# The role of Myc-Interacting Zinc Finger Protein-1 (Miz-1) in T cell development and leukemogenesis

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*To my family and to Mat*

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# ABSTRACT

The Myc-interacting zinc finger protein-1 (Miz-1) is a ubiquitously expressed but poorly studied transcriptional transactivator that can bind to the proto-oncoprotein c-Myc, which is the main driver of a large number of different cancer types. In my thesis, I use murine T cell development as well as mouse models of T cell leukemia to broaden our biochemical understanding of the functions of Miz-1. At the beginning of my Ph.D., our lab had reported that Miz-1 plays an essential role during early stages of T cell development in the thymus, specifically at the β-selection checkpoint, which has important implications for T cell leukemia. Here, Miz-1 deficiency causes apoptosis and a developmental block in cells undergoing β-selection. However, the exact role of Miz-1 during β-selection remained unclear. The work presented here is a compilation of two studies that highlight the important role of Miz-1 during T cell development and transformation and ultimately furthers our understanding of Miz-1 in the context of leukemia research

In the first study, presented in Chapter II, I found that the increased apoptosis in Miz-1-deficient cells is due to deregulated signaling via p53, and this deregulation could be rescued following deletion of p53. Furthermore, I identified a Miz-1 binding site in the promoter of Rpl22, which regulates the synthesis of p53 during β-selection, as well as Miz-1-dependent activation of gene transcription. I also found that Miz-1 deficiency increases the translation of p53 due to the direct binding of Rpl22 to the mRNA of p53. These findings suggest that Miz-1, via the activation of Rpl22, regulates the translation of p53 at the β-selection checkpoint, when DNA damage occurs.

Because I found that Miz-1 regulates the translation of a prominent tumor suppressor in Chapter II specifically at the stage where T cell transformation is thought to occur, in Chapter III I investigated whether loss of Miz-1 affects the development of T cell leukemia. Using two

different T-ALL models, I showed that a strong counter selection occurs during T cell leukemogenesis against the loss of Miz-1, since Miz-1-deficient mice succumb to the disease with a significantly longer latency period than WT animals. Finally, I showed that Miz-1 also regulates p53 activity in this context, since mice deficient for both Miz-1 and p53 develop T-ALL at a rate similar to p53-deficient mice. These findings suggest that Miz-1 is required for the efficient development of T-ALL, and acts as a repressor of p53.

Thus, I demonstrate that loss of Miz-1 significantly hinders both normal T cell development and induction of T-ALL in part through the inability of these cells to repress p53 activity and signaling.

# RÉSUMÉ

La protéine Miz-1 (Myc-interacting zinc finger protein-1) est un facteur de transcription exprimé de façon ubiquitaire, interagissant avec la protéine oncogènique c-Myc, impliquée dans le développement de plusieurs cancers. Pendant ma thèse, j'ai étudié les fonctions biochimiques et moléculaires de Miz-1 dans le développement des cellules T ainsi que son rôle dans différents modèles de leucémies aigues T (T-ALL). Au début de ma thèse, notre équipe a montré que Miz-1 est impliqué dans le développement des cellules T en particulier lors de la β-sélection qui est une étape clé dans l'initiation des leucémies T. En effet, au cours de la β-sélection, l'expression exclusive d'une forme tronquée de Miz-1 (dans les souris Miz-$1^{\Delta POZ}$) induit l'apoptose et cause un défaut de différenciation des lymphocytes T. Cependant, le rôle exact joué par Miz-1 lors de la β-sélection n'est pas clair, ainsi que son impact sur l'initiation et le développement des leucémies T. Le travail présenté ici comprend ainsi deux études qui soulignent l'importance de Miz-1 durant le développement et la transformation des cellules T et améliorent nos connaissances quant à l'implication de Miz-1 dans le domaine de la recherche sur les leucémies T.

Dans notre première étude, présentée dans le deuxième chapitre de cette thèse, j'ai déterminé que l'induction de l'apoptose dans les cellules Miz-$1^{\Delta POZ}$ que nous avions préalablement observée, est causée par une dérégulation de la voie de signalisation de p53.En effet, j'ai montré que Miz-1 se lie au promoteur et active l'expression de *Rpl22*, qui régule la synthèse de p53 durant la β-sélection. Rpl22 peut ainsi se lier à l'ARN messager de p53. Cette liaison favorise une augmentation de la traduction de p53 ainsi qu'une augmentation d'expression de p53 dans les cellules T lors de la β-sélection, augmentant alors l'apoptose médiée par p53. Ces résultats suggèrent une régulation de la traduction de p53 par Miz-1, *via* l'activation de Rpl22, durant la β-sélection des cellules T.

Puisque Miz-1 régule la traduction d'un suppresseur de tumeur important au stade du développement des cellules T où la transformation maligne peut se produire dans les T-ALL, j'ai décidé d'étudier l'effet de la perte de fonction de Miz-1 dans le développement de ces mêmes tumeurs. En utilisant le modèle de surexpression de Notch1 de T-ALL, j'ai montré que les souris exprimant la forme tronquée de Miz-1 ont un meilleur taux de survie par rapport aux souris contrôles, suggérant que Miz-1 est nécessaire pour l'induction des leucémies T. Finalement, j'ai montré que Miz-1 régule aussi l'activité de p53 dans le contexte tumoral, puisque les souris Miz-$1^{\Delta POZ}$ n'exprimant pas p53 développent des T-ALL avec la même rapidité que les souris $p53^{-/-}$ exprimant normalement Miz-1. Ces résultats suggèrent un rôle important de Miz-1 dans le développement des leucémies aigues T en réprimant p53.

En conclusion, je démontre que la perte de fonction de Miz-1 inhibe le développement normal des cellules T ainsi que l'induction des leucémies T en favorisant l'expression de la protéine p53, médiateur important de l'apoptose.

# PREFACE & CONTRIBUTION TO KNOWLEDGE

In accordance with the "Guidelines for Thesis Preparation", the candidate has chosen to present the results of her research in manuscript format. A general introduction is presented in Chapter I. A general discussion is presented in Chapter IV. The materials and methods as well as the results are described in Chapters II and III, and appear in the following published or soon to be submitted manuscripts:

1. **Rashkovan, M**., C. Vadnais, J. Ross, M. Gigoux, W.K. Suh, W. Gu, C. Kosan, and T. Moroy. 2014. Miz-1 regulates translation of Trp53 via ribosomal protein L22 in cells undergoing V(D)J recombination. *Proceedings of the National Academy of Sciences of the United States of America* 111:E5411-5419.
2. **Rashkovan, M**, J. Fraszczak, C. Joly-Beauparlant, C. Vadnais, J. Hébert, E. Haddad, S. Vobecky, A. Droit, and T. Möröy. Notch1-induced T cell leukemogenesis requires the c-Myc cofactor and BTB-POZ domain protein Miz-1 to control the activation of p53. Manuscript in preparation.

The candidate was also involved in the following publication:

Kosan, C., **M. Rashkovan**, J. Ross, A.M. Schaffer, I. Saba, W. Lemsaddek, M. Trudel, and T. Moroy. 2014. The transcription factor Miz-1 is required for embryonic and stress-induced erythropoiesis but dispensable for adult erythropoiesis. *American journal of blood research* 4:7-19.

In this thesis, I present data describing the role of the transcription factor Miz-1 in early T cell development and in T cell transformation. I show that, in both contexts, Miz-1 indirectly regulates the tumor suppressor p53, which is implicated in a broad spectrum of cancers. I show that Miz-1 plays an essential role in T cell development and transformation, and I argue that Miz-

1 may constitute a target for future therapies in the treatment of T-ALL, in order to reduce the harmful secondary effects of chemotherapy and radiation.

# AUTHORS' CONTRIBUTIONS

Described in Chapters II and III are as follows:

**Charles Joly-Beauparlant** performed some bioinformatics analyses on ChIP-seq and RNA-seq data from Chapter III.

**Dr. Charles Vadnais** performed some bioinformatics analyses on ChIP-seq and RNA-seq data from Chapters II and III.

**Dr. Julie Ross** performed ChIP-seq and RNA-seq experiments in Chapter II.

**Dr. Jennifer Fraszczak** analyzed some tumor mice in Chapter III.

**Dr. Mathieu Gigoux** helped perform polysome fractionation experiments in Chapter II.

**Dr. Woong-Kyung Suh** provided reagents for polysome fractionation experiments in Chapter II.

**Dr. Wei Gu** provided p53K117R knock-in mice used in Chapter II.

**Dr. Christian Kosan** generated Miz-$1^{fl/fl}$ and $Myc^{V394D}$ mice used in Chapters II and III, and performed preliminary experiments for Chapter II.

**Dr. Arnaud Droit** provided guidance for bioinformatics analyses from Chapter III.

**Dr. Josée Hébert** provided human T-ALL samples from Chapter III.

**Dr. Elie Haddad** provided human thymus samples from Chapter III.

**Dr. Suzanne Vobecky** provided human thymus samples from Chapter III.

**Dr. Tarik Möröy** was involved in the conception of the project, funding of the project, interpretation of the data, and writing of the manuscripts for Chapters II and III.

I, **Marissa Rashkovan**, performed mouse dissections, flow cytometry, cell cycle and cell death analyses, RNA isolation, qPCRs, polysome fractionation, RNA immunoprecipitation, Western Blots, Actinomycin D treatment, luciferase assays, cloning, retroviral production and infection, *in vitro* T cell differentiation on OP9-DL4 stromal cells, tumorigenesis assays and some

bioinformatics analyses for Chapters II and III. I analyzed all the data from Chapters II and III, and was involved in writing the manuscripts for both chapters.

# TABLE OF CONTENTS

# LIST OF FIGURES

## CHAPTER I: General Introduction



## CHAPTER II: Miz-1 regulates translation of *Trp53* via ribosomal protein L22 in cells undergoing V(D)J recombination

## CHAPTER III: Notch1-induced T cell leukemogenesis requires the c-Myc cofactor and BTB-POZ domain protein Miz-1 to control the activation of p53

## CHAPTER IV: General Discussion & Perspectives

# LIST OF ABBREVIATIONS

**AMPK:** AMP-activated protein kinase

**Arnt:** Aryl hydrocarbon nuclear translocator

**Bcl:** B cell lymphoma

**BCoR:** Bcl6 corepressor

**BCR:** B cell receptor

**bHLH:** Basic helix-loop-helix

**BM:** Bone marrow

**BTB-POZ:** Broad complex, Tramtrack, Bric à brac – Poxvirus and Zinc-finger

**ChIP:** Chromatin Immunoprecipitation

**ChIP-seq:** Chromatin Immunoprecipitation Sequencing

**CLP:** Common lymphoid progenitor

**CTD:** C-terminal domain

**DAG:** Diacylglycerol

**DBD:** DNA-binding domain

**DC:** Dendritic cell

**DL4:** Delta-like 4

**DLBCL:** Diffuse large B cell lymphoma

**DN:** Double negative

**DP:** Double positive

**DSB:** Double strand break

**Epo:** Erythropoietin

**EpoR:** Erythropoietin receptor

**ETP:** Early thymic progenitor

**FACS:** Florescence-activated cell sorting

**FL:** Follicular lymphoma

**GC:** Germinal center

**Gfi1:** Growth factor independence-1

**HD:** Heterodimerization domain

**HSC:** Hematopoietic stem cell

**ICN1:** Intracellular Notch1

**Ig:** Immunoglobulin

**IL-7R:** Interleukin-7 receptor

**Inr:** Initiator

**IP3:** Inositol 1,4,5-trisphosphate

**Jak:** Janus kinase

**MAML:** Mastermind-like

**MEF:** Mouse embryonic fibroblasts

**Miz-1:** Myc-interacting zinc finger protein-1

**MoMuLV:** Moloney Murine Leukemia Virus

**N-CoR:** Nuclear hormone receptor corepressor

**NFAT:** Nuclear factor of activated T cells

**NFκB:** Nuclear factor κB

**NHEJ:** Non-homologous end joining

**NK:** Natural killer

**NPC:** Neuronal progenitor cells

**NPM:** Nucleophosmin

**Pai-1:** Plasminogen activator inhibitor-1

**PI(3)K:** Phosphoinositide 3-kinase

**PKC:** Protein kinase C

**PLCγ:** Phospholipase Cγ

**RIP:** RNA immunoprecipitation

**RNA-seq:** RNA sequencing

**Rpl22:** Ribosomal protein L22

**Rpl22l1:** Ribosomal protein L22-like 1

**Rpl23:** Ribosomal protein L23

**SCF:** Stem cell factor

**SMRT:** Silencing mediator for retinoid and thyroid receptor

**TAD:** Transactivation domain

**T-ALL:** T cell acute lymphoblastic leukemia

**TBP:** TATA binding protein

**TCR:** T cell receptor

**TD:** Tetramerization domain

**TdT:** Terminal deoxynucleotide transferase

**TEC:** Thymic epithelial cell

**Tfh:** T follicular helper cell

**TopBP1:** Topoisomerase IIβ binding protein 1

**WB:** Western blot

**Wif-1:** Wnt inhibitory factor-1

# CHAPTER I

# General Introduction

# 1.0 Leukemia

Cancer is a disease that affects much of the population. In fact, 2 in 5 Canadians will develop cancer in their lifetime, and 1 in 4 Canadians will die from cancer (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015). While most types of cancer generally affect the aging population, leukemia mainly affects children and young adults. Between 2006 and 2010, the most commonly diagnosed cancer in children aged 0-14 was leukemia at 32% in Canada and 30% in the USA (American Cancer Society, 2015; Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015). While survival rates following diagnosis remain high (91% for lymphoid leukemias in Canada and 90% in the USA), chemotherapy and radiotherapy treatments pose a significant risk for children whose bodies are still developing at the time of treatment (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2015; St. Jude Children's Research Hospital, 2015). It is therefore important to understand the molecular mechanisms leading to leukemia induction in order to better treat the children affected by this disease.

# 2.0 T cell development & leukemia

T cells, which develop in the thymus, transit through many stages characterized by the expression of different cell surface markers, the activation of many cell signaling pathways, and several important checkpoints (Figure 2.1). Early precursors of the T lineage are termed ETPs, or "early thymic progenitors" and are part of the DN1 ("double negative" for CD4 and CD8, as well as $cKit^{+}CD44^{+}CD25^{-}$) subset of pro-T cells. The expansion and differentiation of these cells is highly dependent on signaling through cytokine receptors, such as Flt3 and cKit, and through signaling via the Notch1 receptor (Pui et al., 1999; Sambandam et al., 2005; Wilson et al., 2001). Pro-T cell development proceeds from DN1 to DN2a ($cKit^{+}CD44^{+}CD25^{+}$) and the cells are still able to be diverted to other lineages under conditions supporting this diversion, for example, cells cultured *in vitro* or from specific transgenic mouse models (David-Fung et al., 2009). While diversion to the B cell lineage is restricted early after thymic entry, DN2a cells can differentiate into dendritic cells (DCs), natural killer (NK) cells, macrophages or granulocytes (Balciunaite et al., 2005; Heinzel et al., 2007; Lu et al., 2005; Wu et al., 1996). It is between DN2a and DN2b ($cKit^{med}CD44^{+}CD25^{+}$) that the cells become restricted to the T lineage (Moore and Zlotnik, 1995). DN2b pro-T cells subsequently give rise to DN3 cells ($CD44^{-}CD25^{+}$), which can be separated into DN3a and DN3b subsets, and can be identified based on cell size and CD27 expression (Taghon et al., 2006). Transition of cells from DN3a to DN3b depends on the ability to productively rearrange the TCRβ chain during V(D)J recombination. Following the expression of the pre-T cell receptor (pre-TCR) on the cell surface, DN3b cells then undergo massive proliferation to become DN4 ($CD44^{-}CD25^{-}$) cells, before eventually upregulating the CD8α and CD8β chains, followed shortly by CD4. These double positive (DP) cells undergo positive and negative selection in order to select only the DP cells which express functional TCRs, and to remove those DP cells which

strongly recognize self-antigens (Klein et al., 2009; Stefanova et al., 2002; Stritesky et al., 2013). These selected cells then become positive for either CD4 or CD8 (Germain, 2002; Singer et al., 2008). Once this selection process is complete, the cells leave the thymus to enter secondary lymphoid organs. These different stages of T cell development are regulated by a network of transcriptional activators and repressors which promote the T cell fate and commitment to the cell to the T lineage.

T cell acute lymphoblastic leukemia/lymphoma (T-ALL) is an aggressive leukemia that is induced following the malignant transformation of a T cell progenitor. This disease mainly affects children and adolescents, and out of all ALL subtypes, T-ALL prognosis remains the worst. Furthermore, relapse rates remain high and prognosis following relapse is poor (Oudot et al., 2008). There are also many types of genetic abnormalities that can be found in cases of T-ALL, such as chromosomal translocations, deletions, amplifications, duplications and point mutations, affecting many pathways involved in T cell development (Armstrong and Look, 2005; Dai et al., 2014; De Keersmaecker et al., 2005; Grabher et al., 2006; Graux et al., 2006; Sanchez et al., 2014). As is common with most types of malignant transformation, T-ALL occurs when a developing thymocyte incurs many genetic abnormalities leading to uncontrolled cell growth. The DN3 pre-T cell is thought to be the cell which initiates T-ALL, indicating that any process occurring at this step must be tightly controlled. The following chapters will focus on the early stages of T cell development in the thymus, the double negative (DN) stages, as well as the processes that must be disturbed to induce the development of T cell leukemia.

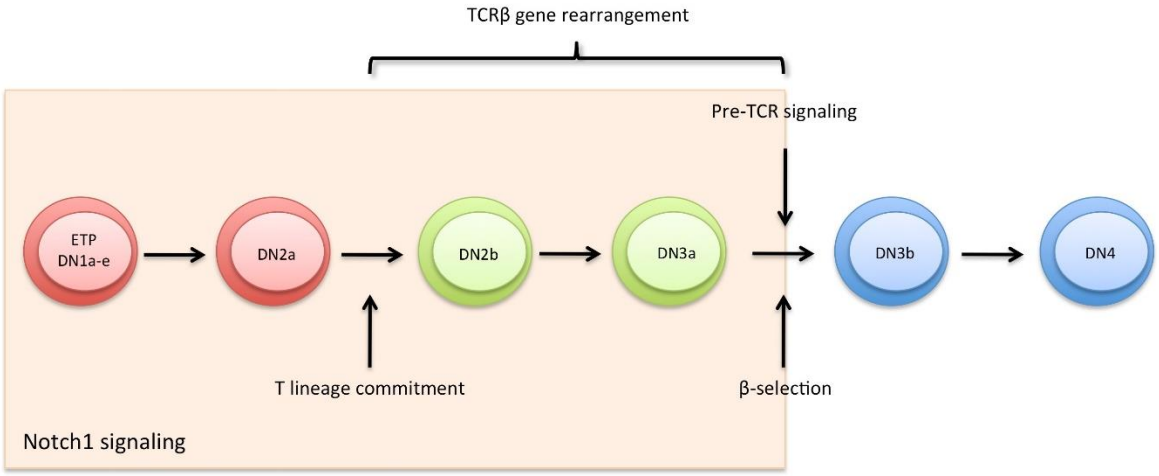


**Figure 2.1.** Early T cell development in the thymus. Early thymic progenitors (ETPs) are part of the DN1 subset of pro-T cells. DN1 cells give rise to DN2a cells. DN2a cells give rise to DN2b cells, which are committed to the T cell lineage. TCRβ rearrangement begins in late DN2b and continues into DN3a, where cells undergo β-selection, and only those cells which have productively rearranged the β chain of the pre-TCR and express it on the cell surface with the pTα surrogate chain enter DN3b. DN3b cells then downregulate CD25 and undergo massive pre-TCR-induced proliferation. From the time the progenitors enter the thymus to signaling through the pre-TCR, the cells are dependent upon Notch1-induced signals (shaded pink box).

# 3.0 The β-selection checkpoint

One of the most important steps in T cell development is the transition from DN3a to DN3b, also known as the β-selection checkpoint. Cells which are able to productively rearrange the β chain of the T cell receptor (TCR) and express this chain on their surface with the preTα surrogate chain are able to survive and proliferate (Falk et al., 2001). Not only must these cells survive the DNA double strand breaks induced by V(D)J recombination, but they must also receive survival signals through the pre-TCR and the Notch1 receptor, which will be discussed in the following sections.

## 3.1 V(D)J Recombination

In order for the immune system to function effectively, both T and B lymphocytes must have a diverse repertoire to recognize a myriad of antigens. This is achieved through somatic rearrangement of the genes encoding the T cell receptor α and β chains and the B cell receptor immunoglobulin heavy and light chains, a process known as V(D)J recombination (Figure 3.1). Recombination begins by joining the D (diversity) and J (joining) segments, followed by the addition of the V (variable) region. During V(D)J recombination, DNA double strand breaks (DSBs) are introduced by the recombinases Rag-1 and Rag-2, specifically at sites of recombination (von Boehmer, 2004). Following cleavage of specific recombination sequences by Rag proteins, the non-homologous end joining DNA repair pathway (NHEJ) proteins Ku70/Ku80 are recruited and bind the broken DNA strands (Gu et al., 1997b; Nussenzweig et al., 1996; Taccioli et al., 1994; Taccioli et al., 1993). Artemis, another NHEJ protein, in association with DNA-PKcs (DNA-dependent protein kinase catalytic subunit), is recruited to mediate opening of the hairpin (Gottlieb and Jackson, 1993; Ma et al., 2002; Roth et al., 1992). The enzyme terminal deoxynucleotide

transferase (TdT) increases the junctional diversity by adding non-templated nucleotides at the cleaved ends, and the DNA DSBs are sealed by XRCC4 and DNA Ligase 4 (Critchlow et al., 1997; Grawunder et al., 1997). If the TCRβ chain is productively rearranged in a DN3a pro-T cell, it is expressed at the cell surface in association with the preTα chain, forming the pre-TCR (Yamasaki et al., 2006). The cell is then said to have passed through β-selection and is referred to as a DN3b pre-T cell. It is after the β-selection step has occurred that the cell receives signals through the pre-TCR to survive and proliferate (Falk et al., 2001; Kreslavsky et al., 2012). V(D)J recombination also occurs later in the development of a T cell, at the CD4/CD8 double positive (DP) stage. It is at this point in development that the α chain of the TCR is rearranged, and, once expressed on the cell surface together with the β-chain, T cells are eliminated based on the affinity of their TCR for self and non-self epitopes via positive and negative selection (Egerton et al., 1990; Kisielow et al., 1988; MacDonald et al., 1988). The newly selected cells then become positive for either CD4 or CD8 and migrate out of the thymus to peripheral lymphoid organs.

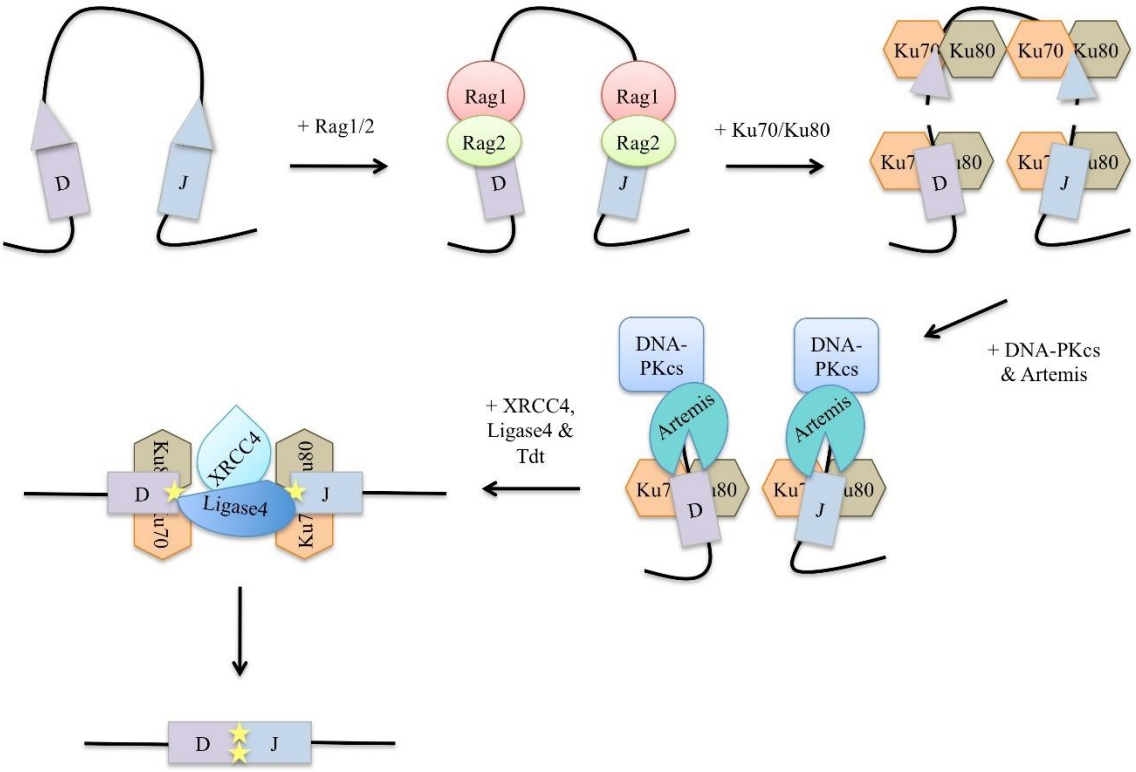


**Figure 3.1.** Schematic representation of V(D)J recombination. The DJ segments of the TCRβ locus are bound by the recombinase enzymes Rag-1 and Rag-2. The Rag1/2 complex cleaves the RSS (recognition signal sequences) at these sites and the Ku70/Ku80 complex is recruited to bind the broken DNA. DNA-PK in complex with Artemis then cleaves the hairpin structure and TdT (terminal deoxynucleotide transferase) adds non-templated nucleotides to increase junctional diversity. Finally, XRCC4 and DNA Ligase 4 repair the cleaved DNA.

## 3.2 Notch Signaling

Notch receptors are transmembrane receptor proteins that are activated upon interaction with canonical Notch ligands, the Delta-like (DL) family and the Jagged family of proteins (D'Souza et al., 2008; Kopan and Ilagan, 2009). In the thymus, these ligands are found on the surface of thymic epithelial cells (TECs) and interact with Notch1, the Notch receptor which is both necessary and

sufficient for T cell development (D'Souza et al., 2008; Pui et al., 1999; Radtke et al., 1999). Interaction between Notch1 and its ligand, Delta-Like-4 (DL4) in the thymus, leads to proteolytic cleavage of the Notch1 transmembrane domain by ADAM metalloproteases and subsequently by the γ-secretase complex (Artavanis-Tsakonas et al., 1999; Fortini et al., 1993; Struhl et al., 1993). This cleavage leads to the release of the intracellular portion of Notch1 (ICN1), which translocates to the nucleus where it can interact with its binding partner RBP-Jκ (also known as CSL) (Radtke et al., 2004). This causes a displacement of co-repressive complexes associated with RBP-Jκ and recruitment of co-activator complexes, including Mastermind-like proteins (MAML) (Figure 3.2) (Wu et al., 2002).

