distance to Inr (bp)
SOCS1_3F	Forward: GAGACGGTTCCTCTAACTTGGTTTC	- 125
SOCS1_3R	Reverse: GAACACAAGATTCCGGTTGGA	- 204
SOCS1_5F	Forward: GGTCAAGACTCCAGCAAGCAT	- 764
SOCS1_5R	Reverse: TCTGACTGCCTGGATCCTAAGC	- 832
SOCS1_7F	Forward: CCCTCTCCCATCCATCACAT	- 1601
SOCS1_7R	Reverse: ATGGAAGGAAGGCAATCAGATC	- 1675
SOCS3_9F	Forward: TCCAGAAGTGGAGGAGACAC	- 656
SOCS 3_9R	Reverse: GTCTGTCACCGAAGAACCAG	- 740
SOCS3_8F	Forward: CCTTCCCTTTCTTTTGATCC	- 447
SOCS3_8R	Reverse: GCTTGGAACTGTACATGAGGA	- 532
SOCS3_5F	Forward: AGGAGAAACCGGGAAAAGC	Intron 1
SOCS3_5R	Reverse: ATTTCGCTTCGGGACTAGGT	Intron 1
SOCS3_4F	Forward: CTCAGTCCGGTGTCCTCAG	Intron 1
SOCS3_4R	Reverse: GCTGGCCTCCTAGAACTGC	Intron 1
GAPDH_F	Forward:GGAGACAACCTGGTCCTCAG	-
GAPDH_R	Reverse:GTGTTCCTACCCCCAATGTG	-

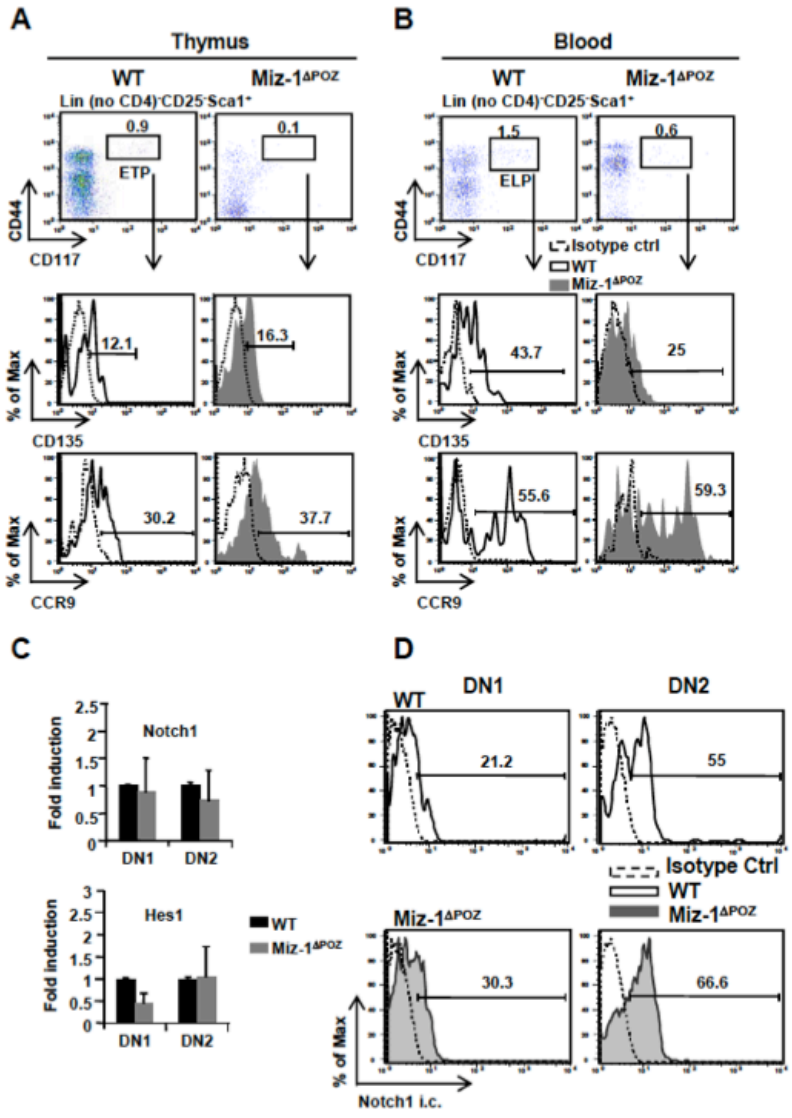
A
Thymus
WT
Miz-1ΔPOZ
Lin (no CD4)-CD25-Sca1+
0.9
0.1
ETP
CD44
CD117
12.1
16.3
% of Max
CD135
30.2
37.7
CCR9
B
Blood
WT
Miz-1ΔPOZ
Lin (no CD4)-CD25-Sca1+
1.5
0.6
ELP
CD44
CD117
Isotype ctrl
WT
Miz-1ΔPOZ
43.7
25
% of Max
CD135
55.6
59.3
% of Max
CCR9
C
Fold Induction
Notch1
DN1
DN2
Hes1
WT
Miz-1ΔPOZ
D
DN1
DN2
WT
21.2
55
Isotype Ctrl
WT
Miz-1ΔPOZ
Miz-1ΔPOZ
30.3
66.6
% of Max
Notch1 i.c.

Figure S1

Figure S1. Miz-1$^{\Delta POZ}$ mice have reduced blood ELPs, but phenotypically normal ETPs, normal thymic DN T-lineage specific gene expression profile, and an intact Notch1 pathway. FACS analysis of thymic ETPs (A) and peripheral blood ELPs (B). ETP/ELP frequencies are indicated on top of the dot plot gate (upper panel), and cells in the ETP/ELP gate were further analyzed for CD135 and CCR9 expression (lower panel). The plots are composed of an overlay of the CD135 or CCR9 staining in black (WT) or in grey (Miz-1$^{\Delta POZ}$) with the matching isotype control antibodies staining (dotted black) (n=4). (C) mRNA expression levels of Notch target genes such as *Hes1* and *Notch1* itself in different thymocyte populations from *Vav*-cre Miz-1$^{\Delta POZ}$ mice (n=3). (D) Notch1 intracellular expression evaluated by flow cytometry in different thymocyte populations from *Vav*-cre Miz-1$^{\Delta POZ}$ mice. The plots are composed of an overlay of the anti-Notch1 staining in black (WT) or in gray (Miz-1$^{\Delta POZ}$) with the matching isotype control antibodies staining (dotted black). Percentages of positive cells are indicated. Data are representative of four independent stainings.

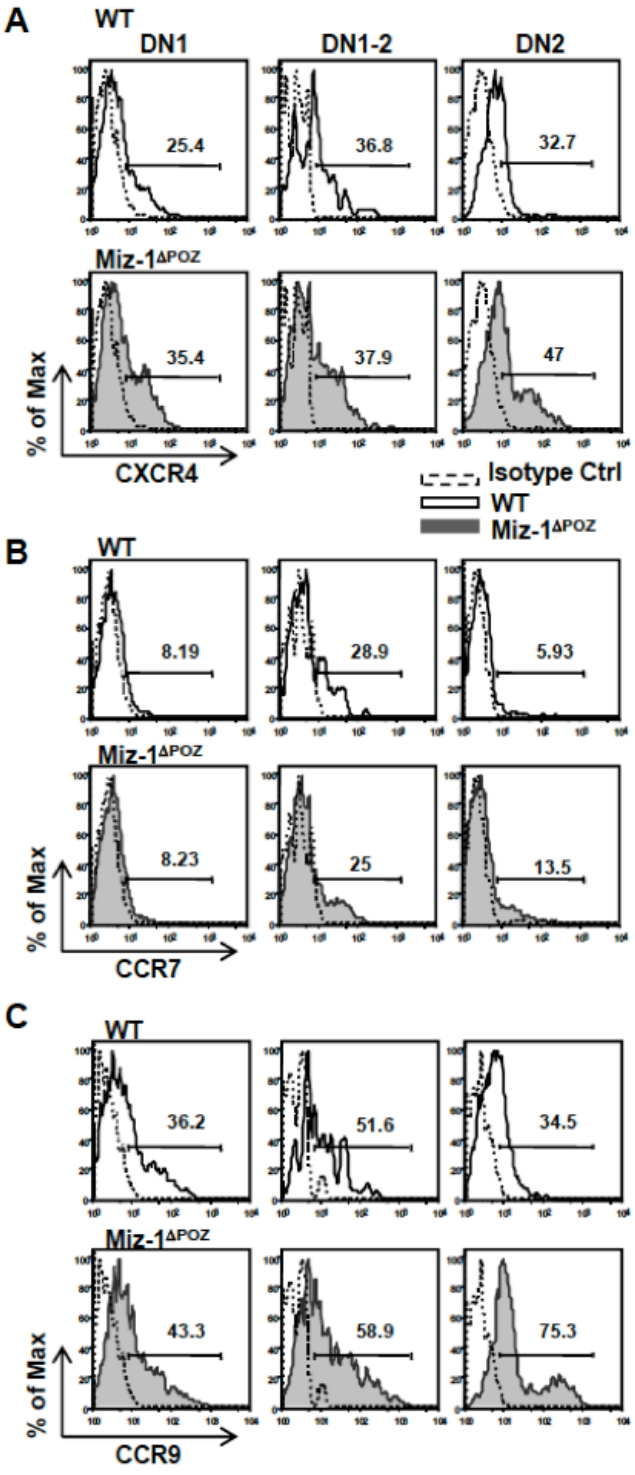
A
WT
DN1
DN1-2
DN2
25.4
36.8
32.7
Miz-1ΔPOZ
35.4
37.9
47
% of Max
CXCR4
Isotype Ctrl
WT
Miz-1ΔPOZ
B
WT
8.19
28.9
5.93
Miz-1ΔPOZ
8.23
25
13.5
% of Max
CCR7
C
WT
36.2
51.6
34.5
Miz-1ΔPOZ
43.3
58.9
75.3
% of Max
CCR9

Figure S2

Figure S2. Chemokine receptors expression during early T-cell development is normal in Miz-1$^{\Delta POZ}$ mice. FACS analysis of CXCR4 (A), CCR7 (B), and CCR9 (C) on identified DN population gated on lineage negative, CD44, and CD25 as indicated. The plots are composed of an overlay of the specific staining in black (WT) or in gray (Miz-1$^{\Delta POZ}$) with the matching isotype control antibodies staining (dotted black). Percentages of positive cells are indicated. Data are representative of three stainings for CCR7 and four independent stainings for CXCR4 and CCR9.

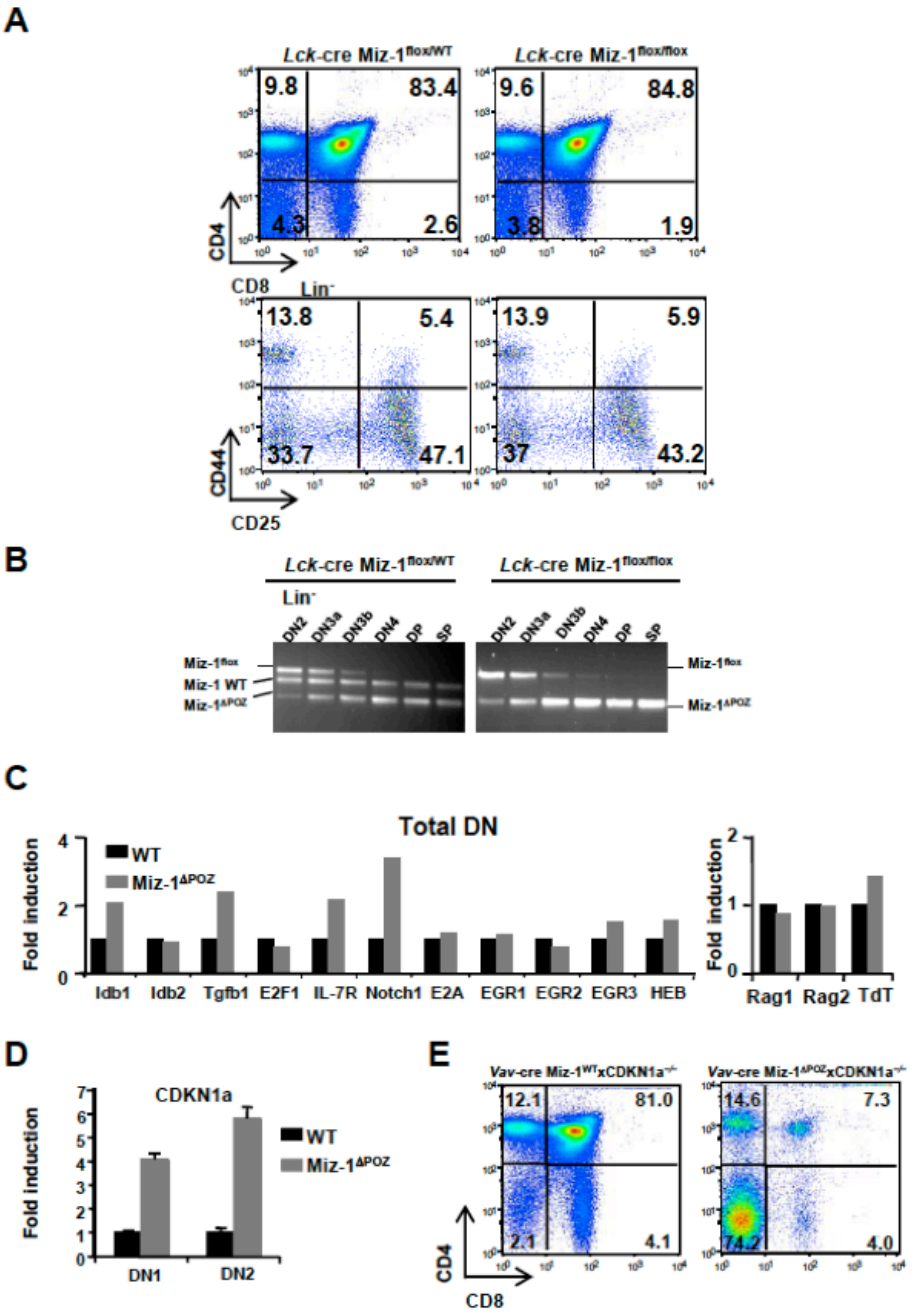
A
Lck-cre Miz-1flox/WT
Lck-cre Miz-1flox/flox
9.8
83.4
4.3
2.6
9.6
84.8
3.8
1.9
CD4
CD8
Lin-
13.8
5.4
33.7
47.1
13.9
5.9
37
43.2
CD44
CD25
B
Lck-cre Miz-1flox/WT
Lck-cre Miz-1flox/flox
Lin-
DN2
DN3a
DN3b
DN4
DP
SP
Miz-1flox
Miz-1 WT
Miz-1ΔPOZ
C
Total DN
WT
Miz-1ΔPOZ
Fold induction
Idb1
Idb2
Tgfb1
E2F1
IL-7R
Notch1
E2A
EGR1
EGR2
EGR3
HEB
Rag1
Rag2
TdT
D
CDKN1a
DN1
DN2
E
Vav-cre Miz-1WTxCDKN1a-/-
Vav-cre Miz-1ΔPOZxCDKN1a-/-
12.1
81.0
2.1
4.1
14.6
7.3
74.2
4.0
CD4
CD8

Figure S3

Figure S3. The deletion of the POZ domain of Miz-1 under the control of *Lck*-cre does not disturb T-cell development at the transition from DN to DP, and CDKN1a regulation is not responsible for the early DN block observed in *Vav*-cre Miz-1$^{\Delta POZ}$ mice. (A) FACS analysis of thymic populations of WT (*Lck*-cre Miz-1$^{flox/WT}$) and Miz-1$^{\Delta POZ}$ (*Lck*-cre Miz-1$^{flox/flox}$) mice. CD4 and CD8 surface staining (upper panel) and lineage negative (Lin$^-$) cells (lower panel), described in Materials and Methods, were analyzed for the surface expression of CD44 and CD25 to assess DN1 to DN4 (n=4). (B) PCR results demonstrating the deletion of exons 3 and 4 encoding for the POZ domain of Miz-1 on one allele or on two alleles of Miz-1 on sorted DN2, DN3a/b, DN4, DP and SP thymic subsets from *Lck*-cre crossings. *Lck*-cre Miz-1$^{flox/WT}$ is still considered as WT, whereas *Lck*-cre Miz-1$^{flox/flox}$ has the POZ domain of Miz-1 deleted on both alleles and is considered Miz-1$^{\Delta POZ}$. (C) Quantitative Real-time PCR analysis of target genes involved in T-cell development. RNA was extracted from sorted total DN (Lin$^-$CD4$^-$CD8$^-$). All values are presented as fold induction relative to values obtained with the respective wild type control. Data are average of triplicates and are representative of two independent experiments for total DN cells. (D) Miz-1$^{\Delta POZ}$ DN1 and DN2 express higher *CDKN1a* as evaluated by quantitative Real-time PCR. Data are average of triplicates ± SD (n=5). (E) Crossing *Vav*-cre Miz-1$^{\Delta POZ}$ mice with mice deficient for CDKN1a (CDKN1a$^{-/-}$) does not restore the DN to DP block. FACS analysis of thymic populations using CD4 and CD8 staining is shown for CDKN1a$^{-/-}$ and Miz-1$^{\Delta POZ}$xCDKN1a$^{-/-}$ mice (n=5).

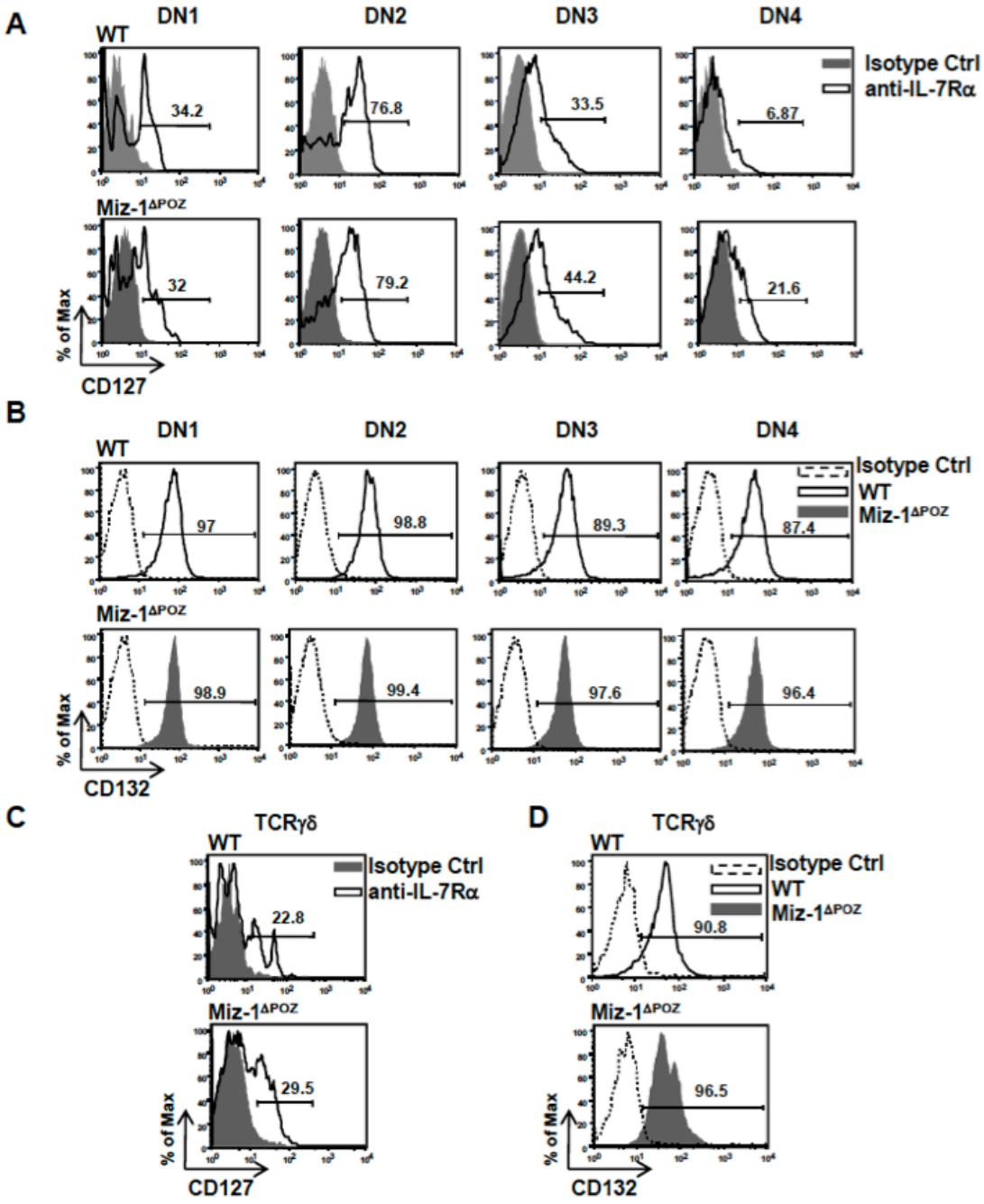
A
DN1
DN2
DN3
DN4
WT
34.2
76.8
33.5
6.87
Isotype Ctrl
anti-IL-7Rα
Miz-1ΔPOZ
32
79.2
44.2
21.6
% of Max
CD127
B
DN1
DN2
DN3
DN4
WT
97
98.8
89.3
87.4
Isotype Ctrl
WT
Miz-1ΔPOZ
Miz-1ΔPOZ
98.9
99.4
97.6
96.4
% of Max
CD132
C
TCRγδ
WT
22.8
Isotype Ctrl
anti-IL-7Rα
Miz-1ΔPOZ
29.5
% of Max
CD127
D
TCRγδ
WT
90.8
Isotype Ctrl
WT
Miz-1ΔPOZ
Miz-1ΔPOZ
96.5
% of Max
CD132

Figure S4

Figure S4. Defects in IL-7/IL-7R pathway are not related to the incorrect expression of IL- 7Rα or the signaling chain γc on the surface of Miz-1$^{\Delta POZ}$ thymocytes. Flow cytometry analysis was used to evaluate surface expression of CD127 (A) and CD132 (B) on gated Lin^{-} DN1-4 thymocytes. The surface expression of these two chains was also evaluated on TCRγδ^{+} cells gated on CD4^{-}CD8^{-}TCRγδ^{+} (C, D). Histograms show percentages of CD127^{+} or CD132^{+} cells in each thymocyte subset. FACS profiles are representative of six independent stainings for CD127 and of four independent stainings for CD132.