Signaling through Notch1 is essential for T cell development, and the loss of Notch1 or RBP-Jκ in adult bone marrow cells leads to impaired T cell development (Radtke et al., 1999). Furthermore, a T cell-specific deletion of Notch1 or RBP-Jκ leads to arrest of T cell development at the DN3 stage, while deletion of Notch1 in ETPs results in differentiation of these cells into dendritic cells (DCs) in the thymus, highlighting the necessity of Notch1 signaling for T cell specification and commitment (Bell and Bhandoola, 2008; Feyerabend et al., 2009; Wada et al., 2008; Wilson et al., 2001). Notch1 is required for early T cell development, namely at the DN1-DN2 transition, as well as for sustained expression of CD25 on the surface of DN2 and DN3 cells, and for the survival of DN2, DN3 and DN4 cells (Ciofani and Zuniga-Pflucker, 2005; Sambandam et al., 2005; Schmitt and Zuniga-Pflucker, 2002; Taghon et al., 2005). Moreover, Notch1 is essential to inhibit the B cell fate; expression of a constitutively active form of Notch1 results in extrathymic T cell development and a block in B cell development in the bone marrow, while deletion of Notch1 in bone marrow progenitors leads to an accumulation of B cells in the thymus, at the expense of T cell development (Heinzel et al., 2007; Izon et al., 2002; Pui et al., 1999;

Schmitt et al., 2004; Wilson et al., 2001). Loss of DL4 on thymic epithelial cells also results in a block in T cell development and an ectopic accumulation of B cells in the thymus, indicating that activated Notch1, and not only the presence of the full length receptor itself, is necessary for T cell development (Hozumi et al., 2008; Koch et al., 2008).

Many genes involved in T cell development and lineage commitment are Notch1 target genes including Hes1, Tcf-1, CD25, preTα, and Notch1 itself (Taghon et al., 2005; Wang et al., 2006; Weber et al., 2011). Some Notch1 target genes act as negative regulators of this signaling pathway, including NRARP and Deltex-1 (Izon et al., 2002; Yun and Bevan, 2003). The proto-oncoprotein c-Myc was also identified as a direct Notch1 target, however the direct association between Notch1 and c-Myc activation has only been characterized in leukemic cells (Chan et al., 2007; Herranz et al., 2014; Palomero et al., 2006; Weng et al., 2006).

Notch1 is also necessary to promote survival, but not proliferation of pre-T cells during β-selection, by activating the PI(3)K/Akt pathway (Ciofani and Zuniga-Pflucker, 2005). In fact, constitutive activation of Akt restores DN3 survival, size and glycolytic rate after Notch signaling withdrawal in an OP9-DL1 culture system, indicating that this pathway is directly or indirectly activated by Notch1 at the β-selection checkpoint. Following β-selection, the cells no longer rely upon Notch1, and Notch1 target genes become repressed. This occurs because pre-T cells are now dependent upon signaling through the newly expressed pre-TCR for survival and proliferation (Falk et al., 2001; Germain, 2002; Kreslavsky et al., 2012).

A role for Notch1 in T-ALL was first postulated when the rare chromosomal translocation t(7;9)(q34;q34.3) was identified as coupling the intracellular portion of Notch1 to the TCRβ locus (Ellisen et al., 1991). In fact, the most common mouse model used to study T-ALL involves bone marrow reconstitution of lethally irradiated mice with cells overexpressing ICN1 (Aster et al.,

2000; Pear et al., 1996). These mice develop aggressive T-ALL with a relatively short latency. Activating mutations in Notch1 have now been identified in over 50% of T-ALL cases, resulting in constitutive Notch1 signaling (Weng et al., 2004). Most Notch1 mutations occur in the heterodimerization domain (HD) or the PEST domain, resulting in ligand-independent cleavage of Notch1 and the release of ICN1, or enhanced ICN1 stability following cleavage, respectively (Chiang et al., 2006; Maillard et al., 2004; Malecki et al., 2006). Mutations in the PEST domain usually lead to a loss of recognition sites for Fbxw7, the E3-ubiquitin ligase that tags ICN1 for degradation (Maser et al., 2007; Thompson et al., 2007). It is therefore not surprising that mutations in Fbxw7 have also been identified in approximately 15% of T-ALL patients, affecting the ability of this protein to recognize degradation sequences in the Notch1 PEST domain and therefore leading to decreased Notch1 degradation (Onoyama et al., 2007).

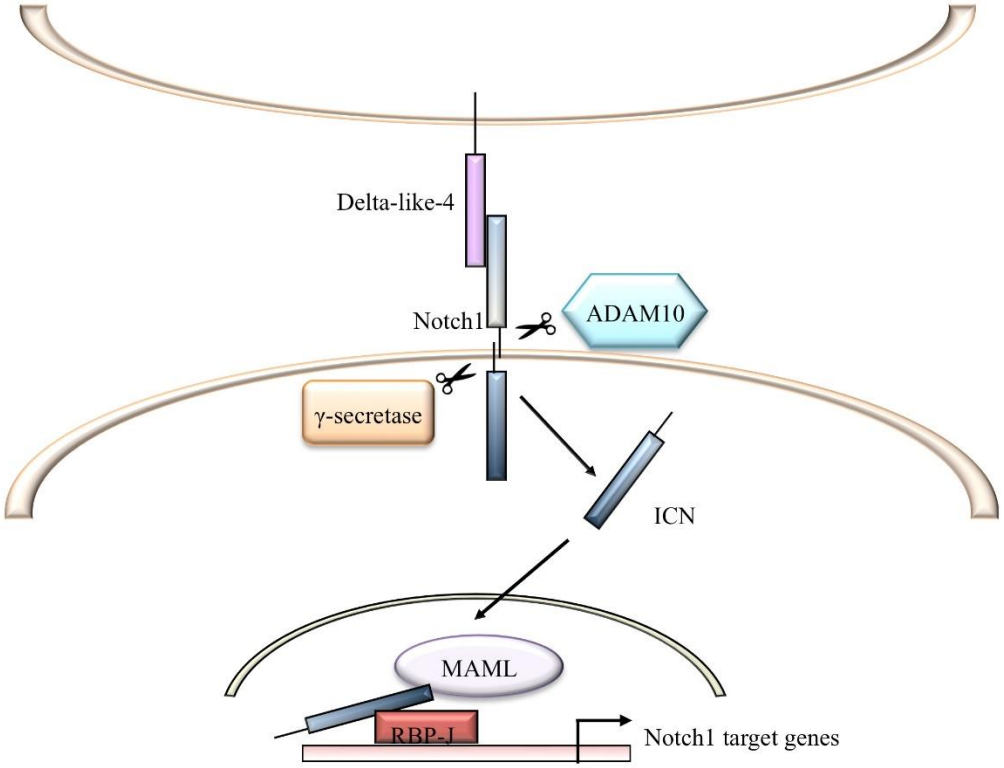


**Figure 3.2.** The Notch1 signaling pathway. Upon interaction with its thymic ligand DL4, Notch1 is cleaved by ADAM metalloproteases and the γ-secretase complex to release the intracellular portion of the receptor. This intracellular Notch1 then translocates to the nucleus where it interacts with RBP-J to recruit co-activators, such as MAML proteins, to activate target gene transcription.

## 3.3 Signaling through the pre-TCR

After a pre-T cell has passed through the β-selection checkpoint, it must receive signals through the pre-TCR, composed of the invariant preTα chain, the newly rearranged TCRβ chain and CD3 signaling molecules (Malissen et al., 1999; von Boehmer and Fehling, 1997). Signaling through the pre-TCR leads to upregulation of CD4 and CD8, downregulation of CD25, inhibition

of apoptosis, a re-entry into the cell cycle and initiation of allelic exclusion to prevent rearrangement of the second TCRβ allele (Dudley et al., 1994; Falk et al., 2001; Hoffman et al., 1996; Michie and Zuniga-Pflucker, 2002). There are many pathways activated downstream of the pre-TCR, including the PI(3)K pathway, the PLCγ pathway and the Ras/Raf/MEK pathway, which I will briefly discuss in the following sections (Aifantis et al., 2006; Kruisbeek et al., 2000; von Boehmer, 2005).

### 3.3.1 The PI(3)K Pathway

One of the pathways that can be activated via the pre-TCR is the phosphoinositide 3-kinase (PI(3)K) pathway. Signaling downstream of PI(3)K mainly leads to phosphorylation and activation of Akt, which then phosphorylates and inhibits transcription factors involved in cell cycle arrest, apoptosis, cell growth, survival, and metabolism, including the BH3-only protein BAD, caspase 9, Nur77 and the forkhead transcription factors (Arden and Biggs, 2002; del Peso et al., 1997; Jones et al., 2000; Mandal et al., 2005; Manning and Cantley, 2007; Pekarsky et al., 2001; Plas and Thompson, 2005; Veis et al., 1993). The Notch1 and pre-TCR signaling pathways therefore converge on Akt to promote cell survival. The importance of the PI(3)K pathway in thymocyte development is illustrated by mice lacking the lipid phosphatase PTEN. PTEN is the enzyme that counteracts PI(3)K, and in the absence of PTEN, Akt and other PI(3)K-activated kinases are localized to the membrane and become constitutively activated (Cantrell, 2001). Loss of PTEN is able to rescue pre-T cell development in mice that are deficient in components of the pre-TCR complex, indicating that the activation of the PI(3)K pathway alone can induce differentiation to the DP stage without further signaling through the pre-TCR (Hagenbeek et al., 2004). Homozygous inactivation of PTEN has also been found in 17% of T-ALL patient samples providing a mechanism for leukemic cells to activate a broad range of pathways downstream of

the pre-TCR (Palomero et al., 2007). These mutations result in hyperactivation of the PI(3)K/Akt pathway and thus enhanced cell size, glucose uptake and proliferation (Palomero et al., 2007).

### 3.3.2 The PLCγ Pathway

Another pathway activated by pre-TCR signaling is the phospholipase Cγ (PLCγ) pathway. PLCγ hydrolyzes $PIP_2$ to produce the signaling molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), leading to the activation of several downstream signaling pathways (Neilson et al., 2004). IP3 release causes mobilization of calcium from the endoplasmic reticulum and activation of the phosphatase calcineurin (Aramburu et al., 2000). Activated calcineurin can then dephosphorylate and activate the NFAT (nuclear factor of activated T cells) family of transcription factors which then translocates to the nucleus to regulate target genes (Kiani et al., 2000; Macian, 2005). Lack of NFAT proteins in the thymus leads to a partial block at the DN3 stage, while overexpression of these transcription factors partially bypasses the need for pre-TCR signaling to passage through β-selection (Oukka et al., 1998). This indicates that NFAT proteins are essential mediators of pre-TCR signaling, and that they are important in thymocyte differentiation. DAG release, on the other hand, leads mainly to activation of protein kinase C (PKC). Downstream of the pre-TCR, PKC activation induces transcription of NF-κB (nuclear factor κB), which is essential for pre-T cell survival (Aifantis et al., 2001; Voll et al., 2000). While mutations in NF- κB have yet to be detected in T-ALL, NF- κB activity has been detected in human T-ALL primary samples, implicating activation of the PLCγ pathway downstream of pre-TCR signaling in the survival, proliferation, differentiation and transformation of β-selected pre-T cells (Kordes et al., 2000).

### 3.3.3 The Ras/Raf/MEK Pathway

The third pathway that can be activated by pre-TCR signaling is the Ras/Raf/MEK pathway. Signaling downstream of the small G-protein Ras is mainly regulated by the Ras guanine nucleotide exchange factors (Ras-GEFs) Sos1 and RasGRP1 in thymocytes (Dower et al., 2000; Kortum et al., 2011; Priatel et al., 2002). Mice deficient for both Sos1 and RasGRP1 in pre-T cells showed a defect in development beyond DN3, suggesting a role for both of these Ras-GEFs during β-selection (Kortum et al., 2012). Moreover, forced transgenic expression of constitutively active Ras ($Ras^{V12}$) or Raf1 (Raf-CAAX) in $Rag^{-/-}$ mice leads to normal proliferation and generation of DP cells, experimentally bypassing pre-TCR-dependent β-selection (Gartner et al., 1999; Iritani et al., 1999). Furthermore, combined deletion of ERK1 and ERK2, phosphorylated downstream of Ras activation, inhibits differentiation of pre-T cells (Fischer et al., 2005; Pages et al., 1999). Ras activation is involved in many transduction pathways, and has been implicated in a variety of malignancies, and activating Ras mutations have been identified in 4-10% of T-ALL cases, implicating signaling downstream of the pre-TCR in the induction of this disease (Campbell et al., 1998; Kawamura et al., 1999; von Lintig et al., 2000; Yokota et al., 1998).

## 3.4 A role for p53

p53 (*Tp53*) is a transcription factor and tumor suppressor protein that is frequently referred to as the "guardian of the genome" because of its role in cancer as well as many aspects of cellular biology (Lane, 1992). Because of the broad spectrum of genes regulated by p53, its activity must be tightly controlled in order to prevent tumor induction. In the following sections, I will describe the structural features of p53, as well as some of the functions of this protein. Furthermore, I will describe some of the mutations associated with the loss of function of p53, highlighting the importance of this protein in the prevention of both solid tumors and T cell leukemia.

### 3.4.1 p53 and cancer

The p53 gene is the most commonly inactivated tumor suppressor in human cancer (Beroud and Soussi, 2003; Hussain and Harris, 1998). In fact, p53 is inactivated by mutations in over 50% of all human cancers (Vogelstein et al., 2000). While loss of p53 eventually leads to cancer development in mouse models, many studies have shown that the expression of p53 mutants in the mouse leads to a change in tumor spectrum and an increase in metastatic potential compared to p53-deficient mice (Donehower et al., 1992; Kim and Deppert, 2004; Lang et al., 2004; Olive et al., 2004; Sigal and Rotter, 2000). Therefore, while complete loss of p53 activity is detrimental to normal cellular activity and eventually leads to cancer, production of a p53 protein that is constitutively active or cannot be degraded might be even more harmful as it can lead to more aggressive tumors.

As I have already discussed, V(D)J recombination induces DNA double strand breaks that must be repaired in order for the pre-T cell to progress through the β-selection checkpoint. Because of these DNA double strand breaks, the p53 tumor suppressor protein must be inactivated during V(D)J recombination. This transcription factor plays a critical role in signal transduction following DNA damage to activate genes involved in apoptosis and cell cycle arrest (Cox and Lane, 1995). The activity of p53 must therefore be restrained following signaling through the pre-TCR in order to prevent the apoptosis of cells in the process of productively rearranging the TCRβ locus (Guidos et al., 1996; Haks et al., 1999). In fact, p53 protein levels are upregulated in *scid* thymocytes, in which there is an inactivating mutation in the active site of DNA-PK, as a consequence of attempted V(D)J recombination (Guidos et al., 1996). Furthermore, in this setting, p53 deficiency enhances survival of both B and T cell precursors undergoing V(D)J recombination. It is therefore not surprising that p53 knockout mice, which are prone to develop

spontaneous tumors, predominantly develop thymic lymphomas (Donehower et al., 1992). Loss of p53 in these lymphomas leads to genomic instability and a high mutation rate, and mutation of PTEN is rapidly selected for early in lymphoma progression (Dudgeon et al., 2014). In humans, however, the mutation rate for TP53 in primary T-ALL is low, and loss of p53 activity is more frequently associated with relapse, as opposed to primary disease onset (Hsiao et al., 1994). While p53 mutation is infrequent at disease onset, more than 70% of primary T-ALL cases present with deletion of the *CDKN2A* (*Ink4A-Arf*) locus (Mullighan et al., 2008). This is important as Arf antagonizes the ubiquitination of p53 by Mdm2, and thus loss of Arf activity results in increased degradation of p53 (Sherr, 2001). Therefore, while p53 mutations rarely occur in primary T-ALL, modulation of the p53 pathway is essential for disease development.

### 3.4.2 Structural features of p53

In the mouse, p53 is a 387 amino acid transcription factor with a molecular weight of 53kDa. In the human, the amino acid sequence of p53 differs, and this transcription factor is composed of 393 amino acids. It is made up of several important domains, necessary for its function and regulation, which are all fairly conserved from mouse to human. The most important of these domains are the transactivation domain (TAD), the DNA-binding domain (DBD), the tetramerization domain (TD) and the C-terminal regulatory domain (CTD), which will be described in the following sections (Figure 3.3).

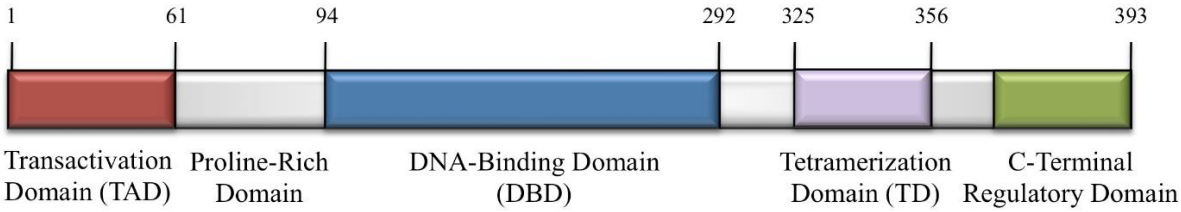


**Figure 3.3.** Structure of human p53. The p53 protein is composed of several regulatory domains. These include the transactivation domain (TAD), the proline-rich domain, the DNA-binding domain (DBD), the tetramerization domain (TD) and the c-terminal regulatory domain. Each domain is necessary for proper regulation of p53 activation and signaling downstream of p53.

#### 3.4.2.1 The Transactivation Domain

The transactivation domain is located at the N-terminus of p53. This acidic domain acts as a binding site for multiple interacting proteins including the transcription machinery, p300/CBP and Mdm2 (Di Lello et al., 2006; Gu et al., 1997a; Kussie et al., 1996; Lu and Levine, 1995; Schon et al., 2002; Teufel et al., 2007; Thut et al., 1995). Recruitment of the basal transcription machinery allows p53 to activate expression of target genes once it has bound to a specific target DNA sequence. Binding of Mdm2 to this region, which targets p53 to the ubiquitin-mediated proteolytic machinery, indicates that the TAD is also important for stability and activity of p53 (Haupt et al., 1997; Kubbutat et al., 1997). Furthermore, both Mdm2 and transcriptional activators bind to the same region in the TAD, preventing p53 from activating transcription when it has been targeted for degradation (Wu et al., 1993). While p53 is ubiquitinated and targeted for degradation by Mdm2, binding to p300 leads to acetylation of specific lysine residues in the TAD of p53 and activation of target gene transcription (Teufel et al., 2007; Zhu et al., 1998). Post-translational modifications of residues in this N-terminal domain also affect the affinity of p53 for different

binding partners, shifting the downstream response to a certain stress signal. For example, upon phosphorylation of Thr-18, the p53-Mdm2 interaction is impaired, while the p53-p300/CBP interaction is strengthened (Dornan and Hupp, 2001; Lai et al., 2000; Sakaguchi et al., 2000; Schon et al., 2002). Furthermore, one residue can be modified by many different enzymes in order to elicit a specific response. This is the case with Ser-15, which can be phosphorylated by ATM, ATR and DNA-PK in response to DNA damage, indicating that the TAD of p53 is largely responsible for mediating the many different responses downstream of p53 (Lambert et al., 1998; Milczarek et al., 1997; Tibbetts et al., 1999).

#### 3.4.2.2 The DNA-Binding Domain

In order to regulate gene transcription, p53 must bind to DNA. The specific binding sequence of p53 consists of two decameric half-site palindromes, separated by 0-13 base pairs (el-Deiry et al., 1992; Ko and Prives, 1996). The specific spacer sequence length, as well as the higher order interaction between the two half-site palindromes dictates the binding affinity of tetrameric p53 (Weinberg et al., 2005). Furthermore, the affinity of p53 for the promoter loosely correlates with function of the gene; promoters for cell cycle arrest and DNA repair genes are described as high-affinity binding sites, while apoptotic response elements are bound with a significantly lower affinity (Schlereth et al., 2010; Timofeev et al., 2013; Weinberg et al., 2005). Crystal structures of p53 tetramers in complex with DNA have also revealed that weak protein-protein interactions are observed between two p53 dimers, leading to transient tetramer formation and interaction with DNA (Kitayner et al., 2006).

While mutations in p53 can occur almost anywhere in the protein, the most common p53 mutations occur at six “hotspot” amino acids in the DBD (Cho et al., 1994; Leroy et al., 2013; Soussi and Lozano, 2005). These mutations may affect the binding of p53 to DNA, but they may

also act as dominant negative proteins to dampen the activity of any wild-type p53 remaining in the cell (Milner et al., 1991; Sigal and Rotter, 2000; Wang et al., 2011b). The DBD therefore plays an essential role in preventing malignant transformation.

#### 3.4.2.3 The Tetramerization Domain

The C-terminal region of p53 contains the tetramerization domain, which allows two p53 dimers to form a tetrameric structure (Clore et al., 1995; Jeffrey et al., 1995; Mittl et al., 1998; Sakaguchi et al., 1997; Veprintsev et al., 2006). While p53 variants lacking the TD are able to bind to DNA and stimulate transcription, the affinity of a p53 monomer for DNA is 10-100 times lower than the affinity of the full length protein, which is able to tetramerize (Balagurumoorthy et al., 1995; Bargonetti et al., 1993; McLure and Lee, 1998; Nagaich et al., 1999; Sang et al., 1994). Because of this, it has been shown that the TD can influence the strength of the interaction between p53 and DNA, as well as the conformation of this complex (McLure and Lee, 1998; McLure and Lee, 1999). *In vitro* p53 biogenesis assays also found that dimer formation occurs co-translationally on the polysome, while tetramerization occurs post-translationally, by dimerization of preformed dimers (Nicholls et al., 2002). In fact, one model suggests that binding of a p53 dimer to one half of the p53 consensus sequence increases the probability for the binding of the second dimer to the adjacent half of the site (McLure and Lee, 1998; McLure and Lee, 1999). This domain is also important for protein-protein interactions; the binding sites for casein kinase 2 and PKC have both been mapped directly to the p53 TD (Delphin et al., 1997; Gotz et al., 1999). Additionally, while other proteins including Mdm2, c-abl and TBP do not bind directly to the TD, they can only bind p53 tetramers, indicating that the TD is essential for most functions of p53 (Liu et al., 1993; Lomax et al., 1998; Marston et al., 1995; Nie et al., 2000).

#### 3.4.2.4 The C-Terminal Regulatory Domain

The C-terminal region of p53 is subject to many post-translational modifications, including acetylation, ubiquitination, phosphorylation, sumoylation, methylation and neddylation (Huang et al., 2010; Huang et al., 2007; Lavin and Gueven, 2006; Toledo and Wahl, 2006). These C-terminal modifications regulate both p53 function and protein levels, however knowledge is still considerably limited with regards to the effect of each of these modifications on downstream processes. Acetylation of C-terminal lysines has been associated with recruitment of co-activators and histone acetyltransferases, required for target gene activation (Barlev et al., 2001; Mujtaba et al., 2004). Mutation of these lysines to arginines prevents modification at these sites but leads to normal levels of p53 before and after DNA damage, indicating that ubiquitination of these residues is not required for p53 degradation (Feng et al., 2005; Krummel et al., 2005). However, it has been shown that deletion of the CTD in the mouse leads to abnormal senescence in the bone marrow, as well as postnatal developmental defects and death within two weeks after birth, pointing to an essential, tissue-specific role for the p53 CTD that has yet to be completely elucidated (Hamard et al., 2013).

### 3.4.3 Functions of p53

Because p53 itself can be modulated by many transcriptional, post-transcriptional and post-translational mechanisms, there are many different pathways that can be activated downstream of this transcription factor (Figure 3.4, (Brady and Attardi, 2010)). Some of these pathways, including apoptosis and cell cycle arrest will be discussed in the following sections.

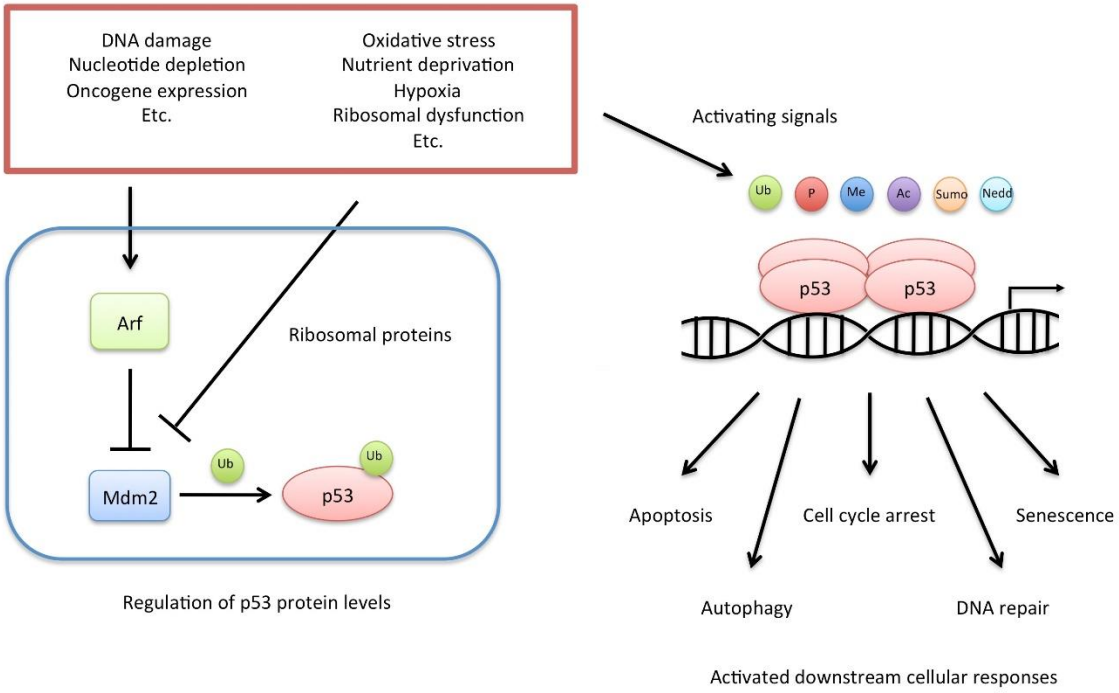


**Figure 3.4.** Signaling via p53. Many extracellular factors and processes induce p53 activation (red box). This leads to either direct inhibition of the E3 ubiquitin ligase Mdm2 or inhibition via Arf. Under normal conditions, Mdm2 ubiquitinates p53, leading to its degradation (blue box). Activating signals also lead to post-translational modification of p53 (including **ub**iquitination, **p**hosphorylation, **me**thylation, **ac**etylation, **sumo**ylation and **nedd**ylation). Active p53 tetramerizes and binds to p53 responsive elements in the DNA to induce many downstream cellular responses. (Adapted from (Brady and Attardi, 2010)).

#### 3.4.3.1 Apoptosis

The cell fate outcomes induced by p53 are thought to be dictated by promoter selectivity, mediated by the p53 consensus binding sequence and the post-translational modifications of p53 itself. Higher levels of p53 protein are also thought to lead to apoptosis, while lower levels lead to cell cycle arrest, although this is not the only determinant for an apoptotic versus cell cycle arrest response downstream of p53 (Chen et al., 1996; Kracikova et al., 2013). One of the first studies to

show that p53 expression could induce apoptosis made use of thymocytes from p53-deficient mice and showed that p53 was required for radiation-induced apoptosis but not apoptosis induced by a variety of other stimuli (Clarke et al., 1993; Lowe et al., 1993). Other studies using knockin mice expressing a transcriptionally dead but DNA-binding proficient p53, show defective induction of apoptosis, indicating that the transactivation domain is necessary for activation of these genes (el-Deiry, 1998; Jimenez et al., 2000; Sax and El-Deiry, 2003). Many direct target genes activated by p53 are members of the Bcl2 family, major regulators of the intrinsic apoptotic pathway (Tsujimoto, 2003). A p53 consensus binding sequence can be found at the promoters of the multidomain Bcl2 family member, *Bax* and the BH3-only domain members *Puma, Noxa* and *Bid* (Miyashita et al., 1994; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002). p53 can also induce apoptosis by activating the transcription of genes involved in the extrinsic apoptotic pathway, including the cell surface receptor *Fas*, the death-domain receptor *DR5*, and the *PERP* gene (Attardi et al., 2000; Muller et al., 1998; Wu et al., 1997). However, while p53 induces the expression of many genes involved in apoptosis, transactivation of these genes is cell-type specific, adding another layer of complexity to the apoptotic signaling cascade activated downstream of p53 (Bouvard et al., 2000; Chong et al., 2000).

#### 3.4.3.2 Cell cycle arrest

In response to DNA damage signals, p53 induces a G1 cell cycle arrest, allowing cells to repair their genome to prevent the propagation of potentially oncogenic mutations (el-Deiry et al., 1993; Lane, 1992). This is accomplished by transactivation of *Cdkn1a* ($p21^{CIP1}$) by p53, and was first shown in $Cdkn1a^{-/-}$ cells which were unable to arrest upon exposure to DNA damage-inducing agents (Brugarolas et al., 1995; Deng et al., 1995; el-Deiry et al., 1993). Other p53 cell cycle arrest target genes include growth arrest and DNA-damage-inducible 45α (*Gadd45α*), protein tyrosine

phosphatase receptor type V (*Ptprv*), and promyelocytic leukemia (*Pml*) (Doumont et al., 2005; Hildesheim et al., 2002; Rego et al., 2001; Tront et al., 2006; Tront et al., 2010; Wang et al., 1999; Wang et al., 1998). While induction of apoptosis is the more common outcome of p53 activation, it is important to note that studies using cell cycle arrest-competent but apoptosis-deficient p53 mutant mouse models have shown that these mutants have the ability to extend tumor latency (Liu et al., 2004; Schlereth et al., 2010; Timofeev et al., 2013). This suggests that, in the context of tumorigenesis, the ability of p53 to induce cell cycle arrest is important for tumor suppression.

# 4.0 Transcription factors in T cell development & leukemia

The transcription factor network regulating T lineage development is complex and numerous transcription factors and signaling pathways are important at different points during early T cell development. Furthermore, improper regulation or expression of many of these transcription factors can result in the development of T cell leukemia. In the following chapter, I will describe some of the functions of several transcription factors involved in early T cell development and the effect of their deregulation on T-ALL development.

## 4.1 Gfi1

The zinc finger transcription factor Gfi1, which functions as a transcriptional repressor, has been implicated in many stages of T cell development (Schmidt et al., 1998; Yucel et al., 2003; Yucel et al., 2004; Zweidler-Mckay et al., 1996). Mice lacking Gfi1 have a severely reduced thymic cellularity, impaired T cell development at the DN1 to DN2 transition, and thymocytes which are highly apoptotic and display a reduced proliferative capacity (Yucel et al., 2003). Furthermore, loss of Gfi1 results in reduced number and function of ETPs, and these cells are unable to induce genes downstream of Notch1 signaling, implicating Gfi1 in the maintenance of lymphoid transcriptional programs activated by Notch1 (Phelan et al., 2013).

Because of these functions in early T cell development, it is not surprising that Gfi1 has been implicated in the development and maintenance of T-ALL. In fact, Gfi1 was first described as the gene activated by proviral insertion of the Moloney Murine Leukemia Virus (MoMuLV). (Akagi et al., 2004; Gilks et al., 1993; Schmidt et al., 1996; Uren et al., 2008; Zornig et al., 1996). Injection of MoMuLV into neonatal mice leads to integration of the virus near oncogenes including c-Myc, Pim1 and Gfi1, leading to activation of these genes and highly aggressive leukemia with

100% penetrance, and a majority of MoMuLV-induced T cell leukemias have integrations at the Gfi1 locus (Cuypers et al., 1984; Fan et al., 1997; Fan et al., 1988; Schmidt et al., 1996; Tsichlis et al., 1989; Zornig et al., 1996). It is therefore surprising that mutations of *GFI1* have not yet been detected in human T-ALL (Zhang et al., 2012). It has, however, been shown that Gfi1 regulates p53-induced cell death in both human and murine leukemic cells, and loss of Gfi1 leads to tumor regression and increased host survival (Khandanpour et al., 2013). It is therefore clear that Gfi1 is important for early T cell development, and deregulation of this transcription factor is a key driver of malignant transformation.

## 4.2 Tcf-1

One of the main T cell-specific targets of Notch1-DL4 signaling is Tcf-1 (encoded by the *Tcf7* gene), also a target of the Wnt/β-catenin signaling pathway (Germar et al., 2011; Weber et al., 2011). This gene is highly expressed in thymocytes throughout pre-T cell development, from the ETP stage onwards, and loss of Tcf-1 leads to several T cell developmental defects (Germar et al., 2011; Verbeek et al., 1995). Tcf-1 is needed for TCRα expression, immediately following β-selection, and for maintenance and renewal of the DN1/2 compartments (Gounari et al., 2001; Ioannidis et al., 2001; Staal et al., 2004). Tcf-1 overexpression, however, can bypass the need for Notch1 signaling to activate a large subset of effectors, but not all Notch1 target genes, indicating that Tcf-1 is one of the more important direct Notch1 target genes needed for proper T cell development (Weber et al., 2011). Finally, while Tcf-1 knockout precursors display severe defects in T cell development, they do not display increased myeloid or B cell potential, indicating that Tcf-1 does not act as an antagonist of non-T cell development (Germar et al., 2011; Weber et al., 2011).

Interestingly, while loss of Tcf-1 impairs T cell development, it has also been shown to function as a tumor suppressor gene in T-ALL, and Tcf-1-deficient mice develop highly aggressive thymic lymphomas (Tiemessen et al., 2012; Yu et al., 2012). TCF-1 has also been reported to play a role in human T-ALL, as the deletion of the *TCF-7* gene was found in two patients (Yu et al., 2012). Tcf-1 is therefore a rare example of a transcription factor whose loss can be detrimental because it can both abrogate T cell development and induce T cell transformation.

## 4.3 E2A/HEB

The two proteins encoded by the *E2A* gene, E12 and E47, play crucial roles in the development of both B and T cells. E12 and E47 are generated by alternative splicing of the *E2A* locus and are members of the basic helix-loop-helix (bHLH) family of transcription factors (Sun and Baltimore, 1991). As E-box binding transcription factors, E12 and E47 are part of the E-protein family of transcription factors, which includes E2-2 and HEB, which are also important for lymphocyte development (Bain et al., 1993; Hu et al., 1992; Zhuang et al., 1996). DN1 cells which are not yet fully committed to the T cell lineage have low levels of E2A expression, and E2A expression is highly upregulated in DN2 cells and remains high in DN3 and DN4 cells (Engel et al., 2001). It is therefore not surprising that T cell development in E2A-deficient mice is almost completely blocked at the DN1 stage (Bain et al., 1997). While E-proteins can form hetero- or homodimers between themselves, they can also bind proteins from the Id family of HLH proteins, which antagonize E-protein activity (Benezra et al., 1990; Sun et al., 1991). Overexpression of Id1, Id2 or Id3 leads to a block in thymocyte development, indicating that the expression level of E-proteins relative to Id proteins is important for T cell differentiation (Blom et al., 1999; Morrow et al., 1999; Sun, 1994). In the thymus, the main binding partner of E2A is HEB. E2A-deficient mice have a partial defect in thymocyte differentiation and reduced thymocyte number, especially

in the DP compartment, while HEB-deficient mice have a similar block in T cell development at the DN-to-DP transition, leading to a decrease in thymic cellularity (Barndt et al., 1999; Takeuchi et al., 2001). E2A-HEB heterodimers are also found at the pre-TCRα promoter indicating that they are required both prior to and following the β-selection checkpoint (Takeuchi et al., 2001).

A role of E2A has also been described during T cell lymphomagenesis. Starting at three months of age, E2A-deficient mice are highly susceptible to developing a malignant T cell lymphoma (Bain et al., 1997; Yan et al., 1997). While genetic alterations in E2A in human T-ALL are uncommon, translocation and subsequent activation of TAL1, an E2A interacting partner and inhibitor, occurs in approximately 25% of patients with T-ALL (Brown et al., 1990; Chen et al., 1990a; Chen et al., 1990b). It is therefore possible that, because TAL1 is overexpressed in a majority of T-ALL cases, inactivation of E2A occurs via this pathway and genetic alterations at the E2A locus are not required to repress the activity of this protein (Ferrando et al., 2004).

## 4.4 c-Myc

c-Myc is a basic helix-loop-helix leucine zipper (bHLH/LZ) transcription factor that activates transcription via oligomerization with its partner Max (Blackwood and Eisenman, 1991). In normal cells, c-Myc target genes include genes involved in regulating the cell cycle, metabolic pathways and cellular proliferation, among others (Fernandez et al., 2003; Grewal et al., 2005; Herold et al., 2002; Iritani and Eisenman, 1999; Johnston et al., 1999; Wu et al., 1999a; Wu et al., 1999b). c-Myc can also repress gene transcription, and mechanisms of c-Myc-mediated gene repression will be discussed in the following sections.

While c-Myc activity does not seem to be required for efficient β-selection, activation of c-Myc following β-selection is necessary for survival and proliferation induced by signaling through the pre-TCR (Dose et al., 2006; Douglas et al., 2001). Furthermore, c-Myc-deficient DN3

cells are able to progress to the DP stage and express the TCR, indicating that c-Myc is not necessary for developmental progression at this checkpoint (Dose et al., 2006).

The expression of c-Myc is not absolutely necessary for T cell development. However, overexpression of c-Myc has severe consequences for both T and B cells. Aberrant expression of c-Myc in either T or B cell precursors leads to development of lymphoid malignancies such as leukemia and lymphoma (Adams et al., 1985; Adams et al., 1999; Graham et al., 1985; Langenau et al., 2003). As previously mentioned, c-Myc is one of the most common integration sites for MoMuLV and c-Myc has been identified as an important direct target of Notch1 in both T cell development and during induction of T-ALL (Cuypers et al., 1984; Fan et al., 1997; Fan et al., 1988; Herranz et al., 2014; Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006).

## 4.5 Myc-Interacting Zinc Finger Protein-1 (Miz-1)

The proto-oncogene c-Myc has been implicated in the regulation of many genes involved in cell cycle and growth arrest, proliferation, and differentiation, especially in the context of cell transformation, as previously discussed (Douglas et al., 2001; Gutierrez et al., 2011). While the interaction of c-Myc with its partner Max induces activation of gene transcription, it was observed that cellular transformation by constitutive c-Myc expression leads to the downregulation of many genes, indicating that c-Myc may also negatively regulate gene expression. To determine c-Myc interacting partners that mediate this function, the Eilers group performed a two-hybrid screen using the carboxy-terminus of c-Myc (Peukert et al., 1997). Using this screen, they discovered a protein which they termed Miz-1, for Myc-interacting zinc finger protein-1. This section will focus on the structure and function of Miz-1, as well as the role of this protein in hematopoiesis and cancer.

### 4.5.1 Structural features of Miz-1

Miz-1 (*Zbtb17*) is an 803 amino acid transcription factor with a molecular weight of 87kDa. It is composed of 13 zinc fingers at its carboxy-terminal end, and a BTB-POZ (Broad complex, Tramtrack, Bric à brac – Poxvirus and Zinc-finger) domain at its amino-terminus (Peukert et al., 1997) (Figure 4.1). As with many POZ domain transcription factors, Miz-1 forms a dimer in solution, and a recently resolved crystal structure of the Miz-1 POZ domain reveals possible dimerization of two POZ domain dimers, leading to the formation of a Miz-1 tetramer (Stead et al., 2007; Stogios et al., 2010). The Miz-1 POZ domain can also mediate interactions between Miz-1 and other BTB-containing proteins, including Bcl6, Nac1 and Zbtb4 (Saito et al., 2009; Stead and Wright, 2014a; Weber et al., 2008).

The thirteen zinc fingers of Miz-1 mediate DNA binding, and the structures of zinc fingers 5-10 and 13 have recently been resolved (Bedard et al., 2012; Bernard et al., 2013; Tremblay et al., 2016). The binding of Miz-1 to c-Myc has been mapped to a specific sequence between zinc fingers 12 and 13 of Miz-1 (Herold et al., 2002). Furthermore, the mutation of a single amino acid, valine 394 to aspartic acid in the helix-loop-helix domain of c-Myc (c-Myc$^{V394D}$), completely abrogates binding of c-Myc to Miz-1 (Gebhardt et al., 2006; Herold et al., 2002; Patel and McMahon, 2006; Wu et al., 2003). It is also known that the zinc fingers of Miz-1 mediate binding to the initiator (Inr) elements of target gene promoters, and a consensus binding sequence for Miz-1 has recently been discovered (Wolf et al., 2013). It has also been proposed that because the 13$^{th}$ zinc finger is somewhat isolated from the other 12 zinc fingers, it might modulate the specificity of DNA binding of Miz-1 or the affinity of Miz-1 binding to c-Myc (Tremblay et al., 2016). However, these hypotheses have yet to be formally tested.

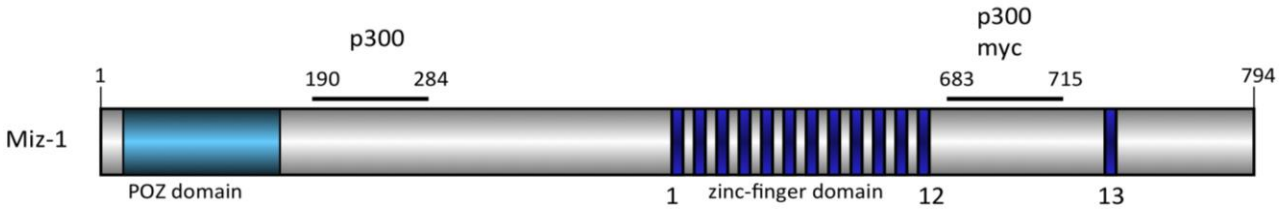


**Figure 4.1.** Schematic representation of Miz-1. Miz-1 is composed of 803 amino acids forming several functional domains. The POZ domain is necessary for protein-protein interaction, tetramerization and binding of Miz-1 to chromatin, while the thirteen zinc fingers mediate binding to DNA. c-Myc binds Miz-1 between zinc fingers 12 and 13.

### 4.5.2 Miz-1 in hematopoiesis

Miz-1 is ubiquitously expressed and is necessary for normal embryonic development during gastrulation in the mouse (Adhikary et al., 2003). Constitutive deletion of Miz-1 leads to a gestation block at day E7.5 of embryonic development, while the expression of Miz-1 lacking the POZ domain (Miz-1$^{\Delta POZ}$) leads to developmental arrest at E13.5 (Kosan et al., 2010). For this reason, published mouse models of Miz-1-deficiency make use of lox-cre technology to remove a floxed Miz-1 POZ domain in specific cell types.

Many BTB-POZ domain transcription factors have been implicated in the development and transformation of hematopoietic cells. Recently, our group and others have discovered that Miz-1 plays an important role in hematopoiesis, specifically in the development of certain hematopoietic cell lineages. Our group has shown that Miz-1 is essential for normal T cell, B cell and erythrocyte development, and it has been speculated by others that Miz-1 is also involved in monocyte development (Alter-Koltunoff et al., 2003; Bowen et al., 2002; Bowen et al., 2003; Kosan et al., 2014; Kosan et al., 2010; Saba et al., 2011a; Saba et al., 2011b).

#### 4.5.2.1 T and B cell development

Many BTB-POZ domain transcription factors have been implicated in both early and late B cell development, and many others play an important role in early T cell development (Basso and Dalla-Favera, 2012; Beaulieu and Sant'Angelo, 2011; Bilic and Ellmeier, 2007; Chevrier et al., 2014; Duy et al., 2010; Ellmeier and Taniuchi, 2014; Maeda et al., 2007; Punwani et al., 2012). It is therefore no surprise that Miz-1 is essential for these processes as well. In order to study the role of Miz-1 in the early stages of T and B cell development, our group generated a mouse model in which conditional deletion of the POZ domain of Miz-1 occurs in hematopoietic cells using a Vav-cre transgenic mouse (Kosan et al., 2010). B cell development in these mice is arrested at the pre-pro-B to pro-B cell transition, while ETPs are severely reduced in the thymi of these mice (Kosan et al., 2010; Saba et al., 2011b) (Figure 4.2). Because early development of both T and B cells is dependent on cytokine signaling specifically through the IL-7 receptor (IL-7R), it was hypothesized that Miz-1 might play a role in this pathway. Indeed, in both cell types, Miz-1 regulates IL-7 signaling by binding to and repressing transcription of the Socs1 promoter, a negative regulator of this pathway (Kosan et al., 2010; Saba et al., 2011b). This promotes Stat5 phosphorylation and upregulation of pro-survival genes in response to IL-7. Furthermore, specifically in B cells, Miz-1 binds to the Bcl2 promoter, inducing the expression of this anti-apoptotic protein (Kosan et al., 2010; Saito et al., 2009). Loss of Miz-1 in B and T cell progenitors therefore leads to increased apoptosis and premature death of these cells, which are unresponsive to IL-7. Interestingly, binding of Miz-1 to both the Socs1 and Bcl2 promoters to regulate this pathway is independent of c-Myc.

A second important checkpoint exists in the development of B and T cells; these cells must undergo V(D)J recombination in order to express receptors which can recognize a multitude of

pathogens. It was found that Miz-1 plays an important role in regulating this checkpoint in DN3 pre-T cells and probably in pro-B cells as well (Saba et al., 2011a) (Figure 4.2). Specifically, Miz-1 is essential to coordinate the expression of p53 target genes induced by the DNA double-strand breaks during V(D)J recombination to ensure survival of DN3 pre-T cells, although the mechanisms through which this regulation occurs were unknown at the start of my Ph.D. (Saba et al., 2011a).

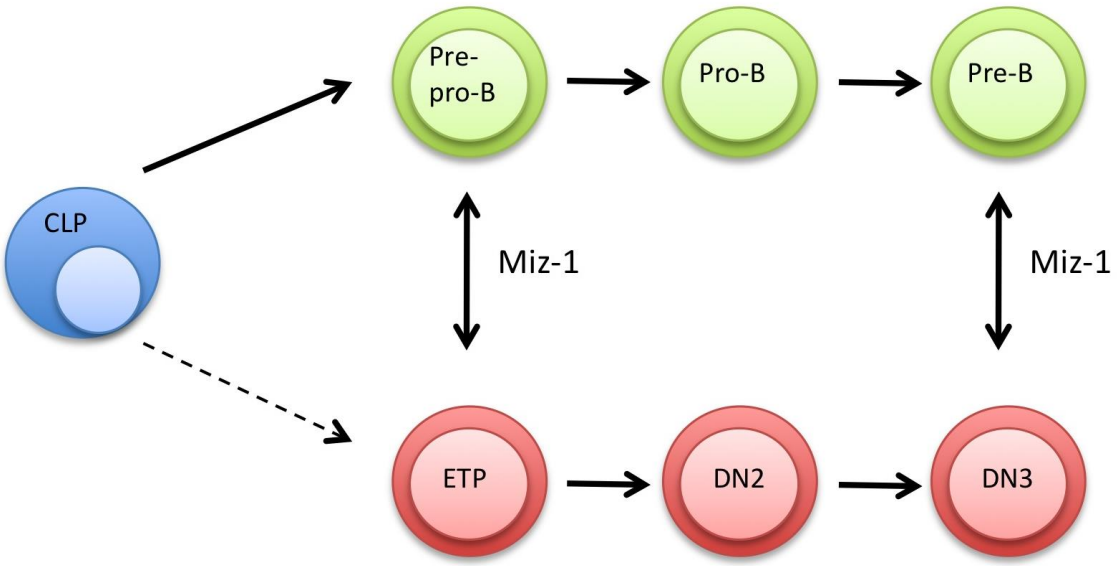


**Figure 4.2.** Miz-1 function in T and B cell development. Miz-1 regulates early cytokine-dependent signaling steps in T and B cell development (at the pre-pro-B cell and ETP stages) as well as later T or B cell receptor-mediated signaling and survival mechanisms (at the pre-B and DN3 stages). (Adapted from (Moroy et al., 2011)).

#### 4.5.2.2 Erythrocytes

Cytokine signaling is also important during erythrocyte development. The main signaling pathway for the differentiation of erythrocytes is initiated by erythropoietin (Epo). Epo signaling

regulates phosphorylation of Stat5 and thus terminal proliferation and survival of red blood cells. We therefore hypothesized that, as Miz-1 regulates Socs1 in lymphoid progenitors, it may play a similar role in erythroid precursors. While loss of Miz-1 induces defects in embryonic erythroid development and stress-induced erythropoiesis, we have found that Miz-1 is not present at the promoter of Socs1 in these cells (Kosan et al., 2014). However, as stress has been shown to induce high levels of Stat5 phosphorylation, a phenotype seen in Miz-1-deficient splenic erythrocytes stimulated with Epo, we hypothesize that the regulation of Socs1 by Miz-1 in these cells is probably indirect. This is in contrast to earlier results obtained in both IL-7-dependent T and B cell development, suggesting that Miz-1 may respond differently following stimulation by different cytokines in distinctive cell types.

#### 4.5.2.3 Monocytes

The Nramp1 gene, a divalent cation transporter specifically expressed in late endosomes of macrophages, plays an important role in the proper function of macrophages during the innate immune response (Atkinson et al., 1997; Gruenheid et al., 1997). Because it was determined that c-Myc negatively regulates the Nramp1 promoter, it was thought that this regulation might occur via interaction with Miz-1. Indeed, Miz-1 binds to and regulates the Nramp1 promoter *in vitro* (Bowen et al., 2002; Bowen et al., 2003). It was also determined that Miz-1 interacts with IRF-8 and PU.1 only in hematopoietic cells, providing a cell type-specific method of Nramp1 regulation by Miz-1 (Alter-Koltunoff et al., 2003). However, while these results hold true in macrophage cell lines and *in vitro* experiments, a role of Miz-1 in macrophage development or function in the mouse has yet to be studied.

### 4.5.3 Miz-1 interaction partners

Depending on the cellular context, Miz-1 has been found to bind to many different proteins at the promoters of its target genes, inducing either activation or repression of transcription (Figure 4.3). Many of these binding partners and their effects on gene transcription by Miz-1 are described in the following sections.

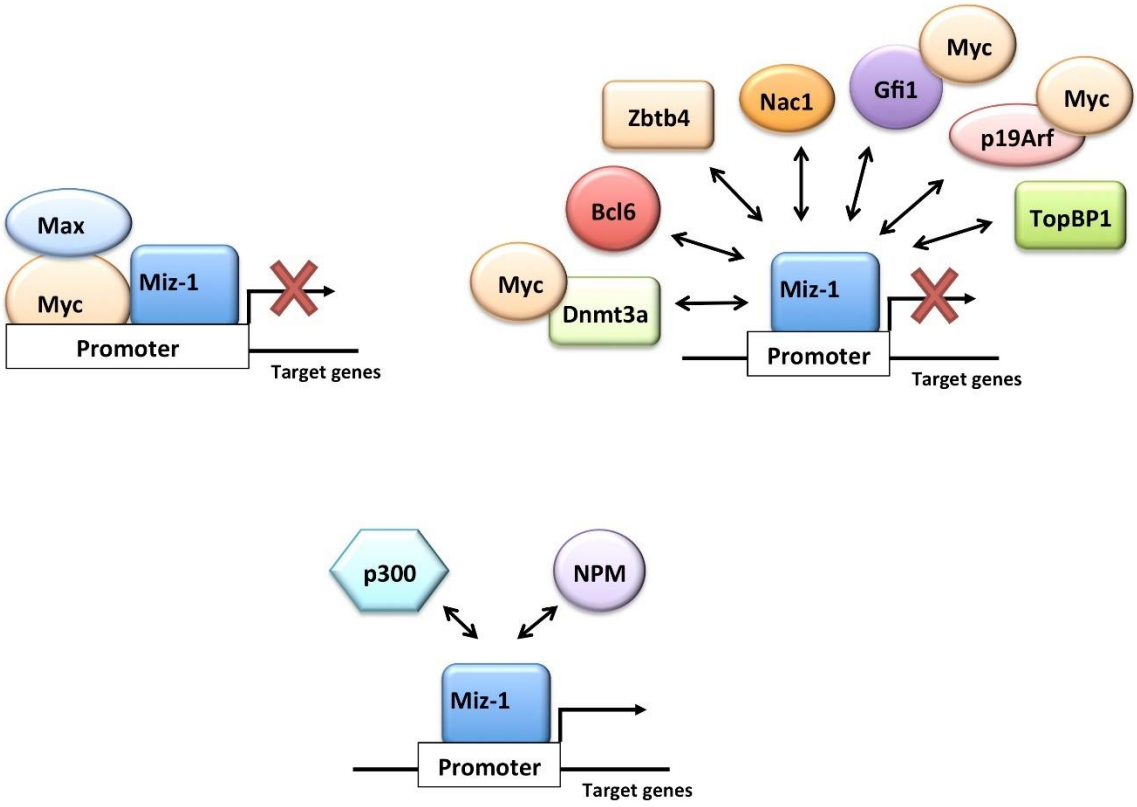


**Figure 4.3.** Overview of Miz-1 interaction partners. Miz-1 interacts with the c-Myc/Max complex to repress a subset of target genes. Miz-1 can also interact with Dnmt3a, Bcl6, Zbtb4, Nac1, Gfi1/c-Myc, p19Arf/c-Myc and TopBP1 to repress target gene transcription. Activation of Miz-1 target genes occurs when Miz-1 is in complex with p300 or nucleophosmin (NPM).

#### 4.5.3.1 The Myc/Max Complex

The transcription factor c-Myc has been well defined in the context of tumorigenesis as an activator of the transcription of its target genes in the presence of one of its binding partners, Max. However, a subset of Myc target genes is repressed when bound by c-Myc, and this repression is mediated by binding of c-Myc to Miz-1 (Peukert et al., 1997). This interaction abrogates Miz-1-mediated cell cycle arrest by repressing transcription of the cell cycle inhibitors *Cdnk1a* and *Cdkn2b* ($p15^{INK4B}$) and converting Miz-1 from a transactivator to a transcriptional repressor (Seoane et al., 2001; Staller et al., 2001; Wu et al., 2003).

Further repression of the *Cdkn2b* promoter can be achieved through Host Cell Factor-1 (HCF-1) binding to Miz-1 in the place of or in addition to c-Myc (Piluso et al., 2002). This regulation is important in the context of TGFβ signaling. TGFβ is an antimitogenic cytokine, and TGFβ-induced signal transduction results in rapid downregulation of c-Myc and transcriptional activation of the cell cycle inhibitors *Cdkn2b* and *Cdkn1a* (Coffey et al., 1988; Fernandez-Pol et al., 1987). In this case, the rapid downregulation of c-Myc transcription induces a reduction in levels of the c-Myc/Miz-1 complex, relieving c-Myc-induced repression of the *Cdkn2b* promoter (Seoane et al., 2001). Furthermore it has been shown in a cell culture model *in vitro* that TGFβ stimulation induces formation of a Miz-1/Smad complex on the *Cdkn2b* promoter leading to activation of this gene. This interaction is mediated mainly via Smad3 binding to the zinc fingers of Miz-1, although Smad4 also interacts weakly with Miz-1 (Seoane et al., 2001). The cellular response to TGFβ is especially important in the context of proliferation of keratinocytes; TGFβ-induced genes involved in extracellular matrix (ECM) adhesion, cell-matrix and cell-cell interactions are regulated by the c-Myc/Miz-1 complex (Gebhardt et al., 2006). The interaction

between Miz-1 and c-Myc must therefore be tightly controlled during normal skin differentiation to prevent the disruption of cell adhesion that could contribute to the development of tumors.