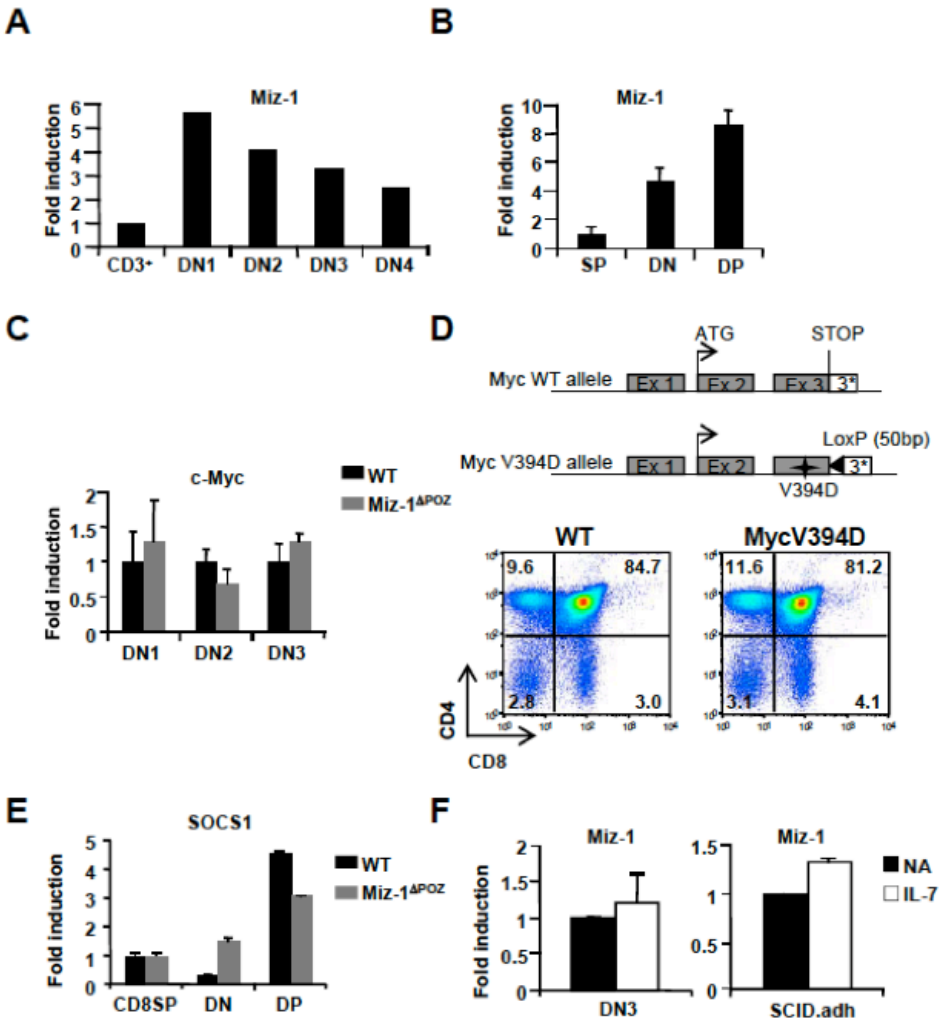
A
Miz-1
Fold induction
CD3+
DN1
DN2
DN3
DN4
B
Miz-1
Fold induction
SP
DN
DP
C
c-Myc
WT
Miz-1ΔPOZ
Fold induction
DN1
DN2
DN3
D
ATG
STOP
Myc WT allele
Ex 1
Ex 2
Ex 3
3'
LoxP (50bp)
Myc V394D allele
V394D
WT
MycV394D
9.6
84.7
2.8
3.0
11.6
81.2
3.1
4.1
CD4
CD8
E
SOCS1
WT
Miz-1ΔPOZ
Fold induction
CD8SP
DN
DP
F
Miz-1
Fold induction
DN3
Miz-1
SCID.adh
NA
IL-7

Figure S5

Figure S5. Miz-1 is highly expressed in immature thymocytes and its implication in T-cell development is likely c-Myc-independent. (A) RNA was extracted from sorted immature double negative (DN) cells, and all values are presented as fold induction relative to values obtained with $CD3^+$ splenic T cells for Miz-1 expression. Data are average of duplicates. (B) RNA was extracted from sorted thymic single positive (SP), double negative (DN) and double positive (DP), and all values are presented as fold induction relative to values obtained with SP cells (n=3). (C) *c-Myc* expression was evaluated in sorted DN cells, and all values are presented as fold induction relative to the respective wild type control (n = 4). (D) Targeting scheme showing the introduction of the knock-in (KI) point mutation into the exon 3 of c-Myc locus that alters the coding sequence to introduce a Valine (V) at position 394 in replacement of an Aspartic Acid (D) (upper panel), and FACS analysis of homozygous KI mice (Myc^{V394D}) (lower panel). CD4 and CD8 surface staining is shown (n=4). (E) *SOCS1* expression was evaluated in sorted thymic CD8 single positive (CD8SP), double negative (DN) and double positive (DP), and all values are presented as fold induction relative to values obtained with CD8SP cells (n=3). (F) Quantitative Real-time PCR analysis of Miz-1 expression in sorted DN3 cells or SCID.adh clone non-activated (NA) or activated with IL-7 for 4 h. Data are presented as fold induction relative to values obtained with the respective NA sample. Average of triplicate values ± SD are shown. Data are representative of two independent experiments.

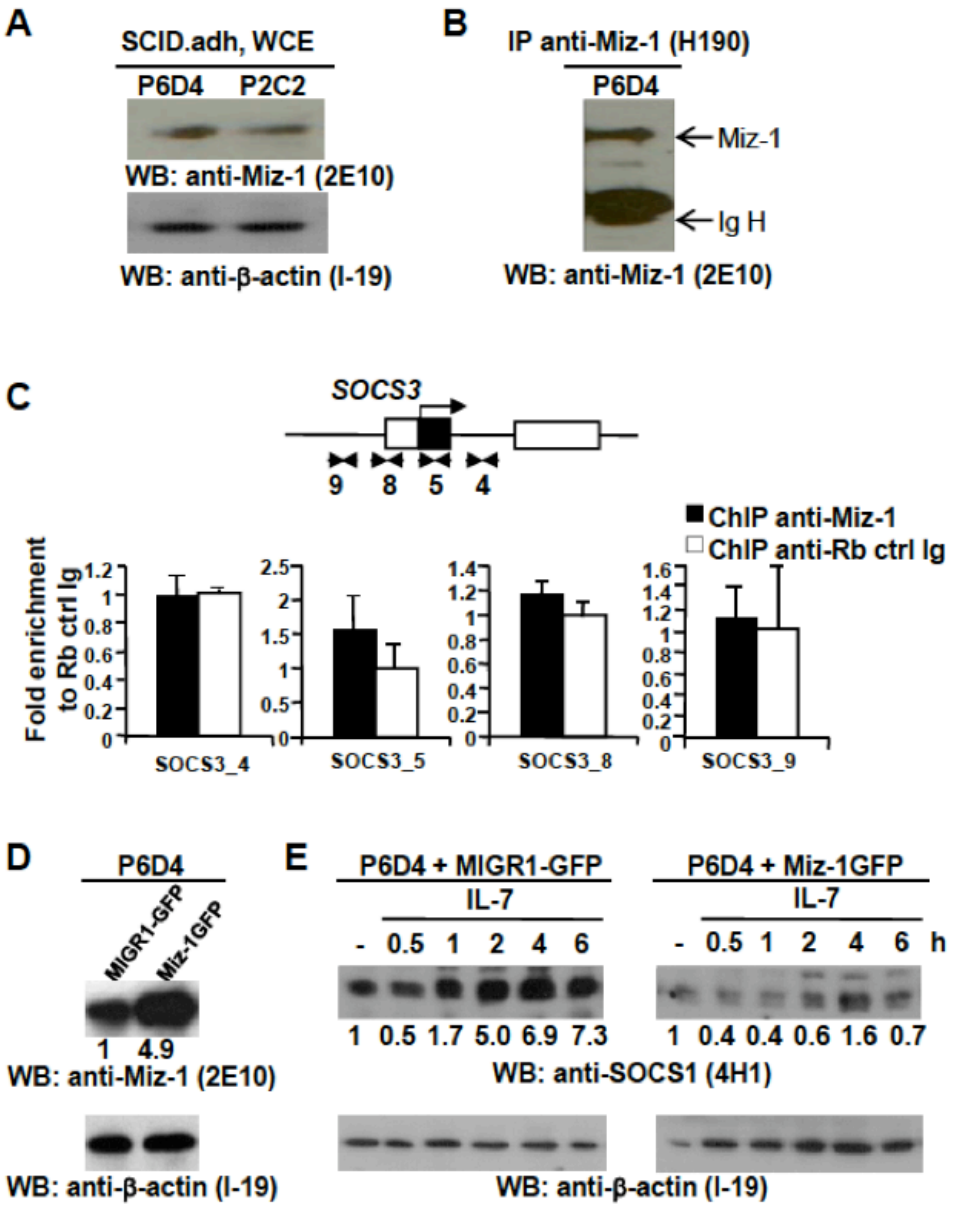
A
SCID.adh, WCE
P6D4
P2C2
WB: anti-Miz-1 (2E10)
WB: anti-β-actin (I-19)
B
IP anti-Miz-1 (H190)
P6D4
Miz-1
Ig H
WB: anti-Miz-1 (2E10)
C
SOCS3
9
8
5
4
ChIP anti-Miz-1
ChIP anti-Rb ctrl Ig
Fold enrichment to Rb ctrl Ig
SOCS3_4
SOCS3_5
SOCS3_8
SOCS3_9
D
P6D4
MIGR1-GFP
Miz-1GFP
1 4.9
WB: anti-Miz-1 (2E10)
WB: anti-β-actin (I-19)
E
P6D4 + MIGR1-GFP
IL-7
- 0.5 1 2 4 6
1 0.5 1.7 5.0 6.9 7.3
P6D4 + Miz-1GFP
IL-7
- 0.5 1 2 4 6 h
1 0.4 0.4 0.6 1.6 0.7
WB: anti-SOCS1 (4H1)
WB: anti-β-actin (I-19)

Figure S6

Figure S6. Overexpression of Miz-1 in SCID.adh murine thymic lymphoma P6D4 clone inhibits SOCS1 up-regulation in response to IL-7. (A) Whole cell extracts (WCE) from P6D4 and P2C2 were evaluated by western blot for the expression of endogenous Miz-1. Upper blot shows Miz-1 expression using anti-Miz-1 antibodies (2E10) and the lower blot shows β-actin loading control. (B) P6D4 clone expressing the highest amount of endogenous Miz-1 was used for Chromatin-immunoprecipitation (ChIP) assay and an immunoprecipitation of Miz-1 using anti-Miz-1 (H190) is shown. (C) ChIP analysis to identify Miz-1 binding to potential sites within SOCS3 promoter. ChIP assays were performed on P6D4 cells. Quantitative Real-time PCR was performed using primers flanking the initiator region of SOCS3 (indicated as arrows in the promoter region). Data are expressed as fold enrichment of specific anti-Miz-1 ChIP over control anti-rabbit IgG isotype ctrl ChIP (set as 1-fold) from triplicates ± SD. Data are representative of four experiments. (D) Miz-1 overexpression was evaluated by western blot on P6D4 cells transduced with an empty retroviral vector (MIGR1-GFP) or with a Miz-1-IRES-GFP virus. Upper blot shows Miz-1 expression using anti-Miz-1 antibodies (2E10) and the lower blot shows the β-actin loading. Images with similar exposures were scanned with Agfa FotoLook and the protein band intensities were quantified using ImageQuant software. Numbers shown bellow the anti-Miz-1 blot are the density of each Miz-1 protein relative to β-actin protein, normalized to the MIGR1-GFP sample, set as 1-fold. Data are representative of 3 independent experiments. (E) Expression of SOCS1 was determined by western blot on P6D4 cells transduced with MIGR1-GFP or with Miz-1-GFP virus after resting (−) or 0.5 to 6h of IL-7 (40 ng/ml) stimulation. Upper blot shows SOCS1 expression

using anti-SOCS1 antibodies (4H1) and the lower blot shows β-actin loading control. Numbers shown bellow the anti-SOCS1 blot are the density of each SOCS1 protein relative to β-actin protein, normalized to the MIGR1-GFP or Miz-1-IRES-GFP untreated sample, set as 1-fold. Data are representative of 2 independent experiments.

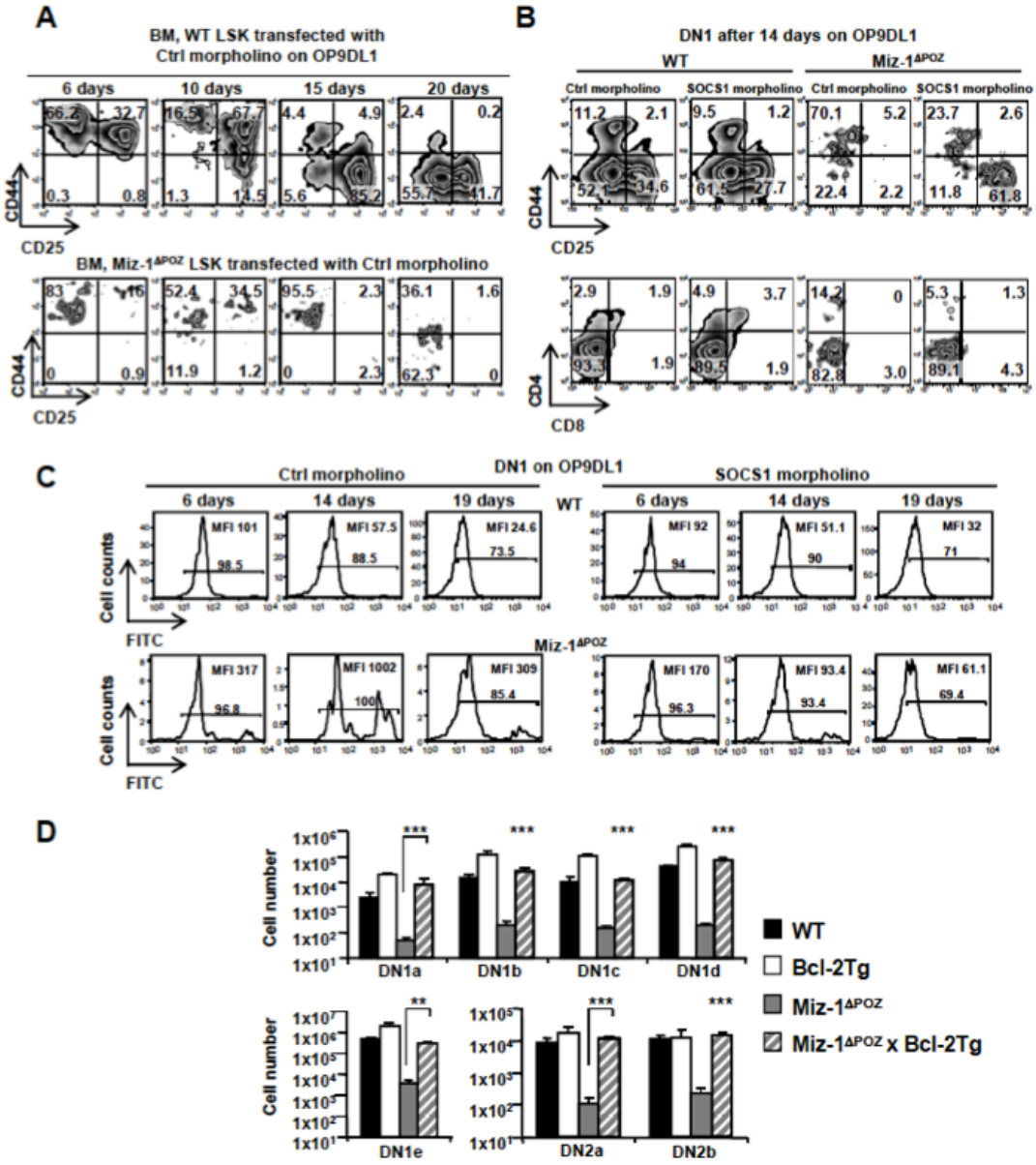
A
BM, WT LSK transfected with Ctrl morpholino on OP9DL1
6 days
10 days
15 days
20 days
CD44
CD25
BM, Miz-1ΔPOZ LSK transfected with Ctrl morpholino
B
DN1 after 14 days on OP9DL1
WT
Miz-1ΔPOZ
Ctrl morpholino
SOCS1 morpholino
CD4
CD8
C
DN1 on OP9DL1
Ctrl morpholino
SOCS1 morpholino
6 days
14 days
19 days
Cell counts
FITC
D
Cell number
DN1a
DN1b
DN1c
DN1d
DN1e
DN2a
DN2b
WT
Bcl-2Tg
Miz-1ΔPOZ
Miz-1ΔPOZ x Bcl-2Tg

Figure S7

Figure S7. *SOCS1* knock-down or Bcl-2 overexpression restored cell numbers of early thymic subpopulations of Miz-1$^{\Delta POZ}$ mice to wild type levels. 1000 sorted LSK (A) or DN1 (B) from WT or Miz-1$^{\Delta POZ}$ were incubated with morpholino against *SOCS1* mRNA or a control (Ctrl) morpholino. CD4, CD8, CD44 and CD25 surface stainings are shown. Cells were analyzed after indicated days of co-culture on OP9DL1, n=4. (C) Morpholino transfection efficiency was monitored by FITC expression. Percentages of positive-FITC morpholino transfected cells and Mean Fluorescence Intensities (MFI) are shown. (D) Total cell numbers of thymic DN populations from Bcl-2Tg mice or Miz-1$^{\Delta POZ}$ crossed with Bcl-2Tg mice (Miz-1$^{\Delta POZ}$ x Bcl-2Tg). Total cell numbers were determined by evaluating the percentage of positive cells for each DN subset and corrected on living cells based on the forward/side scatter profile. Absolute cell counts for DN1a-e (Lin$^-$ no CD4, CD25$^-$, CD44$^+$, and CD24/CD117$^{medium/high}$) and DN2a-b (Lin- no CD4, CD44$^+$ and CD117$^+$, CD25$^{medium/high}$) are shown (n = 4). Error bars indicate the standard deviation. * $p \leq 0.05$.

Chapter III

Miz-1 is Required to Coordinate the Expression of TCRβ and p53 Effector Genes at the Pre-TCR "β-selection" Checkpoint

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Running title: Miz-1 at the β-selection checkpoint

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For this study, I generated all the figures and supplemental figures. Tarik Möröy, Christian Kosan and I designed the research and analyzed the results. I performed all the experiments, Lothar Vassen helped with the gene array analysis. Tarik Möröy and I wrote the paper. Tarik Möröy also provided funding.

Abstract

Miz-1 is a Broad-complex, Tramtrack and Bric-à-brac/Pox virus Zinc finger domain (BTB/POZ) containing protein expressed in lymphoid precursors that can activate or repress transcription. We report in this article that mice expressing a non-functional Miz-1 protein lacking the BTB/POZ domain (Miz-$1^{\Delta POZ}$) have a severe differentiation block at the pre-T cell "β-selection" checkpoint, evident by a drastic reduction of $CD4^-CD8^-$ double-negative-3 (DN3) and DN4 cell numbers. T cell specific genes including *Rag-1, Rag-2, CD3ε, pTα*, and *TCRβ* are expressed in Miz-1-deficient cells and V(D)J recombination is intact, but very few DN3/DN4 cells express a surface pre-TCR. Miz-1-deficient DN3 cells are highly apoptotic and do not divide, which is consistent with enhanced expression of p53 target genes such as *Cdkn1a*, *PUMA* and *Noxa*. However, neither co-expression of the anti-apoptotic protein Bcl-2 nor the deletion of $p21^{CIP1}$ or the combination of both relieved Miz-1-deficient DN3/DN4 cells from their differentiation block. Only the co-expression of rearranged TCRαβ and Bcl-2 fully rescued Miz-1-deficient DN3/DN4 cell numbers and enabled them to differentiate into DN4TCRβ^+ and DP cells. We propose that Miz-1 is a critical factor for the β-selection checkpoint and is required for both the regulation of p53 target genes and proper expression of the pre-TCR to support the proliferative burst of DN3 cells during T-cell development.

INTRODUCTION

T-cell development starts from early thymic precursors (ETP), which progress through four $CD4^-CD8^-$ double negative (DN1-DN4) subsets that differentially express c-Kit, CD25 and CD44 (reviewed in Ref. 1). After cytokine-dependent steps of development and commitment, which also require signaling through the Notch, IL-7R and Wnt pathways, early DNs differentiate into DN3 cells that express T cell specific genes such as *Rag-1*, *Rag-2*, *pTα*, *CD3ε*, *Lck* and *Tdt* (reviewed in Ref. 2). DN3 thymocytes can be subdivided into DN3a and DN3b based on their size and CD27 expression (3, 4). DN3a cells rearrange the T cell receptor (TCR) β chain locus and, if productive, become DN3b blast cells that express a TCRβ chain on the cell surface along with an invariant pTα chain forming the pre-TCR (3, 4). DN3b cells become activated by TCR-dependent signals referred to as the "β-selection" checkpoint, down regulate CD25 and generate DN4 cells, which in turn differentiate into the largest thymic subpopulation, the $CD4^+CD8^+$ double positive (DP) cells (reviewed in Refs. 5, 6). DN3a cells that fail to undergo productive V(D)J recombination at the TCRβ locus are eliminated by apoptosis.

The survival signals that assure proper T cell differentiation are promoted by increasing the expression of anti-apoptotic Bcl-2 and Bcl-x_L, and by redistributing the cell-death proteins Bax and BAD (reviewed in 7). In addition, a role for the p53 tumor suppressor protein in the regulation of cell cycle progression and apoptosis in response to the physiological DNA damage generated by V(D)J recombination at the DN3 stage of pre-TCR cells has been

proposed (8). Interestingly, a differentiation block from DN3/DN4 to DP cells caused by defective pre-TCR signaling, for instance in the absence of Rag-1/2, DNA-PK (SCID) or the CD3γ chain, can be rescued to some extent by the simultaneous loss of p53, suggesting that the β-selection at the DN3 stage depends on a balance between pre-TCR signaling and p53 activation. Importantly, p53 activation has to be contained in the presence of ongoing V(D)J recombination to avoid that developing pre-T cells immediately undergo apoptosis (9, 10, 11, 12). However, the mechanisms to control a p53-dependent DNA damage response in the presence of V(D)J strand breaks have yet to be fully elucidated.

A number of studies showed that the oncoprotein c-Myc plays a role in many cell lineages, including B and T cells, and the generation of conditionally deleted c-Myc alleles in mice has allowed to assess the role of c-Myc in T-cell development more precisely (13, 14). According to these findings, c-Myc is required to ensure pre-TCR-induced proliferation and expansion of both DN3 and DN4 cells (13, 14). It is known that c-Myc protein forms a heterodimeric complex with the transcription factor Max and that the c-Myc/Max complex can activate transcription by binding to E-boxes (CACGTG) in upstream enhancer elements of target genes (15-20). Moreover, c-Myc also interacts with the Broad-complex, Tramtrack and Bric-à-brac/Pox virus Zinc finger domain (BTB/POZ) domain transcription factor Myc-interacting zinc finger protein-1, and a ternary c-Myc/Max/Miz-1 complex has been shown to occupy target gene promoters in an E-box-independent manner. In contrast to c-Myc, which is a helix-loop-helix protein and contains a leucine zipper, Miz-1 is composed of thirteen zinc finger domains at its carboxy-terminus and of a BTB/POZ domain at its N-

terminus (21, 22). Whether Miz-1 activates or represses the transcription of its target genes depends on its interacting partner, but an intact POZ domain is required for both activities. The genes that encode the negative cell cycle regulators Cdkn2b (22, 23) or Cdkn1a (24, 25) have been validated as direct Miz-1 targets. These target genes are activated by Miz-1 and the postive cofactors histone acetyltransferase EP300 (p300) and L23-nucleophosmin and repressed by the c-Myc/Miz-1 complex (22, 23, 26, 27). It has also been shown that c-Myc is recruited to the *Cdkn1a* promoter by Miz-1 and this interaction blocks Cdkn1a induction by p53 and other activators in colon cancer cells. As a result of Miz-1 actions, c-Myc switches the cell fate from cell cycle arrest to apoptosis in response to p53-dependent activation (24).

Since the full knockout of Miz-1 arrests development at an early stage of gastrulation (28), we have generated mice carrying a conditional allele of Miz-1, which produces a truncated protein lacking the functionally critical N-terminal BTB/POZ domain (hereafter named Miz-1$^{\Delta POZ}$). Using these mice we were previously able to demonstrate a new c-Myc-independent function of Miz-1 in early steps of B- and T-lymphoid development, where IL-7R signaling regulates survival and commitment (29, 30). We now report that Miz-1$^{\Delta POZ}$ mice have an additional defect in pre T-cell development at the DN3/DN4 pre-T cell transition. Our data suggest that Miz-1 is important for generating survival signals by counteracting a p53-dependent pathway and assuring the expression of the pre-TCR β chain in order to support the proliferative burst of DN3 cells.

MATERIALS AND METHODS

Mice

Mice have been bred on C57BL/6 background for at least 10 generations and were maintained in Specific-Pathogen-Free Plus environment. All mice used in this study were previously described (29, 30). OTI mice expressing TCRαβ transgenes were purchased from The Jackson Laboratory (C57BL/6-Tg(TCRaTCRb)1100Mjb/J). The Institutional Review Board approved all animal protocols and experimental procedures were performed in compliance with the IRCM guidelines.

Abs and cell lines

OP9DL1 cultures and P6D4 SCID.adh murine thymic lymphoma were used and previously described (30). All antibodies were form BD Bioscience except when indicated. To analyze DN thymic subsets, CD25 (PC61.5 from eBioscience) and CD44 (IM7) plus lineage marker negative cells (Lin^-) were selected by staining thymocytes with the biotinylated-antibodies against CD3ε (145-2C11), CD4 (RM 4-5), CD8α (53-6.7), CD45/B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (Mac-1, M1/70), Ter-119 (Ly-76), NK1.1 (PK136 from eBioscience), Pan-NK (DX5), TCRγδ (GL3) followed by Streptavidin-PerCPCy5.5 or PECy5. When OTI transgenic (Tg) mice were analyzed, the same lineage cocktail without anti-CD3ε was used. Additional staining were performed using CD24 (Heat stable antigen: HSA, M1/69), CD27 (LG.3A10), pTα (2F5) and TCRβ (H57-597) antibodies. Antibody incubation was performed at 4°C for 20 min in PBS buffer with 1% FBS. Cells were

analyzed with a FACSCalibur, FACScan or LSR (Becton-Dickinson). Cell sorting was performed using a MoFlo cell sorter (Cytomation).