Similarly, it has been shown that knockdown of Miz-1 suppresses breast cancer metastasis in a cell culture model. This occurs through regulation of the RhoA gene by Miz-1. *In vitro* knockdown of Miz-1 reduces RhoA protein expression and knockdown of any component of the Miz-1/c-Myc/p300 transcriptional complex impairs RhoA expression, leading to defects in cell migration and invasion (Chan et al., 2010).

It has also been proposed that the repressive Miz-1/c-Myc/Max complex and an activating complex of Miz-1/Hifα/Arnt (aryl hydrocarbon receptor nuclear translocator) are present at the *Cdkn2b* promoter under normoxic conditions to ensure adequate levels of this gene. However, upon induction of hypoxia, the complexes are released and Miz-1 binds the *Cdkn2b* promoter in the presence of as of yet unknown factors to repress gene transcription (Aesoy et al., 2014).

The regulation of the *Cdkn1a* promoter is similar, but seemingly less complex; Miz-1 represses *Cdkn1a* when in complex with c-Myc, promoting cell growth and inhibiting differentiation. As c-Myc is downregulated, Miz-1 can recruit other cofactors to activate transcription of this cell cycle inhibitor (Wu et al., 2003). This regulation, however, may only be important to regulate the cell cycle in the context of DNA damage; Miz-1 binding to the *Cdkn1a* promoter is not required in the developing mouse embryo, where the main Miz-1 target regulating the cell cycle in this context is $p57^{KIP2}$ (*Cdkn1c*) (Adhikary et al., 2003). Furthermore, while repression of Miz-1 transactivation by c-Myc is dispensable for the induction of cell cycle progression and transformation, the ability of c-Myc to bind to and inactivate Miz-1-mediated transcription is essential for induction of apoptosis by this proto-oncoprotein (Patel and McMahon,

2006). This is because Miz-1 normally activates transcription of Bcl2, and Bcl2 activation blocks c-Myc-induced apoptosis (Patel and McMahon, 2007; Saito et al., 2009).

Miz-1 has also been implicated in the regulation of Wnt pathway genes. Because Wif-1 (Wnt inhibitory factor-1) and c-Myc expression are inversely correlated in bladder cancer, it was hypothesized that Wif-1 is part of the set of genes repressed by c-Myc in the context of malignant transformation (Tang et al., 2009). Indeed, Miz-1 activates Wif-1 transcription alone, but represses its transcription in the presence of c-Myc (Licchesi et al., 2010). Not only is this the case in cells stably overexpressing c-Myc, but also in cells where the Wnt pathway is activated, leading to physiological upregulation of c-Myc (Licchesi et al., 2010).

The Miz-1/c-Myc complex is also important in embryonic stem (ES) cell differentiation. In this context, the Miz-1/c-Myc complex represses *Hox* genes (Varlakhanova et al., 2011). However, the binding sites for Miz-1 and c-Myc in these cells were determined using ChIP-chip, and indicate that Miz-1 binds almost exclusively away from transcription start sites, contrary to most published Miz-1 data, which indicates that Miz-1 binds almost exclusively to Inr elements in the core promoters of its target genes.

In the cases described above, Miz-1 acts as a transcriptional transactivator on its own and a transcriptional repressor in the presence of c-Myc. The regulation of the C/EBPδ promoter is an exception to this method of regulation. Miz-1 is required for c-Myc mediated repression of C/EBPδ in nontransformed mammary epithelial cells, but does not activate transcription of this gene in the absence of c-Myc in these same cells (Si et al., 2010).

Regulation of gene transcription by the c-Myc/Miz-1 complex is multifaceted and probably context-dependent. It is therefore important to keep this in mind when studying the functions of both c-Myc and Miz-1 in the context of cellular development and transformation.

#### 4.5.3.2 Transcriptional co-activators

The Miz-1/c-Myc complex has been well established as a transcriptional repressor. However, when Miz-1 is dissociated from this complex, it acts as a transcriptional transactivator, probably via the recruitment of a co-activating complex. The main co-activators recruited by Miz-1 are the histone acetyltransferase p300, and nucleophosmin (NPM), discussed below. However, as most functions of Miz-1 *in vivo* are known to be c-Myc-independent, it is possible that other as yet unidentified co-activators can be recruited to DNA by Miz-1.

As p300 has been implicated in the regulation of *Cdkn2b*, it was thought that this protein could play a role in transcriptional activation by Miz-1. Indeed, Miz-1 interacts with the cysteine/histidine-rich region 3 of p300 via two distinct binding domains in the Miz-1 protein (Staller et al., 2001). One of these p300 interaction domains overlaps with the c-Myc-binding domain of Miz-1, indicating that binding of Miz-1 to p300 is abrogated by c-Myc. Furthermore, this domain also interacts with HCF-1, indicating that either protein can inhibit Miz-1-mediated transactivation induced by binding to p300 (Piluso et al., 2002).

Nucleophosmin (NPM) has been implicated in many cellular processes, including protein formation, DNA replication, and progression through the cell cycle (Pelletier et al., 2007; Rubbi and Milner, 2003; Zeller et al., 2001). It can act as a transcriptional co-activator of p53 and NF-κB, and it is localized in the nucleolus when in complex with the ribosomal protein L23 (Rpl23) (Colombo et al., 2002; Dhar et al., 2004; Jin et al., 2004). Rpl23 has been implicated in the regulation of p53 via Mdm2 inhibition, and the gene encoding this protein is a direct target of c-Myc (Guo et al., 2000; Kim et al., 2000; Menssen and Hermeking, 2002). In both stressed and unstressed cells, Rpl23 sequesters NPM in the nucleolus, preventing the interaction of Miz-1 with NPM at the promoters of the Miz-1 target genes *Cdkn2b* and *Cdkn1a* (Wanzel et al., 2008).

Therefore, in the absence of Rpl23, NPM acts as a co-activator of Miz-1, leading to activation of *Cdkn1a* transcription and cell cycle arrest in a p53-independent manner (Wanzel et al., 2008).

#### 4.5.3.3 Transcriptional co-repressors

While the most widely studied Miz-1 co-repressor is c-Myc, Miz-1 can also interact with other proteins to repress target gene transcription. Interaction with these co-repressors is context-specific and can occur either in the presence of or in place of c-Myc. However, repression via Miz-1 has mainly been shown to occur at the typical Miz-1 target genes *Cdkn1a* and *Cdkn2b*, and this mainly *in vitro*.

There are many epigenetic modifications in eukaryotic cells that control gene expression. The most abundant of these is the methylation of DNA at CpG dinucleotides, which is associated with transcriptional repression and is both gene and tissue specific (Sinsheimer, 1955; Stein et al., 1983). Three DNA methyltransferases have been identified in mammals; Dnmt1, Dnmt3a and Dnmt3b (Bestor et al., 1988; Okano et al., 1998). Dnmt1 acts mainly to maintain the methylation pattern on a newly synthesized strand of DNA, while Dnmt3a and Dnmt3b are required for *de novo* DNA methylation (Li et al., 1992; Okano et al., 1999). Dnmt3a associates with c-Myc at the *Cdkn1a* promoter, leading to enhanced DNA methylation and thus gene silencing. This interaction occurs only in the presence of Miz-1, forming a trimeric Miz-1-c-Myc-Dnmt3a complex and switching Miz-1 from a transcriptional activator to a repressor (Brenner et al., 2005).

The POZ domain of Miz-1 interacts with several other POZ domain-containing transcription factors, including Bcl6 (B cell lymphoma 6) (Phan et al., 2005; Stead and Wright, 2014b). Bcl6 was first described as a target for chromosomal translocations in B cell Non-Hodgkin's Lymphoma and plays an essential role in germinal center (GC) B cell and follicular helper T (Tfh) cell development (Baron et al., 1993; Johnston et al., 2009; Niu et al., 2003; Nurieva

et al., 2009; Shaffer et al., 2000; Tunyaplin et al., 2004; Ye et al., 1993b; Yu et al., 2009). It is a 95kDa protein that functions as a transcriptional repressor by binding to DNA sequences through its zinc finger domain, and recruiting corepressor complexes via its POZ domain (Lemercier et al., 2002; Wong and Privalsky, 1998; Ye et al., 1993a). The recruitment of corepressor complexes such as SMRT (silencing mediator for retinoid and thyroid receptor), N-CoR (nuclear hormone receptor corepressor) and BCoR (Bcl6 corepressor) occurs through a lateral groove motif that is formed upon dimerization of the POZ domain (Dhordain et al., 1997; Huynh and Bardwell, 1998; Huynh et al., 2000; Wong and Privalsky, 1998). Other repressor complexes such as the NuRD (nucleosome remodeling and deacetylase) complex, are recruited via the RD2 (repression domain 2) of Bcl6, and lead to chromatin modulation and repressed gene expression (Fujita et al., 2004). The interaction between Miz-1 and Bcl6 was first reported in the context of the GC reaction; recruitment of Bcl6 to the *Cdkn1a* promoter in GC B cells by Miz-1 leads to inhibition of Miz-1-mediated activation of this gene, and this in a c-Myc independent manner (Phan et al., 2005). In this context, repression of *Cdkn1a* transcription facilitates proliferation of GC B cells. Miz-1 also acts as an intermediate for the interaction of Bcl6 with the Bcl2 promoter, leading to repression of Bcl2 in normal GC B cells (Saito et al., 2009). The regulation of both genes by Miz-1 and Bcl6 in GC B cells may have clinical significance, as Bcl6 is a proto-oncoprotein that is overexpressed in most cases of diffuse large B cell lymphoma (DLBCL), and Bcl2 translocation leading to ectopic activation of this gene is present in the majority of follicular lymphoma (FL) patients (Ci et al., 2009; Saito et al., 2009). However, the interaction between Miz-1 and Bcl6 has only been shown *in vitro* in transient transfection assays and would need to be validated in primary human or mouse B cells and B cell lymphoma.

Zbtb4 is another BTB-POZ domain protein that has been shown to interact with Miz-1 (Weber et al., 2008). Zbtb4, also a transcriptional repressor, is downregulated in human tumors, specifically breast, prostate and lung carcinoma, compared to normal tissue (Kim et al., 2012a; Kim et al., 2012b). Zbtb4 binds directly to methylated DNA to induce repression of the genes regulated by these sequences (Filion et al., 2006). Furthermore, in response to p53 activation, Zbtb4 has been shown to suppress cell cycle arrest at the G1 phase by repressing the transcription of *Cdkn1a*. This repression is due to direct binding of Zbtb4 to the *Cdkn1a* promoter in the presence of Miz-1 in response to vincristine-induced activation of p53 (Weber et al., 2008). The Miz-1/Zbtb4 complex has also been shown to recruit histone deacetylases through interaction with the Sin3 adaptor protein to repress gene transcription (Weber et al., 2008).

Nac1 (nucleus accumbens1, encoded by the gene Nacc1) is also a BTB-POZ domain transcription factor that was first described as the protein product of a cocaine-inducible transcript in the nucleus accumbens of the rat brain (Cha et al., 1997). It also functions in embryonic stem cell self-renewal, and high levels of this gene have been found in ovarian, cervical and uterine cancers (Ishikawa et al., 2010; Rahman et al., 2012; Shih Ie et al., 2011; Yeasmin et al., 2012). Nac1 can act as a repressor in both neuronal and non-neuronal cells, by interacting with the corepressor CoREST and the histone deacetylases HDAC3 and HDAC4 (Korutla et al., 2007; Korutla et al., 2005). The heterodimeric interaction between the POZ domains of Miz-1 and Nac1 has been shown to repress *Cdkn1a* in ovarian cancer cells (Stead and Wright, 2014a). It has therefore been proposed that this interaction may be analogous to the interaction between Miz-1 and Bcl6 in DLBCL. Furthermore, given that Miz-1 also activates the expression of Nac1 through direct binding to the Nac1 promoter, this interaction may function as an autoregulatory feedback loop to control cellular levels of Nac1 (Wolf et al., 2013).

As already discussed, Gfi1 is a transcriptional repressor that plays an important role in the development of cells of the hematopoietic system, especially hematopoietic stem cells (HSCs), granulocytes, T cells and B cells (Hock et al., 2004; Hock et al., 2003; Karsunky et al., 2002; Yucel et al., 2003; Zeng et al., 2004). Gfi1 binds to upstream sequences in the *Cdkn1a* promoter, making this gene a direct Gfi1 target. Furthermore, Gfi1 binding to the *Cdkn1a* promoter is mediated by Miz-1, and Gfi1, Miz-1 and c-Myc form a ternary complex on this promoter to repress transcription of the *Cdkn1a* gene (Liu et al., 2010). This regulation is especially relevant in the context of TGFβ-induced activation of *Cdkn1a*. In the absence of TGFβ, Gfi1 and c-Myc are recruited to the *Cdkn1a* promoter through Miz-1 to repress transcription and induce cell growth. In the presence of TGFβ, Gfi1 and c-Myc are downregulated and Miz-1 binds to the *Cdkn1a* promoter and activates transcription of this gene to limit cell growth. This mechanism also holds true for the regulation of the cell cycle inhibitor *Cdkn2b*, another Miz-1 target gene (Basu et al., 2009). The authors of both studies indicate that this Miz-1-dependent regulation of *Cdkn1a* and *Cdkn2b* by Gfi1 may play a significant role in Gfi1-mediated tumorigenesis.

p19Arf in the mouse, or p14Arf in humans, is a tumor suppressor encoded by the alternative reading frame of the INK4a/Arf locus and is also known as *Cdkn2a* (Quelle et al., 1995). This protein, however, is not expressed under physiological conditions but is induced in response to oncogenic stress signals (Zindy et al., 2003). Arf binds to and inhibits the activity of the ubiquitin ligase Mdm2, leading to stabilization and activation of p53. Miz-1 has been shown to bind Arf through its two separate zinc finger regions, and repress the transcriptional activation of p53 by direct binding to the DNA binding region of p53 in an *in vitro* cell culture model (Miao et al., 2010). This interaction reduces the binding of p53 to the promoters of Bax and Puma, specifically in H1299 cells and when both proteins are overexpressed. Furthermore, the authors conclude that,

when Arf is in excess, it competes with p53 for binding to Miz-1 and excess p53 is released to activate gene transcription. The interaction between Miz-1 and Arf was validated by a second group in both human and mouse cells also *in vitro* (Herkert et al., 2010). The data presented by this group indicate that Arf binding to Miz-1 renders Miz-1 insoluble, leading to a redistribution of Miz-1 in the cell. This induces the local formation of heterochromatin at Miz-1 binding sites. Furthermore, the ability of Arf to inhibit transactivation by Miz-1 is dependent on c-Myc binding independently to the Arf protein. The formation of this ternary complex leads to the sumoylation of Miz-1, however while sumoylation of transcription factors can mediate the recruitment of corepressor complexes, the function of this post-translational modification of Miz-1 has yet to be determined (Ouyang and Gill, 2009; Ouyang et al., 2009; Stielow et al., 2008). In addition, while Miao et al. showed regulation of p53 target genes by Miz-1, Herkert et al. did not observe regulation of these genes, indicating that transcriptional control of gene expression may vary in different cell types and that the models presented may be context-specific.

Topoisomerase IIβ binding protein (TopBP1) is a BRCT-domain (BRCA1 carboxyl-terminal) containing protein, which localizes to the sites of DNA damage and arrested replication forks (Makiniemi et al., 2001; Yamane et al., 2002). This protein contains 8 BRCT domains, and domains 7 and 8 interact specifically with the POZ domain of Miz-1 (Herold et al., 2002). Binding of TopBP1 to Miz-1 inhibits Miz-1 transactivation of the *Cdkn2b* promoter, and these proteins interact in both *in vitro* pull-down assays and *in vivo* transient transfection assays in cell lines. However, while UV irradiation induces TopBP1 redistribution to distinct foci, the distribution of Miz-1 is unchanged following this stress signal. This suggests that Miz-1 and TopBP1 dissociate in response to UV irradiation, allowing for TopBP1 relocalization to sites of DNA damage, and leading to Miz-1-dependent activation of the cell cycle inhibitor *Cdkn2b* (Herold et al., 2002).

However, while the UV response induces activation of c-Myc, the critical target gene of the Miz-1/c-Myc complex in this context is *Cdkn1a* and not *Cdkn2b*, as the latter is not induced in mouse embryonic fibroblasts (MEFs) following UV irradiation. Furthermore, in the absence of c-Myc, or in the presence of $Myc^{V394D}$, Miz-1 is able to activate the transcription of its target genes, specifically *Cdkn1a*, following UV irradiation (Herold et al., 2002). This suggests that c-Myc represses induction of *Cdkn1a* upon UV irradiation by inhibiting Miz-1 function at the *Cdkn1a* promoter. Therefore, in response to UV irradiation, Miz-1 is released from its complex with TopBP1 and binds to the promoter of C*dkn1a* to induce cell cycle arrest. However, in unstressed cells, Miz-1 is required for the binding of a portion of TopBP1 to chromatin, preventing the ubiquitination of TopBP1 by HectH9 and its subsequent degradation by the proteasome (Herold et al., 2008). This prevents binding of Miz-1 to c-Myc and subsequent repression of *Cdkn1a* transcription.

### 4.5.4 c-Myc-independent Miz-1 promoter regulation

Early data relating to the function of Miz-1 presented in the preceding sections indicated that most genes bound by this transcription factor are actively transcribed, and these genes can be repressed via binding of the c-Myc/Max complex to Miz-1. There is, however, a subset of Miz-1 target genes whose regulation is independent of c-Myc. The first published Miz-1 ChIP-seq was performed in neuronal progenitor cells (NPCs) and showed that Miz-1 binds to ~260 target sites (Wolf et al., 2013). While it was previously known that Miz-1 binds to the Inr element of target gene promoters, this experiment allowed Wolf et al. to describe an extended non-palindromic consensus sequence for Miz-1, as well as direct Miz-1 targets whose expression is independent of c-Myc. A second group later confirmed this consensus binding sequence (Barrilleaux et al., 2014). While it is clear that Miz-1 does not regulate one specific functional group of genes, many direct

Miz-1 targets are involved in the early and late stages of autophagy and in vesicle trafficking within the cell (Wolf et al., 2013). The loss of these mediators of autophagy accounts for the cerebellar neurodegeneration in mice in which Miz-1 is selectively rendered nonfunctional in neuronal cells by virtue of a nestin-cre recombination strategy. Furthermore, the authors propose that the trafficking defects that arise when Miz-1 is nonfunctional could explain phenotypes in other Miz-$1^{\Delta POZ}$ cell types. For example, Miz-1 also regulates vesicle trafficking in the mammary gland epithelium (Sanz-Moreno et al., 2014). Lower levels of phosphorylated Stat5, leading to an inability of this protein to effectively activate target gene transcription, cause a lactation defect in these Miz-$1^{\Delta POZ}$ mice. In these cells, the Jak2-Stat5 pathway is activated by the prolactin receptor, which is downregulated in Miz-1-deficient mammary gland cells, probably due to defective trafficking of this receptor to the plasma membrane.

While deletion of the Miz-1 POZ domain in cerebellum and mammary gland epithelium affects vesicular trafficking and autophagy, this is not the case in Schwann cells, myelin-forming cells which are part of the peripheral nervous system (Mirsky et al., 2008; Svaren and Meijer, 2008; Woodhoo and Sommer, 2008). Ablation of the Miz-1 POZ domain in these cells leads not to defective myelination, but to demyelination around three months of age, followed by remyelination (Sanz-Moreno et al., 2015). The authors propose that the phenotype observed in these mice is a result of premature cellular senescence, induced by upregulation of the Miz-1 target gene *Cdkn1a*. However, while *Cdkn1a* is upregulated in Miz-1-deficient Schwann cells, these cells also downregulate other vesicle trafficking-related genes, such as Vamp4, indicating that the mechanism of action of Miz-1 in these cells may be complex. Together, these studies suggest that both c-Myc-dependent and c-Myc-independent functions of Miz-1 may be necessary for the proper development and function of a single cell type.

### 4.5.5 Regulation of Miz-1 activity

While the function of Miz-1 in regulating the expression of its target genes has been studied extensively, little is known about how Miz-1 itself is regulated at both the gene and protein levels. It has, however, been shown that phosphorylation of Miz-1 by Akt regulates the recovery of cells from DNA damage (Wanzel et al., 2005). Cell lines overexpressing a mutant Miz-1 that cannot be phosphorylated enter an extended G1 phase arrest after DNA damage. Furthermore, Miz-1 binds 14-3-3η, but only when phosphorylated by Akt. This binding inhibits the interaction of Miz-1 with the promoters of a subset of its target genes (Wanzel et al., 2005). Additionally, ERK activation contributes to Miz-1 activity through phosphorylation, leading to nuclear accumulation of Miz-1 and increased regulation of Miz-1 target genes (Ziegelbauer et al., 2004).

It is well known that transcription factors must be localized in the nucleus to bind to and regulate DNA transcription. However, Miz-1 has also been detected in the cytoplasm. It has therefore been proposed that Miz-1 is normally sequestered in the cytoplasm through association with microtubules and, upon disruption of this interaction, Miz-1 translocates to the nucleus where it can bind to and regulate target gene promoters (Ziegelbauer et al., 2001). Furthermore, as microtubule changes occur at specific stages of the cell cycle, cell cycle signaling might control this localization of Miz-1. Therefore, both post-transcriptional modifications and cellular localization might control the activity of Miz-1 as well as the recruitment of co-activators or co-repressors, depending on the cellular context.

### 4.5.6 Miz-1 and cancer

Because of its interaction with c-Myc, a prominent proto-oncoprotein, it has been hypothesized that Miz-1 may also play an important role in tumorigenesis. In fact, Miz-1 has been implicated in the development of several types of tumors. Increased expression of Miz-1 correlates

with favorable outcome in cases of neuroblastoma. Specifically, Miz-1 can induce *Cdkn1a* and *Cdkn2b* in neuroblastoma cell lines, triggering both apoptosis and growth arrest when expressed at high levels (Ikegaki et al., 2007). Furthermore, transient overexpression of Miz-1 by transfection in primary patient-derived neuroblastoma cells enhances the expression of other genes associated with favorable outcome in neuroblastoma and leads to growth arrest or cell death. However, while there is a correlation between high Miz-1 expression and increased patient survival, this was only found in patients lacking N-Myc amplification, suggesting that high levels of both N-Myc and Miz-1 may lead to massive amounts of cell death, which are not favorable for tumor development (Ikegaki et al., 2007).

The opposite is true in many other tumor models however; expression of a non-functional Miz-1 protein, or a Miz-1 protein unable to interact with c-Myc, increases the latency of tumors, and loss of Miz-1 is therefore favorable. For example, induction of lymphoma in mice overexpressing $Myc^{V394D}$ is significantly delayed relative to lymphomagenesis induced by overexpression of wild-type c-Myc (van Riggelen et al., 2010). This is in part due to the fact that the interaction between Miz-1 and c-Myc antagonizes the ability of TGFβ to suppress proliferation, and in part due to the inability of the Miz-1/c-Myc complex to repress the cell cycle regulator *Cdkn2b*.

Miz-1 also plays a role in skin tumorigenesis. It had previously been shown that keratinocytes lacking a functional Miz-1 exhibit an impaired morphogenesis of hair follicles and pigment alterations in older animals (Gebhardt et al., 2007). In mice lacking a functional Miz-1 specifically in keratinocytes, application of a tumor-promoting agent on the skin leads to increased differentiation and reduced proliferation of keratinocytes and strongly decreased papilloma formation (Honnemann et al., 2012). This occurs because Miz-1 can no longer bind the *Cdkn1a*

promoter in conjunction with a repressor complex, leading to expression of this cell cycle regulator. Furthermore, proliferation is completely restored in a *Cdkn1a* null background, suggesting that the increase in *Cdkn1a* expression in Miz-1-deficient mice is responsible for the increased tumor latency. A second model also implicates Miz-1 in skin tumorigenesis; Mule (HectH9) ubiquitinates and therefore targets for degradation the c-Myc/Miz-1 complex, preventing downregulation of *Cdkn1a* (Inoue et al., 2013). In this model, the tumor-promoting agent applied to the skin acts via the Ras pathway, and Mule is necessary to suppress oncogenic signaling via Ras. Loss of Mule leads to c-Myc/Miz-1-mediated repression of *Cdkn1a* and tumor progression.

The final model supporting a role of Miz-1 in tumorigenesis focuses mainly on the effect of c-Myc overexpression on c-Myc/Miz-1 promoter binding and regulation. Overexpression of c-Myc in U2OS cells, a bone osteosarcoma cell line which expresses only low levels of this proto-oncoprotein, allows for identification of changes in Myc occupancy at promoters upon overexpression, mimicking possible changes in gene expression during tumor development (Walz et al., 2014). Furthermore, in this context it was shown that the c-Myc/Miz-1 ratio at each promoter affects the direction of the response to c-Myc, when c-Myc levels are oncogenic; the ratio of c-Myc to Miz-1 at c-Myc activated genes was higher than this ratio at c-Myc repressed promoters. This is consistent with a pancreatic tumor mouse model where Kras is oncogenic and p53 is mutated, and removal of even one functional allele of Miz-1 strongly delays tumorigenesis, suggesting again that Miz-1 is necessary for tumor formation.

Therefore, while Miz-1 clearly plays an important role in tumor formation and progression, the effect of Miz-1 inactivation or overexpression may vary depending on the type of tumor. Furthermore, Miz-1 function can be either c-Myc-dependent or independent, suggesting that Miz-1 may exert different functions based on tumor cell type and other context-specific factors.

# 5.0 Hypotheses and Rationale

The expression of the Miz-1 POZ domain is essential for normal T cell development in the mouse. In mice lacking this domain in early hematopoietic progenitors, T cell development is abrogated at the ETP stage of differentiation, due to defective signaling via the IL-7 receptor. A second developmental block exists at the DN3 stage of differentation, at the β-selection checkpoint. Our lab had previously found that these cells exhibit high levels of apoptosis and elevated expression of p53 target genes, including Bax, Puma and p21, but not p53 itself. However, the molecular mechanisms leading to this block in T cell development remained unclear. *I therefore hypothesized that, during normal T cell development, Miz-1 regulates the p53 pathway.*

Because Miz-1 interacts with c-Myc, an important driver of T-ALL, and regulates p53 signaling during β-selection, the stage at which T cell transformation is thought to occur, I sought to determine the role of Miz-1 in the development of T-ALL. Because loss of Miz-1 leads to increased apoptosis via p53 signaling in DN3 pre-T cells, *I hypothesized that Miz-1 regulates the p53 pathway during T cell transformation and that Miz-1 is necessary for the development of T-ALL.* While we have yet to discover mutations in the Miz-1 gene in primary T-ALL samples, we have found that Miz-1 is upregulated in a subset of T-ALL patients. Because the Miz-1 POZ domain forms tetramers and is a structure unique to this protein, it would be possible to create an inhibitor which could specifically disrupt this tetramerization, and thus the function of Miz-1. Furthermore, because Miz-1 regulates p53 in T-ALL, and p53 is rarely mutated in primary T-ALL, inactivating Miz-1 would be an interesting target to activate p53 in these patients.

# CHAPTER II

# Miz-1 regulates translation of *Trp53* via ribosomal protein L22 in cells undergoing V(D)J recombination

The transcription factor Miz-1 plays an important role in the early differentiation of both T and B lymphocytes. A role for Miz-1 in signaling downstream of the IL-7 receptor has been found in both cell types, early in the differentiation process. However, loss of a functional Miz-1 also plays a role later in lymphocyte development, during V(D)J recombination. In this manuscript, we identify the ribosomal protein L22 (Rpl22) as a direct target of Miz-1 transactivation, regulating p53 translation specifically during V(D)J recombination in both pre-T and pro-B cells.

Chapter II presents original work published in the *Proceedings of the National Academy of Sciences of the United States of America,* 2014 Dec 16; 111(50):E5411-9. Reprint available in **Appendix I**.

# Miz-1 regulates translation of *Trp53* via ribosomal protein L22 in cells undergoing V(D)J recombination

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# 1.0 Abstract

To be effective, the adaptive immune response requires a large repertoire of antigen receptors which are generated through V(D)J recombination in lymphoid precursors. However, these precursors must be protected from DNA damage-induced cell death since V(D)J recombination generates double strand breaks and may activate p53. Here we show that the BTB-POZ-domain protein Miz-1 restricts p53-dependent induction of apoptosis in both pro-B and DN3a pre-T cells that actively rearrange antigen receptor genes. Miz-1 exerts this function by directly activating the gene for ribosomal protein L22 (*Rpl22*), which binds to p53 mRNA and negatively regulates its translation. This mechanism limits p53 expression levels and thus contains its apoptosis-inducing functions in lymphocytes, precisely at differentiation stages where V(D)J recombination occurs.

# 2.0 Significance Statement

V(D)J recombination occurs in lymphoid precursors to enable their maturation, but at the same time induces DNA damage. It has therefore been proposed that the activity of the tumor suppressor and gatekeeper protein p53 must be controlled during this process in order to prevent premature induction of apoptosis. In this study, we show that the transcription factor Miz-1 can exert such a function. Miz-1 activates expression of the ribosomal protein Rpl22, which in turn controls the translation of p53 specifically in lymphoid precursors. We propose that this Miz-1-Rpl22-p53 pathway prevents p53 from inducing cell death as a response to V(D)J recombination in lymphoid precursors from both the T- and B-lineage.

# 3.0 Introduction

The development of T lymphocytes starts with early thymic progenitors (ETPs) that first enter the thymus after transiting through the blood stream from the bone marrow. These progenitor cells differentiate through four $CD4^{-}CD8^{-}$ double negative (DN1-4) stages of development before becoming first $CD4^{+}CD8^{+}$ double positive (DP) cells, and then either $CD4^{+}$ or $CD8^{+}$ single positive T cells. The four DN subsets are differentiated based on their expression of the cell surface markers CD44 and CD25. Cells at the DN3 ($CD44^{-}CD25^{+}$) stage are fully committed to the T cell lineage and require signaling through cytokine receptors and Notch1 to survive (Michie and Zuniga-Pflucker, 2002). This DN3 population can be further subdivided into DN3a and DN3b by their size and CD27 surface expression. Importantly, DN3a cells ($FSC^{lo}CD27^{lo}$) are actively rearranging the genes encoding the T cell receptor β (TCRβ) chain through V(D)J recombination (Germain, 2002; Michie and Zuniga-Pflucker, 2002; Rothenberg and Pant, 2004). Those DN3a cells that have not productively rearranged the TCRβ locus on both alleles are eliminated by apoptosis. In contrast, cells that have productively rearranged their TCRβ chain genes are selected and become DN3b cells ($FSC^{hi}CD27^{hi}$), which express a pre-T cell receptor (pre-TCR), a heterodimer between the TCRβ chain and a pTα chain. DN3b cells grow in size due, in part, to increased metabolic activity (Taghon et al., 2006) and give rise to the ($CD44^{-}CD25^{-}$) DN4 subset, which in turn is rapidly expanding to produce $CD4^{+}CD8^{+}$ double positive (DP) cells.

Since V(D)J recombination involves DNA double strand breaks, it has been speculated that the activity of p53 as a DNA damage response factor must be contained and regulated during this process, in order to prevent DN3a cells from prematurely undergoing apoptosis (Dujka et al., 2010; Guidos et al., 1996). V(D)J recombination occurs in DN3a cells when they are in G1 and a

checkpoint might exist at this stage which specifically protects against DNA damage-induced p53-mediated apoptosis. Similar to pre-T cells, early B cell precursors in the bone marrow, the so-called pro-B cells, begin to rearrange the genes encoding the immunoglobulin (Ig) heavy chain also through V(D)J recombination (Melchers et al., 2000). A similar mechanism as for pre-T cells might thus also exist for pro-B cells at this stage to protect them from DNA damage-induced p53-mediated apoptosis (Lu et al., 1999). As for pre-T cells, pro-B cells that have productively rearranged the IgH locus become pre-B cells and express a pre-B cell receptor (pre-BCR) on their surface, which consists of the Ig heavy chain and two surrogate light chains (Hardy et al., 1991). Those pro-B cells that have not productively rearranged the Ig heavy chain genes die by apoptosis, as is the case for DN3a cells that fail to productively rearrange their TCRβ chain genes, mainly because the pre-T cell or pre-B cell receptor mediated proliferation and survival signals are lacking.

The Myc-Interacting Zinc-finger protein-1 (Miz-1) is an 87kDa protein that was first described as an interaction partner for the proto-oncoprotein c-Myc (Peukert et al., 1997). It contains thirteen $C_2H_2$ type zinc finger domains at its C-terminus, and a BTB-POZ domain at its N-terminus. This POZ domain is required for Miz-1 function, since it mediates the formation of homotetramers and interaction with a number of partner proteins (Stead et al., 2007). In addition, loss of this domain renders Miz-1 incapable of stably binding to chromatin (Staller et al., 2001). Among the validated Miz-1 target genes are those that encode the negative cell cycle regulators *Cdkn2b* (p15) (Seoane et al., 2001; Staller et al., 2001) and *Cdkn1a* (p21) (Herold et al., 2002; Phan et al., 2005). Furthermore, a number of upstream factors regulate Miz-1 activity such as Bcl-6 (Phan et al., 2005; Saito et al., 2009), which is itself a POZ domain transcription factor directly binding to Miz-1, the

DNA damage sensor protein TopBP1 (Herold et al., 2002), the E3 ubiquitin ligase HectH9 (Adhikary et al., 2005; Herold et al., 2008) and the proto-oncoprotein Akt (Peukert et al., 1997; Wanzel et al., 2005). However, it has also been shown that recruitment of the c-Myc/Max complex by Miz-1 inhibits transcriptional activation very likely by interfering with the formation of an activating Miz-1/p300 complex (Staller et al., 2001).

We have previously described a Miz-1-deficient mouse, in which the POZ domain of Miz-1 is conditionally deleted specifically in hematopoietic cells (Miz-$1^{flox/flox}$ x Vav-Cre, hereafter referred to as Miz-$1^{\Delta POZ}$ animals). This model has allowed us to identify a role of Miz-1 in the regulation of IL-7 receptor signaling in early steps of T and B cell development (Kosan et al., 2010; Saba et al., 2011b). We could also demonstrate that Miz-1 regulates expression of p53 effector genes in DN3a cells undergoing pre-TCR selection to generate DN3b cells (Saba et al., 2011a). Although Miz-1 does not affect V(D)J recombination, Miz-1-deficient DN3a cells die and show increased transcriptional activation of a subset of p53 target genes that are known for inducing apoptosis (Saba et al., 2011a).

In this study, we present evidence for a novel mechanism that controls p53 activity in pre-T and pro-B cells when V(D)J recombination occurs. Our results indicate that, in these cells, Miz-1 activates transcription of the gene encoding ribosomal protein L22 (*Rpl22*), a component of the 60S ribosomal subunit. Since our data indicate that Rpl22 binds directly to p53 mRNA and negatively controls its translation, we propose that Miz-1 controls p53 protein expression levels via Rpl22 at stages of lymphoid development when p53 action has to be contained.

# 4.0 Results

## 4.1 Ablation of *Trp53* restores the development of Miz-1-deficient αβ-lineage pre-T and pro-B cells

To determine whether the developmental block and increased apoptosis seen in Miz-1-deficient pre-T cells and pro-B cells are due to increased p53 signaling, we generated p53-deficient Miz-$1^{\Delta POZ}$ mice. We found a significant increase in both the percentage and absolute number of DN3b and DN4 cells in Miz-$1^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice and also an almost completely restored thymic cellularity compared to Miz-$1^{\Delta POZ}$ mice (Figure 4.1A,B). Deletion of p53 also partially restored the ability of Miz-1-deficient DN3 pre-T cells to transition to the DN4 stage *in vitro* on an OP9-DL4 stromal layer, while proliferation of these cells after DN4 is still impaired (Figure S4.1A). Miz-$1^{\Delta POZ}$ DN3a pre-T cells had previously been shown to exhibit increased levels of apoptosis and to be defective in their ability to progress through the cell cycle (Saba et al., 2011a). The increase in apoptosis in Miz-$1^{\Delta POZ}$ DN3a pre-T cells is completely abolished in Miz-$1^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice, as shown by Annexin V staining (Figure 4.1C,D). Furthermore, ablation of p53 rescues the ability of Miz-1-deficient DN3 pre-T cells to progress through the cell cycle, as shown by both propidium iodide and BrdU stainings (Figure S4.1B,C). The development of Miz-1-deficient pro-B cells was also rescued in Miz-$1^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice, as indicated by the three-fold increase in the percentage of pro-B cells expressing CD19 on the surface and the three-fold increase in the absolute number of pre-B cells in the bone marrow (Figure 4.2A,B). These results suggest that the developmental defects in Miz-$1^{\Delta POZ}$ pre-T and pro-B cells are linked to both p53-mediated cell cycle arrest and apoptotic cell death.

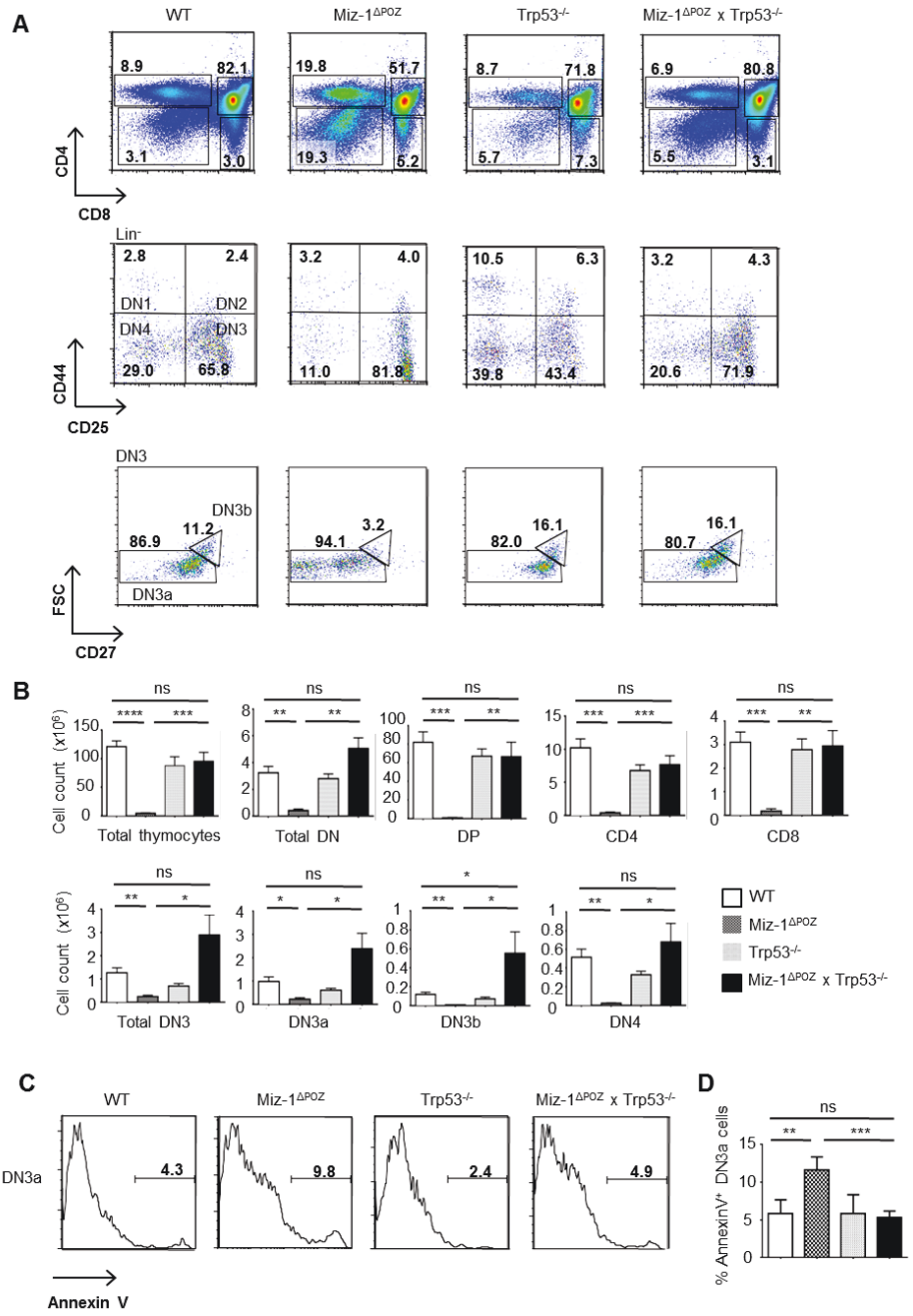


**Figure 4.1** Deletion of p53 restores pre-T cell development in Miz-1$^{\Delta POZ}$ mice. (A) FACS analysis of thymic subsets from WT, Miz-1$^{\Delta POZ}$, *Trp53*$^{-/-}$ *and* Miz-1$^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice. FACS plots are a representative example of four independent experiments. (B) Absolute cell counts of thymocyte subsets from (A). Data are averaged from four independent experiments and are presented as mean ± SD. (C) FACS analysis of Annexin V staining on DN3 pre-T cells. FACS plots are a representative example of three independent experiments. (D) Quantification of percentage of Annexin V$^{+}$ cells from staining in (C). Data are averaged from three independent experiments and are presented as mean ± SD.

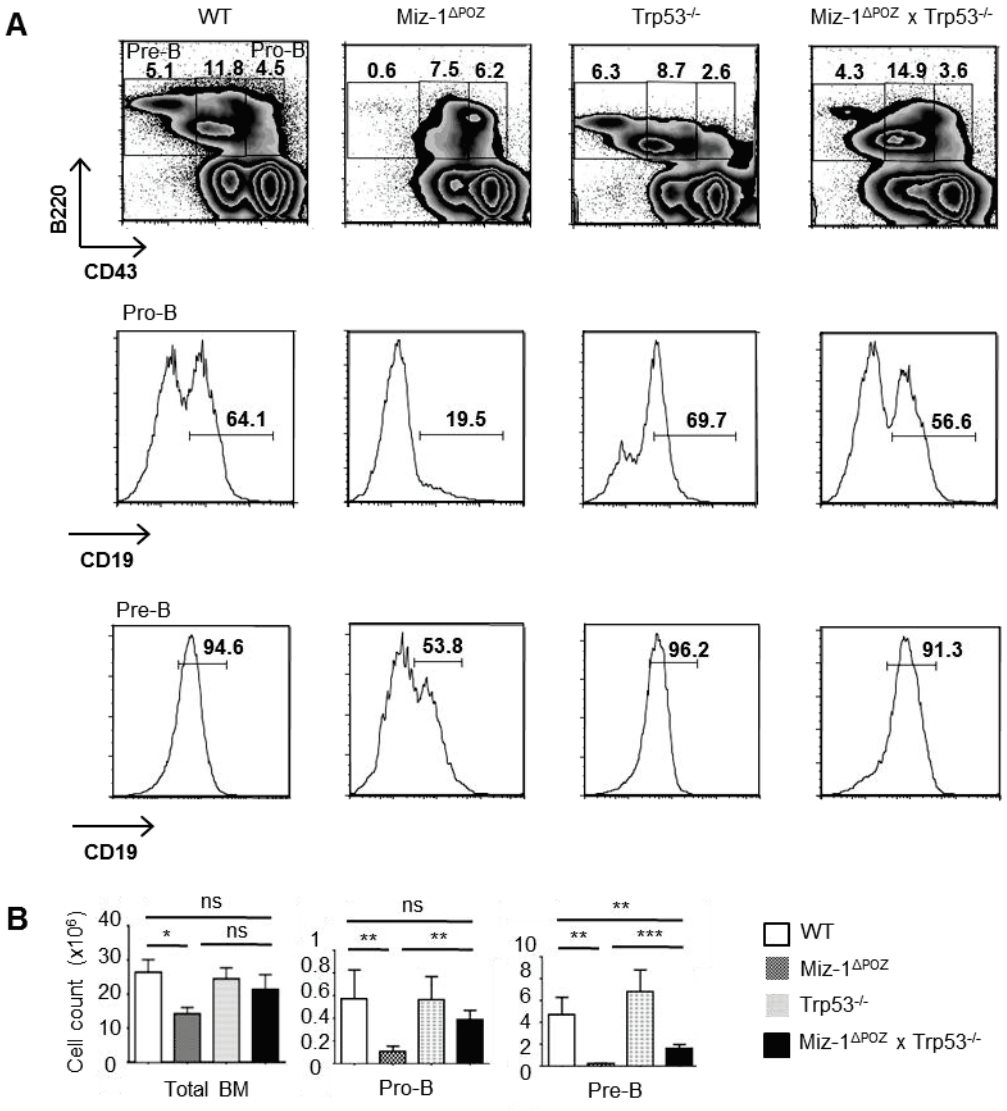


**Figure 4.2** Deletion of p53 restores early steps of B cell development in Miz-1$^{\Delta POZ}$ mice. (A) FACS analysis of B cell subsets from BM of WT, Miz-1$^{\Delta POZ}$, *Trp53*$^{-/-}$ and Miz-1$^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice. FACS plots are a representative example of four independent experiments. (B) Absolute cell counts of B cell subsets from (A). Data are averaged from four independent experiments and are presented as mean ± SD.

## 4.2 The developmental arrest in Miz-1-deficient pre-T cells and pro-B cells is associated with induction of *Puma* and *Bax*

Ablation of p53 in Miz-1$^{\Delta POZ}$ DN3 pre-T cells and pro-B cells also restored the over-expression of p53 target genes *Cdkn1a* (p21), *Bax*, and *Bbc3* (*Puma*) to WT levels (Figure 4.3A, 4.4A). We had previously shown that deletion of *Cdkn1a* fails to rescue the transition from DN3a to DN3b (Saba et al., 2011a), which excludes the possibility that p53-mediated cell cycle arrest through activation of *Cdkn1a* (p21) is responsible for this differentiation block. We therefore attempted to restore pre-T cell development by preventing activation of the pro-apoptotic p53 targets *Puma* and *Bax*, using p53$^{K117R}$ knock-in mice in which p53 is specifically rendered unable to activate transcription of both *Puma* and *Bax* (Li et al., 2012; Sykes et al., 2006; Tang et al., 2006). Lymphocyte development in Miz-1-deficient p53$^{K117R}$ (Miz-1$^{\Delta POZ}$ x p53$^{K117R}$) mice is partially restored as indicated by the decreased percentage of total DN cells compared to the Miz-1-deficient thymus (Figure 4.3B). Furthermore, there is a five-fold decrease in the percentage of DN3a cells undergoing apoptosis compared to Miz-1$^{\Delta POZ}$ DN3a cells, as shown by Annexin V staining (Figure 4.3B). Total thymic cellularity and absolute numbers of DN3b cells are also increased in Miz-1$^{\Delta POZ}$ x p53$^{K117R}$ mice compared to Miz-1$^{\Delta POZ}$ mice (Figure 4.3C). Development of pro-B cells is also partially restored in Miz-1$^{\Delta POZ}$ x p53$^{K117R}$ mice compared to Miz-1$^{\Delta POZ}$ mice, as indicated by the two-fold increase in the percentage of pre-B cells expressing CD19 on the surface and the absolute number of pre-B cells in the bone marrow of Miz-1$^{\Delta POZ}$ x p53$^{K117R}$ mice (Figure 4.4B,C).

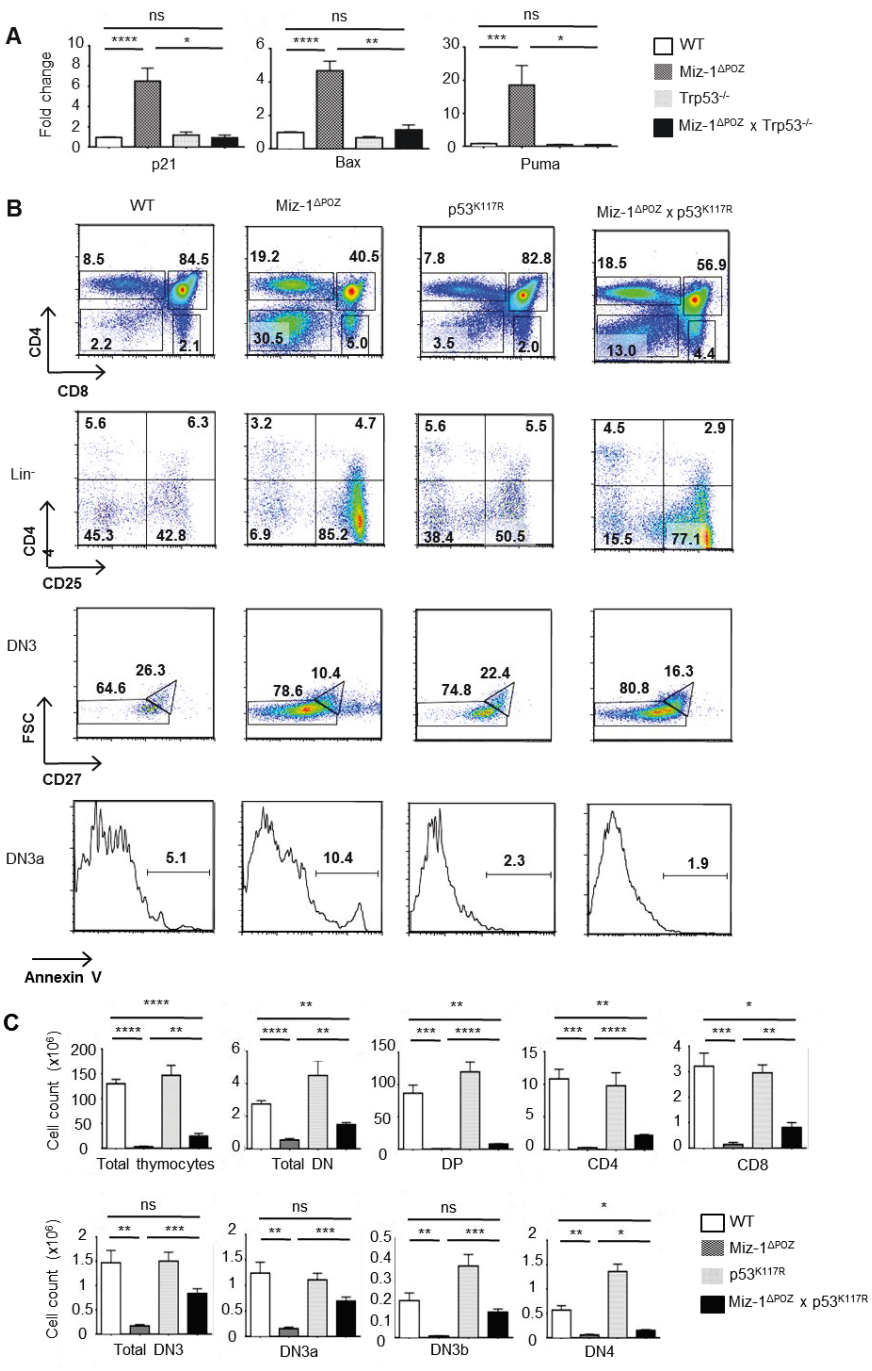


**Figure 4.3** The developmental arrest in Miz-1-deficient thymocytes is associated with the overexpression of several p53 effectors. (A) Analysis of p53 target gene expression by RT-qPCR in sorted DN3 pre-T cells from WT, Miz-1$^{\Delta POZ}$, *Trp53*$^{-/-}$ and Miz-1$^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice. Data are average fold change over *Gapdh* from three independent experiments and are presented as mean ± SD. (B) FACS analysis of thymic subsets from WT, Miz-1$^{\Delta POZ}$, p53$^{K117R}$ and Miz-1$^{\Delta POZ}$ x p53$^{K117R}$ mice. FACS plots are a representative example of three independent experiments. (C) Absolute cell counts of thymocyte subsets from (B).

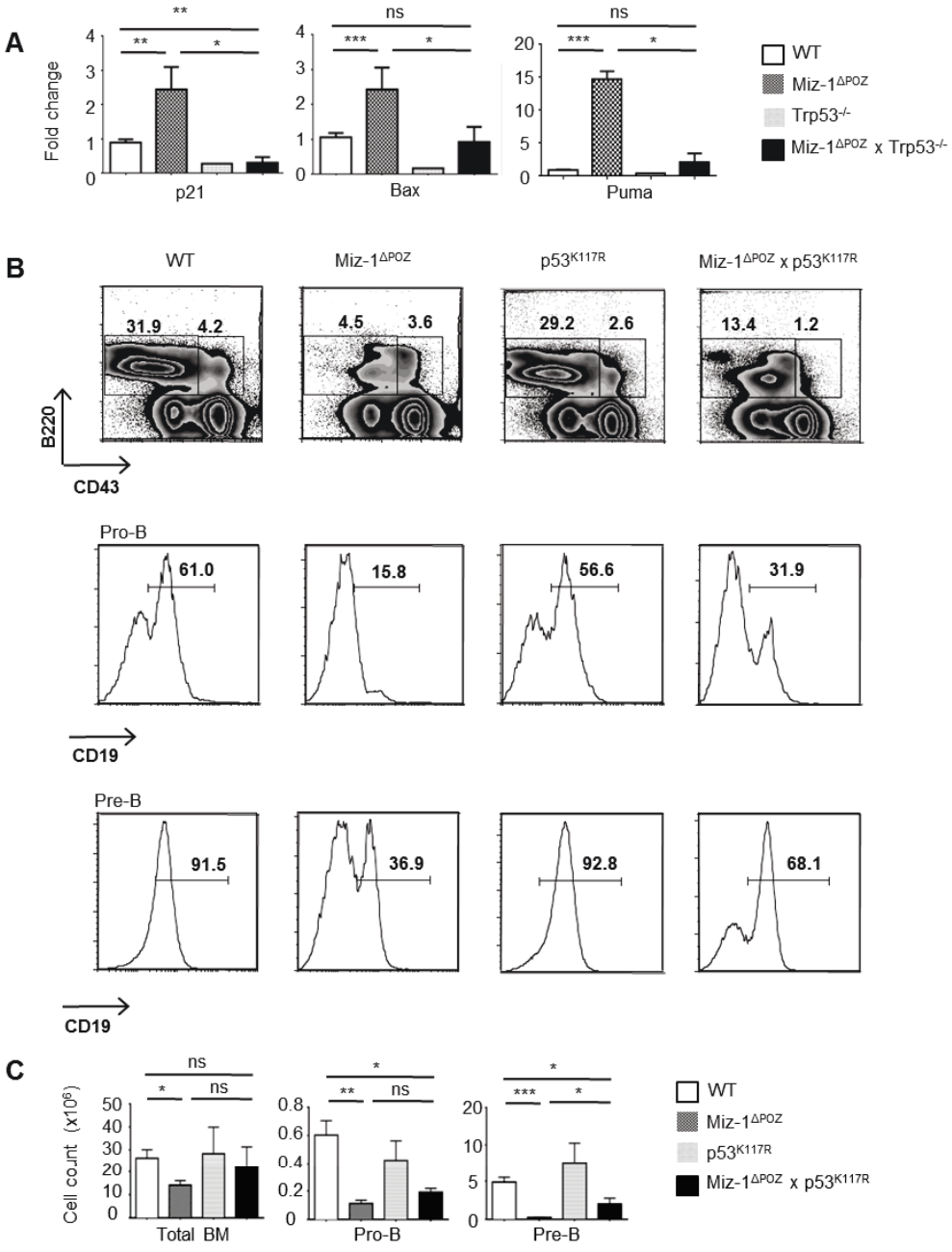


**Figure 4.4** The developmental arrest in Miz-1-deficient pre-B cells is associated with several p53 effectors. (A) Analysis of p53 target gene expression by RT-qPCR in sorted pro-B cells from WT, Miz-1$^{\Delta POZ}$, *Trp53*$^{-/-}$ and Miz-1$^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice. Data are average fold change over *Gapdh* from two independent experiments and are presented as mean ± SD. (B) FACS analysis of B cell subsets from BM of WT, Miz-1$^{\Delta POZ}$, p53$^{K117R}$ and Miz-1$^{\Delta POZ}$ x p53$^{K117R}$ mice. FACS plots are a representative example of three independent experiments. (C) Absolute cell counts of B cell subsets from (B).