TCRβ intracellular staining and immunoblot analysis

Cells were fixed with formaldehyde (BD cytofix 554655), blocked with 5μg of Armenian Hamster Ig2, λ1 (Ha4/8), washed and permeabilized with BD Cytofix Fixation/Permeabilization kit (Becton Dickinson). Intracellular TCRβ staining was performed and surface staining was done after thereafter. For immunoblot analysis, cells were sorted and lysed in 1% Nonidet P-40 (NP-40) containing 20 mM Tris-HCl pH 7.5, 420 mM NaCl, 2mM EDTA, 1mM $MgCl_2$ and 1mM EGTA in the presence of protease and phophatase inhibitors. Immunoblotting was performed using anti-Cdkn1a (556431 BD biosciences) or anti-βactin (I-19, Santa Cruz).

Calcium mobilization

2 x 10^6 cells were incubated in HBSS buffer containing 0.001 M CaCl2, 0.001 M MgCl2, 0.001 M Hepes, 0.1 g BSA. Cells were loaded with Indo1-AM dye (Invitrogen) at 31 °C. After washing, and surface staining, the cells were coated with biotinylated anti-CD3 (145-2C11) or biotin anti-TCRβ. Warm HBSS buffer was added and cells were aquired for 30 sec. Avidin (Sigma) was added for the crosslinking (represented by the arrow in Supplemental Fig. 2C) and data recording was performed for a total time of 5 min. As positive control for Ca^{++} mobilization, ionomycin, was used at 4 μM and cells were

acquired for 2 min total after a first fluorescence at 405 nm (Bound Ca^{++}) versus 510 nm (Free Ca^{++}).

Cell stimulation

For pERK1/2 staining, following stimulation (CD3/CD28 biotin at 10 μg/ml followed by avidin crosslinking at 20μg/ml, or 25 ng/ml PMA/ 1μM ionomycin), cells were fixed with formaldehyde (BD cytofix 554655) and additionally permeabilized with methanol (BD phosflow Perm III 558050). Samples were then stained with anti-phospho-p44/42 MAPK (Erk1/2) Thr202/Tyr 204 mouse Ab (E10), Alexa Fluor 488 conjugate (Cell Signaling) or isotype control. Surface staining was performed after the intracellular staining.

Cell cycle and cell death analysis

Cell cycle analysis was performed on sorted DN3-DN4. Cells were directly sorted in modified Krishan buffer (0.1% sodium citrate, 0.3% NP-40) containing 0.05 mg/ml Propidium Iodide (PI) and 0.02 mg/ml RNase and analyzed after 30 min of incubation**.** Apoptosis rates were measured by AnnexinV staining (BD Pharmingen, AnnexinV-allophycocyanin kit, BD Pharmingen).

RNA isolation, real-time PCR and morpholino oligo knock-down.

For RNA isolation, cells were FACS-sorted directly in TRIZOL Reagent (Invitrogen). RT-PCR was performed using Superscript II (Invitrogen). Real time PCR was performed in

triplicates on the Invitrogen Mx3005 in 20µl reactions using TaqMan Universal PCR Master Mix (Applied Biosystems). The expression of the gene of interest was calculated relative to *GAPDH* mRNA (Δ Threshold cycle (ΔC_T)) and is presented as "fold induction" relative to values obtained with the respective control (set as "1-fold"). Primers used for the experiments are available upon request. FITC-Morpholino oligo against *Cdkn1a* mRNA (GTCGGACATCACCAGGATTGGACAT 3' Fluorescein) or a control Morpholino oligo (Gene Tools, LLC, USA) were used on DN3a sorted cells or P6D4 clone as previously described (30).

V(D)J recombination

DN3 from wild-type (WT) and Miz-$1^{\Delta POZ}$ thymi were sorted in 0.05% Tween-20, 0.05% NP-40, 50 µg/ml proteinase K and incubated overnight at 56 °C. After proteinase K inactivation, genomic DNA was amplified by PCR with TaKaRa Ex Taq (TAKARA BIO INC.). The amplification protocol (1 min 94°C, 1 min 63°C, 2 min 72°C) for Dβ2-Jβ2, Vβ5-Jβ2, Vβ8-Jβ2, Vβ11-Jβ2 and eF1 (primers described in Ref. 31) was repeated 31 cycles. The amplification protocol (1 min 94°C, 1 min 63°C, 2 min 72°C) for Dβ1.1-Jβ1.7 and Dβ2.1-Jβ2.7 was first performed with external primers and repeated 20 times. A 0.5 µL from the first amplification was used for a second PCR reaction with nested internal primers (1 min 94°C, 1 min 63°C, 2 min 72°C) for additional 31 cycles (primers described in Ref. 32). An aliquot of the PCR product for each reaction was fractionated on a 1.8 % agarose gel.

DNA micro-array analysis

10 μg of cRNA from sorted DN3 cells (triplicates) were hybridized on Affymetrix GeneChip MG-430_2.0 arrays (GPL1261). After washing and staining, GeneChips were scanned using the Affymetrix GeneChip scanner 3000 (G7 update) and data were analyzed with GCOS 1.4 software using affymetrix default analysis settings and global scaling as normalization method. The data has been deposited in the public database Gene Expression Omnibus repository (National Center for Biotechnology Information; accession number GSE28342; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28342).

Statistical analysis

Two tailed student's t-test was used to calculate p-values where indicated. A p-value ≤ 0.05 was considered as statistically significant: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

RESULTS

Block of pre-T-cell development at the β-selection checkpoint in Miz-1$^{\Delta POZ}$ mice

Flow cytometric analysis showed a strongly reduced thymic cellularity affecting all T cell subsets, and an increase in the relative percentages of DN cells at the expense of DP and CD4 or CD8 single positive (SP) cells in Miz-1$^{\Delta POZ}$ mice compared to control littermates (Fig. 1A, 1B). In addition, it revealed a block at the transition from DN3 to DN4 cells in Miz-1$^{\Delta POZ}$ mice, most noticeable because of the relative increased frequencies of DN3 at the expense of DN4 cells and a strong relative accumulation of DN3a versus DN3b cells (Fig. 1A). In absolute cell counts and compared to WT controls, Miz-1-deficient DN3a cells were reduced by 13-fold, and DN3b and DN4 cells were reduced by 100- and 180-fold, respectively (Fig. 1B). This loss of DN3 and DN4 cells and the block at DN3/DN4/DP transition, in combination with the recently described early ETP/DN1/DN2 differentiation block (30), dramatically reduced the absolute cell counts of DP, CD4 and CD8 SP cells in Miz-1$^{\Delta POZ}$ mice >100-fold compared to WT controls (Fig. 1B).

DN4TCRβ^{+} pre-T cells are almost absent in Miz-1$^{\Delta POZ}$ mice but are able to produce DP and SP cells with intact TCR signaling

DN3b cells, which are larger in size than DN3a cells and up-regulate CD27, were less frequent in Miz-1$^{\Delta POZ}$ mice than in WT controls (Fig. 1A). In addition, DN3b cells expressing surface TCRβ were decreased in frequency and numbers (Fig. 1B) and their TCRβ expression levels were reduced, evident by a lower mean fluorescence intensity

(MFI) compared to WT DN3b cells (Fig. 2A). Consequently, very few Miz-1$^{\Delta POZ}$ DN4 cells expressed TCRβ on their surface compared to the respective WT subset (Fig. 2A). Also, the frequencies of Miz-1-deficient DN3b and DN4 cells expressing a cytoplasmic (intracellular; i.c.) TCRβ chain were reduced by 2 and 2.5-fold compared to WT controls (Fig. 2B). The expression of CD3ε on the surface of the different thymoyte subsets (Supplemental Fig. 1), as well as the expression of *TCRβ* and *pTα* mRNA and of other genes required for V(D)J recombination such as *Rag1*, *Rag2* and *Tdt* were at WT levels in Miz-1$^{\Delta POZ}$ thymic subsets (Fig. 2C-E). In addition, no defects in Dβ2-Jβ2, Dβ1.1-Jβ1.7 or Dβ2.1-Jβ2.7 rearrangement or in Vβ5-Jβ2, Vβ8-Jβ2 or Vβ11-Jβ2 recombination were detected (Fig. 2F), indicating that all prerequisites for a proper pre-TCR selection are present in Miz-1-deficient cells.

The pre-T cell differentiation block in Miz-1$^{\Delta POZ}$ mice is partially overcome by the introduction of rearranged OTI TCRαβ transgenes.

DP and CD4 and CD8 SP cells are still found in Miz-1$^{\Delta POZ}$ mice, albeit at drastically reduced numbers (Fig. 1B) and expressed normal levels of CD3 and TCRβ on their surface (Supplemental Fig. 1A, 1B). Also, Miz-1-deficient thymocytes did not show defects in TCR-dependent signaling, as evaluated by the phosphorylation of the MAPK ERK1/2 (Supplemental Fig. 2A, 2B) and the mobilization of intracellular Ca^{++} (Supplemental Fig. 2C). Finally, the frequency of CD4 and CD8 cells within selected populations defined by the expression of CD69 and TCRβ (33, 34) was comparable between WT and Miz-1$^{\Delta POZ}$

mice (Supplemental Fig. 3). This indicated that commitment to T cell lineage, mRNA expression of T cell specific genes including the TCRβ chain, V(D)J recombination, TCR signaling and positive/negative selection are intact in Miz-1$^{\Delta POZ}$ mice.

To further investigate the block at the Miz-1 dependent DN3/DN4 transition, we generated Miz-1-deficient mice expressing a transgene encoding for rearranged TCRαβ chains (OTI Tg x Miz-1$^{\Delta POZ}$). In these mice, the frequencies and absolute numbers of DN3b, DN4 and DN4TCRβ^{+} cells were significantly increased compared to Miz-1$^{\Delta POZ}$ thymocytes (Fig. 3), but the overall cellularity of the thymus and of DN4 or DN4 TCRβ^{+} cells did not reach WT or OTI Tg levels (Fig. 3B). This indicated that a functional TCRαβ receptor can be expressed and processed from the cytoplasm to the cell surface of Miz-1$^{\Delta POZ}$ pre-T cells and confirmed that TCR signals driving DN3 to DN4 differentiation can be initiated in Miz-1-deficient mice. It also suggests that the absence of TCRβ surface expression is not solely responsible for the DN3/DN4 transitional block in Miz-1$^{\Delta POZ}$ mice. Additionally, the positive and negative selection was evaluated by the expression of CD69 and TCRβ in OTI Tg x Miz-1$^{\Delta POZ}$ mice expressing a less diverse TCR repertoire compared to Miz-1$^{\Delta POZ}$ mice. The FACS analyses of CD4 and CD8 expression did not reveal any obvious defects in the positive or negative selection processes (Supplemental Fig. 3). The efficiency of the positive/negative selection was also evaluated in previously described *Lck*-cre Miz-1$^{\Delta POZ}$ mice, where the deletion of the BTB/POZ domain of Miz-1 starts at the DN2/DN3 transitional stage of T-cell development but is complete only at the DN4 and subsequent

stages (30). Consistent with a normal DN/DP T-cell development in *Lck*-cre Miz-1$^{\Delta POZ}$ mice (30), the positive selection was intact in mice expressing Miz-1$^{\Delta POZ}$ at a later point of T cell differentiation (Supplemental Fig. 3).

The higher apoptotic rate of Miz-1$^{\Delta POZ}$ DN3 could result from enhanced expression of p53-dependent genes.

AnnexinV staining that allows detecting cells undergoing programmed cell death was used to evaluate DN3a cells from Miz-1-deficient mice. Our data showed that 37.6% of Miz-1$^{\Delta POZ}$ DN3a cells were apoptotic compared to only 6.8% of WT cells (Fig. 4A). In contrast, the DN3b subpopulation showed only 9.2% of Annexin-positive cells and the DN4 subsets did not contain any apoptotic cells (Fig. 4A). Consistent with this, DN3a cells sorted from Miz-1$^{\Delta POZ}$ or OTI Tg x Miz-1$^{\Delta POZ}$ mice were unable to differentiate on OP9DL1 cultures and showed low frequencies of live cells, as determined by the FSC/SSC gate, which is indicative of apoptosis (Supplemental Fig. 4A). This suggested that enhanced spontaneous cell death occurs in Miz-1$^{\Delta POZ}$ DN3a at the moment where V(D)J recombination and the β−selection checkpoint take place.

Next, we compared genome wide expression profiles from Miz-1$^{\Delta POZ}$ and WT DN3 cells and observed that differently regulated genes between those two cell populations belonged to specific gene ontology and KEGG pathways specific for senescence, cell cycle arrest and cell death. As illustrated by a heat map, we noticed in particular, that the p53 target genes *Bax*, *Noxa* (*Pmaip1*), *Atr*, *Apaf1*, *Atm* and *PUMA* (*Bbc3*) that induce apoptosis in response

to a DNA damage signal were up regulated in Miz-1-deficient DN3 cells (Fig. 4B). We re-analyzed a subset of this group by RT-PCR and confirmed up-regulation of *PUMA, Noxa* and *Bax* in Miz-1-deficient DN3a cells (Fig. 4C). Because the expression of *transformation-related protein 53* itself was at comparable levels to WT (Fig. 4C), these findings suggested that Miz-1 might be involved in controlling the activity of p53 or the expression of its effector genes that mediate cell death in DN3 pre-T cells. We reasoned that constitutive Bcl-2 expression should counteract the enhanced cell death and restore the numbers of Miz-1-deficient DN3 and DN4 subsets. Indeed, co-expression of a previously described *H2K*-Bcl-2 transgene that confers pan-hematopoietic expression of Bcl-2 (35) significantly increased frequencies (Fig. 5A) and numbers (Fig. 5B) of DN3 and DN4 cells, but did not restore the numbers of DN3, DN4 (Fig. 5B) and DN4TCRβ^+ cells (not shown) to WT levels, nor rescued the block at the DN3/DN4 transition in Miz-1$^{\Delta POZ}$ mice. Thus, although accelerated apoptosis may cause the low numbers of DN3 cells in Miz-1-deficient mice, it does not explain the block at the DN3/DN4 transition.

A defect in cell cycle progression in Miz-1$^{\Delta POZ}$ pre-T cells concurs with the up-regulation of *Cdkn1a*

Our expression array data also showed that the G1 specific cyclin-dependent kinase inhibitor *Cdkn1a,* which is a downstream target of p53, was up-regulated in Miz-1$^{\Delta POZ}$ DN3 cells (Fig. 4B). This could be confirmed by RT-PCR and western blot analysis on Miz-1-deficient DN3 cells (Fig. 6A, 6B). We also found significant defects in cell cycle progression in Miz-1$^{\Delta POZ}$ DN3 and DN4 cells (Fig. 6C). To test whether the differentiation

block observed at the DN3/DN4 transition in Miz-1$^{\Delta POZ}$ mice could be attributable to the defect in cell cycle progression and the up-regulation of *Cdkn1a* expression, we generated combinatorial mutants deficient for both Miz-1 and Cdkn1a (Cdkn1a$^{-/-}$ x Miz-1$^{\Delta POZ}$). However, we found that the DN3/DN4 differentiation block and the accumulation of DN3a cells at the expense of DN3b cells were not rescued in Cdkn1a$^{-/-}$ x Miz-1$^{\Delta POZ}$ mice (Fig. 6D).

To test whether a combination of *Cdkn1a* inhibition and ectopic TCR expression could rescue the Miz-1-deficient phenotype, we sorted DN3a cells from OTI Tg and OTI Tg x Miz-1$^{\Delta POZ}$, transfected them with FITC-labeled morpholino-oligonucleotide against *Cdkn1a* mRNA, or a control morpholino, and co-cultured them on OP9DL1 cells. Although the knockdown of Cdkn1a was efficient (Supplemental Fig. 4B), OTI Tg x Miz-1$^{\Delta POZ}$ DN3a cells treated with the morpholino oligo against *Cdkn1a* were still arrested, as assessed by the intensity of the FITC labeling, the poor survival on OP9DL1 cells and the generation of only 5.6 % of DP cells compared to 20.1 % from OTI Tg DN3a cells (Supplemental Fig. 4C, 4D). Similarly, a rescue of the DN/DP differentiation block was not obtained when we treated DN3 cells sorted from Bcl-2 x Miz-1$^{\Delta POZ}$ mice with the morpholino oligo against *Cdkn1a* (Supplemental Fig. 4C, 4D). Thus, high levels of Cdkn1a are not responsible for the observed block of differentiation at the β-selection checkpoint seen in Miz-1$^{\Delta POZ}$ DN3 cells.

Bcl-2 and OTI TCR expression rescues the pre-T cell differentiation block in Miz-1$^{\Delta POZ}$ mice

We next examined whether the block at the β-selection checkpoint can be restored in Miz-1$^{\Delta POZ}$ mice by providing both the pro-survival protein Bcl-2 and the rearranged TCRαβ transgenes. Indeed, co-expression of *H2K*-Bcl-2 and OTI TCRαβ transgenes relieved the DN to DP block observed in Miz-1$^{\Delta POZ}$ mice and significantly enhanced the transition from DN3 to DN4 cells compared to controls (Fig. 7A). Both frequencies and absolute numbers of DN3b and DN4 cells were increased in Bcl-2 Tg x OTI Tg x Miz-1$^{\Delta POZ}$ compared to OTI x Miz-1$^{\Delta POZ}$ mice, and over 90% of gated DN4 cells now expressed TCRβ on their cell surface (Fig. 7A). The full rescue of the defect in pre-T cell differentiation by the introduction of both transgenes was also evident by the overall cellularity of the thymus, and most importantly by the re-appearance of DN3b and DN4 cells, which were almost undetectable in Miz-1$^{\Delta POZ}$ mice and now reached OTI Tg x Bcl-2 Tg levels (Fig. 7B). To further show that the rescue provided by the expression of both Bcl-2 and OTI transgenes was at the β-selection checkpoint, we compared HSA expression in DN3, DN4 and DP cells, as HSA is a marker that gets downregulated as thymocytes mature from DN4/DP to SP (36). The downregulation of HSA was much more noticeable, both in percentages and mean fluorescence intensities, as DN3 cells mature to DN4 and DP cells in Bcl-2 Tg x OTI Tg x Miz-1$^{\Delta POZ}$ mice compared to OTI Tg x Miz-1$^{\Delta POZ}$ mice (Fig. 7C). The accelerated transition from DN3b to DN4 in Bcl-2 Tg x OTI Tg x Miz-1$^{\Delta POZ}$ mice suggested that the introduction of the TCRαβ transgenes allowed the cells to deliver a differentiation and

proliferation signal, and that, together with Bcl-2 which counteracts apoptosis, these signals were sufficient to neutralize the effect of Miz-1 deficiency on cell survival.

DISCUSSION

We have previously shown that the BTB/POZ transcription factor Miz-1 regulates IL-7R signaling by monitoring the expression levels of both SOCS1 and Bcl-2. This protects ETP/DN1/DN2 cells from apoptosis and enables their differentiation (30). In the present study, we show that Miz-1 has an additional function later in pre-T cell differentiation, at the β-selection stage of DN3 cells, which is the first critical checkpoint in the maturation of pre-T cells. DN3 cells passing this checkpoint actively rearrange the TCRβ locus and thus have to tightly control DNA damage response pathways such as the one induced by the activation of p53. After productive rearrangement, DN3 cells activate allelic exclusion (37) and become pre-TCR$^+$ DN3b cells that undergo a massive proliferative expansion, differentiate into DN4 cells (3) and escape apoptosis. Here, we present evidence that the transcription factor Miz-1 is essential to coordinate the steps that assure survival of DN3 cells and the expansion and differentiation of DN3 and DN4 cells. Our data suggest that Miz-1 coordinates expression of the pre-TCR and may be involved in controlling p53 target genes possibly induced by DNA double strand breaks initiated upon V(D)J recombination in DN3 cells.

Miz-1 deficiency affects expansion of TCRβ chain expressing DN3b and DN4 pre-T cells

FACS analyses demonstrated that DN cell differentiation is blocked in Miz-$1^{\Delta POZ}$ mice at the β-selection checkpoint. However, *Rag-1*, *Rag-2*, *Tdt* and *TCRβ* genes are expressed, and pTα and CD3ε are present in Miz-$1^{\Delta POZ}$ pre-T cells. Moreover, V(D)J recombination and TCR mediated signaling appeared to be intact in the absence of a functional Miz-1, at least in the cells that still emerged in Miz-1-deficient mice. Despite this, only very few DN3b or DN4 cells that express the TCRβ protein in the cytoplasm or at the surface were present in Miz-1-deficient mice. This phenotype is only detected in *Vav*-cre Miz-$1^{\Delta POZ}$ mice and not in the previously described *Lck*-cre Miz-$1^{\Delta POZ}$ model, where the deletion of the BTB/POZ domain starts occurring in the DN2/DN3 transitional stage of T-cell development (38). We reported that the deletion in this mouse strain started at the DN2 stage, reached 50% in DN3a cells, and was complete at the DN4 stage (30). The residual expression of Miz-1 in the DN3a/b cells of *Lck*-cre Miz-$1^{\Delta POZ}$ mice may be sufficient to overcome the T cell differentiation block at the β-selection checkpoint. Consistent with this report, no obvious DN/DP defects in T-cell development were noted (30), and the positive and negative selection processes were not affected by Miz-1 deficiency in *Lck*-cre Miz-$1^{\Delta POZ}$ mice.

TCRβ gene is expressed at normal levels in Miz-1-deficient DN3 or DN4 cells, possibly implicating Miz-1 in the control of a post-transcriptional step affecting translation, stability or membrane transport of the TCRβ protein. Transport to the membrane and surface expression of TCRβ is necessary to trigger proliferation and the differentiation of DN to DP

cells (39). However, the introduction of a rearranged TCR receptor transgene led to a stable, high-level surface expression of ectopic TCRαβ in Miz-1-deficient cells. In addition, the DP and SP cells that still develop in Miz-1$^{\Delta POZ}$ mice expressed normal levels of TCRβ chain on their surface. Further, the frequency of CD4 and CD8 cells within selected populations defined by the expression of CD69 and TCRβ (33, 34) was comparable between WT and Miz-1$^{\Delta POZ}$ mice. As selected thymic populations could be better evaluated in mice expressing a less diverse repertoire like TCR transgenic mice, we could show that positive/negative selection is not affected by Miz-1 deficiency in OTI Tg x Miz-1$^{\Delta POZ}$ mice. Finally Miz-1-deficient cells did up-regulate CD69 and CD5 in response to TCR stimulation at comparable levels to WT controls (data not show). These data rather support the view that Miz-1 deficiency does not alter the expression, stability or processing of the pre-TCR *per se*.

Lack of cell cycle progression in Miz-1-deficient DN3 and DN4 cells

The presence of a transgenic OTI TCR led to an expansion of DN3 cells in Miz-1$^{\Delta POZ}$ mice but failed to rescue the DN3/DN4 block, indicating that even if the TCRβ chain is expressed at the cell surface and is able to transmit the appropriate signal, the cells still encounter a proliferative block mediated by other signals. One of the most striking findings that could possibly explain this observation was the significant up-regulation of Cdkn1a expression in Miz-1$^{\Delta POZ}$ DN3 cells. At high Cdkn1a levels, Miz-1-deficient DN3 cells would be unable to react to the proliferative burst induced by the pre-TCR signaling. Although compelling, a rescue attempt showed that the DN3/DN4 block and the

accumulation of DN3a cells at the expense of DN3b persisted in Cdkn1a$^{-/-}$ x Miz-1$^{\Delta POZ}$ mice. Therefore, although the *Cdkn1a* gene is a *bona fide* Miz-1 target and is occupied by Miz-1 on its promoter in DN thymocytes (data not shown), the de-regulation of *Cdkn1a* by Miz-1 cannot be the only cause of the Miz-1$^{\Delta POZ}$ DN3 cell arrest at the β-selection checkpoint. The Cdkn1a up-regulation may rather be a consequence of the cell cycle progression arrest that is caused by both the lack of surface pre-TCR expression and the up-regulated p53-target genes at this stage.

Evidence for an accelerated p53 response in Miz-1-deficient DN3 cells

Analysis of genome wide expression array data indicated that Miz-1-deficient DN3 cells over-expressed not only genes mediating cell cycle arrest such as *Cdkn1a* but also a whole set of p53 target genes that can initiate apoptosis such as *PUMA*, *Noxa* and *Bax*. It is known that p53 is required for the maintenance of genomic stability and regulates apoptosis and growth arrest in response to DNA damage, in particular to DNA double-stranded breaks (DSBs; reviewed in Refs. 40, 41). Moreover, it has been shown that pre-TCR signaling inhibits the p53 response after a successful V(D)J recombination . Such regulation is crucial for pre-TCR selection and DN/DP differentiation and survival (9-12).

As Miz-1-deficient cells fail to express TCRβ proteins on their cell surface, it is possible that these cells cannot induce pre-TCR-dependent inactivation of p53 when they have completed a productive rearrangement which generates physiological DSBs. One effect of this failure to inactivate p53 could result in the induction of pro-apoptotic genes such as

Bax (42) and the regulation of Bcl-2 (43). Miz-1-deficient DN3 cells express high *Bcl-2* levels (not shown) that should decline in developing αβ DN3 cells and also display high *Bax* expression, which most likely counteracts the Bcl-2 function and provokes higher apoptosis rates. Interestingly, the introduction of the anti-apoptotic Bcl-2 protein partially restored DN3a numbers but did not rescue the DN3/DN4 block in Miz-$1^{\Delta POZ}$ mice, which is consistent with the finding that Bcl-2 expression alone does not promote differentiation of pre-TCR deficient SCID DN3 cells (44).

Miz-1 as a potential regulator of the p53 response during pre-TCR selection

During V(D)J recombination, DSBs are generated and any p53 response that may initiate apoptosis has to be contained until a pre-TCR signal shuts down the p53 pathway and enables selected cells to expand. In this model, cells that fail to receive a pre-TCR signal die, which is in line with evidence showing that loss of p53 in pre-TCR deficient thymocytes can restore their development and survival (9-12). How the p53 response is controlled during V(D)J recombination is not fully understood, but our data point to the possibility that Miz-1 is involved in this process. Although alternative possibilities exist and further experimentation is needed to fully support this hypothesis, our finding that the ectopic expression of Bcl-2 and OTI transgenic TCR fully rescued Miz-1 deficiency supports this view, since both Bcl-2 and TCR can counteract the p53 initiated apoptosis and growth arrest in DN3 cells. A balance between the role of p53 in inducing apoptosis versus promoting cell survival has been shown to contribute to the normal development of the cells (45). Accordingly, low levels of p53 are maintained under physiological conditions

and normal cell proliferation to promote the induction of temporary cell cycle arrest in stress situations to allow for instance the repair of DNA damage. If a damage signal persists, a p53 mediated apoptosis program is activated to eliminate these cells. Since the expression levels of *p53* is comparable to wild-type in Miz-1 $^{\Delta POZ}$ cells and as it has been shown that Miz-1 can bind to p53 (46), it is possible that Miz-1 is one of the factors that dampens the part of p53 response that promotes cell death. The precise regulation of p53 activity by Miz-1 is presently unknown. It is possible that in the absence of a functional Miz-1 protein, p53-mediated cell death response may be induced and the disturbed expression levels of *Bax* and *PUMA* as seen in DN3 cells from Miz-1$^{\Delta POZ}$ mice are consistent with this view. Experiments that enable the detection of a co-occupation of DNA damage response gene promoters by p53 and Miz-1 may help clarify this point.

A recent report that directly implicates Miz-1 as a mediator of the p19ARF-p53 pathway would also be consistent with this hypothesis. In this study, evidence show that Miz-1 is able to bind to p19ARF and to interfere with p53 stability. The same study also demonstrated that Miz-1 interacts directly with p53 and that this interaction diminishes the binding of p53 to its target promoters and inhibits p53-mediated gene transcription (46). It is therefore conceivable that Miz-1 plays a role in the regulation of p53 in pre-T cells and that a functional form of Miz-1 is necessary to control p53 activity at the specific β-selection checkpoint to allow V(D)J recombination to occur without initiating apoptosis. In the absence of a functional form of Miz-1, particularly lacking its BTB/POZ domain, the

p53 response to the V(D)J recombination events is enhanced causing cell cycle arrest and apoptosis and this prevents TCRβ^+ cells from expanding and initiating their differentiation into DP thymocytes. In order to understand this regulation, more experiments need to be done to identify the particular p53 target genes that may be dependent on the expression of a functional Miz-1 protein. Taken together, the data presented in this report help explain the DN3/DN4 block observed in Miz-1-deficient mice and would establish the BTB/POZ domain protein Miz-1 as a new regulator of the pre-TCR β–selection checkpoint. Whether this regulation is mediated by co-factors or by post-transcriptional events remains to be explored.

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CONFLICT-OF-INTEREST STATEMENTS

Conflict-of-interest disclosure: The authors declare no competing financial interests.

FOOTNOTES

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2. The sequences presented in this article have been submitted to National Center for Biotechnology Information under accession number GSE28342

3. Address correspondence and reprint requests to Dr. Tarik Möröy, Institut de Recherches Cliniques de Montréal, 110 des Pins West Avenue, Montréal, Québec, H2W 1R7, Canada.

4. Abbreviations used in this article: BTB, Broad-complex, Tramtrack and Bric-à brac; DN, double-negative; DP, double-positive; DSB, double-stranded break; ETP, early thymic precursor; FSC, Forward scatter; SSC, Side scatter; MFI, mean fluorescence intensity; NP-40, Nonidet P-40; POZ, Pox virus Zinc finger; SP, single-positive; Tg, transgenic; WT, wild-type

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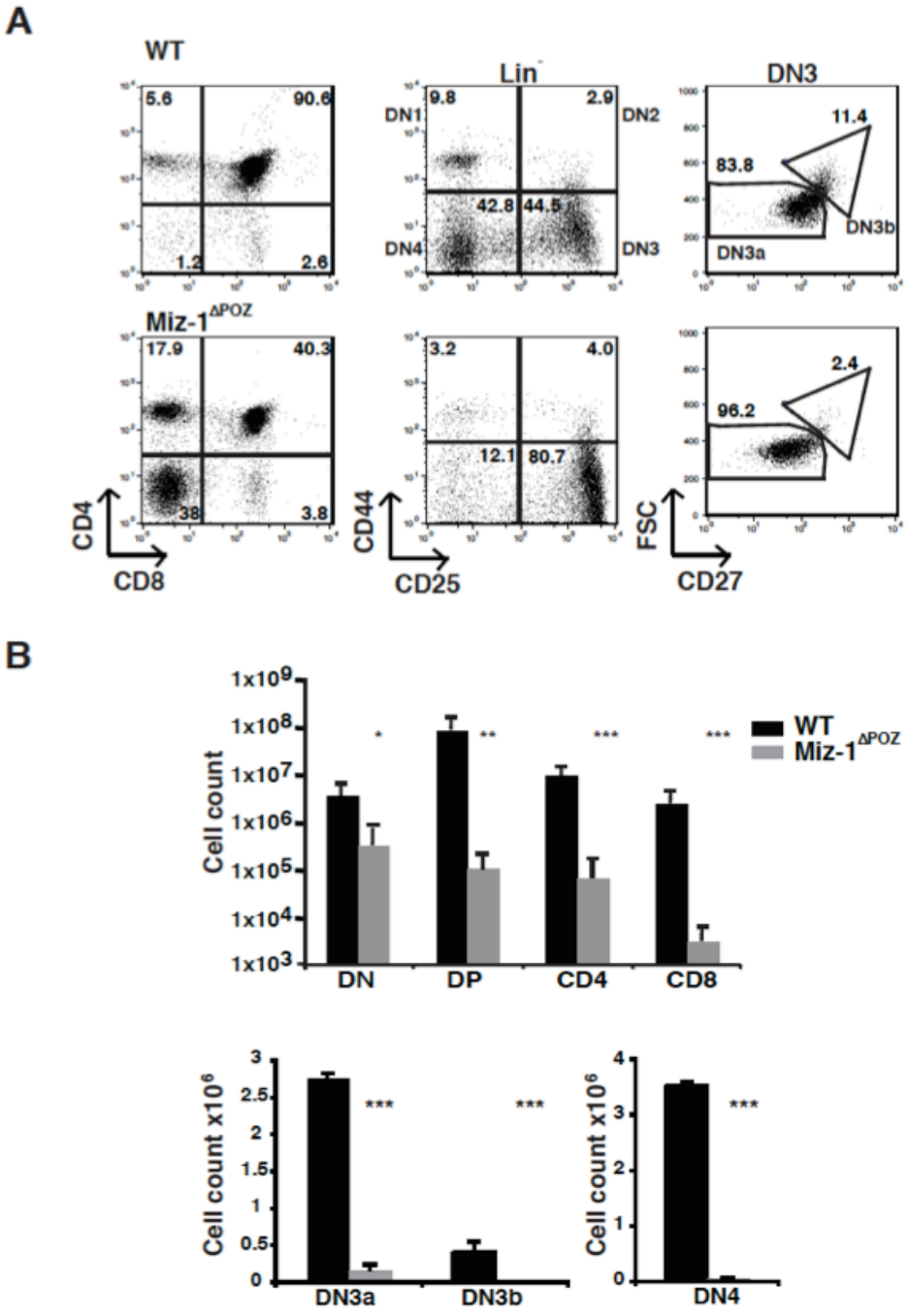

A
WT
5.6
90.6
1.2
2.6
Lin-
DN1
9.8
2.9
DN2
42.8
44.5
DN4
DN3
DN3
11.4
83.8
DN3b
DN3a
Miz-1ΔPOZ
17.9
40.3
38
3.8
3.2
4.0
12.1
80.7
2.4
96.2
CD4
CD8
CD44
CD25
FSC
CD27
B
Cell count
1x10^9
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
*
**
***
***
WT
Miz-1ΔPOZ
DN
DP
CD4
CD8
Cell count x10^6
3
2.5
2
1.5
1
0.5
0
***
***
DN3a
DN3b
Cell count x10^6
4
3
2
1
0
***
DN4

Figure 1

Figure 1. Miz-1$^{\Delta POZ}$ DN cells have a severe block at the β-selection check point between DN3a/DN3b to DN4 transition.

Comparative thymocytes FACS analyses (**A**) and absolute cell counts of thymocyte subsets (**B**) from Miz-1$^{\Delta POZ}$ and WT littermates. (**A**) CD4 and CD8 expression was analyzed for each mouse of the indicated genotype (left panel). Gated lineage negative cells were further analyzed for the expression of CD44 and CD25 to assess DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$) and DN4 ($CD44^{-}CD25^{-}$) (middle panel). Gated DN3 subset was further fractionated into DN3a ($FSC^{low}CD27^{-}$) and DN3b ($FSC^{high}CD27^{+}$) (right panel). The percentage of positive cells for each quadrant or gate is indicated. (**B**) Percentages in (**A**) are calculated relative to the total live cells (FSC/SSC gate) of the thymus and expressed as absolute cell counts. Data are representative of at least 8 independent experiments. Mean average and SD are shown. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

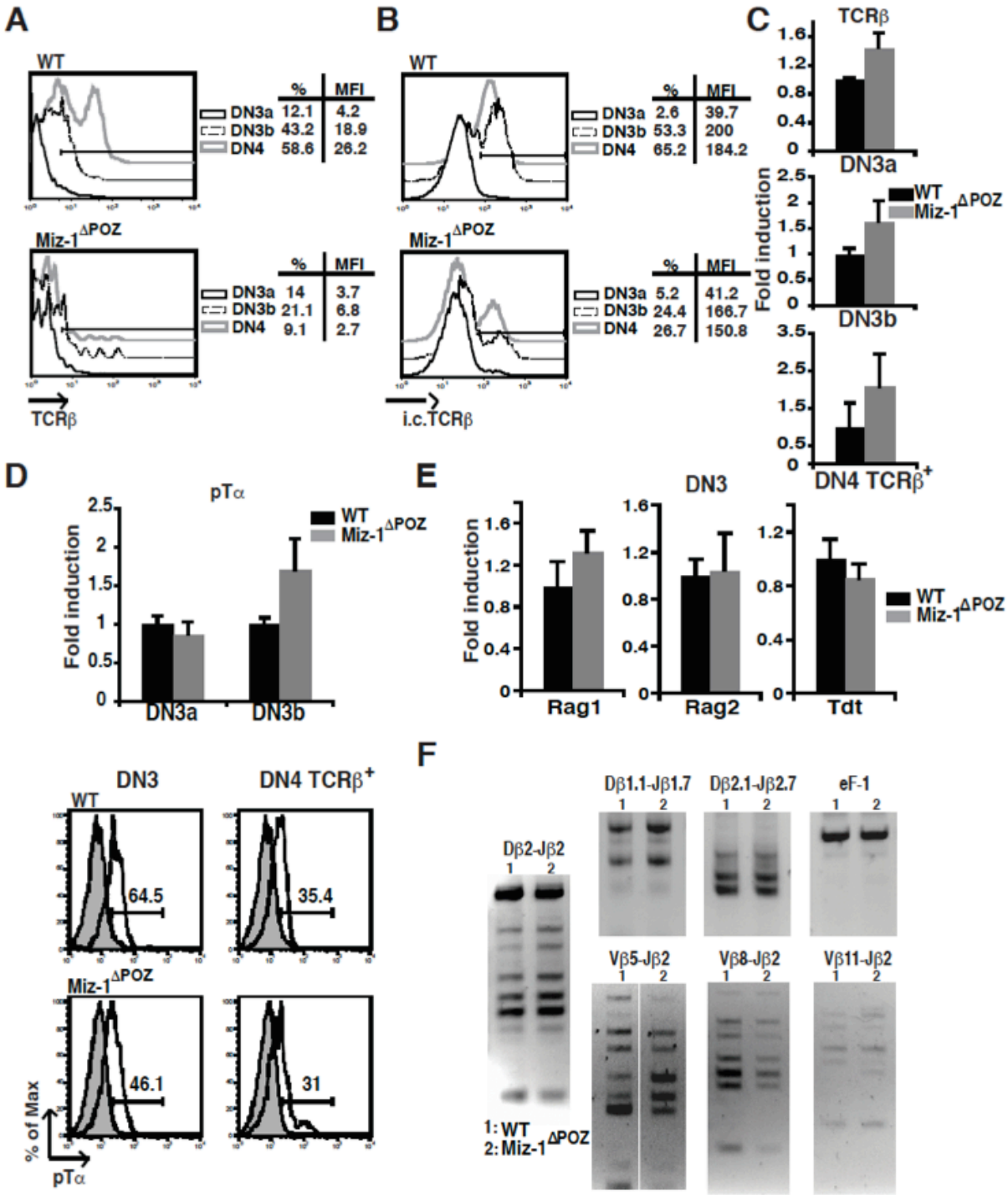

A
WT
% MFI
DN3a 12.1 4.2
DN3b 43.2 18.9
DN4 58.6 26.2
Miz-1ΔPOZ
DN3a 14 3.7
DN3b 21.1 6.8
DN4 9.1 2.7
TCRβ
B
WT
DN3a 2.6 39.7
DN3b 53.3 200
DN4 65.2 184.2
Miz-1ΔPOZ
DN3a 5.2 41.2
DN3b 24.4 166.7
DN4 26.7 150.8
i.c.TCRβ
C
TCRβ
DN3a
DN3b
DN4 TCRβ+
Fold induction
WT
Miz-1ΔPOZ
D
pTα
Fold induction
DN3a
DN3b
E
DN3
Fold induction
Rag1
Rag2
Tdt
F
DN3
DN4 TCRβ+
WT
64.5
35.4
Miz-1ΔPOZ
46.1
31
% of Max
pTα
Dβ2-Jβ2
Dβ1.1-Jβ1.7
Dβ2.1-Jβ2.7
eF-1
Vβ5-Jβ2
Vβ8-Jβ2
Vβ11-Jβ2
1: WT
2: Miz-1ΔPOZ

Figure 2

Figure 2. Lack of TCRβ expression in Miz-1$^{\Delta POZ}$ DN4 pre-T cells.

Offset histograms showing surface staining (**A**) and intracellular staining (i.c.) (**B**) of TCRβ expression on gated DN3a ($Lin^-CD44^-CD25^+FSC^{low}CD27^-$), DN3b ($Lin^-CD44^-CD25^+FSC^{high}CD27^+$), and DN4 ($Lin^-CD44^-CD25^-$) thymocytes. The percentages shown are % of $TCR\beta^+$ cells. Mean fluorescence intensities (MFI) are also indicated. Data represent five independent experiments for the surface expression and three independent experiments for the intracellular staining. *TCRβ* mRNA (**C**), *pTα* (**D,** upper panel) mRNA expression and protein levels (D, lower panel) were assessed in DN3a, DN3b and DN4 cells. The plots are composed of an overlay of the pTα staining (black line) with the matching isotype isotype control Ig1κ (gray filled histogram). The numbers in the histograms are percentages of $pT\alpha^+$ cells. Data represent five independent experiments for the surface expression and three independent experiments for mRNA quantification in triplicates. (**E**) Quantitative Real-time PCR analysis of genes involved in the V(D)J recombination. RNA was extracted from 50,000 sorted DN3 cells from WT and Miz-1$^{\Delta POZ}$ mice. Average of triplicate values and SD are shown. Data are representative of five independent experiments. (**F**) Genomic DNA PCR analyses of the extent of Dβ-to-Jβ rearrangement or V(D)Jβ recombination in sorted 50,000 DN3 cells from WT (1) and Miz-1$^{\Delta POZ}$ (2) mice. The amplification of eF-1α fragment was used as input control. Results are representative of three independent experiments.

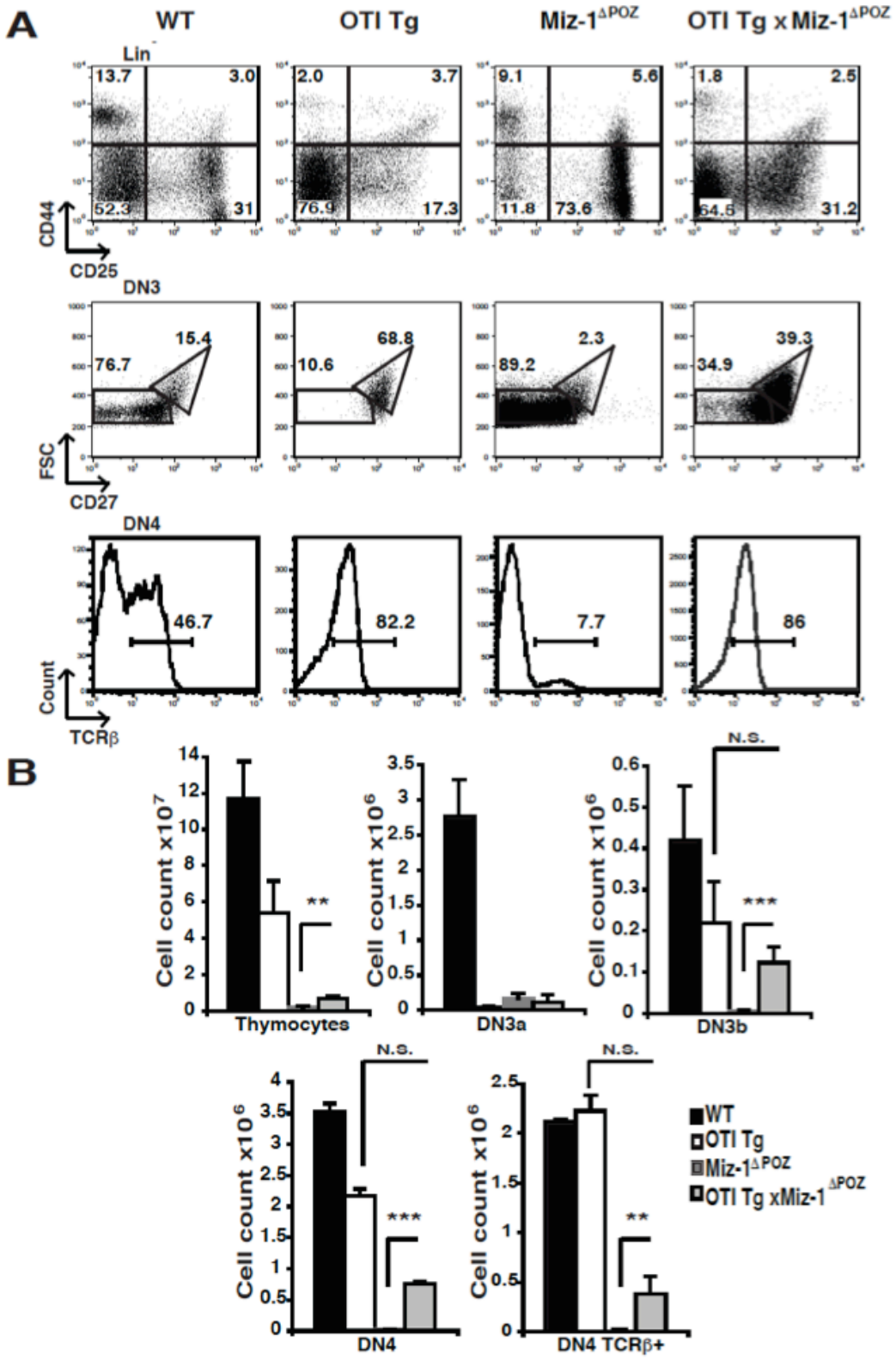

A
WT
OTI Tg
Miz-1ΔPOZ
OTI Tg x Miz-1ΔPOZ
Lin-
13.7
3.0
52.3
31
2.0
3.7
76.9
17.3
9.1
5.6
11.8
73.6
1.8
2.5
64.6
31.2
CD44
CD25
DN3
15.4
76.7
68.8
10.6
2.3
89.2
39.3
34.9
FSC
CD27
DN4
46.7
82.2
7.7
86
Count
TCRβ
B
Cell count x10^7
Thymocytes
**
Cell count x10^6
DN3a
Cell count x10^6
DN3b
N.S.
***
Cell count x10^6
DN4
N.S.
***
Cell count x10^6
DN4 TCRβ+
N.S.
**
WT
OTI Tg
Miz-1ΔPOZ
OTI Tg xMiz-1ΔPOZ

Figure 3

Figure 3: The pre-T cell differentiation block in Miz-1$^{\Delta POZ}$ mice is partially overcome by the introduction of rearranged OTI TCRαβ transgenes in vivo.

FACS analyses (**A**) and total cell numbers (**B**) of thymic subsets from WT and Miz-1$^{\Delta POZ}$ or OTI Tg and OTI Tg x Miz-1$^{\Delta POZ}$ mice (n=5). (**A**) Lineage negative cells were analyzed for the surface expression of CD44 and CD25 to assess the four DN populations (upper panel). Within the DN populations, DN3 (middle panel) were analyzed for DN3a ($FSC^{low}CD27^{-}$) and DN3b ($FSC^{high}CD27^{+}$) subsets, and DN4 (lower panel) for the surface expression of TCRβ. (**B**) Percentages in (**A**) are calculated relative to the total live cells (FSC/SSC gate) of the thymus and expressed as absolute cell counts. Mean average and SD are shown. ** $p \leq 0.01$, *** $p \leq 0.001$.