## 4.3 Miz-1-deficient DN3 cells progress to DN4 in the absence of V(D)J recombination

To determine whether V(D)J recombination-induced DNA damage might be responsible for the p53-mediated differentiation block of Miz-1-deficient pre-T cells at the DN3a stage, we crossed Miz-$1^{\Delta POZ}$ mice with *Rag1*$^{-/-}$ mice that are defective in rearranging TCR or Ig genes. We injected *Rag1*$^{-/-}$ x Miz-$1^{\Delta POZ}$ mice with αCD3 antibodies to mimic pre-TCR signaling and followed the differentiation of DN3 cells into the DN4 and DP stages as described (Shinkai and Alt, 1994). Whereas αCD3 stimulation had no effect on Miz-1-deficient pre-T cells with a functional *Rag1* gene (Figure S4.2), the ablation of *Rag1* in Miz-$1^{\Delta POZ}$ mice clearly allowed a full differentiation of Miz-1-deficient DN3 cells to DN4 cells (Figure 4.5A). However, while *Rag1*$^{-/-}$ thymocytes were able to further differentiate into DP cells 72 hours post-αCD3 injection, *Rag1*$^{-/-}$ x Miz-$1^{\Delta POZ}$ thymocytes were blocked at the DN4 stage (Figure 4.5A) and were unable to proliferate to the same extent as *Rag1*$^{-/-}$ thymocytes (Figure 4.5B). Since c-Myc is a Miz-1 co-factor and has previously been identified as a mediator of the DN to DP transition, we tested whether the ability of c-Myc to bind to Miz-1 is critical for this function. We used Myc$^{V394D}$ knock-in mice, in which c-Myc is unable to interact with Miz-1 due to a mutation in its helix-loop-helix domain, but can still dimerize with Max (Gebhardt et al., 2006; Herold et al., 2002). As expected, stimulation of both *Rag1*$^{-/-}$ x Myc$^{V394D}$ and *Rag1*$^{-/-}$ thymocytes with αCD3 led to full differentiation of DN3 cells to the DN4 stage (Figure 4.5C). However, although both *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ x Myc$^{V394D}$ thymocytes were able to differentiate into DP cells 72 hours post-injection, this transition was less efficient in *Rag1*$^{-/-}$ x Myc$^{V394D}$ animals, as indicated by the lower absolute cell counts and DP cell percentages from these mice post-stimulation (Figure 4.5C,D). Two c-Myc effectors, CD71 and CD98, which are highly expressed post-pre-TCR selection, were not up-regulated on the surface of αCD3 stimulated *Rag1*$^{-/-}$ x Miz-$1^{\Delta POZ}$ T cells (Figure 4.5E). Furthermore, both CD71 and CD98

were less efficiently up-regulated on the surface of *Rag1*$^{-/-}$ x Myc$^{V394D}$ T cell stimulated with αCD3 compared to *Rag1*$^{-/-}$ stimulated T cells (Figure 4.5F). These results indicate that in the absence of V(D)J recombination, Miz-1-deficient thymocytes regain the ability to pass through pre-TCR selection and give rise to DN4 cells, but are still unable to differentiate further to become DP thymocytes, since they probably require a functional Miz-1/c-Myc complex for this step.

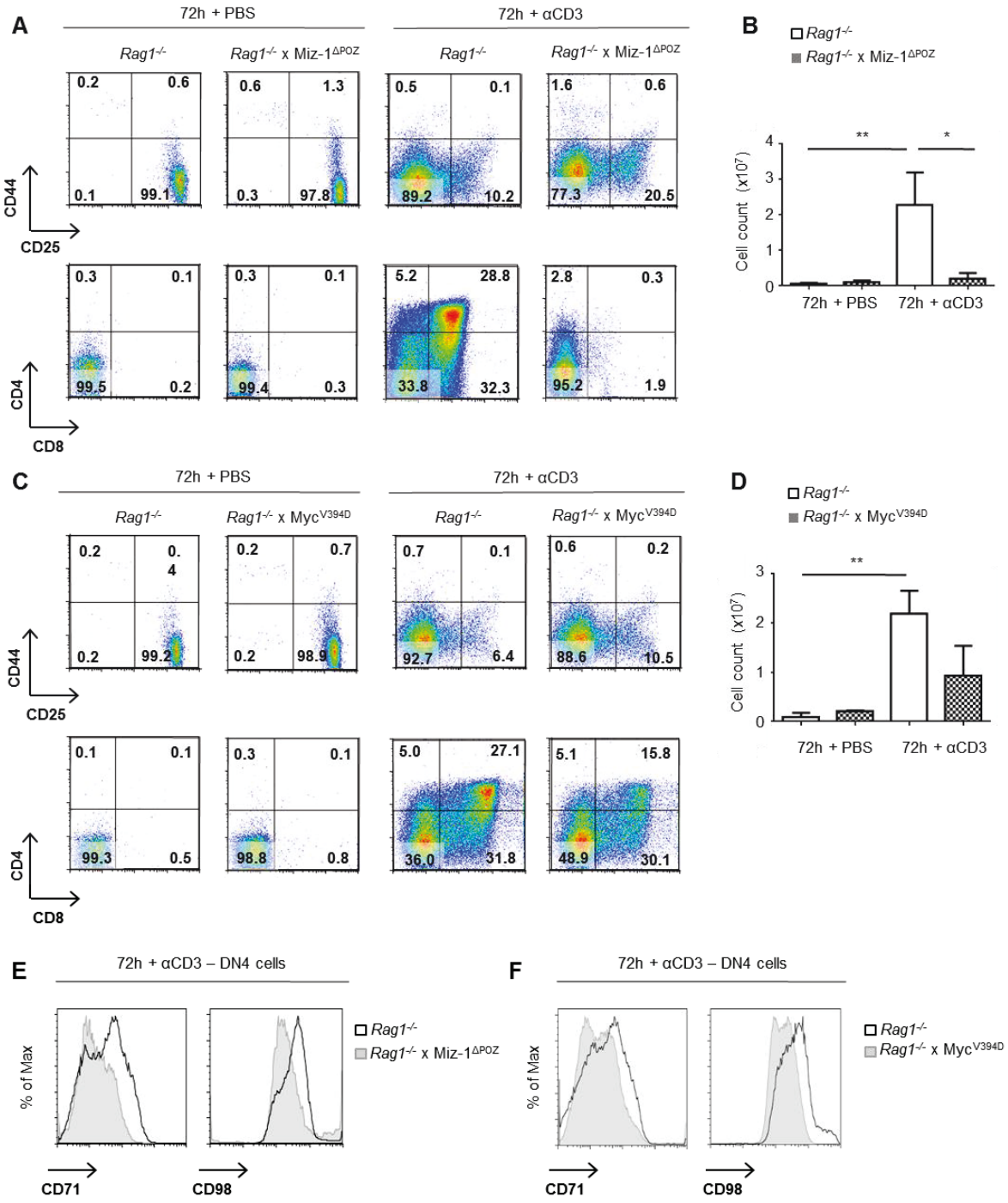


**Figure 4.5** Mitogenic stimulation of *Rag1*$^{-/-}$, *Rag1*$^{-/-}$ x Miz-1$^{\Delta POZ}$ and *Rag1*$^{-/-}$ x Myc$^{V394D}$ KI pre-T cells. (A) FACS analysis of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ x Miz-1$^{\Delta POZ}$ mice 72h after injection with PBS or αCD3 antibodies. FACS plots are a representative example of three independent experiments. (B) Absolute cell counts of total thymocytes from (A). Data are averaged from three independent experiments and are presented as mean ± SD. (C) FACS analysis of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ x Myc$^{V394D}$ KI mice 72h after injection with PBS or αCD3. FACS plots are a representative example of

three independent experiments. (D) Absolute cell counts of total thymocytes from (B). Data are averaged from three independent experiments and are presented as mean ± SD. (E) FACS analysis of CD71 and CD98 on *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ x Miz-1$^{\Delta POZ}$ thymocytes 72h after injection with αCD3. FACS plots are a representative example of three independent experiments. (F) FACS analysis of CD71 and CD98 on *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ x Myc$^{V394D}$ thymocytes 72h after injection with αCD3. FACS plots are a representative example of two independent experiments.

## 4.4 Miz-1 binds to the promoter of *Rpl22* and regulates its expression in DN3 pre-T cells and pro-B cells

ChIP-seq experiments in P6D4 cells, a DN3 pre-T cell line, and 70Z/3 cells, a pre-B cell line, showed that Miz-1 does not bind to the promoters of p53 target genes such as *Cdkn1a*, *Bax* or *Puma* in these cells, although they are deregulated in Miz-1$^{\Delta POZ}$ cells (Figure S4.3A, S4.4A). However p53 target genes are being actively transcribed in these cells, as demonstrated by the presence of the histone activation marks in the ChIP-seq experiment and active transcription in the RNA-seq experiment performed in the same cells. The *Vamp4* gene promoter contains a Miz-1 binding site, and has previously been shown to be a bona fide Miz-1 target (Wolf et al., 2013) and is used as a control. The data from the Miz-1 ChIP-seq experiments were confirmed by ChIP-qPCR with P6D4 cells (Figure S4.3B) and with sorted primary WT DN3 pre-T cells (Figure S4.3C), as well as with 70Z/3 cells (Figure S4.4B). These results suggest that the expression of the pro-apoptotic p53 target genes *Puma* and *Bax*, which are partially responsible for the developmental block in Miz-1$^{\Delta POZ}$ DN3 pre-T cells and pro-B cells (Figure 4.3,4.4), is not directly regulated by Miz-1.

The analysis of a microarray dataset from Miz-1-deficient DN3 pre-T cells (Saba et al., 2011a) showed *Rpl22* as the most down-regulated gene in these cells compared to WT DN3 pre-T cell controls. Both DN3 pre-T cells and CD19$^{+}$ pro-B cells were sorted from WT and Miz-1$^{\Delta POZ}$

littermates and *Rpl22* mRNA expression levels were confirmed to be significantly down-regulated in both cell types (Figure 4.6A). Furthermore, ChIP-seq data from P6D4 and 70Z/3 cells indicated that Miz-1 occupies the promoter of *Rpl22*. A combination of the presence of active histone marks (ChIP-seq) and high levels of mRNA (RNA-seq) indicated that this gene is actively transcribed in both P6D4 pre-T cells and 70Z/3 pre-B cells (Figure 4.6B). Miz-1 binding to the *Rpl22* promoter was validated by ChIP-qPCR in P6D4 cells, sorted WT DN3 pre-T cells and 70Z/3 cells (Figure 4.6C**)** using different primer pairs (Figure 4.6B**,** primer pair 2**,** primer pair 3), indicating that *Rpl22* is a direct Miz-1 target gene. To confirm activation of the *Rpl22* promoter by Miz-1, 293T cells were co-transfected with the human *Rpl22* promoter fused to luciferase and increasing amounts of human Miz-1. We found that increasing amounts of Miz-1 led to increased activation of the *Rpl22* promoter (Figure 4.6D). This suggests that, not only does Miz-1 bind to the *Rpl22* promoter in DN3 pre-T cells and pre-B cells, but it also favors transcriptional activation of this gene.

We have previously shown that overexpression of Bcl2 in Miz-1$^{\Delta POZ}$ mice (Miz-1$^{\Delta POZ}$ x Bcl2 Tg) rescues the apoptosis of Miz-1-deficient ETPs and partially rescues total thymic cellularity, but had no effect on the developmental block of Miz-1-deficient DN3 pre-T cells (Saba et al., 2011b). Since Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice have increased numbers of thymocytes compared to Miz-1$^{\Delta POZ}$ animals, we used them to test the effect of Miz-1 deficiency on the expression levels of p53 protein. Thymocyte extracts from Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice showed increased p53 protein levels compared to Bcl2 Tg mice (Figure 4.6E). This is consistent with a previous report that loss of *Rpl22* correlates with an increased synthesis of p53 protein (Anderson et al., 2007). Furthermore, this suggests that loss of Miz-1 leads to down-regulation of *Rpl22*, which increases the expression of p53 protein in DN3 pre-T cells. To determine whether overexpression of Rpl22 could rescue

the phenotype of Miz-1$^{\Delta POZ}$ pre-T cells, DN3a cells from WT and Miz-1$^{\Delta POZ}$ mice were infected with a retrovirus expressing *Rpl22*. Overexpression of *Rpl22* partially restores the ability of these cells to survive and differentiate into DN4 cells *in vitro* on OP9-DL4 stromal cells after 4 days (Figure 4.7A). However, while the absolute numbers of both live cells and GFP$^{+}$DN4 cells could be clearly restored upon forced expression of Rpl22 (Figure 4.7B), the cells are still unable to proliferate and differentiate past the DN4 stage into DP cells (Figure 4.7A). Due to the limited number of DN4 cells produced on the OP9-DL4 stromal layer in this experiment, it is not possible to measure the effect of Rpl22 overexpression on the levels of p53 protein. Such an experiment, however, would be necessary to further support a direct link between Miz-1, Rpl22 and p53 protein levels.

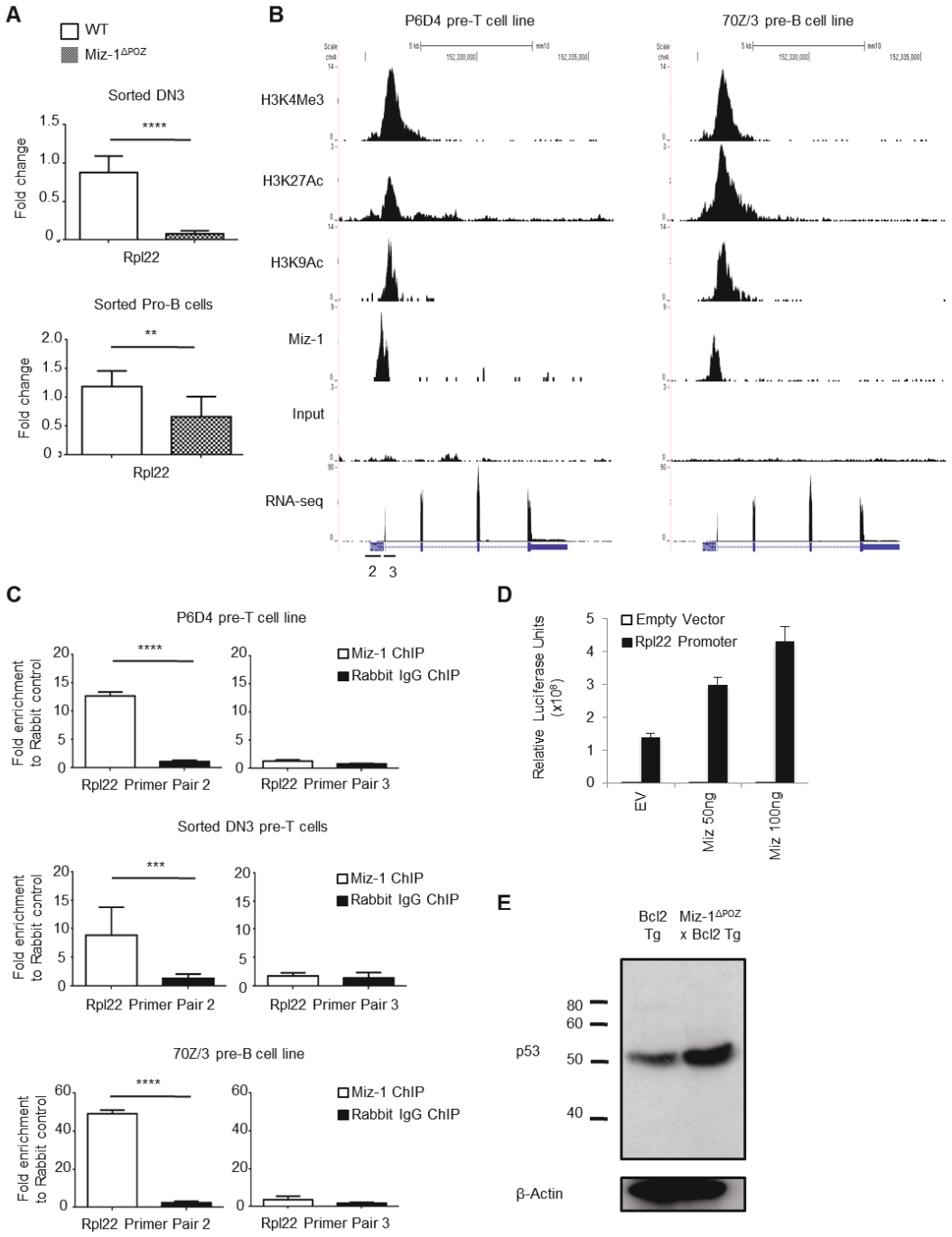


**Figure 4.6** Miz-1 regulates the expression of *Rpl22* in DN3 pre-T cells and pro-B cells. (A) *Rpl22* mRNA expression was assessed in sorted DN3 pre-T cells (top) and sorted pro-B cells (bottom) from WT and Miz-1$^{\Delta POZ}$ mice. Data are averaged from three independent experiments and are presented as mean ± SD. (B) ChIP-seq experiments for Miz-1 and histone activation marks from P6D4 murine pre-T cells (right) 70Z/3 murine pre-B cells (left). Shown is the *Rpl22* locus. Scale is in number of reads per million reads. Primer pairs were designed in the promoter (2) or first exon (3) of *Rpl22*

to determine Miz-1 binding by ChIP. (C) ChIP-qPCR experiments to determine binding of Miz-1 to the promoter of *Rpl22* in P6D4 murine pre-T cells (top), sorted WT DN3 pre-T cells (middle), and 70Z/3 murine pre-B cells (bottom). Graphs show fold enrichment of anti-Miz-1 ChIP over Rabbit IgG control ChIP. Data are represented as average fold change ± SD from at least three independent experiments. (D) 293T cells were transfected with the human *Rpl22* promoter fused to luciferase and pcDNA3.1 empty vector (EV) or pcDNA3.1 with human Miz-1 in varying concentrations. Data are normalized for transfection using β-galactosidase. Data are presented as average relative luciferase units ± SD and are representative of three independent experiments. (E) Whole protein extracts from total thymus of Bcl2 Tg or Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice were evaluated for p53 expression by Western Blot. Data are representative of at least three independent experiments.

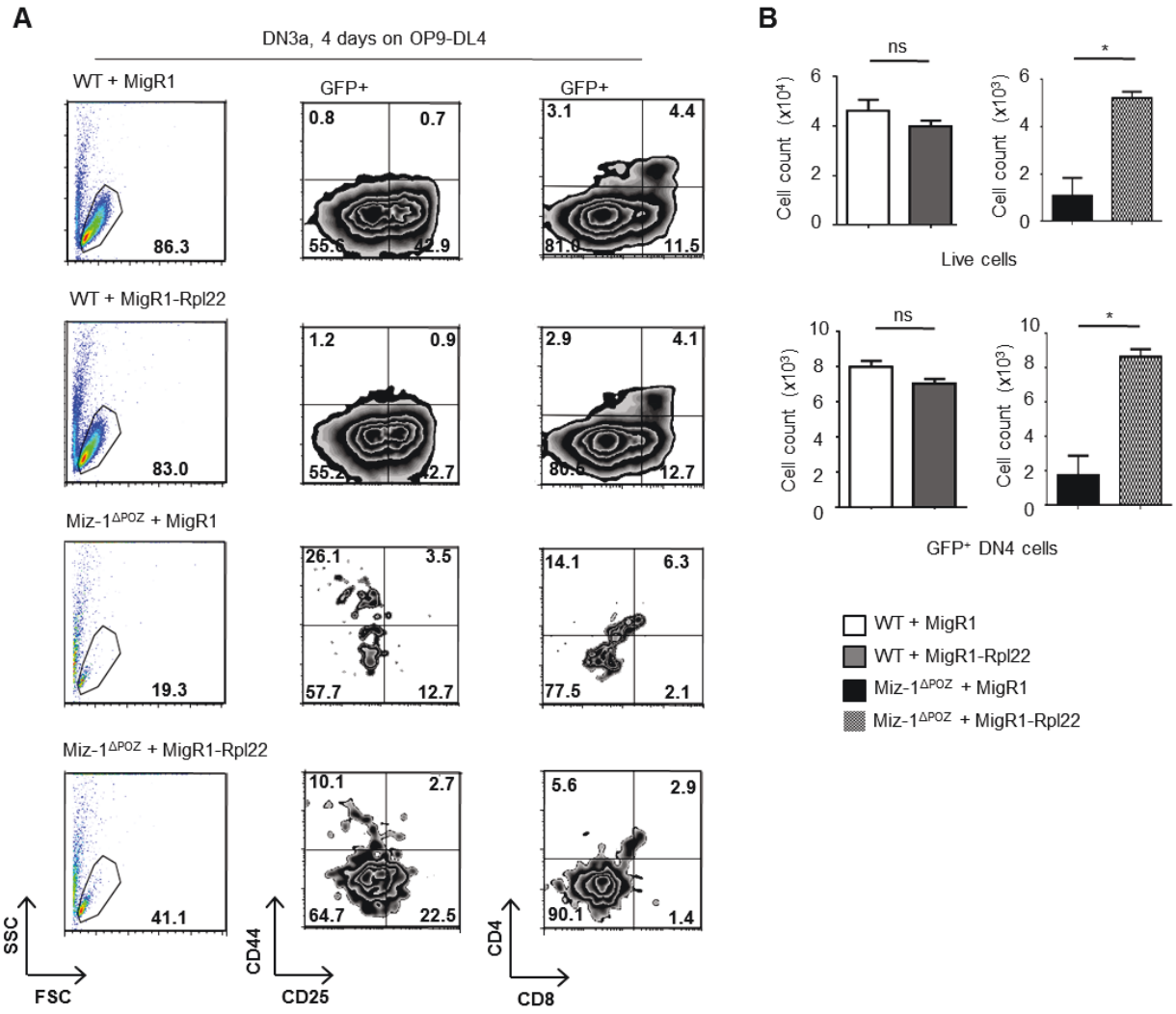


**Figure 4.7** Overexpression of Rpl22 in Miz-1-deficient DN3a cells partially restores survival and differentiation. (A) Flow cytometry analysis of sorted WT and Miz-1$^{\Delta POZ}$ DN3a cells infected with MigR1 or MigR1-Rpl22 after co-culture for 4 days on OP9-DL4. Indicated graphs are gated on GFP+ population. Data are representative of two independent experiments. (B) Absolute total live cell number (top) and total GFP$^{+}$DN4 cell number (bottom) from OP9-DL4 experiments in (A).

## 4.5 Rpl22 binds to p53 mRNA to regulate translation

To test whether Miz-1 deficiency affects the stability of p53 mRNA, DN3 pre-T cells were sorted from WT and Miz-1$^{\Delta POZ}$ littermates and treated with Actinomycin D, an inhibitor of RNA Polymerase II, over four hours (Figure 4.8A). The degradation of p53 mRNA over this time period is comparable in both WT and Miz-1-deficient cells, indicating that the increase in p53 protein is not due to an increase in stability of p53 mRNA. To determine whether loss of Miz-1 leads to increased incorporation of p53 mRNA into actively translating ribosomes, we again used Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice because the low number of thymocytes in Miz-1$^{\Delta POZ}$ animals limits experimentation. Thymocytes from Bcl2 Tg and Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice were isolated and lysates were sedimented through sucrose gradients. RNA from the fractionated gradients was extracted and analyzed by RT-qPCR for the presence of p53 mRNA. In Miz-1-deficient samples, the percentage of p53 mRNA in polysome fractions (Fractions 8-12) is significantly increased compared to WT controls (Figure 4.8B). Since the percentage of *β-actin* mRNA in polysomes is not significantly different in Miz-1-deficient or WT thymocytes (Figure 4.8C), a global shift in translation in Miz-1-deficient cells can be ruled out, suggesting that Miz-1 is required to specifically regulate p53 protein levels by controlling the incorporation of p53 mRNA into actively translating ribosomes, probably via Rpl22.

To determine whether the increased translation of p53 is due to direct interaction of p53 mRNA with Rpl22 protein, an RNA immunoprecipitation (RIP) was performed in P6D4 pre-T cells. Using this technique, we found a 25-fold increase in the amount of p53 mRNA bound by the Rpl22 protein compared to the rabbit IgG control (Figure 4.8D). Rpl22 has previously been shown to bind the mRNA of its paralog, *Rpl22l1* (O'Leary et al., 2013), which was used as a positive control

for the Rpl22 RIP. We found a 10-fold increase in the amount of *Rpl22l1* mRNA bound by Rpl22 compared to the rabbit IgG control (Figure 4.8D), confirming that both *Rpl22l1* and p53 mRNA are bound by Rpl22. Furthermore, the presence of β-actin mRNA in the RIP was assessed and it was found that, while translation of this gene is not significantly different in Miz-1-deficient thymocytes compared to WT, it is bound by Rpl22.