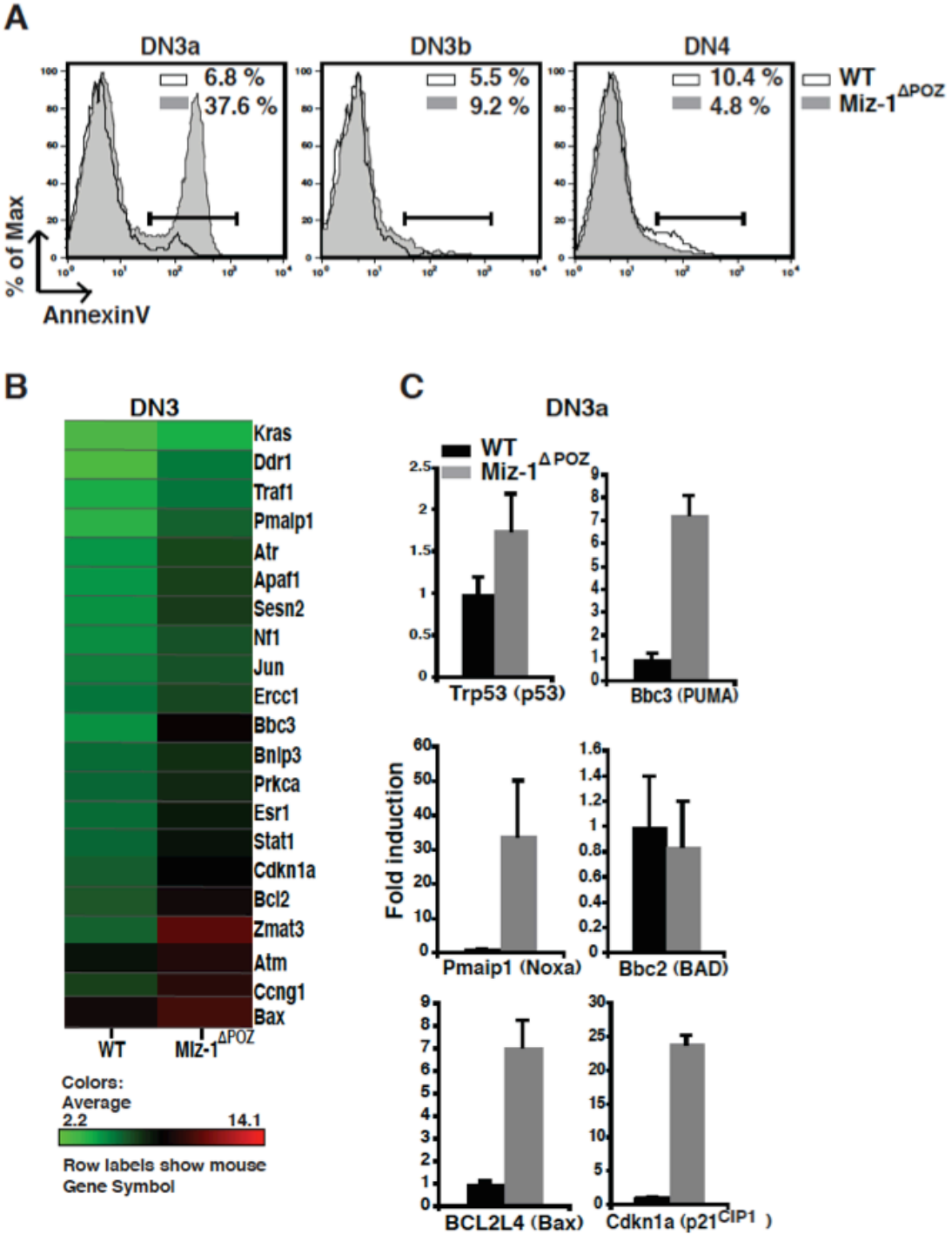

A
DN3a
DN3b
DN4
6.8 %
37.6 %
5.5 %
9.2 %
10.4 %
4.8 %
WT
Miz-1ΔPOZ
% of Max
AnnexinV
B
DN3
Kras
Ddr1
Traf1
Pmaip1
Atr
Apaf1
Sesn2
Nf1
Jun
Ercc1
Bbc3
Bnip3
Prkca
Esr1
Stat1
Cdkn1a
Bcl2
Zmat3
Atm
Ccng1
Bax
WT
Miz-1ΔPOZ
Colors:
Average
2.2
14.1
Row labels show mouse
Gene Symbol
C
DN3a
WT
Miz-1ΔPOZ
Trp53 (p53)
Bbc3 (PUMA)
Fold induction
Pmaip1 (Noxa)
Bbc2 (BAD)
BCL2L4 (Bax)
Cdkn1a (p21CIP1)

Figure 4

Figure 4: DN3 cells from Miz-1$^{\Delta POZ}$ mice are highly apoptotic and show enhanced p53-target gene expression.

(**A**) Single-cell suspensions of thymocytes were stained with antibodies against lineage markers, CD44 and CD25 followed by AnnexinV staining. Percentages of AnnexinV-positive cells are indicated for DN3a, DN3b and DN4 cells (n=3). (**B**) RNA from sorted DN3 cells from WT and Miz-1$^{\Delta POZ}$ thymi were isolated and subjected to expression profiling analysis. An overview of genes differentially expressed is shown in a "heat map". Red bars represent relatively high expression levels, and green bars represent low expression levels of triplicates. (**C**) Validation of some p53-dependent target genes by quantitative Real-time PCR. RNA was extracted from 50, 000 sorted DN3a cells of WT and Miz-1$^{\Delta POZ}$ mice. Mean average of triplicate values and SD are shown. Data are representative of four independent experiments.

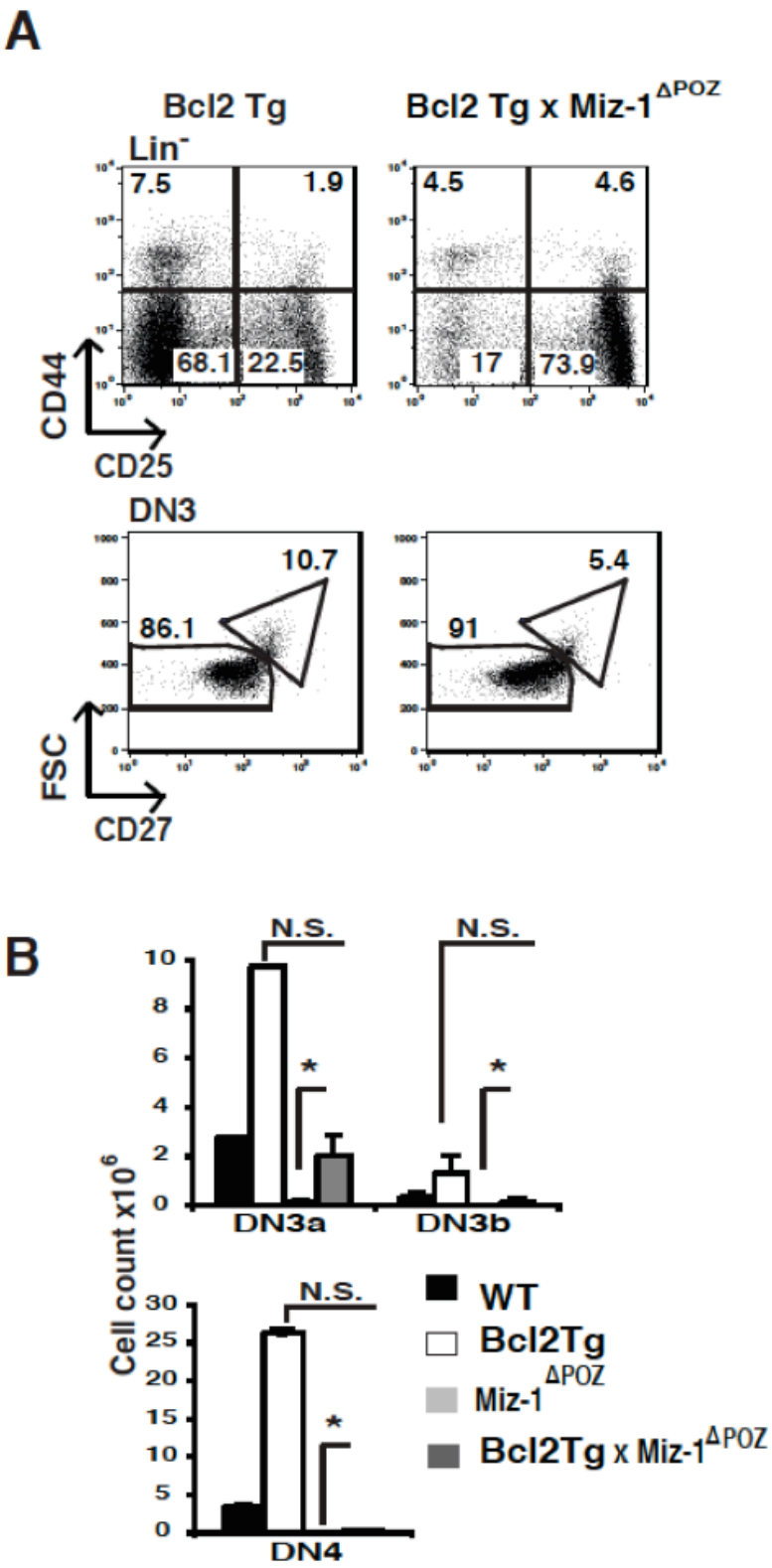

A
Bcl2 Tg
Bcl2 Tg x Miz-1ΔPOZ
Lin-
7.5
1.9
68.1
22.5
4.5
4.6
17
73.9
CD44
CD25
DN3
10.7
86.1
5.4
91
FSC
CD27
B
N.S.
N.S.
*
*
DN3a
DN3b
Cell count x10^6
N.S.
*
DN4
WT
Bcl2Tg
Miz-1ΔPOZ
Bcl2Tg x Miz-1ΔPOZ

Figure 5

Figure 5: The DN3/DN4 transitional block in Miz-1$^{\Delta POZ}$ mice is not rescued by the overexpression of the Bcl-2 transgene.

FACS analyses (**A**) and total cell numbers (**B**) of thymic subsets from Bcl-2 Tg and Bcl-2 Tg x Miz-1$^{\Delta POZ}$ (n=7). (**A**) Lineage negative cells were analyzed for the surface expression of CD44 and CD25 to assess the four DN populations (upper panel). Within the DN populations, DN3 cells were analyzed for DN3a ($FSC^{low}CD27^{-}$) and DN3b ($FSC^{high}CD27^{+}$) subsets (lower panel). (**B**) Percentages in (**A**) are calculated relative to the total live cells (FSC/SSC gate) of the thymus and expressed as absolute cell counts. Mean average and SD are shown. * $p \leq 0.05$.

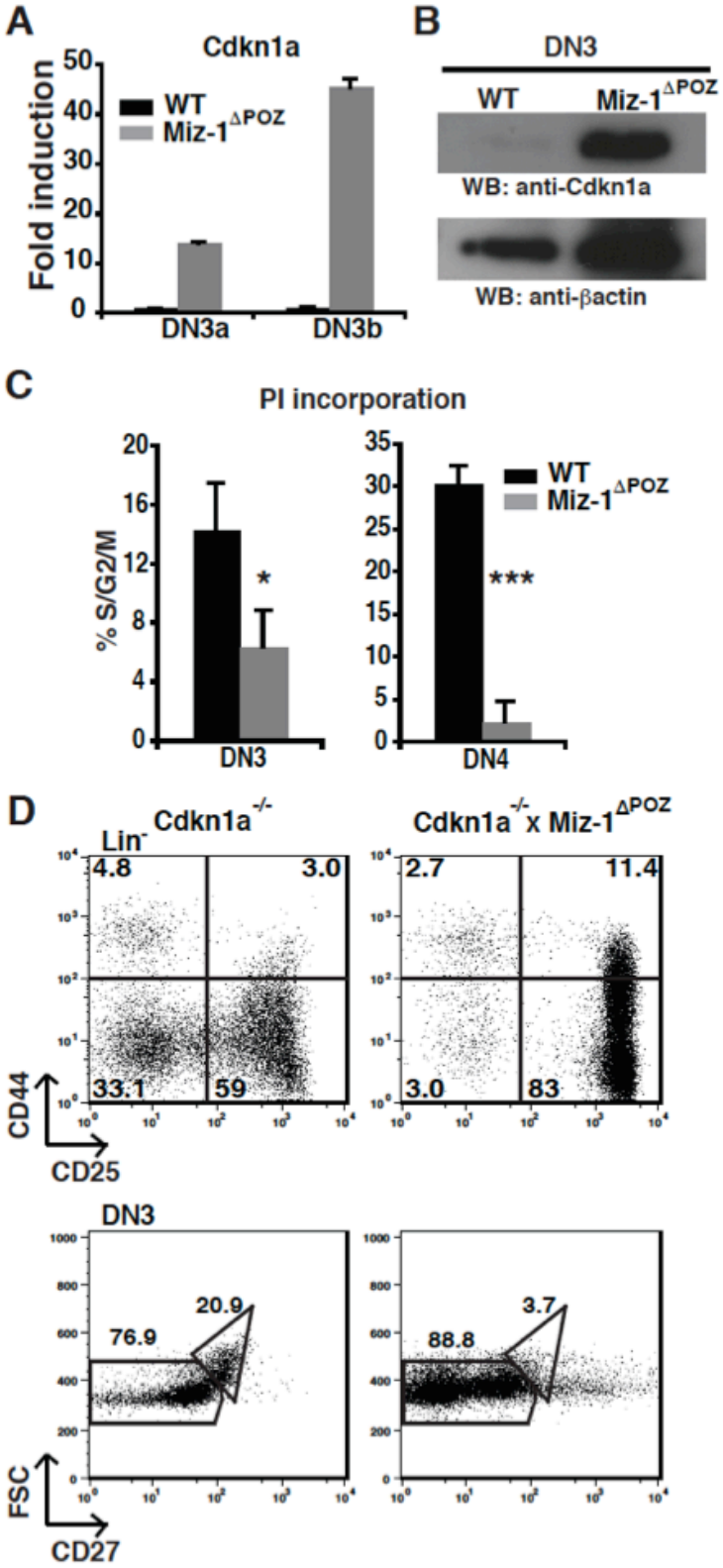

A
Cdkn1a
Fold induction
50
40
30
20
10
0
WT
Miz-1ΔPOZ
DN3a
DN3b
B
DN3
WT
Miz-1ΔPOZ
WB: anti-Cdkn1a
WB: anti-βactin
C
PI incorporation
% S/G2/M
20
16
12
8
4
0
*
DN3
35
30
25
20
15
10
5
0
***
DN4
WT
Miz-1ΔPOZ
D
Cdkn1a-/-
Cdkn1a-/- x Miz-1ΔPOZ
Lin-
4.8
3.0
33.1
59
2.7
11.4
3.0
83
CD44
CD25
DN3
20.9
76.9
3.7
88.8
FSC
CD27

Figure 6

Figure 6: The cell cycle progression defect in Miz-1$^{\Delta POZ}$ pre-T cells is Cdkn1a-independent.

(**A**) *Cdkn1a* mRNA expression was assessed in DN3a and DN3b cells from WT and Miz-1$^{\Delta POZ}$ mice and is representative of five independent experiments done in triplicates. (**B**) Whole protein extracts from sorted DN3 cells were evaluated for Cdkn1a by western blot. βactin was used as loading control. (**C**) Cell cycle analysis using propidium iodide staining was performed on permeabilized, sorted DN3 and DN4 cells. Data show percentage of cells in S/G2/M phase ± SD, and are representative of four independent experiments. (**D**) Thymic DN analysis of Miz-1$^{\Delta POZ}$ mice crossed with *Cdkn1a* deficient mice (Cdkn1a$^{-/-}$). FACS data of gated Lin^{-} cells were analyzed for CD44 and CD25 expression (upper panel) and the gated DN3 subset was further fractionated into DN3a (FSC^{low}CD27^{-}) and DN3b (FSC^{high}CD27^{+}) (lower panel). The stainings for Cdkn1a$^{-/-}$ and Cdkn1a$^{-/-}$ x Miz-1$^{\Delta POZ}$ are representative of 5 independent experiments. * $p \leq 0.05$, *** $p \leq 0.001$.

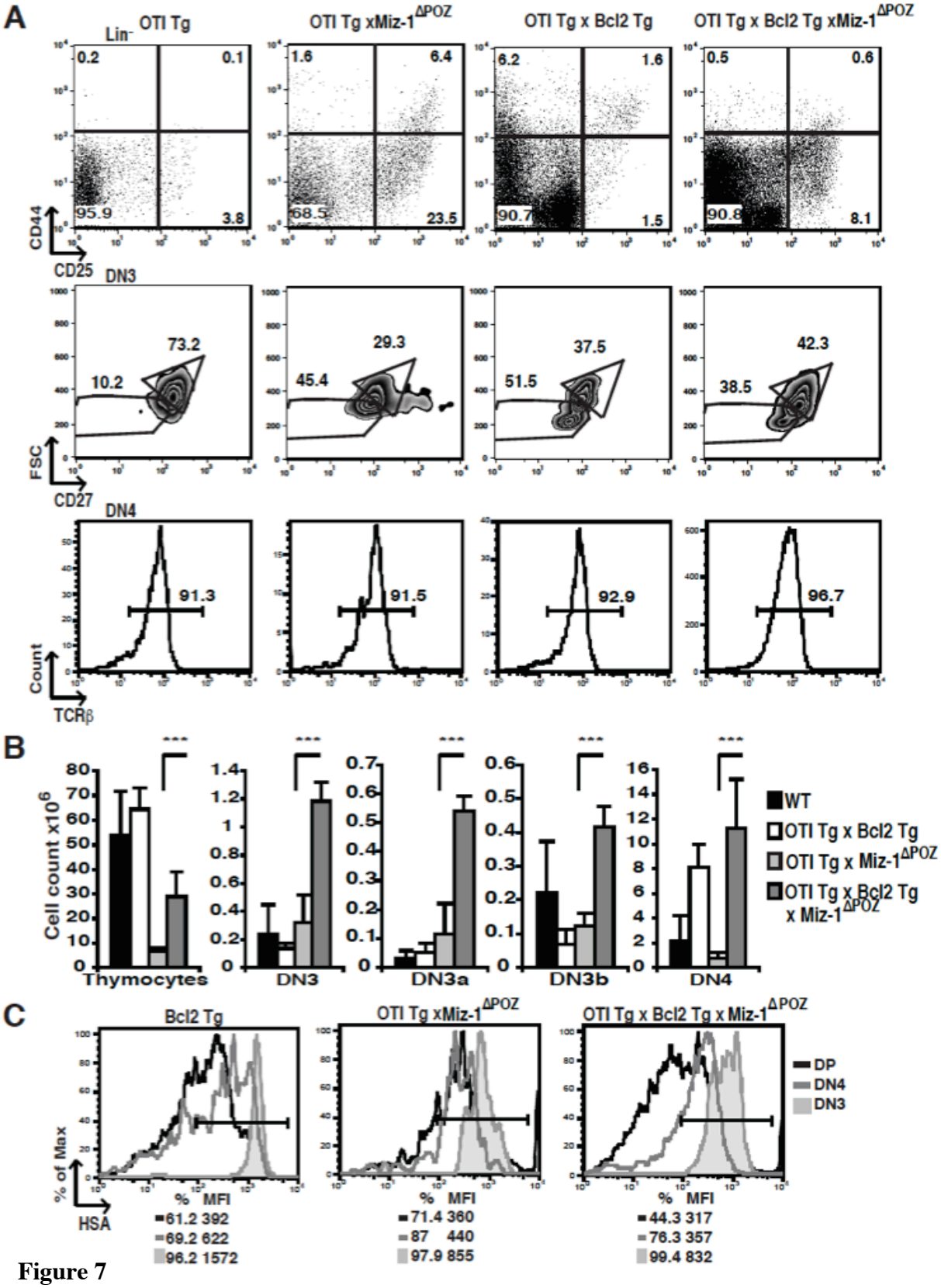

A
Lin-
OTI Tg
OTI Tg xMiz-1ΔPOZ
OTI Tg x Bcl2 Tg
OTI Tg x Bcl2 Tg xMiz-1ΔPOZ
0.2
0.1
95.9
3.8
1.6
6.4
68.5
23.5
6.2
1.6
90.7
1.5
0.5
0.6
90.8
8.1
CD44
CD25
DN3
73.2
10.2
29.3
45.4
37.5
51.5
42.3
38.5
FSC
CD27
DN4
91.3
91.5
92.9
96.7
Count
TCRβ
B
Cell count x10^6
Thymocytes
DN3
DN3a
DN3b
DN4
WT
OTI Tg x Bcl2 Tg
OTI Tg x Miz-1ΔPOZ
OTI Tg x Bcl2 Tg x Miz-1ΔPOZ
C
Bcl2 Tg
OTI Tg xMiz-1ΔPOZ
OTI Tg x Bcl2 Tg x Miz-1ΔPOZ
% of Max
HSA
% MFI
61.2 392
69.2 622
96.2 1572
% MFI
71.4 360
87 440
97.9 855
% MFI
44.3 317
76.3 357
99.4 832
DP
DN4
DN3

Figure 7

Figure 7: The pre-T cell differentiation block in Miz-1$^{\Delta POZ}$ mice is rescued by the overexpression of the anti-apoptotic protein Bcl-2 and the rearranged OTI TCRαβ transgenes in vivo.

FACS analyses (**A**, **C**) and total cell numbers (**B**) of thymic subsets from OTI Tg and OTI Tg x Miz-1$^{\Delta POZ}$ or OTI Tg x Bcl-2 Tg and OTI Tg x Bcl-2 Tg x Miz-1$^{\Delta POZ}$ mice (n=3). (**A**) Lineage negative cells were analyzed for the surface expression of CD44 and CD25 to assess the four DN populations (upper panel). Within the DN populations, DN3 cells were analyzed for DN3a (FSC^{low}CD27^{-}) and DN3b (FSC^{high}CD27^{+}) populations (middle panel), and DN4 for the surface expression of surface TCRβ (lower panel). (**B**) Percentages in (**A**) are calculated relative to the total live cells (FSC/SSC gate) of the thymus and expressed as absolute cell counts. Mean average and SD are shown. (**C**) HSA (CD24) staining on gated DN3, DN4 and DP cells from Bcl-2 Tg, OTI Tg x Miz-1$^{\Delta POZ}$ or OTI Tg x Bcl-2 Tg x Miz-1$^{\Delta POZ}$ mice (n=2). Histograms are composed of an overlay of HSA expression profile on gated DN3 (filled histogram), DN4 (gray line) and DP (black line). Percentages (%) of HSA-positive cells and mean fluorescence intensities (MFI) are indicated at the bottom of each histogram. *** $p \leq 0.001$.

Supplementary materials

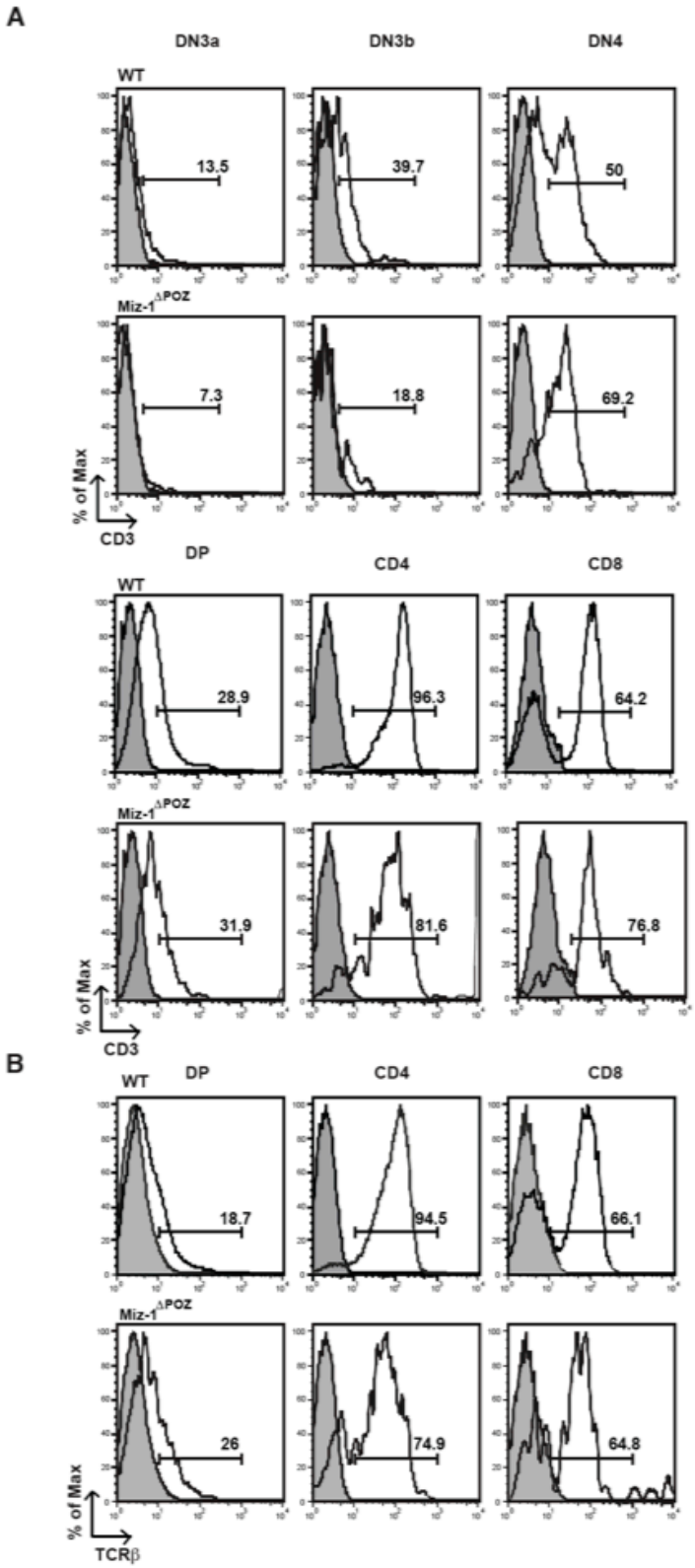
Miz-1 is Required to Coordinate the Expression of TCRβ and p53 Effector Genes at the Pre-TCR "β-selection" Checkpoint

Ingrid Saba, Christian Kosan, Lothar Vassen, Ludger Klein-Hitpass and Tarik Möröy

Inventory

Supplemental Figures:

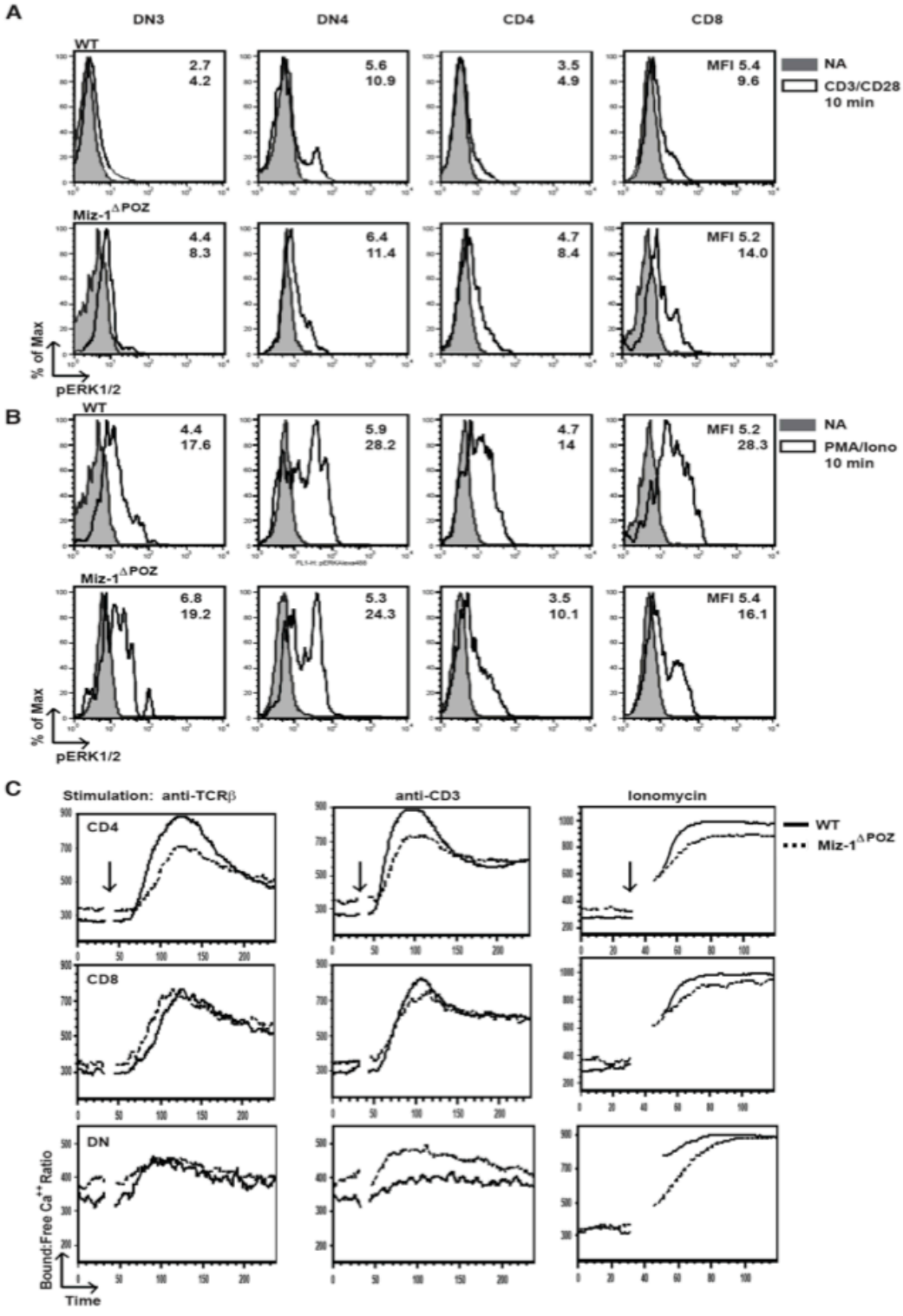
Figure S1 to S4



Supplemental Figure 1

Supplemental Figure 1. CD3 and TCRβ expressions during T-cell development are normal in Miz-1$^{\Delta POZ}$ mice.

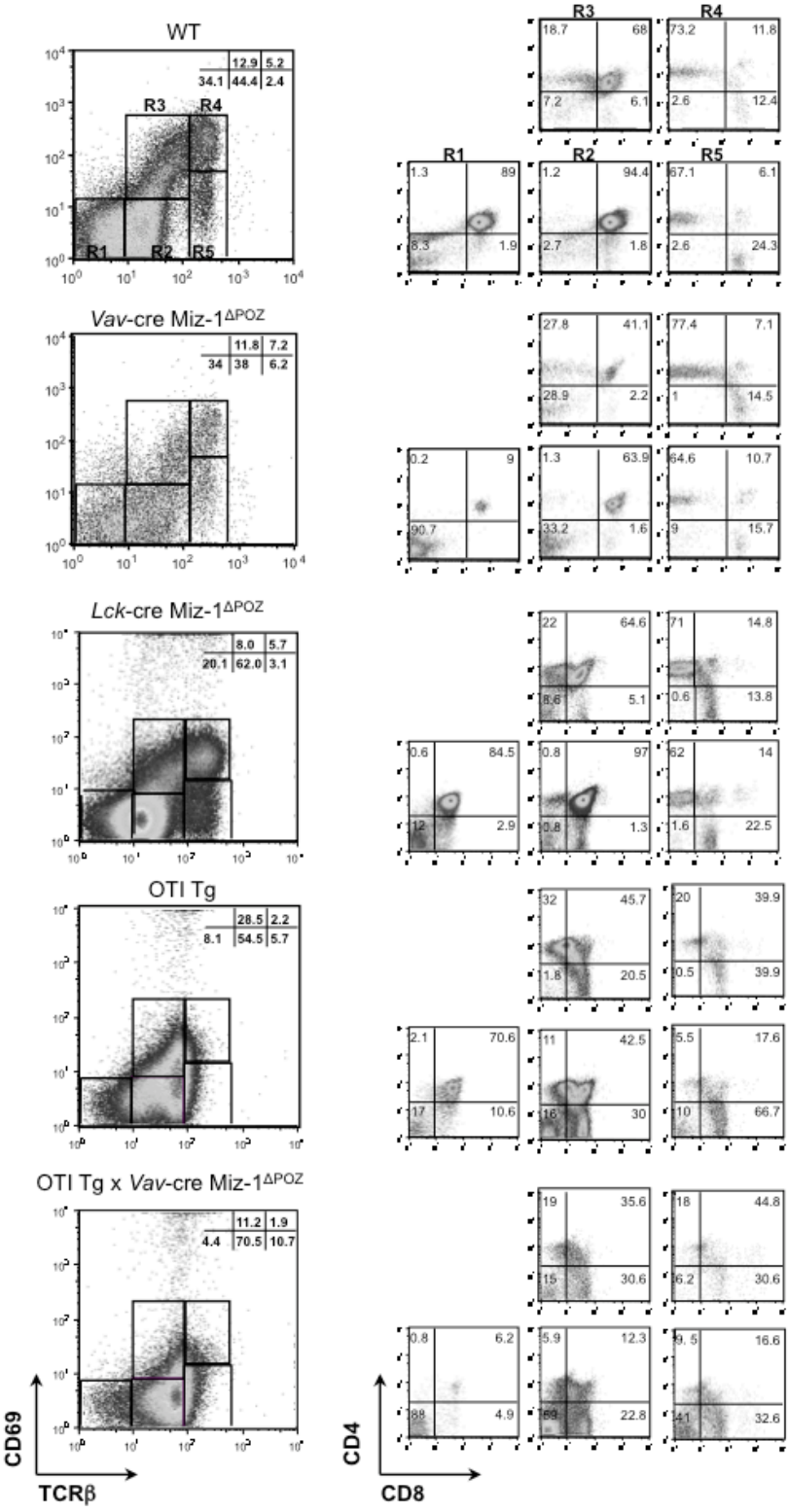
FACS analyses of CD3 (**A**), and TCRβ (**B**) expression on the surface of identified DN population from WT or Miz-1$^{\Delta POZ}$ mice. The cells were gated on lineage negative, CD44 and CD25 as indicated. DP and single positive CD4 or CD8 cells were also analyzed. The plots are composed of an overlay of the specific staining (black line) with the matching isotype control antibodies staining (filled histogram). Percentages of positive cells are indicated. Data are representative of five independent experiments.



Supplemental Figure 2

Supplemental Figure 2. Miz-1$^{\Delta POZ}$ pre-T cells and mature single positive cells do not have a defect in pre-TCR-dependent signaling.

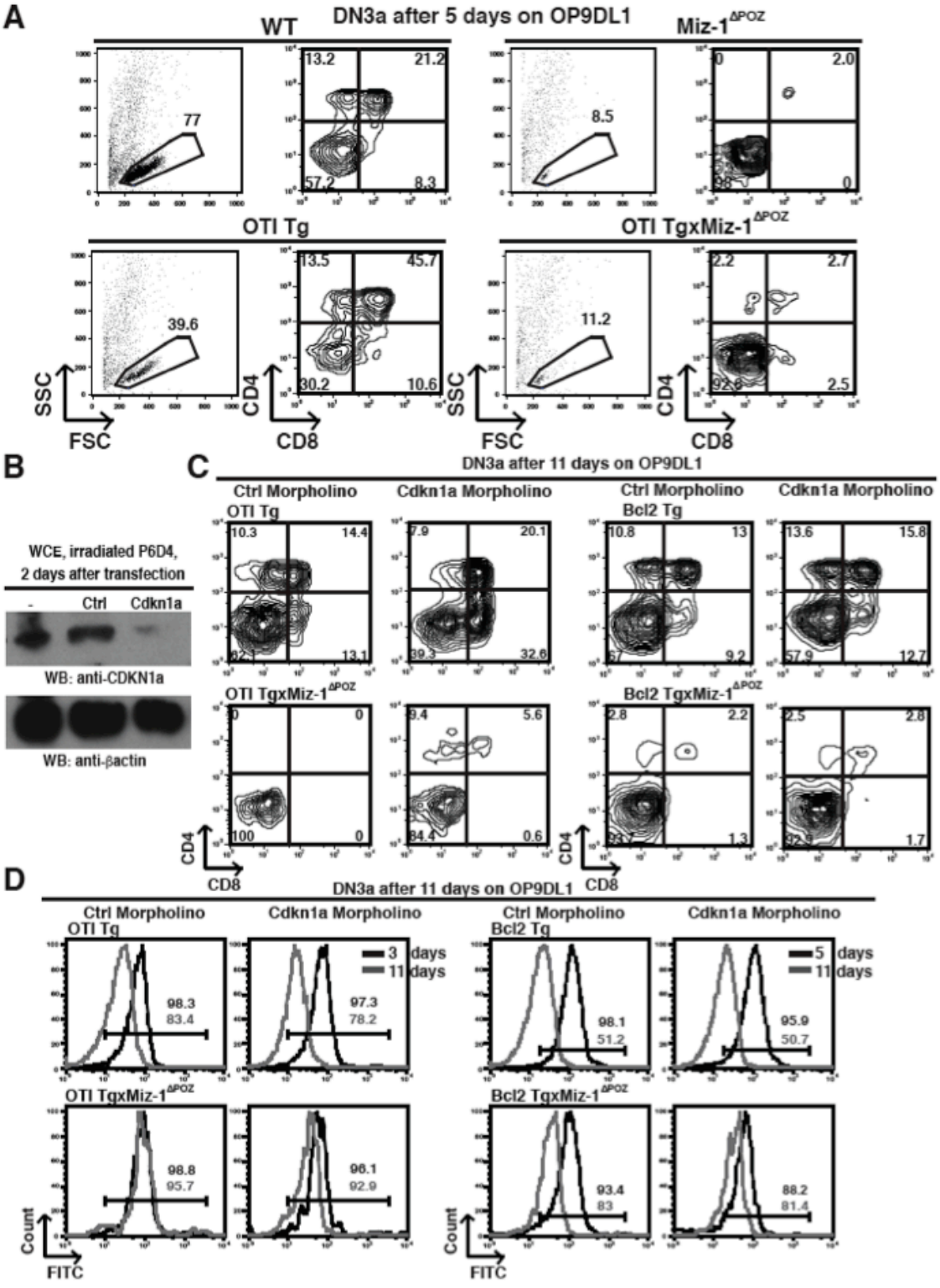
Intracellular pERK1/2 detection in WT and Miz-1$^{\Delta POZ}$ thymocytes after ex vivo stimulation with anti-CD3/CD28 (A) or PMA/Iono (B). Histograms show non-activated (NA) staining in gray, and pERK1/2 antibodies staining after 10 min of stimulation at 37°C. Mean fluorescence intensities are indicated, n=2. (C) Intracellular calcium (Ca^{++}) concentration was assessed by calculating the ratio between bound to free Ca^{++} after thymocytes were loaded with Indo1 at 31 °C and stimulated by avidin-induced crosslinking of biotinylated antibodies. Arrows indicate the addition of avidin or ionomycin at 30 s. The solid line represents WT, the dotted line represents Miz-1$^{\Delta POZ}$. Data are representative of two independent experiments



Supplemental Figure 3

Supplemental Figure 3. Thymic positive and negative selection does not seem affected by Miz-1 deficiency.

Total thymocytes from different mice with the indicated genotype were stained for CD69, TCRβ and gated as followed: R1 ($TCR^{neg}CD69^{neg}$), R2 ($TCR^{int}CD69^{neg}$), R3 ($TCR^{int}CD69^{+}$), R4 ($TCR^{+}CD69^{+}$) and R5 ($TCR^{+}CD69^{int/-}$) as indicated (left panel). The expression of CD4 and CD8 was analyzed on cells falling in each gate defined by CD69/TCRβ expression (right panel). Data represent three independent experiments of each mouse strain, except for OTI Tg and OTI Tg x *Vav*-cre Miz-$1^{\Delta POZ}$, n=2.



Supplemental Figure 4

Supplemental Figure 4. DN3 cells from Miz-1$^{\Delta POZ}$ mice lack in vitro differentiation and/or survival signals on OP9DL1 co-cultures even in the presence of a transgenic TCR (OTI Tg), Bcl-2 Tg or *Cdkn1a* knock-down.

(**A**) Comparative differentiation kinetics of 500 sorted DN3a cells from WT and Miz-1$^{\Delta POZ}$ (n=6) or OTI Tg and OTI Tg x Miz-1$^{\Delta POZ}$ (n=3) after 5 days of co-culture on OP9DL1 stromal cells. Gated live cells (according to FSC/SSC) were analyzed for CD4 and CD8 surface. The numbers in dot plots are percentages of cells. (**B**) Morpholino knock-down efficiency was evaluated by western blot analysis. SCID.Adh murine thymic lymphoma P6D4 cells were transfected with morpholino oligo against *Cdkn1a* mRNA or a control morpholino (Ctrl) and after 48h, the cells were irradiated for 5 min at 5Gy. After 1 h of irradiation, whole protein cell extracts (WCE) were evaluated for Cdkn1a by western blot and βactin was used as loading control. (**C**) 5,000 sorted DN3a from OTI Tg or OTI Tg x Miz-1$^{\Delta POZ}$ or from Bcl-2 Tg or Bcl-2 Tg x Miz-1$^{\Delta POZ}$ were incubated with *Cdkn1a* or *ctrl* morpholino oligos. CD4 and CD8 surface staining is shown after 11 days of co-culture on OP9DL1, n=2 for OTI Tg mice and n=1 for Bcl-2 Tg mice. (**D**) Morpholino transfection efficiency of sorted DN3a cells was monitored by flow cytometry, measuring FITC expression after 3, 5 or 11 days of co-culture on OP9DL1. Percentages of FITC-positive morpholino transfected cells are shown for the indicated days.

Discussion

1. Miz-1 is required for embryonic development

The implication of Miz-1 in regulating embryonic development was previously suggested by the complete deletion of the *Zbtb17* gene, which led to lethality at E7.5 [503]. To overcome this limitation, mutant mice were generated by gene targeting, in which the sequence coding for the BTB/POZ domain can be deleted. This strategy was chosen since previous studies had shown that a truncated Miz-1 protein lacking its BTB/POZ domain is non-functional [138, 478]. Surprisingly, embryos expressing two deleted $Zbtb17^{\Delta POZ}$ alleles arrested their development at around E14, showing again the critical requirement of a functional Miz-1 protein in embryonic development processes. Although this truncated form is non-functional as a transcription factor [138, 478], the remaining part of the Miz-1 protein is still encoded. The presence of the deleted form of Miz-1 is probably sufficient for the enhanced survival of the embryos, but the expression of a functional full form of the protein is critical for the embryonic development. Moreover, when embryonic stem cells expressing the two deleted $Zbtb17^{\Delta POZ}$ alleles were used to generate chimeras, they did not contribute to the repopulation of B and T cells, but contributed to all the other hematopoietic cell lineages in peripheral organs [511]. These observations provided the rationale for the hypothesis that the BTB/POZ domain of Miz-1 is required for B- and T-cell development and were the premises to begin this research project.

2. Miz-1$^{\Delta POZ}$ mice have a normal HSC pool

Consistent with the embryonic characterization, severe defects in B- and T-cell development were observed in the conditional *Zbtb17*$^{fl/fl}$ mice crossed with *Vav*-cre transgenic mice [511-513], which enabled the deletion of the BTB/POZ domain in all hematopoietic cells [505]. To clarify whether these deficiencies in B and T cells were due to decreased frequencies of lymphoid progenitors, their presence in *Vav*-cre-*Zbtb17*$^{fl/fl}$ mice was evaluated. The frequencies of hematopoietic progenitors including HSC, MPP, LMPP [511], ELP and the ALP and BLP subsets of CLP [512] were not altered, and even present at higher frequencies in Miz-1$^{\Delta POZ}$ mice. ELP, the precursors of CLP, showed almost normal expression of specific markers and expressed B- and T-lineage specific genes such as *GATA3, Notch1, Rag-1, Tdt*, *E2A, Ikaros*, *c-Myb* and *Pu.1*. These observations suggested that Miz-1 deficiency does not alter the expression program related to B- or T-lineage specification from precursor cells. A homing defect based for example on substandard stromal cells or imperfect supporting microenvironment of the lymphoid progenitors was also excluded because the lymphopenic phenotype typical for Miz-1$^{\Delta POZ}$ mice was obtained upon adoptive transfer of *Miz-1*-deficient bone marrow into syngenic recipients. Miz-1$^{\Delta POZ}$ progenitors also expressed normal levels of different chemokine receptors such as CXCR4, CCR7 and CCR9 that are involved in migration and homing. The opposite experiment using control bone marrow HSC transferred into Miz-1$^{\Delta POZ}$ mice resulted in a normal lymphoid and myeloid reconstitution, indicating that the disruption of B- and T-cell development is a cell-autonomous phenotype [511, 512].

Although the numbers of Miz-1$^{\Delta POZ}$ HSC are unchanged, the numbers of LSK cells are increased in *Miz-1*-deficient mice. These LSK cells are also less efficient at competing with normal wild-type cells in transplantation assays [Kosan, C. and Möröy, T., unpublished data]. Moreover, most of the progenitors from *Miz-1*-deficient mice failed to differentiate into more mature B and T cells in vitro on OP9 or OP9DL1 co-cultures, but still differentiated into myeloid cells when co-cultured on OP9 with the appropriate mix of cytokines cocktail [511, 512]. These observations pointed to a role of Miz-1 in regulating survival signals and differentiation of hematopoietic stem cells. It is still not clear whether all lymphoid progenitors depend on IL-7R signaling for survival, but early B- and T-cell expansion is severely reduced in *IL-7*$^{-/-}$ and *IL-7R*$^{-/-}$ mice [37, 38]. More recent findings showed that ETP or their progenitors, although negative for the expression of IL-7R on their surface, have encountered IL-7/IL-7R priming throughout their development [39]. This history of IL-7R signaling may be responsible for the requirement of IL-7 accessibility in the bone marrow in order for progenitor cells to benefit from this IL-7 to survive [514]. A functional Miz-1 may be needed for bone marrow precursors that migrate through the blood before reaching the thymus to regulate *Bcl-2* and help the cells benefit from IL-7 availability in order to survive. This would explain the lack of survival of bone marrow progenitors, blood ELP and thymic ETP in Miz-1$^{\Delta POZ}$ mice that can be rescued by Bcl-2 overexpression [511, 512].

3. The importance of the BTB/POZ domain

The Miz-1$^{\Delta POZ}$ mouse model clearly suggested a role of Miz-1 in the commitment and differentiation of the T- and B- cell lineages. These functions are specifically linked to the BTB/POZ domain of Miz-1 protein. The BTB/POZ domain of Miz-1 primarily serves as a protein-protein binding motif important for homo- and hetero-oligomerisation with other POZ domain-expressing proteins [470, 472, 473]. It is most likely not involved in a direct interaction of Miz-1 with DNA, but it is required for a stable binding of Miz-1 to the chromatin and to regulate the transcription of some target genes like *p15*^{INK4B} [478, 511]. Although the explanation for this BTB/POZ-dependent loss of activity is unclear, it is possible that, based on the crystal structure of the BTB/POZ domain [474], the ability of Miz-1 to form dimers and tetramers is lost when lacking its BTB/POZ domain. The oligomerization of the POZ domain may stabilize Miz-1 protein complexes creating conformational changes that allow Miz-1 to bind to the initiator sites of its target gene promoter. Consequently, Miz-1$^{\Delta POZ}$ can no longer form either homo- or tetramers and this absence of oligomerization affects Miz-1 capacity to bind DNA or to form a complex with other co-factors required for transcriptional regulation of target genes (proposed model in Figure 12).

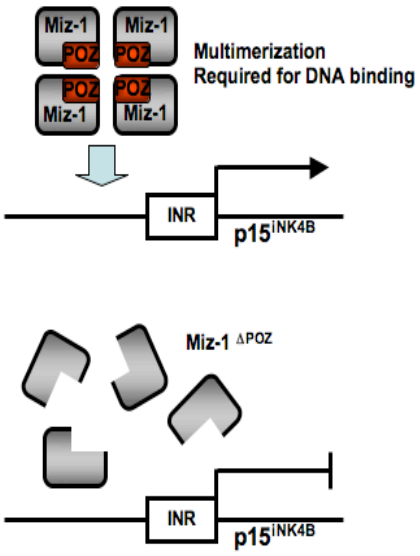


Figure 12. The BTB/POZ domain of Miz-1 influences the oligomerization capacity of the protein. A view of the proposed model for the importance of Miz-1 tetramer formation via the BTB/POZ domain to assure transactivation of target genes (upper panel). In the absence of the BTB/POZ domain, Miz-1 may not be able to bind to the DNA. The absence of the tetrameric complex may also influence the capacity of Miz-1 to interact with other co-factors required for its binding to the DNA resulting in transcriptional repression (lower panel) (adapted from [515]).

4. Miz-1 in early B-cell development

B- and T-cell developmental events are orchestrated by the interplay between cytokine signaling (IL-7/IL-7R), V(D)J recombination and transcription factor mediated regulation [179, 180, 190]. Similar to the reported phenotypes of *IL-7* or *IL-7R*-deficient mice [185, 516], most residual peripheral B cells in Miz-1$^{\Delta POZ}$ mice are marginal zone B cells [511].

These cells can reside in the marginal zone for a long time and are less affected by the absence of IL-7 signaling [516, 517], which explains their accumulation in *Miz-1*-deficient mice. In contrast, the loss of follicular B cells, but not marginal zone B cells, pointed to the possible role of Miz-1 as a downstream effector needed for the IL-7R signaling cascade. In line with these observations, the expression of the non-functional Miz-1$^{\Delta POZ}$ protein generates a block precisely at the pre-pro-B to pro-B cell transition [511], which was also consistent with the block reported for *IL-7* or *IL-7R*-deficient mice [161, 162].