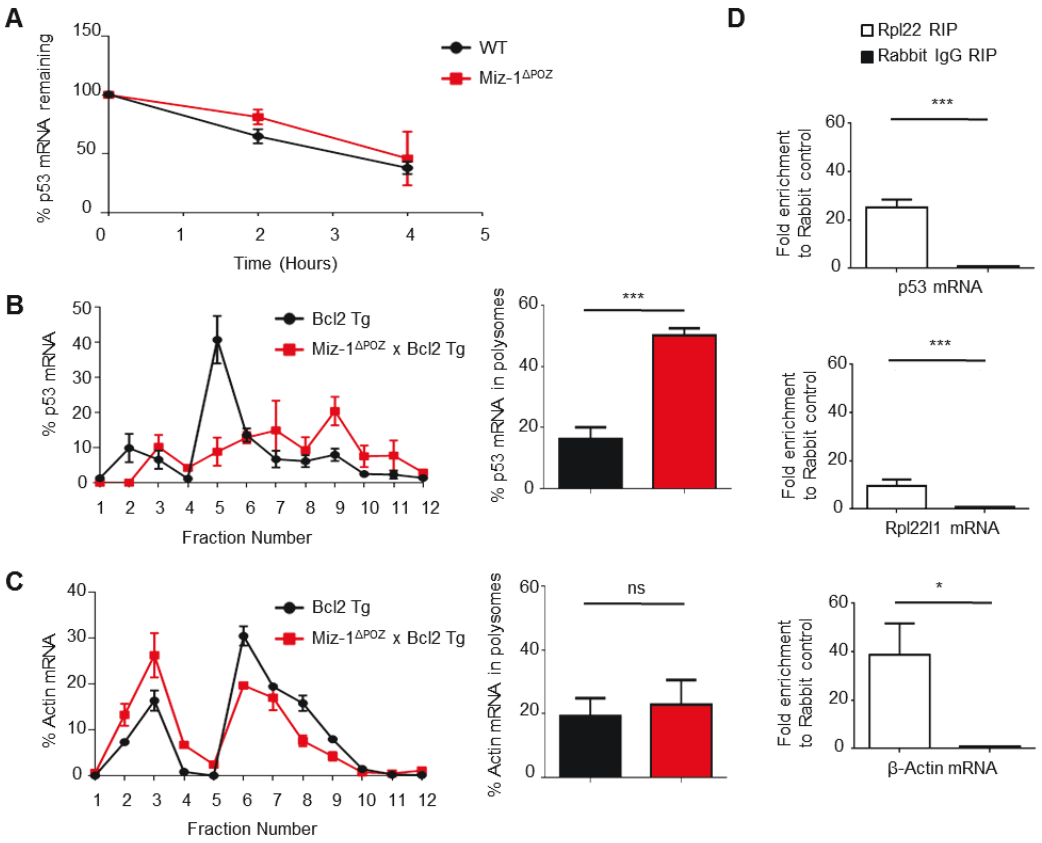


**Figure 4.8** Rpl22 regulates the translation of p53. (A) Sorted DN3 cells from WT or Miz-1$^{\Delta POZ}$ mice were treated with 5ug/mL Actinomycin D and harvested at the indicated time points. The percentage of p53 mRNA remaining at each time point was assessed by RT-qPCR, and normalized to *Gapdh*. Data are averaged from three independent experiments and are presented as mean ± SD. (B) (Left) Total thymic extracts from Bcl2 Tg and Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice were sedimented through a sucrose gradient and fractionated. qRT-PCR was performed to measure p53 mRNA in the fractions collected. Data are presented as the percentage of p53 mRNA in each fraction. Graph is representative of three independent experiments. (Right) Quantification of percentage of p53 mRNA in polysomes (Fractions 8-12) from left. Data are averaged from three independent experiments and are presented as mean ± SD. (C) (Left) Total thymic extracts from Bcl2 Tg and Miz-1$^{\Delta POZ}$ x Bcl2 Tg mice were sedimented on a sucrose gradient and fractionated. qRT-PCR was performed for *β-actin* on the fractions collected. Data are presented as the percentage of *β-actin* mRNA in each fraction. Graph is representative of three independent experiments. (Right) Quantification of percentage of *β-actin* mRNA in polysomes (Fractions 8-12) from left. Data are averaged from three independent experiments and are presented as mean ± SD. (D) RNA-IP of Rpl22 in P6D4 pre-T cells. Graph shows fold enrichment of anti-Rpl22 RIP over Rabbit IgG control RIP. *Rpl22l1* mRNA is used as a positive control for Rpl22 RIP. *B-Actin* mRNA is also enriched in anti-Rpl22 RIP. Data are represented as average fold change ± SD from three independent experiments.

# 5.0 Discussion

V(D)J recombination is necessary to rearrange TCR or Ig gene segments and to ensure the generation of a large repertoire of antigen receptors. T and B lymphocytes, which carry one antigen-specific TCR or immunoglobulin at their cell surface, require such a repertoire to ensure recognition of a very large spectrum of antigens. The process of V(D)J recombination itself, however, needs to be tightly regulated. It has to be coordinated with DNA replication and mitosis to avoid genomic damage. This is achieved in part by linking V(D)J recombination to cell cycle progression by periodic phosphorylation and destruction of the Rag-2 recombinase that breaks and rejoins Ig or TCR gene segments with the effect that V(D)J recombination occurs only in cells at G1 (Lin and Desiderio, 1995). In addition, however, it has been put forward that a DNA damage response pathway that could be initiated as a consequence of the occurring double strand breaks during V(D)J recombination has to be controlled to avoid the induction of p53-mediated apoptosis (Dujka et al., 2010; Guidos et al., 1996). Here we provide evidence that the BTB-POZ domain transcription factor Miz-1 exerts such a function in both T- and B-lineage cells by restricting the expression of p53 through the inhibition of its translation via the ribosomal protein Rpl22 specifically in cells that undergo V(D)J recombination.

Miz-1-deficient pre-T cells express all the necessary components to properly undergo pre-TCR selection (Saba et al., 2011a), but are blocked at the DN3 stage and do not give rise to significant numbers of DN4 cells. In addition, DN3a cells lacking a functional Miz-1 show increased rates of apoptosis and several experiments in this study suggest that this differentiation block and the increased cell death rate in DN3 pre-T cells is mediated by an overactive p53. First, as shown previously and confirmed here, a number of direct, known p53 target genes such as *Cdkn1a*, *Bax*

and *Puma* are up-regulated in Miz-1-deficient cells. Second, deletion of p53 not only restores the numbers of DN3 pre-T cells but also proper expression levels of *Cdkn1a*, *Bax* and *Puma* and overall thymic cellularity, indicating a full rescue of T cell differentiation. In addition, p53 deletion also partially restores the numbers of pre-B cells that otherwise do not develop from pro-B cells in Miz-1-deficient mice. While there is a complete rescue of T cell differentiation in the thymus, loss of p53 expression only partially restores B cell development in Miz-1-deficient mice. This may be due to the fact that the previously reported defect in IL7 signaling has a stronger impact on B cell precursors than on T cell precursors (Kosan et al., 2010). The fact that both pre-T cells and pro-B cells are affected by Miz-1 ablation and that they can regain their differentiation ability after concomitant p53 deletion precisely at stages where V(D)J recombination occurs, supports the hypothesis that this function of Miz-1 represents a general mechanism controlling potentially damaging consequences of V(D)J recombination regardless of cell lineage.

While p53 affects both cell cycle progression and apoptosis, our data suggest that the pro-apoptotic p53 effectors *Puma* (*Bbc3*) and *Bax* are mainly responsible for the developmental block of Miz-1-deficient pre-T and pro-B cells. This is supported by our observation that Miz-1$^{\Delta POZ}$ mice that carry a p53$^{K117R}$ allele, in which p53 has specifically lost its ability to activate expression of both *Puma* and *Bax*, show a noticeable rescue of their pre-T and pro-B cell developmental defects. While the loss of the acetylation site at K117 in mouse p53 completely abolishes p53-mediated apoptosis, it has no effect on p53 translation or expression levels, nor does it affect p53-dependent cell cycle arrest or senescence (Li et al., 2012; Sykes et al., 2006; Tang et al., 2006). Moreover, total thymic cellularity is only partially restored in these mice, as is the percentage of DN3b pre-T cells. Furthermore, in the bone marrow B cell compartment, absolute numbers of pre-B cells are

partially restored. This indicates that other functions of p53 may also be partially responsible for the pre-T and pro-B cell differentiation block in Miz-1-deficient mice.

Our findings are consistent with the hypothesis that Miz-1 is involved in regulating a p53-mediated response initiated by V(D)J-induced DNA damage in pre-T or pro-B cells. Further evidence that supports this contention is provided by our finding that Miz-1 deficient DN3 pre-T cells fully develop into DN4 cells in the absence of V(D)J recombination. Indeed, our experiments with *Rag1*$^{-/-}$ x Miz-1$^{\Delta POZ}$ mice showed massive accumulation of DN4 cells upon CD3 receptor stimulation to the same extent as seen in *Rag1*$^{-/-}$ mice that express a functional Miz-1. However, at this stage, the role of Miz-1 may not be limited to regulating the p53 pathway, as Miz-1$^{\Delta POZ}$ x *Trp53*$^{-/-}$ DN3 pre-T cells progress to DN4, but these DN4 cells are still less efficient than WT DN4 in their ability to proliferate and differentiate further to DP cells *in vitro*. In addition, although *Rag1*$^{-/-}$ x Miz-1$^{\Delta POZ}$ mice efficiently generate DN4 cells upon αCD3 stimulation, they do not differentiate into DP cells. As the Miz-1 binding partner c-Myc is necessary for the transition of DN4 to DP (Dose et al., 2006), and because the *c-Myc* gene is activated by pre-TCR signaling, we reasoned that the interaction of c-Myc with Miz-1 might be required for an efficient DN4-DP transition. This is supported by data from *Rag1*$^{-/-}$ x Myc$^{V394D}$ mice which, when stimulated with αCD3 antibodies, were less efficient in producing DP cells than *Rag1*$^{-/-}$ mice with a functional c-Myc protein that still binds to Miz-1. Also, CD71 and CD98, key metabolic genes up-regulated at the DN-DP transition, and direct targets of c-Myc (O'Donnell et al., 2006; Wang et al., 2011a), are not up-regulated on *Rag1*$^{-/-}$ x Miz-1$^{\Delta POZ}$ DN4 cells after mitogenic stimulation. We therefore propose that, in contrast to the differentiation of DN3 cells to DN4, the DN4 to DP transition represents a step that requires a functional c-Myc/Miz-1 complex. It is likely that this is necessary to activate key

metabolic pathways to support cell growth and differentiation of DN4 cells to generate a large number of DP thymocytes.

While p53 mediates effectors involved in both cell cycle progression and regulation of apoptosis, it is unlikely that V(D)J recombination leads to p53-induced cell cycle arrest, because the dissociation between DNA replication and V(D)J recombination is ensured by cell cycle stage specific expression of Rag-2. We have found increased expression of *Cdkn1a* (p21), which can arrest cell cycle progression and is a p53 target gene, in Miz-1-deficient pre-T and pro-B cells. However, we have shown previously that deletion of *Cdkn1a* in Miz-1-deficient cells did not rescue the defects caused by ablation of Miz-1, indicating that this part of the p53 pathway, although affected by Miz-1-dependent regulation of p53, is not responsible for the phenotype seen in Miz-1-deficient pre-T and pro-B cells. We propose therefore that p53 can be activated to induce apoptosis in response to the DNA strand breaks that occur during V(D)J recombination, and that this activation of p53 is at least partially mediated through a mechanism that involves Miz-1 and its effector, the ribosomal protein Rpl22. It is however likely that other additional mechanisms to activate p53, for instance through posttranslational mechanisms such as phosphorylation, or mechanisms that do not involve p53 are also affected by Miz-1 and also play an important role at this critical point in pre-T cell and pro-B cell differentiation. It is for instance possible that Rpl22 also acts upon other proteins and not only via p53 and that forced expression of Rpl22 alone may not be sufficient to completely restore physiological p53 expression levels in Miz-1-deficient cells. Future experiments will be necessary to clarify the nature of these additional mechanisms and their link to Miz-1.

The regulation of p53 can occur at many levels. P53 expression is most commonly regulated at the post-translational level, and ribosomal proteins have previously been shown to play a role in this regulation; Rpl5 (Marechal et al., 1994), Rpl11 (Lohrum et al., 2003; Zhang et al., 2003), Rpl23 (Dai et al., 2004; Jin et al., 2004) and Rpl26 (Ofir-Rosenfeld et al., 2008) interact directly with the p53 E3 ubiquitin ligase Mdm2 and inhibit its activity, leading indirectly to enhanced stability of p53 under conditions of ribosomal biogenesis stress. Furthermore, Rpl26 has been shown to bind to both the 5' and 3' UTR of p53 mRNA to increase its translation and induction after DNA damage, while nucleolin binds specifically to the p53 5' UTR after DNA damage (Chen and Kastan, 2010; Takagi et al., 2005). Our results indicate that, unlike other ribosomal proteins that have been shown to either activate p53 translation or act as Mdm2 antagonists under physiological conditions, Rpl22 directly suppresses p53 translation, and this in a cell-type and developmental stage specific manner, namely in cells undergoing V(D)J recombination. This is in agreement with previous reports, which have shown that *Rpl22*-deficient mice present no defects in global translation, but are defective specifically in regulation of p53 expression during pro-B and pre-T cell development (Anderson et al., 2007; O'Leary et al., 2013; Stadanlick et al., 2011). The data presented here strongly suggest that this regulation occurs through Miz-1 and that Miz-1 is a direct upstream regulator of the *Rpl22* gene. Furthermore, the regulation of Rpl22 presented here provide evidence for a novel regulation of p53 translation by a ribosomal protein, in that Rpl22 would be the only example of such a protein to, under physiological conditions, repress the translation of p53 by decreasing its incorporation in actively translating ribosomes. In addition, the results presented here indicate that Rpl22 may bind many mRNA transcripts, including Rpl22l1 and β-actin as part of its function in the ribosomal complex. However, based on our polysome fractionation experiment, the interaction between Rpl22 and p53 mRNA increases the

incorporation of this mRNA specifically into polysomes and not other mRNA transcripts. How this specificity is achieved will be subject to further investigation.

The expression of p53 must be tightly regulated in order to promote survival of cells that rearrange the TCRβ or IgH loci. These cells have to break and join DNA ends, a signal that normally initiates a p53-dependent DNA damage response. Our data support a function of Miz-1 that, via Rpl22, acts as a regulator of the translation of p53 in those cells that are at risk to be eliminated by p53-dependent cell death since they are undergoing V(D)J recombination.

# 6.0 Materials and Methods

**Mice**

Mice had been bred on C57BL/6 background for at least 10 generations and were maintained in Specific-Pathogen-Free Plus environment. Miz-1$^{\Delta POZ}$ mice were previously described (Kosan et al., 2010). Trp53$^{-/-}$ mice were purchased from The Jackson Laboratory. p53$^{K117R}$ mice were provided by Dr. Wei Gu (Li et al., 2012). The IRCM Animal Care Committee (ACC) has approved the animal protocols under which all animal experiments in this study have been performed (protocol 2013-01), and all animal experimental procedures were performed in compliance with the guidelines of the Canadian Council of Animal Care (www.ccac.ca).

**Flow Cytometry Antibodies and Cell Lines**

OP9-DL4/OP9 cultures and P6D4 SCID.adh murine thymic lymphoma and 70Z/3 pre-B cells were used as previously described (Kosan et al., 2010; Saba et al., 2011b). All antibodies were from BD Bioscience except when indicated. DN thymic subsets were analyzed using CD25 (PC61.5 from

eBioscience) and CD44 (IM7) plus lineage marker-negative cells ($Lin^-$) 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 PE. The same staining was performed with CD27 (LG.3A10) to differentiate between DN3a and DN3b subsets. Pre-B and pro-B cells were analyzed using B220 (RA3-6B2), CD43 (S7) and CD19 (1D3). Antibody incubations were performed at 4°C for 20 minutes in PBS buffer. Cells were analyzed with a LSR (Becton-Dickson). Cell sorting was performed using a MoFlo cell sorter (Cytomation).

**Cell Cycle and Cell Death Analysis**

Cell cycle analysis was performed on sorted DN3 and DN4 cells. Cells were sorted directly into modified Krishan buffer (0.1% sodium citrate, 0.3% NP-40) containing 0.05mg/mL propidium iodide (PI) and 0.02mg/mL RNase and analyzed after 30 minutes of incubation on a FACSCalibur (Beckton-Dickson). For bromodeoxyuridine (BrdU) analysis, mice were injected intraperitoneally with 100mg/kg BrdU in PBS and sacrificed after 16h. Staining was performed using a FITC BrdU kit (BD Pharmingen) according to the manufacturer's instructions. Apoptosis rates were measured by Annexin V staining (Annexin V-FITC Apoptosis detection kit, BD Pharmingen).

**RNA Isolation and Real-Time PCR**

For RNA isolation, cells were FACS-sorted directly into RLT buffer with β-mercaptoethanol (RNEasy Micro Kit, Qiagen). RT-PCR was performed using Superscript II (Invitrogen). Real-time PCR was performed in triplicates on the ViiA7 using SYBR green reagent (Applied Biosystems)

and indicated primers. The expression of the gene of interest was calculated relative to Gapdh mRNA ($\Delta C_T$) and is presented as fold induction relative to values obtained with the respective control (set as 1-fold). Primer sequences can be found in Table S6.1.

**Polysome Fractionation**

Polysome fractionation was performed as previously described (Mamane et al., 2007). Briefly, single cell suspensions of $10x10^6$ cells from total thymus were treated with cycloheximide (100ug/mL) for 15 minutes at 37ºC. Cells were washed in cold PBS containing 100ug/mL cycloheximide and then lysed in ice-cold hypotonic lysis buffer (5mM Tris-HCl pH 7.5, 2.5mM $MgCl_2$, 1.5mM KCl, 1X protease inhibitor cocktail (complete Mini, Roche Diagnostics), 400U/mL RNaseIN (Promega)). The lysates were immediately adjusted to 100ug/mL cycloheximide, 2mM DTT, 0.5% Triton X-100 and 0.5%sodium deoxycholate. Cell extracts were centrifuged for 5 minutes at 14,000 rpm and the supernatants were collected and loaded onto a 10-50% sucrose gradient. Gradients were placed in a Beckman SW40Ti rotor and centrifuged at 38,000rpm for 2 hours at 4ºC. Fractions were collected (12 fractions of 1mL each) using a tube piercer system (Brandel) and a fraction collector. Samples were incubated with Proteinase K and RNA was extracted using phenol/chloroform. RT-PCR was performed using Superscript II (Invitrogen). Real-time PCR was performed in triplicates on the ViiA7 using SYBR green reagent (Applied Biosystems) and indicated primers.

**RNA Immunoprecipitation**

Single cell suspensions of $10x10^6$ P6D4 cells were treated with cycloheximide (100ug/mL) for 15 minutes at 37ºC. Cells were washed in cold PBS containing 100ug/mL cycloheximide and then lysed in ice-cold hypotonic lysis buffer (5mM Tris-HCl pH 7.5, 2.5mM $MgCl_2$, 1.5mM KCl, 1mM

DTT, 1% Triton X-100, 1% sodium deoxycholate, 100ug/mL cycloheximide, 1X protease inhibitor cocktail (complete Mini, Roche Diagnostics) and 400U/mL RNaseIN (Promega)) for 10 minutes. Cell extracts were centrifuged for 10 minutes at 14,000 rpm and the supernatants were collected and pre-cleared with 10uL Protein A/G Dynabeads (Life Technologies) for 30 minutes. Immunoprecipitation was performed using 10ug anti-Rpl22 (H-106, Santa Cruz Biotechnology) or rabbit control IgG antibodies (Santa Cruz Biotechnology). Washes were performed using RIP wash buffer (50mM Tris pH 7.5, 300mM KCl, 12mM $MgCl_2$, 1% Triton X-100, 1mM DTT, 200ug/mL cycloheximide). RNA was extracted using the RNEasy Mini Kit (Qiagen), followed by RT-PCR and qPCR.

**Immunoblot Analysis**

For immunoblot analysis, cells were lysed in RIPA buffer containing protease inhibitors (complete Mini; Roche Diagnostics) on ice for 20 minutes and then sonicated for 10 minutes in a water bath (Branson 5510). Immunoblotting was performed using anti-p53 (1C12, Cell Signaling), or anti-β-actin (AC-15, Sigma Aldrich).

**Chromatin Immunoprecipitation Sequencing and RNA Sequencing**

Assays were performed on sorted primary DN3 cells, P6D4 SCID.adh, or 70Z/3 as indicated. Cells were fixed with 1% formaldehyde. Cell lysis was performed using the following buffers: total cell lysis - 5mM PIPES pH 8, 85mM KCl, 0.5% NP-40, 1X protease inhibitor cocktail, 1mM PMSF, and nuclear lysis – 50mM Tris, 10mM EDTA, 1% SDS, 1X protease inhibitor cocktail, 1mM PMSF. After sonication (Covaris E220 sonicator), immunoprecipitation was performed using Protein A/G Dynabeads (Life Technologies) and 10ug of rabbit anti-Miz-1 (H-190, Santa Cruz

Biotechnology), H3K4Me3 (Abcam), H3K27Ac (Abcam), H3K9Ac (Millipore) or rabbit control IgG antibodies (Santa Cruz Biotechnology). For ChIP-qPCR experiments, primers can be found in Table S6.2.

For ChIP-Seq experiments, sequencing libraries were prepared from immunoprecipitated chromatin using the TruSeq DNA kit from Illumina according to the manufacturer's instructions and sequenced using the TruSeq PE Clusterkit v3-cBot-HS on an Illumina HiSeq 2000 system. Sequencing reads were aligned to the mm10 genome using Bowtie2 v2.10 (Langmead and Salzberg, 2012). Reads were processed and duplicates were removed using Samtools and a genome coverage file was generated and scaled to reads per million reads (RPM) obtained for each sample using Bedtools (Li et al., 2009; Quinlan and Hall, 2010).

For RNA-Seq, a biological triplicate of sequencing libraries were prepared from RNA extracts using the TruSeq Stranded mRNA kit from Illumina according to the manufacturer's instructions and sequenced using the TruSeq PE Clusterkit v3-cBot-HS on an Illumina HiSeq 2000 system. Sequencing reads were aligned to the mm10 genome using Tophat v2.0.10. Reads were processed with Samtools and then mapped to Ensembl transcripts using HTSeq. Differential expression was tested using the DESeq R package (R Development Core Team, 2010). A genome coverage file was generated and scaled to reads per million reads (RPM) using Bedtools (Li et al., 2009; Quinlan and Hall, 2010).

GEO Accession: GSE57694

**Actinomycin D Treatment**

DN3 cells were FACS sorted into serum-free OptiMEM media (Gibco) and rested at 37°C for 30 minutes. Cells were then treated with 5ug/mL Actinomycin D at 37°C and lysed in RLT buffer with β-mercaptoethanol. RNA was extracted using the RNEasy Mini Kit (Qiagen), followed by RT-PCR and qPCR.

### *Rpl22* Reporter Assay

293T cells were transfected with 250ng human *Rpl22* promoter fused to luciferase (Switchgear Genomics), beta-galactosidase as well as either pcDNA3.1 alone or with 0.5ug or 1ug of human Miz-1 in pcDNA3.1. Cells were lysed after 48h and analyzed for Renilla luciferase expression and beta-galactosidase expression for normalization, using LightSwitch Luciferase Assay Reagent (Switchgear Genomics), and ONPG, respectively.

### Anti-CD3 Injections

4-6 week old Miz-1$^{\Delta POZ}$ x Rag1$^{-/-}$, MycV394D x Rag1$^{-/-}$ and Rag1$^{-/-}$ mice were injected intraperitoneally (i.p.) with 50ug anti-CD3 (145-2C11) per mouse or with PBS alone (Dose et al., 2006). Mice were sacrificed at the indicated times and analyzed by flow cytometry.

### Retroviral Transfection

Rpl22 was cloned into the MigR1 vector using the following primers: 5'-CGACTCGAGATGGCGCCTGTGAAAAAGCTTG-3' and 5'-CGAGAATTCTTAATCCTCGTCTTCCTCCTCCTC-3'. MigR1 and MigR1-Rpl22 were generated using Phoenix Eco cells. DN3a cells were sorted and resuspended in viral supernatant in the presence of 8μg/mL polybrene. Cells were centrifuged at 1600rpm for 90 minutes. Media

was changed 4 hours after infection and cells were plated onto OP9-DL4 stromal cells with 5ng/mL Flt3L and 1ng/mL IL-7 for 4 days.

**Statistical Analysis**

Quantitative data are presented as mean ±SD and were analyzed using a one-way ANOVA or two-tailed student's *t* test. A p-value ≤0.05 was indicated as statistically significant ( * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

# 7.0 Acknowledgements

We are indebted to Mathieu Lapointe for technical assistance and Marie-Claude Lavallée, Mélanie St-Germain and Jade Dussureault for excellent animal care, Eric Massicotte and Julie Lord for FACS and cell sorting and Odile Neyret for molecular biology assistance. Marissa Rashkovan was supported by a fellowship from the Cole Foundation, Julie Ross and Mathieu Gigoux by fellowships from the Canadian Institute for Health Research (CIHR), Charles Vadnais was supported by a fellowship from the „Fonds recherche Québec-Santé" (FRQ-S). Tarik Möröy holds a Canada Research Chair (Tier 1) and a grant from the CIHR (MOP-115202) that supported this work. We thank Martin Eilers, Biozentrum, Universität Würzburg, and the members of his laboratory for communicating results on Miz-1 promoter binding prior to publication.

# 8.0 Author Contributions

M.R. and T.M. designed research; M.R., J.R., M.G., and C.K. performed research; W.-K.S. and W.G. contributed new reagents/analytic tools; M.R. and C.V. analyzed data; and M.R. and T.M. wrote the paper.