The high expression levels of the JAK inhibitor *SOCS1* in *Miz-1*-deficient CLP provided a first explanation for the lack of IL-7 responsiveness in Miz-1$^{\Delta POZ}$ mice. In addition to this observation, chromatin immunoprecipitation (ChIP) experiments suggested that Miz-1 represses *SOCS1* transcription through a direct binding to its initiator site, which inhibits STAT5 phosphorylation and the subsequent up-regulation of IL-7-dependent target genes. Parallel to these findings, it was demonstrated that uncommitted B cells need to up-regulate Bcl-2 expression in order to survive [518]. In the absence of a functional Miz-1 protein, *Bcl-2* expression cannot be up-regulated in response to IL-7 in CLP [511]. Furthermore, ChIP experiments demonstrated that Miz-1 binds directly to the *Bcl-2* promoter, not at the initiator site but in a region farther upstream, in immature B cells [511]. These data suggested that Miz-1 exerts two functions in regulating the IL-7R pathway: it represses *SOCS1* and activates *Bcl-2* transcription. This dual role is not surprising since it has been reported by many groups that Miz-1 can be either a transactivator or a transrepressor depending on its interacting partner [135, 138, 469, 478, 480, 491, 492]. A role for Miz-1

as an activator of *Bcl-2* transcription, probably in response to IL-7, is also in agreement with two other studies reporting that *Bcl-2* is a direct effector gene of Miz-1 [491, 494]. Nevertheless, neither the overexpression of Bcl-2 nor the knock-down of SOCS1 expression were sufficient to relieve the pre-pro-B to pro-B cell transitional defect in Miz-$1^{\Delta POZ}$ mice. Although these rescue attempts increased the survival of immature B cells, they indicated that Miz-1 is required for another B-cell specific regulatory pathway, different from the one initiated by IL-7.

Based on the known expression patterns of B-cell specific genes, it was possible to identify B-cell differentiation signals that are affected by Miz-1 deficiency. EBF1 and E2A were prime candidates, as the deletion of these genes leads to a complete block at the pre-pro-B to pro-B transition [266, 519], which phenocopies the effects seen in Miz-$1^{\Delta POZ}$ mice. E2A expression is absolutely required for CLP generation, which is greatly reduced in *E2A*-deficient mice [520, 521]. The expression of E2A is essential for the up-regulation of EBF1 and the ectopic expression of EBF1 in *E2A*-deficient progenitors rescued B-cell differentiation [519, 522]. However, CLP numbers were normal in *Miz-1*-deficient mice and *E2A* expression was only reduced by half. This reduced expression is probably sufficient to maintain the CLP pool in *Miz-1*-deficient mice, and is possibly responsible for the reduced *EBF1* expression in Miz-$1^{\Delta POZ}$ cells. The expression levels of *E2A*, *EBF1*, *Pax-5*, *Rag-1* and *Rag-2* were all reduced in CLP from Miz-$1^{\Delta POZ}$ mice, suggesting that Miz-1 acts upstream of E2A to regulate the differentiation signals initiated by the E2A/EBF1/Pax-5/Rag-1/2 axis [511]. However, it is unlikely that Miz-1 itself directly regulates *EBF1*

expression, as evidence for a direct binding of Miz-1 to the *EBF1* promoter could not be readily generated [Kosan, C., Saba, I. and Möröy, T., unpublished data]. Whether the regulation of EBF1 is IL-7-dependent or independent is unclear. First, the ectopic expression of EBF1 in Miz-$1^{\Delta POZ}$ progenitors did not rescue the B-cell differentiation block. Second, and consistent with the observations made in the first chapter of the results section of this thesis, the expression of EBF1 in *IL-7R*$^{-/-}$ progenitors allowed the development of some $B220^{+}CD19^{+}IgM^{+}$ cells, but did not help them overcome their defect in cell survival [162]. Third and more recently, Malin and colleagues showed that the IL-7/STAT5 signaling pathway mainly controls cell survival in pro-B cell rather than B-cell development *per se* [207]. Consistent with these findings, data from the experiments shown in the first results chapter also indicated that *EBF1* cannot be induced by IL-7 stimulation and that the ectopic re-expression of *EBF1* in *Miz-1*-deficient cells cannot rescue their defective B-cell development. As a consequence, these findings corroborate the study of Malin and his collaborators and confirm the divergence between B-cell survival and B-cell differentiation [511]. Furthermore, in vitro experiments in a stroma-free cell culture system demonstrated that CLP have B-cell potential, can develop into $CD19^{+}$ cells but do not divide in response to IL-7. This lack of cell proliferation was not due to a cycling defect as originally expected since Miz-1 is know to regulate cyclin-dependent kinase inhibitors [511]. Taken together, these observations indicated that IL-7 relays signals for survival and differentiation, but not for the proliferative expansion of B cell progenitors [27].

A full rescue of B-cell development in *Miz-1*-deficient mice was achieved when both Bcl-2 and EBF1 were co-expressed in Miz-1$^{\Delta POZ}$ cells [511]. It is thus likely that survival and differentiation signals, mediated by Bcl-2 and EBF1 respectively, are initiated by the IL-7R signaling and can also be controlled by other pathways. These data support a model where Miz-1 exerts a function in regulating the IL-7-independent E2A/EBF1/Pax-5/Rag-1/2 axis responsible for the induction of B-cell differentiation signals, and the IL-7-dependent up-regulation of Bcl-2 required for the survival of B cell progenitors. Both elements need to be coordinated by Miz-1 to allow the full lineage commitment and differentiation of B cells (Figure 13).

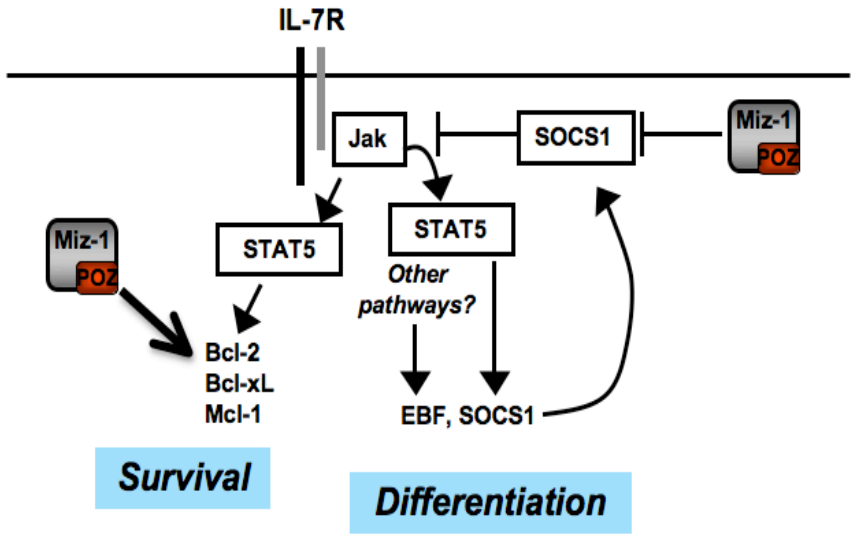


Figure 13. Miz-1 is required for early B-cell development. Miz-1 directly represses the JAK inhibitor SOCS1 and up-regulates Bcl-2 upon IL-7 stimulation. Both the IL-7-independent E2A/EBF1/Pax-5/Rag1/2 axis responsible for the induction of B-cell differentiation signals and the IL-7-dependent IL-7/STAT5/Bcl-2 axis required for the survival of B cell are shown (adapted from [511]).

5. Miz-1 in early T-cell development

Similar to its role in regulating IL-7R-dependent B-cell differentiation, Miz-1 controls early T-cell differentiation, at stages where the IL-7/IL-7R signaling coordinates survival and lineage commitment [512]. IL-7/IL-7R pathway not only assures the proliferation, survival and progression of pro-T cells very similarly to the early B-cell differentiation steps [342-346], but it also induces the rearrangement and transcription of the TCRγ locus [523, 524].

The part of IL-7R signaling that is involved in protecting cells from apoptosis and promoting survival in early T cell subsets of Miz-$1^{\Delta POZ}$ mice is severely altered due to an unbalanced *Bcl-2*-to-*Bax* ratio [512]. The early T cell block described in Miz-$1^{\Delta POZ}$ mice is a direct consequence of Bcl-2 deregulation, since Bcl-2 cannot be up-regulated upon IL-7 stimulation in all Miz-$1^{\Delta POZ}$ pro-T cells. The thymic block induced by IL-7R deficiency can be reversed by overexpressing Bcl-2 protein [342, 343] or by deleting the *Bax* gene [525]. Similarly, the overexpression of Bcl-2 in *Miz-1*-deficient cells restored most of the early αβ T cells numbers to wild-type levels [512]. It is known that *Bax* is a target gene of the Gfi1 transcriptional repressor and it has been described that Miz-1 can recruit Gfi1 to target gene promoters [134, 139, 526]. Therefore, it is possible that the up-regulation of *Bax* in Miz-$1^{\Delta POZ}$ cells is the result of a disruption of the Miz-1/Gfi1 complex. This disruption may be caused by conformational changes in the mutant Miz-1 protein lacking its BTB/POZ domain or by the absence of the recruitment of a co-factor to the *Bax* promoter due to the

lack of the BTB/POZ domain of Miz-1. Future work would be required to clarify the details of this regulatory mechanism.

The development of γδ-T cells strictly depends on IL-7 for its lineage-specific maintenance [353, 527]. IL7Rα expression is also sustained on γδ-T cells both in OP9DL1 culture [290] and in vivo [527]. Nevertheless, γδ-T cell lineage development is less dramatically altered when compared to the αβ-T cell lineage in Miz-$1^{\Delta POZ}$ [512]. Bcl-2 expression levels may represent one explanation why γδ-T cells are less affected since *Bcl-2* mRNA levels are maintained in emerging γδ T cells, whereas it declines in developing αβ DN3 cells [290]. On the contrary, *Bcl-*X_L transcription tends to decline in γδ-T cells, but remains stable in αβ DN3 cells, generating a lower *Bcl-2*-to-*Bcl-*X_L ratio in αβ-T cells compared to γδ-T cells [290]. DN3 from Miz-$1^{\Delta POZ}$ maintain a high level of *Bcl-2* transcripts and normal *Bcl-*X_L levels [Saba, I. and Möröy, T., unpublished data], suggesting that the higher *Bcl-2*-to-*Bcl-*X_L ratio favors a relatively normal selection of γδ-T cells.

Contrary to what was observed in B cell precursors, Miz-1 does not seem to directly bind to the regulatory regions of *Bcl-2* in immature T cells [Saba, I. and Möröy, T., unpublished data]. Still, high expression levels of *Bcl-2* are detected in *Miz-1*-deficient pro-T cells, possibly maintained through an IL-7-independent signal. This may provide a selection advantage for DN2/DN3 cells allowing them to escape the lack of survival signals caused by Miz-1 deficiency at the ETP/DN1 stage. It is also possible that a residual and very inefficient IL-7-dependent signal could be responsible for the elevated *Bcl-2* expression

since DN2 cells from Miz-1$^{\Delta POZ}$ still had a low level of STAT5 phosphorylation in response to IL-7 stimulation despite elevated *SOCS1* expression levels [512]. Taken together, it is likely that Miz-1 deficiency interrupts the IL-7/IL-7R/STAT5/Bcl-2 axis, which assures T-cell survival.

Moreover, as in early B cells, Miz-1 binds to the *SOCS1* promoter in pro-T cells and *SOCS1* is overexpressed in *Miz-1*-deficient cells. Furthermore, the overexpression of Miz-1 in DN3 pro-T cell line (SCID.adh murine thymic lymphoma) efficiently repressed SOCS1 expression in response to IL-7 stimulation. These observations suggest that the lack of a functional Miz-1 leads to a de-repression of the *SOCS1* gene [512]. Contrary to what was observed in the B cell compartment, transgenic overexpression of Bcl-2 in vivo or inhibition of *SOCS1* using a morpholino oligo knock-down approach in vitro fully restored pro-T cell numbers and their ability to differentiate into more mature DP cells [512]. A definitive experiment to show SOCS1 prerequisite and its capacity to fully rescue the differentiation in vivo would have required testing *SOCS1*- and *Miz-1*- double deficient mice. However, parts of the effects of SOCS1 deficiency on thymocytes maturation are mediated by IFNγ [352, 528]. Therefore, *SOCS1*-deficient mice must also be *IFNγ*-deficient in order to be able to study T-cell development [352, 528, 529]. The crossing of *Vav*-cre Miz-1$^{\Delta POZ}$ to *SOCS1*- and *IFNγ*- double deficient mice, which represents over five alleles, would have required many mice generations before being able to analyze the first animal. For these reasons, the morpholino knock-down experiment was privileged. Furthermore, Miz-1 is the only factor required to regulate the expression of SOCS1 downstream of IL-7R

signaling in pro-T cells, whereas in early B-cells, another pathway is required to regulate EBF1. The regulation of SOCS1 by Miz-1 controls the activation of STAT5 phosphorylation in response to IL-7 and enables the gauging of *Bcl-2* levels required for the survival and further development of ETP/DN1/DN2 subsets (Figure 14).

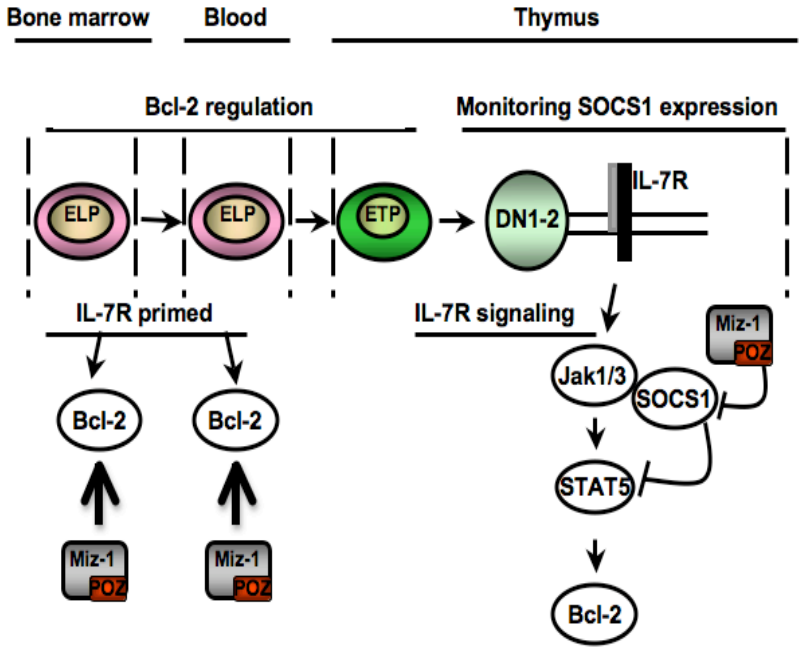


Figure 14. Miz-1 is required for early T-cell development. Miz-1 directly represses the JAK inhibitor SOCS1 and up-regulates Bcl-2 upon IL-7 stimulation. The IL-7-dependent IL-7/STAT5/Bcl-2 axis required for the survival and differentiation of ETP/DN1/DN2 pro-T cells is shown (adapted from [512]).

6. Miz-1 functions in early B and T cells are independent of c-Myc

Miz-1 was originally discovered as a c-Myc interacting protein, allowing c-Myc to repress the transcription of genes coding for cell cycle regulators [136-138, 481, 492]. However, no evidence for a disturbed cell cycle progression in *Miz-1*-deficient B- and T-cell progenitors were observed, suggesting that Miz-1 does not regulate cell division or proliferation as originally thought in these subsets. Both early B- and T-cell differentiation are not altered in knock-in mice homozygous for c-Myc^{V394D} alleles, a form of c-Myc that no longer interacts with Miz-1 [135, 469]. These data indicate that the function of Miz-1 in early B and T cells is most likely c-Myc-independent [511, 512]. It could be argued that in the absence of a functional Miz-1, free c-Myc levels *i.e.* not bound to Miz-1 increase and drive *Miz-1*-deficient cells into cycling and thus initiate cell death, which could explain some of the phenotypes observed in Miz-1$^{\Delta POZ}$ mice. Nonetheless, c-Myc expression levels in *Miz-1*-deficient pro-T cells are normal and it has be reported that constitutive expression or overexpression of c-Myc in T cells leads to T cell lymphomas rather than to T cell depletion [448]. Moreover, higher c-Myc activity leads to a higher proliferation of pre-T cells [530], which is not observed in Miz-1$^{\Delta POZ}$ mice, arguing against such a hypothesis.

7. Miz-1 at the β-selection checkpoint

Miz-1 protein must have an additional function later in pre-T cell differentiation, namely at the β-selection stage of DN3 cells, which is the first critical checkpoint in the maturation of pre-T cells [513]. The massive block of T-cell differentiation at the DN3/DN4 transition in *Miz-1*-deficient mice is the evidence for such implication, which in contrast to the earlier block at the ETP/DN1/DN2 stage, cannot be restored by Bcl-2 overexpression [512, 513]. DN3 cells passing this checkpoint actively rearrange the TCRβ locus and thus have to tightly control DNA damage response pathways initiated by the V(D)J recombination events. After productive rearrangement, DN3 cells activate allelic exclusion [402] and become pre-TCR$^+$ DN3b cells that undergo a massive proliferative expansion, differentiate into DN4 cells [289] and escape apoptosis. The findings described in the third results chapter of this thesis suggest that Miz-1 is essential to coordinate the steps that assure survival of DN3 cells and their expansion/differentiation into DN4/DP cells. In particular, Miz-1 seems important to coordinate the expression of the pre-TCR and to control p53 expression and activity in the presence of DNA double strand breaks generated by V(D)J recombination events in DN3 cells [513].

All the observations described in the third results chapter of this thesis point to the conclusion that Miz-1 deficiency does not affect the expression, stability or processing of the pre-TCR *per se*, nor does it alter TCR-dependent signaling in thymocytes [513]. The

absence of a pre-T cell rescue when a transgene coding for rearranged TCRαβ chains was introduced in Miz-1$^{\Delta POZ}$ mice indicated that the lack or low expression levels of surface TCRβ is not solely responsible for the block of T-cell differentiation at the DN3/DN4 transition [513]. A first indication to the nature of the defect in *Miz-1*-deficient pre-T cells was that enhanced spontaneous cell death occurs in Miz-1$^{\Delta POZ}$ DN3a cells, at the moment where V(D)J recombination and β−selection checkpoint take place [513]. This correlated with defects in cell cycle progression in Miz-1$^{\Delta POZ}$ DN3 and DN4 cells and with the up-regulation of p53 target genes that control apoptosis such as *Bax*, *PUMA* and *Noxa* and those that control cell cycle progression such as *p21^{CIP1}* [426]. This evidence suggested that Miz-1 exert a different function in DN3 cells compared to ETP/DN1/DN2 cells where it controls the IL-7R signaling pathway. This new function is related to the control of p53 response at a cell stage implicating the generation of DNA damage response and p53 activation that needs to be under control during V(D)J recombination events.

8. p53 and the balance between survival and cell death

The G1 specific negative regulator of cell cycle progression p21^{CIP1} is a p53-target gene [531] that plays a central role in the activation of p53-dependent cell cycle arrest. A balance between the well-established roles of p53 in inducing cell cycle arrest and apoptosis and its more recent implications in promoting cell survival [426, 532] is required to maintain the normal development of cells, especially to control responses to stress and DNA damage. Accordingly, low level of p53 activity is maintained under conditions of normal cell

proliferation and in the absence of stress signals. A short and transient activation of p53, for example when DNA damage occurs, allows a temporary cell cycle arrest of stressed cells for DNA double strand breaks repair. If the damage or stress signal persists, p53 activation will lead to the induction of genes that promote cell death to eliminate cells with damaged DNA, which represent a potential source of malignancies [532]. Clearly, the expression levels of *p53* itself in Miz-1$^{\Delta POZ}$ cells was comparable to wild-type cells, but a number of p53 target genes was up-regulated suggesting that Miz-1 affects the regulation of p53 transcriptional machinery either directly or indirectly [513]. *Miz-1*-deficient DN3 cells overexpress genes such as *Bax*, *Noxa and PUMA*, which probably results from damage or stress signals that persist. This sustained p53 activation leads to the induction of cell cycle arrest and cell death (Figure 15).

A recent report that directly implicates Miz-1 as a mediator of the p19^{ARF}-p53 pathway is also consistent with the potential regulation of p53 by Miz-1. In this study, it was shown that Miz-1 is able to bind to p19^{ARF} and to interfere with p53 stability. The same study also demonstrated that Miz-1 interacts directly with p53 and that this interaction diminishes the binding of p53 to its target gene promoters and inhibits p53-mediated gene transcription [502]. It is therefore conceivable that Miz-1 plays a role in the regulation of p53 in pre-T cells and that a functional form of Miz-1 is necessary to control p53 activity at the β-selection checkpoint to allow V(D)J recombination to occur without initiating apoptosis. To understand this regulation more deeply, more experiments need to be done. For example, experiments that enable the detection of a co-occupation of Miz-1 and p53 on the promoter

of DNA damage response genes may help clarify this point. Taken together, all the observations described here, together with published data, can help explain the DN3/DN4 differentiation block observed in *Miz-1*-deficient mice and would establish the BTB/POZ domain protein Miz-1 as a new regulator of the pre-TCR β-selection checkpoint (Figure 15).

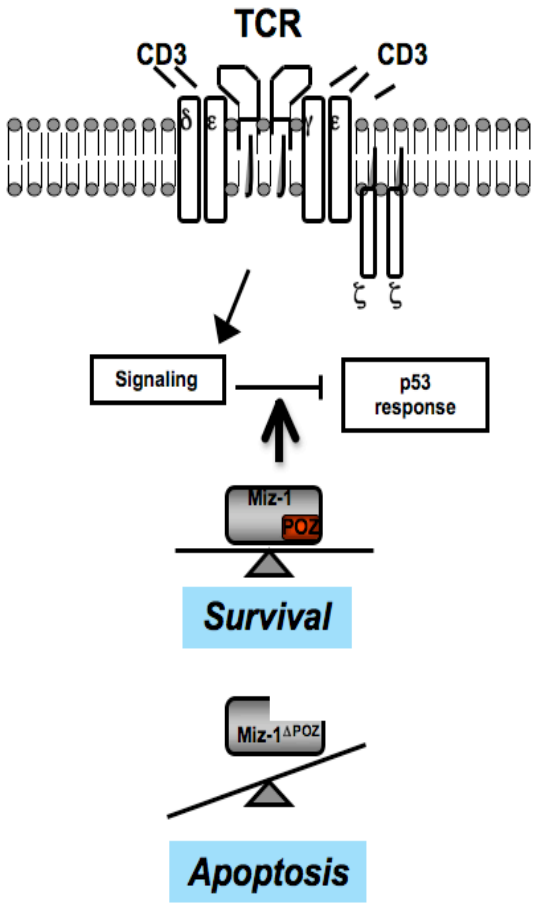


Figure 15. Implication of Miz-1 during the β-selection checkpoint. Miz-1 is required to regulate the p53 response during pre-TCR DN3 selection. The expression of Miz-1 lacking the BTB/POZ domain shifts the balance between pre-TCR signals and p53 response toward apoptosis.

9. Miz-1 regulation at the β-selection through direct protein-protein interaction?

The availability of a given transcription factor to interact with DNA can be influenced by post-translational modifications, and by interaction with other proteins. These modifications can lead to the sequestration, inactivation or degradation of a transcription factor or a complex of transcription factors that alter gene regulation [61]. Miz-1 seems to be involved in other physiological processes than transcriptional regulation. The characterization of the c-Myc/Miz-1 binding showed that Miz-1 is expressed both in the cytoplasm and in the nucleus, indicating that it may be involved in the nuclear/cytoplasmic shuttling of c-Myc [469]. A recent study pointed to a possible cytoplasmic function of Miz-1 by showing that it can interact with the c-Jun N-terminal kinase (JNK1). This interaction seems to mediate specifically the TNF-α-dependent JNK1 activation and the subsequent induction of cell death by the inhibition of TRAF2 K63-linked polyubiquitination [533].

Another example is the SLUG protein, an inhibitor of *PUMA* expression, which is also encoded by a p53 target gene. SLUG is able to antagonize p53-mediated apoptosis in hematopoietic progenitors by repressing *PUMA* [430]. Whether Miz-1 can bind to SLUG or to other co-factor that facilitates SLUG-induced repression of PUMA can represent a mechanism that implicates the cytoplasmic function of Miz-1. This cytoplasmic activity of Miz-1 in pre-T cells may be dependent or independent of the BTB/POZ domain. It is conceivable that the absence of the BTB/POZ domain of Miz-1 may inhibit a Miz-1/p53 interaction as suggested by Miao and collaborators [502], or other Miz-1 mediated

interactions involved in the transcriptional regulation of p53 target genes. More experimentation is needed to explore this other role of Miz-1 as a cytoplasmic protein-protein interacting regulator.

Perspectives

The mouse models generated for this study allowed making a number of new observations regarding the implication of Miz-1 in T- and B-cell development. Several questions remain to be address in order to understand the molecular mechanisms by which Miz-1 exerts these newly discovered functions. Moreover, preliminary findings suggest that Miz-1 is involved in malignant transformation of lymphoid cells and thus may be a critical element necessary for the development and maintenance of c-Myc-dependent and -independent lymphoma. Some of these data and the perspectives on how to investigate these implications may help to elucidate whether Miz-1 can be a suitable target for future therapies.

1. Implication of Miz-1 in p53 target gene regulation

Since some target genes such as *PUMA*, *Noxa* and *Cdkn1a* are deregulated in Miz-1$^{\Delta POZ}$ DN3 deficient cells, it can be argued that Miz-1 regulates the p53 pathway at least in specific subsets of T cells. It would be interesting to perform a ChIP-seq experiment to identify the sites in the genome that are occupied by Miz-1 and p53, for instance sites in p53-dependent target genes. This would help elucidate whether Miz-1 can directly bind to the promoter of these genes and thus could regulate their transcription. Moreover, the occupancy of p53 targets by Miz-1 may be tested under conditions where p53 is at a steady state or activated state by exposing the cells to irradiation or other agents that induce DNA damage. Non-irradiated or irradiated DN3 cells could also be tested for the activation of

p53 by western blot analysis to detect phosphorylated forms of the protein in the presence or absence of a functional Miz-1 protein to conclude whether Miz-1 interferes with p53 activation. Furthermore, comparing wild-type and Miz-$1^{\Delta POZ}$ DN3 cells before and after irradiation may determine if p53 activity behaves differently in the presence or absence of a functional Miz-1 protein. For example, p53 may occupy different binding sites in Miz-$1^{\Delta POZ}$ DN3 compared to wild-type cells. Additionally, it is conceivable that Miz-1 acts as a p53 inhibitor, as proposed by Miao and collaborators [502] and that p53 becomes activated in DN3 cells that are undergoing β-selection checkpoint in the presence of a non-functional Miz-1 protein. Using *p53*-deficient mice crossed with *Vav-cre* Miz-$1^{fl/fl}$, one could confirm that the ablation of p53 can rescue the DN3/DN4 pre-T cell differentiation block in Miz-$1^{\Delta POZ}$ mice. These experiments would strengthen a new role of Miz-1 as a regulator of the activation of p53 in immature thymocytes under a physiological DNA damage response induced, in this case, by V(D)J recombination events.

Miz-1 involvement in the p53 pathway is suggested by data presented in this thesis and was also proposed by another study showing that p53 competes with $p19^{ARF}$ for the binding of Miz-1 and that Miz-1/p53 complex prevents p53 from activating target genes [502]. The validation of such interaction in vivo and whether Miz-1 does indeed form a complex with the p53 protein in pre-T cells by co-immunoprecipitation would be of importance. If confirmed, this would strongly support the view that Miz-1 binds to p53 and attenuates its activity by interfering with its DNA binding capacity. Many tumors develop by being selected for the inactivation of p53-dependent target genes [534, 535]. The implication of

Miz-1 in such regulation of p53 activity may help explain at a the molecular level how the loss of this pathway accelerates c-Myc-induced tumorigenesis

2. The role of Miz-1 in mature B cells

The few follicular B cells or mature T cells that manage to populate peripheral lymphoid organs in Miz-1$^{\Delta POZ}$ mice did not show a proliferation defect despite the fact that they emerged from a disturbed early B- and T- cell development [Kosan, C., Saba, I. and Möröy, T., unpublished data]. Furthermore, mature B cells from *Vav*-cre Miz-1$^{\Delta POZ}$ have higher IgM titers before and after immunization. These cells are also able to do class switching to IgG [Kosan, C. and Möröy, T., unpublished data]. To obtain further insight into the role of Miz-1 in peripheral mature B cells, other Cre deleter mice strains will have to be used. For example, *CD19*-cre [506] or *Mb1*-cre [536] mice could be used to delete the BTB/POZ domain of Miz-1 at the pro-B cell or slightly earlier at the pre-pro-B cell stage, respectively. The frequencies and numbers of B cells in the spleen and lymph nodes of these pan-B cell-specific Cre deleter mice can then be evaluated without the effect of Miz-1 disruption in earlier precursors. This would allow studying the function of Miz-1 in germinal center formation after in vivo antigenic stimulation, for example. Moreover, class switch recombination could be compared between wild-type and Miz-1$^{\Delta POZ}$ cells after in vivo antigenic stimulation or after in vitro stimulation with LPS and LPS/IL-4. The implication of Miz-1 in germinal center formation has previously been suggested. In fact, Miz-1 and Bcl-6, another BTB/POZ domain protein with defined roles in germinal center

formation, form a complex that binds to the *Cdkn1a* promoter and represses its transcription [492]. During these processes, germinal center B cells are released from cell cycle arrest and undergo massive proliferation, class switch recombination and somatic hypermutation [227]. As a consequence, and similar to the events occurring in DN3 pre-T cells, a p53-dependent DNA damage response is induced. It has been suggested that Bcl-6 is a key-player required to coordinate this p53-dependent DNA damage response [537-539]. In particular, it has been proposed that Bcl-6 is necessary to downregulate p53-dependent DNA damage response genes. For example, Bcl-6 can directly bind to p53 promoter and repress its transcription [540]. It remains to be explored how other p53 target genes and effectors may be controlled during physiological DNA damage responses, but the functions of Bcl-6 and of the Bcl-6/Miz-1 complex are compelling examples for future work. These studies could help understand how germinal center B cells may be protected from the consequences of a DNA-damage if this occurs during important physiological process such as class switch recombination or somatic hypermutation. Such a protection against DNA damage responses has to be tightly controlled to prevent malignant transformation as a consequence of the introduction of mutations. The observation that Miz-1 is one of the key factors regulating this process suggests that it may protect lymphoid cells from developing into leukemia or lymphoma.

3. Miz-1 implication in human B cell production?

IL-7 is important for mouse B cell generation. However its role in human B lymphopoiesis is controversial [541]. In vitro studies of human B-cell development were carried out using fetal hematopoietic cells [542-548] or a hybrid co-culture system of human progenitors on murine stromal layers [549-551]. Moreover, in vivo data from clinical reports indicated that patients with severe combined immunodeficiency due to defects in the IL-7/IL-7R signaling lacked T cells, but had peripheral blood B cells [552-554]. These studies showed that IL-7 had little effect on human B cell production. The major difficulty interpreting these studies resides in the inability to differentiate human cord blood or adult bone marrow HSC without murine stroma. Moreover, samples from immunocompromised patients were not very abundant to conduct rigourous studies and most of the clinical data obtained from patients with defective IL-7/IL-7R signaling were acquired early in life. This is consistent with observations that fetal and neonatal B cell production is intact in mice with IL-7 defects [185]. A recent study has established that IL-7 is essential for human B cell generation. Using a human-only culture model, Parrish and collaborators provided the first evidence that B-cell development from human HSC in adult bone marrow is dependent on IL-7 [555]. The authors also showed that IL-7-induced expansion of human pro-B cells is increasingly critical as the precursors progress through their developemental steps of ontogeny [555]. Human IL-7 effects were mediated through the direct action of the cytokine on human $CD19^+$ pro-B cells to increase proliferation of both $CD34^+$ and $CD34^-$ stages of B-cell development [555]. The work presented in this thesis suggests that Miz-1 exerts a function in regulating the IL-7-independent E2A/EBF1/Pax-5/Rag-1/2 axis

responsible for the induction of adult B-cell differentiation signals in mice, and the IL-7-dependent up-regulation of Bcl-2 required for the survival of B cell progenitors. Both elements need to be coordinated by Miz-1 to allow the full lineage commitment and differentiation of murine B cells [511]. The characterization of Miz-1 in coordinating IL-7/IL-7R signaling in adult human HSC should elucidate the implication of Miz-1 in lineage commitment and differentiation of human B cells. For instance, human HSC expressing functional and non-functional Miz-1 could be co-cultured on human stroma cell lines. The differentiation of HSC towards the B lineage may be monitored by the expression of EBF, Pax-5 and the capacity of the developing cells to respond to IL-7 by proliferating and upregulating CD19. These future studies would have implications for the immune reconstitution following stem cell transplantation whether cord blood or bone marrow HSC are used in a clinical setting.

4. The role of Miz-1 in peripheral T cells

Although few T cells survive and populate the peripheral organs in Miz-1$^{\Delta POZ}$ mice, the proportion of mature CD4 to CD8 single positive cells is altered [Saba, I., Kosan, C. and Möröy, T., unpublished data]. This unbalanced ratio of mature cells probably results from the abnormal IL-7 signaling reported in the thymus [512]. As Miz-1 regulates SOCS1 expression, a role of SOCS1 in modulating T cell ratio and functions in the periphery cannot be ruled out. In fact, SOCS1 is a critical player during T-cell development and maturation within the thymus, but it can also regulate mature T lymphocytes. The

peripheral T cell pool of SOCS1$^{-/-}$IFNγ$^{-/-}$ mice displays defective homeostasis of CD8^{+} cells due to their accumulation, which causes a decrease in CD4 to CD8 ratio [556, 557]. The functions of Miz-1 in mature T cells could be studied in Miz-1$^{\Delta POZ}$ mice where the deletion of the BTB/POZ domain is induced by *CD4*-cre mice [558]. This deletion occurs as the thymocytes enter the double positive stage and avoids all the early T-cell development defects reported with the *Vav*-cre deleter mouse strain. IL-7 signaling can therefore be studied in the mature T cell compartement of a mouse strain devoid of the early pro-T cell block caused by Miz-1 deficiency. The magnitude and kinetics of IL-7 induced STAT5 phosphorylation and Bcl-2 upregulation, as well as the expression and implication of SOCS1, can be studied in the *CD4*-cre *Miz-1*-deficient mouse. The cellularity, frequency, proportion of cells and their homeostasis and capacity to proliferate may also be evaluated.

After stimulation, naïve CD4^{+} cells differentiate into helper CD4^{+} T cells like the Th1 and Th2 subset. Th1 cells are regulated by the transcription factor T-bet, mainly secrete IFNγ and participate in cellular immunity processes. Th2 cells express the transcription factors GATA3 and c-Maf, and produce IL-4, IL-5 and IL-13 [559]. Cell proliferation, cytokine secretion and Th polarization that produce different cytokines could be evaluated to study the role of Miz-1 in the differentiation of mature T cells. In vivo or in vitro antigenic responses could also be studied in different Miz-1-deficient mice crossed with transgenic mice expressing a TCR that specifically recognizes a peptide and that is MHC class I or II restricted, like the OTI and OTII tg mice.

T follicular helper cells (Tfh) have been defined as a subset of helper $CD4^+$ T cells that is activated but non-polarized [560]. B cells are crucial for the development of these Tfh cells that express high levels of Bcl-6 and secrete IL-21 [561-563]. The cognate interaction between these cells occurs in the germinal centers [564], where germinal center B cells require T cell help to produce antibodies with high affinity. The up-regulation of Bcl-6 in the T cell zone is necessary for Tfh to migrate to the B cell follicule. Since, Miz-1 can interact with Bcl-6, this T-B cell border movement may be influenced by the expression of a functional or mutated Miz-1 protein. Such a hypothesis, if validated, may implicate Miz-1 in regulating the differentiation and expansion of B and T cells in germinal centers. As Miz-1 is a crucial factor required for early B- and T-cell commitment, survival and differentiation [511-513], it would be interesting to extend the study of the role of Miz-1 in the survival and maturation of Tfh and B cells in the germinal center. Many groups have recently contributed to the identification of mechanisms that may explain how B cells support Tfh cell survival and maintenance [565-567]. Collectively, these efforts may advance our understanding of the immune response in germinal centers in order to improve vaccine designs.

5. Conditional full deletion of the Miz-1 protein

To further characterize the entire range of Miz-1 functions early and later in lymphoid development, and to clarify whether the implications of Miz-1 in regulating B- and T-cell commitment, survival and differentiation are entirely dependent on the presence of the

BTB/POZ domain of Miz-1 [511-513], mice that lack the entire Miz-1 protein have been generated. Briefly, the gene targeting strategy that was chosen will allow to conditionally invert the exon containing the translational initiation codon after Cre-recombinase expression. This strategy should prevent any read-through of the *Zbtb17* mRNA and thus assures the conditional full ablation of Miz-1. The generation of a complete conditional Miz-1 knock-out mouse with this strategy will hopefully circumvent the embryonic lethality previously reported [503]. This new mouse model will also help understand the differences between the complete deletion of Miz-1 and the expression of Miz-$1^{\Delta POZ}$ alleles in regulating lymphoid development.

6. Miz-1 and lymphomagenesis

Miz-1 interacts with c-Myc and Bcl-6, both being proto-oncogenic proteins implicated in the emergence of leukemia and lymphoma if deregulated [433, 568]. The c-Myc/Miz-1 and the Miz-1/Bcl-6 complexes were shown to play important roles in cell cycle progression by repressing the negative cell cycle regulator $p21^{CIP1}$ [492, 496]. This strongly suggested that Miz-1 is involved in malignant transformation. Both *c-Myc* and *Bcl-6* genes undergo chromosomal translocations with the immune globulin loci. Translocations of heterologous chromosomes to the coding region of Bcl-6 are frequently reported in diffuse large B cell lymphomas that originate in germinal centers [568-574]. These translocations lead to an activated form of Bcl-6 which downregulates $p21^{CIP1}$ via Miz-1 and cause acceleration in proliferation [492]. In addition to the elevated Bcl-6 expression, some DLBCL show high expression levels of Bcl-2 that prevent cells from undergoing apoptosis [575-577]. It was

shown in human germinal center centroblats and in primary biopsies from patients diagnosed with DLBCL that Bcl-6 does not bind directly to the Bcl-2 promoter, but was able to suppress its transcription via Miz-1 [494]. Bcl-6-mediated suppression of Bcl-2 can be altered in primary DLBCL by an additional translocation t(14;18) or by deregulated Miz-1 expression [494]. On the one hand, the insufficient expression of Miz-1 may inhibit Bcl-6 from reaching Bcl-2 promoter and repressing its transcription [494]. On the other hand, high Miz-1 expression levels, as observed in one third of $Bcl\text{-}2^{+}Bcl\text{-}6^{+}$ DLBCL, may out-titrate the capacity of Bcl-6 to suppress Bcl-2 by generating a lot of Miz-1 protein unbound to Bcl-6 [494]. The co-expression of Bcl-6 and Bcl-2 in DLBCL was reported and may identify patients with unfavorable prognosis [578]. The abnormal expression of Bcl-2 may increase the pool of cells that can be targeted by genetic modifications in the germinal center. Therefore targeting the oncogenic activities of Bcl-6 and the anti-apoptotic function of Bcl-2 [579, 580] via Miz-1 may represent a strategy for treating some DLBCL.

c-Myc has been implicated in the generation of the Burkitt-type B cell lymphoma in human and mice models. These studies showed that the *c-Myc* gene becomes deregulated after a t(8;14) translocation juxtaposes it close to the transcriptional regulatory elements of the immunoglobulin heavy chain locus [431-434]. In collaboration with the Eilers and Felsher groups, the implication of the c-Myc/Miz-1 complex in c-Myc-induced tumorigenesis was studied by using transgenic mice that overexpress c-Myc or the $c\text{-}Myc^{V394D}$ mutant that no longer interacts with Miz-1 [581]. This study showed that tumor development in transgenic mice overexpressing $c\text{-}Myc^{V394D}$ is delayed compared to mice expressing the wild-type

allele of c-Myc [581]. These data hint to the fact that the formation and maintenance of c-Myc-induced lymphomas is dependent on the interaction between Miz-1 and c-Myc and very likely on the repressive function of this complex. In accordance with this proposed model, preliminary results with *CD19*-cre x Miz-1$^{\Delta POZ}$ x *Eμ*-Myc mice show that the ablation of Miz-1 impedes the development of malignancies in the lymphoma prone *Eμ*-Myc transgenic mouse model [444, 450, 582, 583] and Kosan, C. and Möröy, T., unpublished data]. Finding candidate genes that are critical for Eμ-Myc driven B-cell lymphomagenesis and that are regulated by Miz-1 may provide insights into the role of Miz-1 in c-Myc- or Bcl-6-dependent B-cell lymphomagenesis. The molecular mechanisms that are exerted by Miz-1 in the development and maintenance of these tumors may help obtain information on whether Miz-1 may become a suitable target for therapy.

c-Myc also plays an important role in the development of T-cell lymphomas and been recognized as a critical element in T-cell acute lymphoblastic leukemia that develop as a consequence of activated Notch signaling and mutations in the *Notch1* gene [455, 457, 458]. Recently though, the role of c-Myc in Notch- induced T-ALL has been debated after demonstrating that Notch rather than c-Myc is the dominant oncogene in T-ALL [460]. Evidence that Miz-1 regulates Notch1 signaling was obtained when studying *Miz-1*-deficient cells in which Notch target genes such as *Hes-1*, *Dtx-1*, *Dtx-2*, *Cdkn1a* and *Cyclin D1* were deregulated [Saba, I. and Möröy, T., unpublished data]. Moreover, in a tumor prone Notch1 transgenic mouse model expressing a form of Notch that lacks its c-terminal domain (Notch1$^{\Delta CT}$) [584], preliminary results were obtained showing that Miz-1 plays a role in the development of Notch-induced T-ALL [Rashkovan, M., Saba, I. and Möröy, T.,

unpublished data]. Interestingly, this role is different from the one we observed in B-cell lymphomagenesis. These findings point to a different requirement for Miz-1 in c-Myc-dependent versus Notch-dependent tumors. To further understand these differences, it will be necessary to identify the genes that are critical for Notch-induced malignant transformation and that are at the same time regulated by Miz-1. These targets may later be used to design strategies aiming to suppress Notch-induced T-cell lymphomagenesis.

Conclusions

B- and T-cell differentiation is coordinated by cytokine signaling, V(D)J recombination and transcription factor regulation. The signal transduction cascade initiated by IL-7 and its direct and indirect downstream effectors are essential for the earliest commitment stages of these cells. Additionally, transcription factors such as EBF and Pax-5 cooperate with the IL-7R signaling to ensure the coordination and regulation of these early commitment and differentiation stages. This study showed that the BTB/POZ-domain protein Miz-1 is a novel and essential regulatory factor for both B- and T-cell development. The data presented in this thesis suggest that Miz-1 is involved in regulating Bcl-2 expression in bone marrow precursors that migrate through the blood before reaching the thymus to help the cells benefit from IL-7 availability to survive. In early B- and T- cells, Miz-1 regulates the survival signals induced by IL-7 signaling by monitoring, on one hand, the expression levels of *SOCS1*, a negative regulator of IL-7/IL-7R signaling and, on the other hand, Bcl-2, an anti-apoptotic protein and a positive effector of the IL-7/IL-7R signaling. The IL-7/STAT5/Bcl-2 axis requires the expression of a functional form of Miz-1 protein that exerts its transcriptional activities in a cell specific manner at the ETP/DN1/DN2 stages of T-cell development and in immature B cells. In addition to the IL-7/STAT5/Bcl-2 axis regulation in B cells that is responsible for generating survival signals, Miz-1 is required for the proper expression of EBF to induce differentiation signals of committed B cells. Therefore, Miz-1 assures the functioning of both the IL-7/STAT5/Bcl-2 and the EBF/Pax-5/Rag-1/2 axis for the proper commitment and differentiation of the T- and B- cell lineage. Miz-1 is also a critical factor for the β-selection checkpoint in differentiating pre-T cells. It

is required for both the regulation of the p53 response and the proper expression of the pre-TCR to support the proliferative burst of pre-T cells (Figure 16). The regulation exerted by Miz-1 in B and T cells is mostly likely independent of its interacting partner c-Myc, and seems specifically linked to the BTB/POZ domain of Miz-1.

Cancer in the immune and hematopoietic system manifests as lymphoma and leukemia. It is a consequence of a deregulation of particular constituents in the signaling pathways that control differentiation and proliferation of B and T cells. Many of these signaling pathways regulate the activity of transcription factors and chromatin modifiers and thus indirectly influence gene expression patterns. The findings exposed in this thesis on c-Myc and Miz-1 functions may contribute to better understand the mechanisms responsible for the emergence of leukemia and lymphoma. These transcription factors were originally found to regulate cell cycle progression. The data reported here show that they also control cell survival and lineage commitment and differentiation of B and T cells. As transcription factors constitute the endpoint of specific hematopoietic signaling pathways, they have been causally implicated in the altered genetic programming found in hematopoietic malignancies. This study may therefore provide new knowledge important for the design of future therapeutic strategies against cancer.

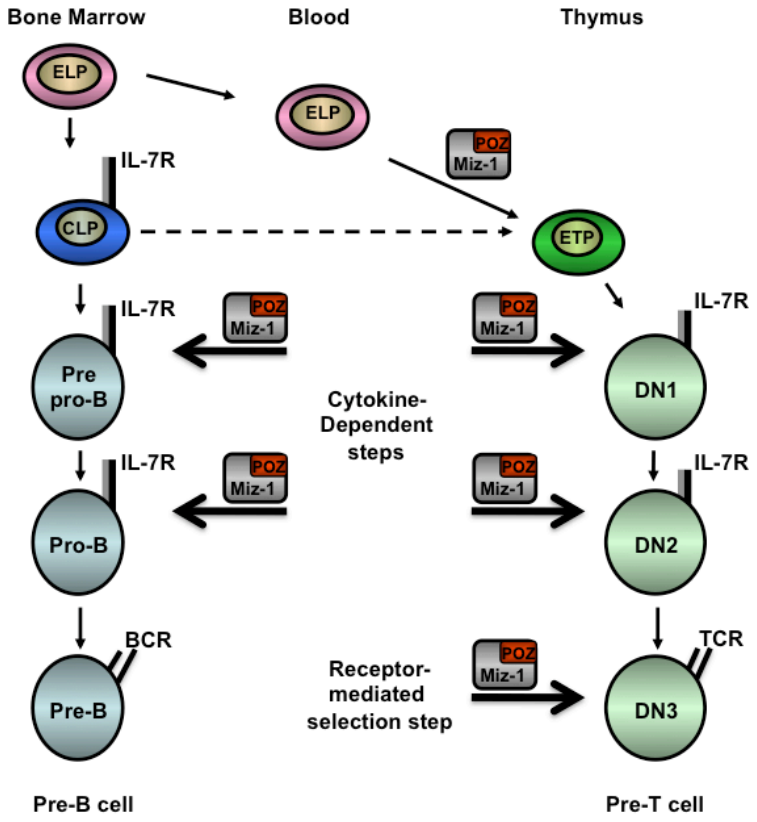


Figure 16. A functional Miz-1 protein is required to regulate lymphoid precursors and early B- and T-cells functions. Miz-1 regulates Bcl-2 expression in ELP, and *Bcl-2* and *SOCS1* expression in ETP/DN1/DN2 and in pre-proB to pre-B cell transition. Miz-1 is required for both the IL-7-independent E2A/EBF1/Pax-5/Rag-1/2 axis responsible for the induction of B cell differentiation signals, and the IL-7-dependent IL-7/STAT5/Bcl-2 axis required for the survival of B and T cell at the cytokine-dependent steps of the development. An additional role of Miz-1 in regulating the pre-TCR and p53-target genes expression is necessary for pre-T-cell differentiation (adapted from [515]).

Other contributions

- **Related to the Miz-1 project**

1- Möröy T, **Saba I**, Kosan C. The role of the transcription factor Miz-1 in lymphocyte development and lymphomagenesis – Binding Myc makes the difference. *Seminars in Immunology*. 2011 Oct;23(5):379-87. Epub 2011 Oct 13.

- **Related to projects implicating Gfi1**

1- Heyd F, Chen R, Afshar K, **Saba I**, Lazure C, Fiolka K, Möröy T. The p150 subunit of the histone chaperone Caf-1 interacts with the transcriptional repressor Gfi1. *Biochim Biophys Acta.* 2011 Apr-Jun;1809(4-6):255-61. Epub 2011 May 1.

2- Ichiyama K, Hashimoto M, Sekiya T, Nakagawa R, Wakabayashi Y, Sugiyama Y, Komai K, **Saba I**, Möröy T, Yoshimura A. Gfi1 negatively regulates T(h)17 differentiation by inhibiting RORgammat activity. *Int Immunol.* 2009 Jul;21(7):881-9. Epub 2009 Jun 7.

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