# 9.0 Supplemental Information

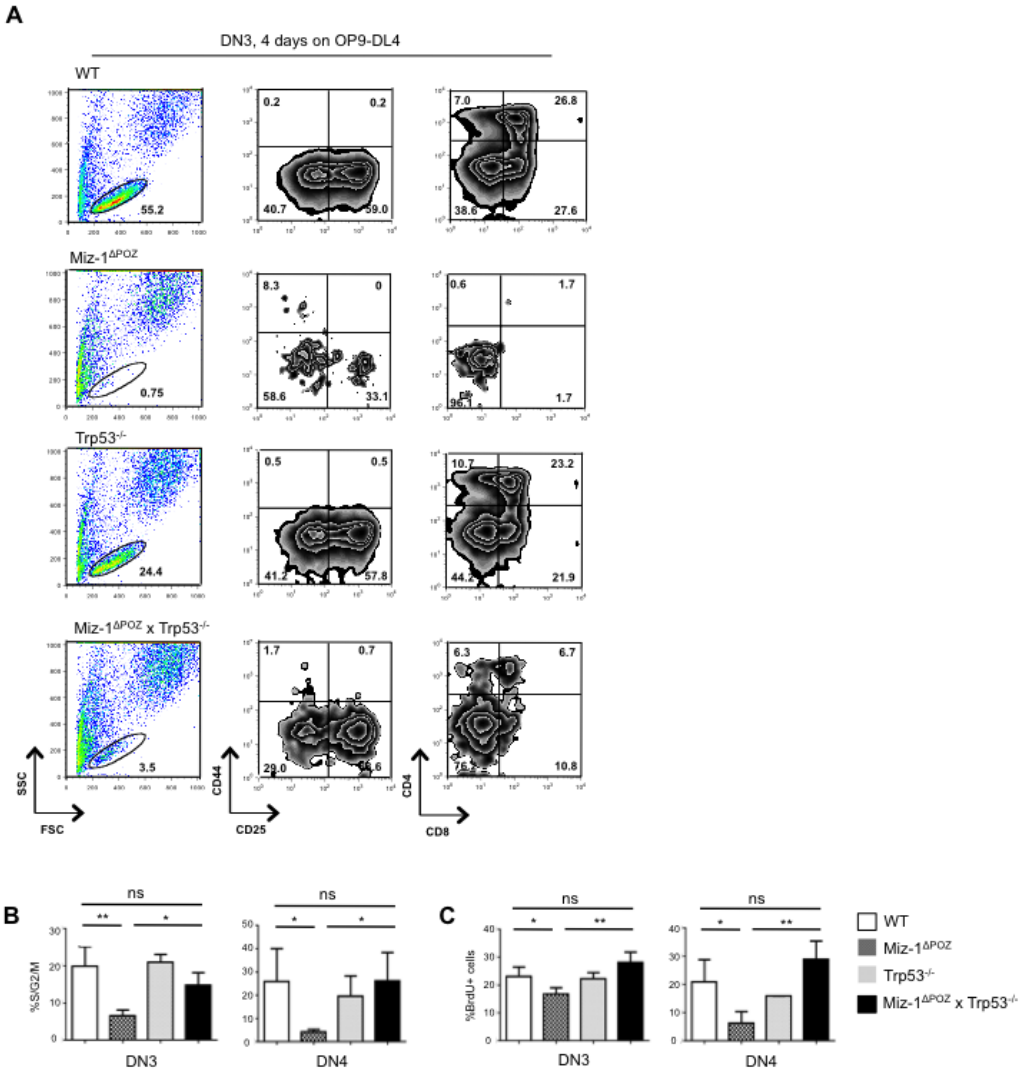


**Figure S4.1** Deletion of p53 restores the ability of Miz-1$^{\Delta POZ}$ pre-T cells to differentiate *in vitro*. (A) DN3 pre-T cells from WT, Miz-1$^{\Delta POZ}$, *Trp53*$^{-/-}$ and Miz-1$^{\Delta POZ}$ x *Trp53*$^{-/-}$ mice were sorted onto OP9-DL4 and analyzed for CD25, CD44, CD4 and CD8 surface expression after 4 days in culture. Data are representative of three independent experiments. (B) Cell cycle analysis using propidium iodide (PI) staining performed on sorted, permeabilized DN3 and DN4 cells. Graph shows percentage of cells in S/G2/M phases of the cell cycle. Data are averaged from three independent experiments and are presented as mean ± SD. (C) Cell cycle analysis after *in vivo* BrdU labeling. Graph shows percentage of BrdU$^{+}$ DN3 and DN4 cells. Data are averaged from three independent experiments and are presented as mean ± SD.

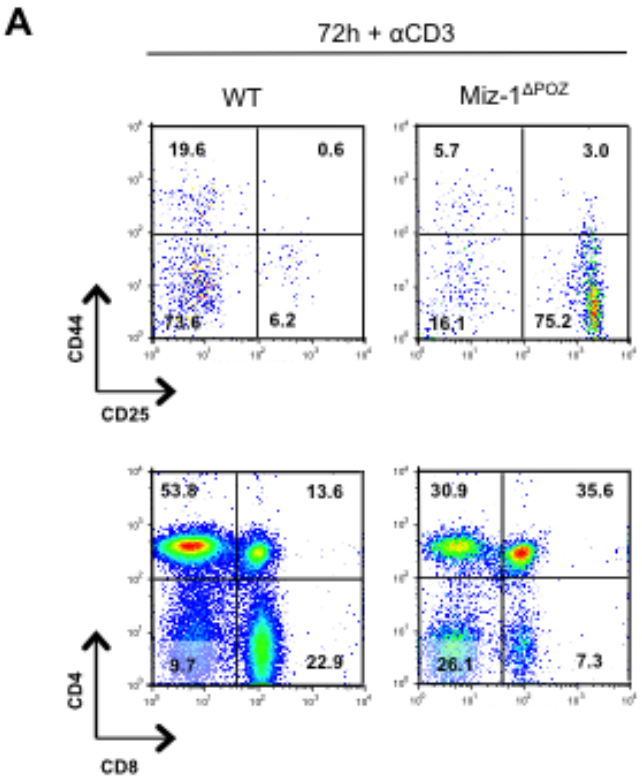


**Figure S4.2** Mitogenic stimulation of WT and Miz-1$^{\Delta POZ}$ pre-T cells. (A) FACS analysis of WT and Miz-1$^{\Delta POZ}$ pre-T cells 72h after injection with αCD3.

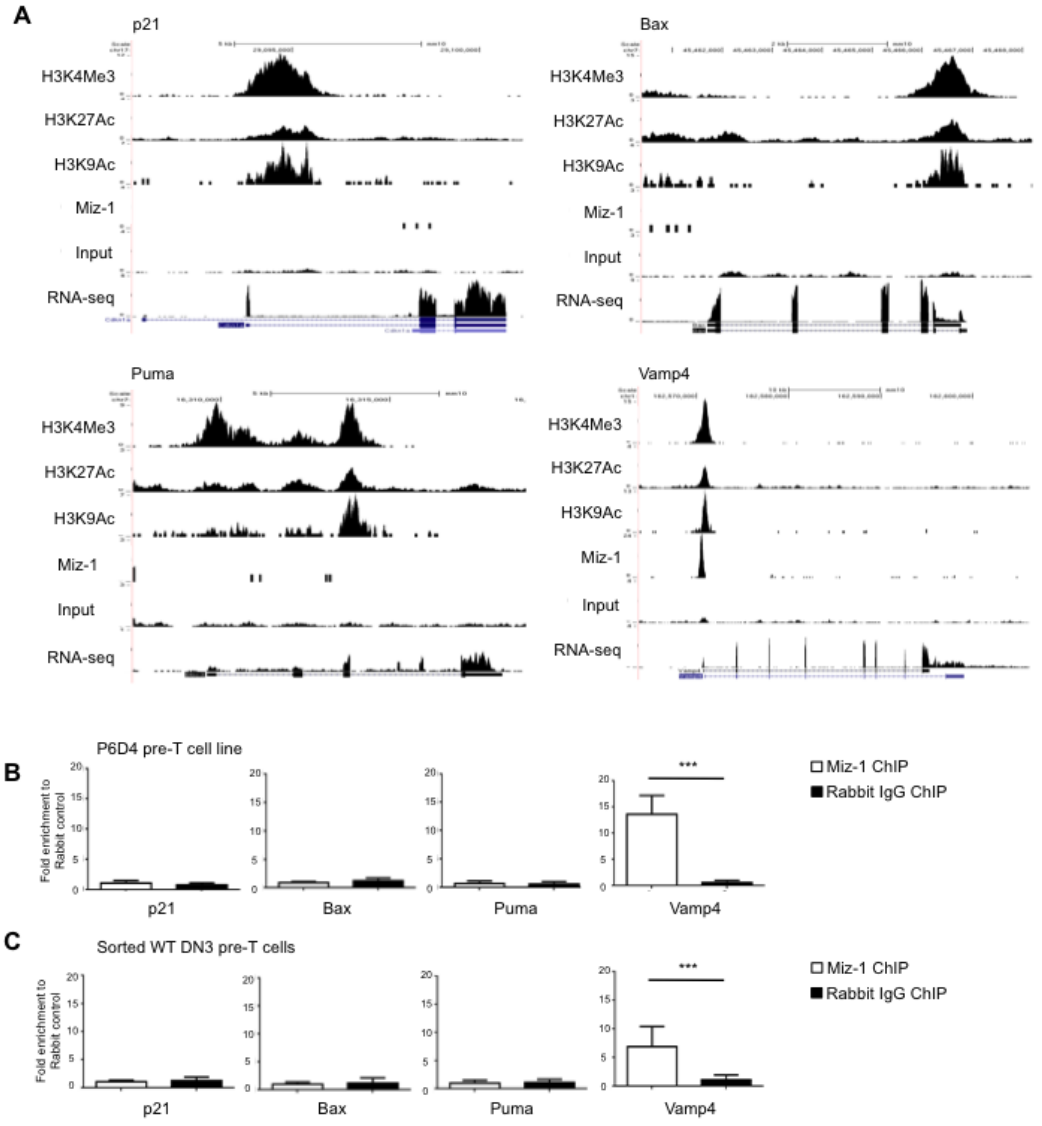


**Figure S4.3** Miz-1 does not directly regulate the expression of p53 target genes in DN3 pre-T cells. (A) ChIP-seq experiments for Miz-1 and histone activation marks (H3K4Me3, H3K27Ac, H3K9Ac) in P6D4 murine pre-T cells. Shown are p53 target genes (*p21*, *Bax*, *Puma*) and a positive control for Miz-1 binding and activation (*Vamp4*). Scale is in number of reads per million reads. (B) ChIP-qPCR experiments to determine possible binding of Miz-1 to the promoters of p53 target genes in murine P6D4 pre-T cells. Graph shows fold enrichment of anti-Miz-1 ChIP over Rabbit IgG control ChIP. The *Vamp4* promoter contains a Miz-1 binding site and is used as a positive control for the Miz-1 ChIP. Data are represented as average fold change ± SD from at least three independent experiments. (C) ChIP-qPCR experiments to determine possible binding of Miz-1 to the promoters of p53 target genes in sorted primary DN3 cells. Graph shows fold enrichment of anti-Miz-1 ChIP over Rabbit IgG control ChIP. Data are represented as average fold change ± SD from at least three independent experiments.

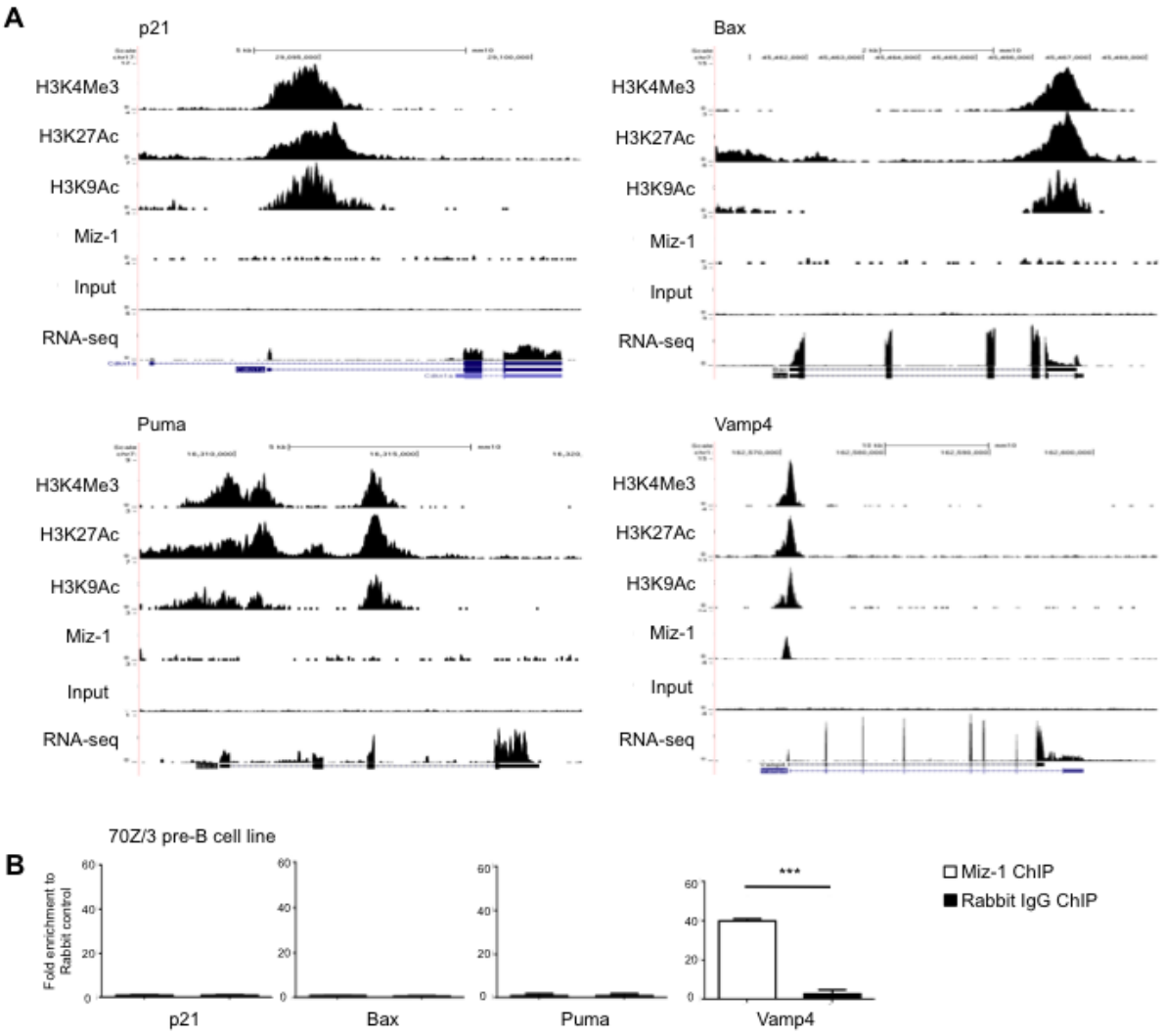


**Figure S4.4** Miz-1 does not directly regulate the expression of p53 target genes in pre-B cells. (A) ChIP-seq experiments for Miz-1 and histone activation marks (H3K4Me3, H3K27Ac, H3K9Ac) in 70Z/3 pre-B cell line. Shown are p53 target genes (*p21*, *Bax*, *Puma*) and a positive control for Miz-1 binding and activation (*Vamp4*). Scale is in number of reads per million reads. (B) ChIP-qPCR experiments to determine possible binding of Miz-1 to the promoters of p53 target genes in 70Z/3 pre-B cells. Graph shows fold enrichment of anti-Miz-1 ChIP over Rabbit IgG control ChIP. The *Vamp4* promoter contains a Miz-1 binding site and is used as a positive control for the Miz-1 ChIP. Data are represented as average fold change ± SD from at least three independent experiments.

**Table S6.1** qPCR Primer Sequences

| Primer | Sequence | Reference |
|---|---|---|
| Cdkn1a (p21) F | AGATCCACAGCGATATCCAGAC | (Li et al., 2012) |
| Cdkn1a (p21) R | ACCGAAGAGACAACGGCACACT | |
| Puma (Bbc3) F | ACGACCTCAACGCGCAGTACG | (Li et al., 2012) |
| Puma (Bbc3) R | GAGGAGTCCCATGAAGAGATTG | |
| Bax F | CAGGATGCGTCCACCAAGAA | (Li et al., 2012) |
| Bax R | AGTCCGTGTCCACGTCAGCA | |
| Gapdh F | TTCCGTGTTCCTACCCCCAATG | (Kosan et al., 2010) |
| Gapdh R | GGAGTTGCTGTTGAAGTCGCAG | |
| P53 F | AAGACAGGCAGACTTTTCGCC | (Hattangadi et al., 2010) |
| P53 R | CGGGTGGCTCATAAGGTACC | |
| Actin F | CTCTGGCTCCTAGCACCATGAAGA | (Stephens et al., 2011) |
| Actin R | GTAAAACGCAGCTCAGTAACAGTCCG | |
| Rpl22 F | AGGTGCCTTTCTCCAAAAGGTATT | This study |
| Rpl22 R | AAACCACCGGTTTTGTTCCT | |
| Rpl22l1 F | TGGAGGTTTCATTTGGACCTTAC | (O'Leary et al., 2013) |
| Rpl22l1 R | TTTCCAGTTTTTCCATTGACTTTAAC | |

**Table S6.2** ChIP-qPCR Primer Sequences

| Primer | Sequence | Reference |
|---|---|---|
| Cdkn1a (p21) F | CGCTGCGTGACAAGAGAATA | (Khandanpour et al., 2013) |
| Cdkn1a (p21) R | CCTCCCCTCTGGGAATCTAA | |
| Puma (Bbc3) F | CTTGTGCCCCAGCTTTCAT | (Khandanpour et al., 2013) |
| Puma (Bbc3) R | GAGTCCCAGGTGCTTCCTTC | |
| Bax F | CGGCAATTCTGCTTTAACCT | (Khandanpour et al., 2013) |
| Bax R | CGCCCCCATTATTTCTTCTT | |
| Gapdh F | GTGTTCCTACCCCCAATGTG | This study |
| Gapdh R | GGAGACAACCTGGTCCTCAG | |
| Vamp4 F | AGTCACCCTTTCAGCTCCAG | This study |
| Vamp4 R | TCAGATCCGATGGAGGAGCA | |
| Rpl22_2 F | TCCCTGAGTCATTCGCAGT | This study |
| Rpl22_2 R | CTTTTCCCAGGGCGAAGT | |
| Rpl22_3 F | CAGTTCCTAACTGGCGTTGG | This study |
| Rpl22_3 R | AGCCTCAGCCCAGAGAATG | |

# CHAPTER III

# Notch1-induced T cell leukemogenesis requires the c-Myc cofactor and BTB-POZ domain protein Miz-1 to control the activation of p53

As described in Chapter II, the transcription factor Miz-1 regulates T cell development via transactivation of the ribosomal protein L22 (Rpl22), which then regulates p53 translation. Because Miz-1 exerts this function at DN3, where T cell transformation is thought to occur, we sought to determine the role of Miz-1 in T cell leukemogenesis. In this manuscript, we identify Miz-1 as a key regulator of p53 during Notch1-induced T cell leukemogenesis and propose that Miz-1 would be a viable target for drug development in the treatment of T-ALL.

Chapter III presents original work to be submitted for publication following submission of the thesis.

# Notch1-induced T cell leukemogenesis requires the c-Myc cofactor and BTB-POZ domain protein Miz-1 to control the activation of p53

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# 1.0 Abstract

T cell acute lymphoblastic leukemia/lymphoma is an aggressive disease mainly affecting children and adolescents. Relapse rates are high and toxicity due to treatment at a young age causes many health problems later in life, underlining the need for targeted therapy. We show here that a strong counter selection occurs during Notch1-induced T cell leukemogenesis against the loss of Miz-1, a c-Myc cofactor, since Miz-1-deficient mice succumb to the disease with a significantly longer latency period than WT animals. This likely occurs through regulation of p53 activity, since mice deficient for both Miz-1 and p53 develop T-ALL at a rate similar to $p53^{-/-}$ mice. Based on these results, we conclude that Miz-1 is required for the efficient development of T-ALL and that loss of Miz-1 significantly hinders T-ALL development in part through the inability of these cells to repress p53 activity and signaling.

# 2.0 Introduction

T cell acute lymphoblastic leukemia/lymphoma (T-ALL) is an aggressive hematopoietic malignancy that affects adults, but mainly arises in children. It accounts for 10-15% of pediatric and 25% of adult cases (Ferrando et al., 2002). While the clinical outcome of this disease has greatly improved with the introduction of intensified chemotherapy regimens, relapse rates remain high and prognosis following relapse is poor (Oudot et al., 2008). T-ALL patients are most often children whose bodies are still developing at the time of treatment. These patients often suffer from therapy-induced side effects following treatment as well as later in life (Armstrong et al., 2009; Feig, 2001; Mertens et al., 2001; Moller et al., 2001; Neglia et al., 2001). Targeted therapies are therefore needed in order to increase survival rates and reduce therapy-induced morbidity and mortality.

There are many types of chromosomal abnormalities that can occur to induce the development of T-ALL, the most common of which include mutations inducing activation of Notch1, which occur in over 50% of T-ALL cases (Weng et al., 2004). Hyperactivation of the Notch1 pathway, for instance by truncated forms of Notch1 that represent only the intracellular part (ICN) can initiate T-cell leukemogenesis. One commonly used experimental model for the induction of T-ALL in mice is the overexpression of the intracellular portion of Notch1 (ICN1), which mimics the situation that occurs in human T-ALL. In this model, a GFP-expressing virus is produced containing human ICN1 lacking the PEST domain. Therefore, ICN1 cannot be targeted for proteasomal degradation and this hyperactivation of Notch1 leads to leukemia in 100% of the animals infected with the virus (Pear et al., 1996).

Notch1 signaling leads to activation of the proto-oncoprotein c-Myc, a direct Notch1 target gene in T-ALL (Herranz et al., 2014). Moreover, amplification of c-Myc is among the most common alterations in cancer genomes and on average 50% of human cancers display increased c-Myc expression (Beroukhim et al., 2010; Vita and Henriksson, 2006). This can be attributed to the ability of c-Myc to regulate many cellular processes including proliferation, cell cycle progression, DNA replication, metabolism, and apoptosis, which are often deregulated in cancer cells (Reviewed in Tansey,2014). And while c-Myc would be an attractive target for drug development because of its prominent role in disease, transcription factor inhibitors remain elusive (Darnell, 2002). c-Myc inhibitors including Omomyc, a dominant negative c-Myc peptide which competitively binds c-Myc, have been proposed to treat c-Myc amplified cancers (Delmore et al., 2011; Savino et al., 2011; Soucek et al., 2008). However, these drugs have not yet been proven

clinically efficient, and it therefore remains necessary to determine other ways to target the functions of c-Myc.

The transcription factor Miz-1 (Myc-Interacting Zinc-finger protein-1) is a BTB-POZ domain zinc finger transcription factor that was first described as an interaction partner for c-Myc (Peukert et al., 1997). While c-Myc is mainly known as an activator of gene transcription in association with its partner Max, the interaction of c-Myc with Miz-1 represses target gene transcription (Peukert et al., 1997). Furthermore, Miz-1 alone transactivates target genes by recruiting p300 and other co-activators to promoter regions (Staller et al., 2001). While Miz-1 has been implicated in skin carcinogenesis and neuroblastoma, a role of this protein has yet to be determined in hematopoietic malignancies (Honnemann et al., 2012; Inoue et al., 2013; Iraci et al., 2011). Miz-1 plays an essential role in early T cell development, specifically at the DN3 stage where V(D)J recombination occurs (Rashkovan et al., 2014; Saba et al., 2011a). The ability of Miz-1 to regulate p53 activity at the DN3 stage, where T cell transformation is thought to occur, prompted us to explore the role of Miz-1 in the initiation of T-ALL.

In this study, we show that Miz-1 is required for the development of Notch1-induced T-ALL, since loss of Miz-1 significantly increases leukemia latency and significantly decreases leukemia incidence in mice transplanted with ICN1-transduced T cells. Furthermore, we show that this effect of Miz-1 ablation is lost in cells that are also lacking p53, indicating that Miz-1 regulates p53 signaling during T cell transformation. Similarly abrogation of the Miz-1/c-Myc interaction significantly increases ICN1-induced leukemia latency, indicating that binding to Miz-1 is necessary for Myc to exert some of its oncogenic functions. Finally, we show that Miz-1 is highly

expressed in some primary T-ALL patient samples, as well as in T-ALL cell lines. The strong requirement for Miz-1 in Notch1-induced T-ALL formation in mice suggests that this c-Myc cofactor could represent an attractive target for therapies to treat T-ALL.

# 3.0 Results

## 3.1 Miz-1 regulates normal T cell development

To determine the role of Miz-1 in T-cell leukemia, we chose to inactivate it or to disrupt its ability to interact with c-Myc during the process of leukemogenesis. We used animals that had previously been generated using gene targeting, in which Miz-1 is non-functional, because the sequences that encode its BTB-POZ domain are either deleted in all hematopoietic lineages (Miz-$1^{fl/fl}$ x Vav-Cre mice) or during T cell development from the DN3 stage onwards (Miz-$1^{fl/fl}$ x Lck-Cre mice) (Kosan et al., 2010) (Figure S3.1A). We also used $Myc^{V394D}$ knockin mice, in which the Miz-1 binding site in c-Myc is mutated in such a way that Miz-1 is no longer able to bind c-Myc (Herold et al., 2002; Kosan et al., 2010). Loss of Miz-1 function in all hematopoietic lineages had a profound effect on T cell development and both thymic and splenic cellularity are reduced as previously described (Rashkovan et al., 2014; Saba et al., 2011a; Saba et al., 2011b) (Figure S3.1B,C). However, if Miz-1 deletion occurs at or after the DN3 pre-T cell stage (Miz-$1^{fl/fl}$ x Lck-Cre mice), the effect is less severe and no significant changes in T cell subsets in both the thymus and spleen are detected (Figure S3.1B,C). We found that, while the Miz-1 POZ domain deletion was complete in all sorted T cell subsets in Miz-$1^{fl/fl}$ x Vav-Cre mice, Cre-mediated recombination was less efficient in Miz-$1^{fl/fl}$ x Lck-Cre mice, even when one allele of the Miz-1 POZ domain was

deleted in the germline, possibly accounting for the lack of phenotypic differences compared to WT (Figure S3.1D).

## 3.2 Miz-1 is required for Notch1-induced T-ALL in mice

Lineage negative cells from WT, Miz-1$^{fl/fl}$ x Vav-Cre, Miz-1$^{fl/fl}$ x Lck-Cre and Myc$^{V394D}$ bone marrow were isolated, infected with MigR1-ICN1 and GFP$^{+}$ (i.e. ICN1-expressing) cells were transplanted into lethally irradiated hosts. Mice transplanted with Miz-1$^{fl/fl}$ x Vav-Cre cells exhibited significantly increased leukemia latency compared to WT (Figure 3.1A, top survival curve). Furthermore, leukemia incidence was significantly decreased in Miz-1$^{fl/fl}$ x Vav-Cre mice as only 4 animals developed T cell leukemia (n = 17), and one of these mice was only found to have leukemia (defined as splenomegaly and the presence of GFP$^{+}$ cells in lymphoid organs) upon dissection of the mouse at the end of the experiment (Day 100). In mice transplanted with Miz-1$^{fl/fl}$ x Lck-Cre cells, leukemia was able to develop from cells that had both completely (n = 4) and incompletely (n = 7) lost expression of the Miz-1 POZ domain, and in both cases, leukemia development was significantly delayed compared to WT controls (Figure 1A, middle survival curve, Figure 3.1B). In this model, both carrier cells and residual cells from the transplanted host do not contain floxed Miz-1 POZ domain encoding sequences, making complete excision of the POZ domain in leukemic cells easier to detect (Figure 3.1B). However, a significant number of mice transplanted with Miz-1$^{fl/fl}$ x Lck-Cre cells did not develop leukemia and were sacrificed at 100 days post-transplantation (n = 9). These mice did not display signs of illness, and neither GFP$^{+}$ nor Miz-1 POZ floxed or deleted alleles were detectable in lymphoid organs (data not shown). T-ALL onset was also significantly delayed in the Myc$^{V394D}$ model with a median leukemia latency of 70 days in these mice compared to 35 days in WT mice (Figure 3.1A, bottom survival curve). Furthermore, only 9 mice (n = 12) developed T-ALL, and two of these mice did not display signs

of illness prior to the end of the experiment, and leukemia was only discovered upon final dissection at day 100. These results indicate that, in this setting, both the interaction between Miz-1 and c-Myc, and the presence of Miz-1 itself, are necessary for an efficient malignant transformation by activated Notch1.

ICN1-induced leukemia developed with increased latency and decreased incidence in Miz-1-deficient animals compared to WT, but the phenotypical characteristics of these leukemic cells were similar to WT controls (Figure 3.1C,D). Moreover, dissemination of white blood cells (WBCs) and blasts in the blood, and the percentage of Notch1-expressing (i.e. $GFP^+$) cells in the thymus, bone marrow, spleen and blood was not significantly different between all genotypes (Figure S3.2A,B). Spleen weight and cell count also did not vary significantly between samples (Figure S3.2C). These results directly implicate Miz-1 in ICN1-mediated T-ALL, and also demonstrate the critical function of the Miz-1/c-Myc interaction in the development of this disease.

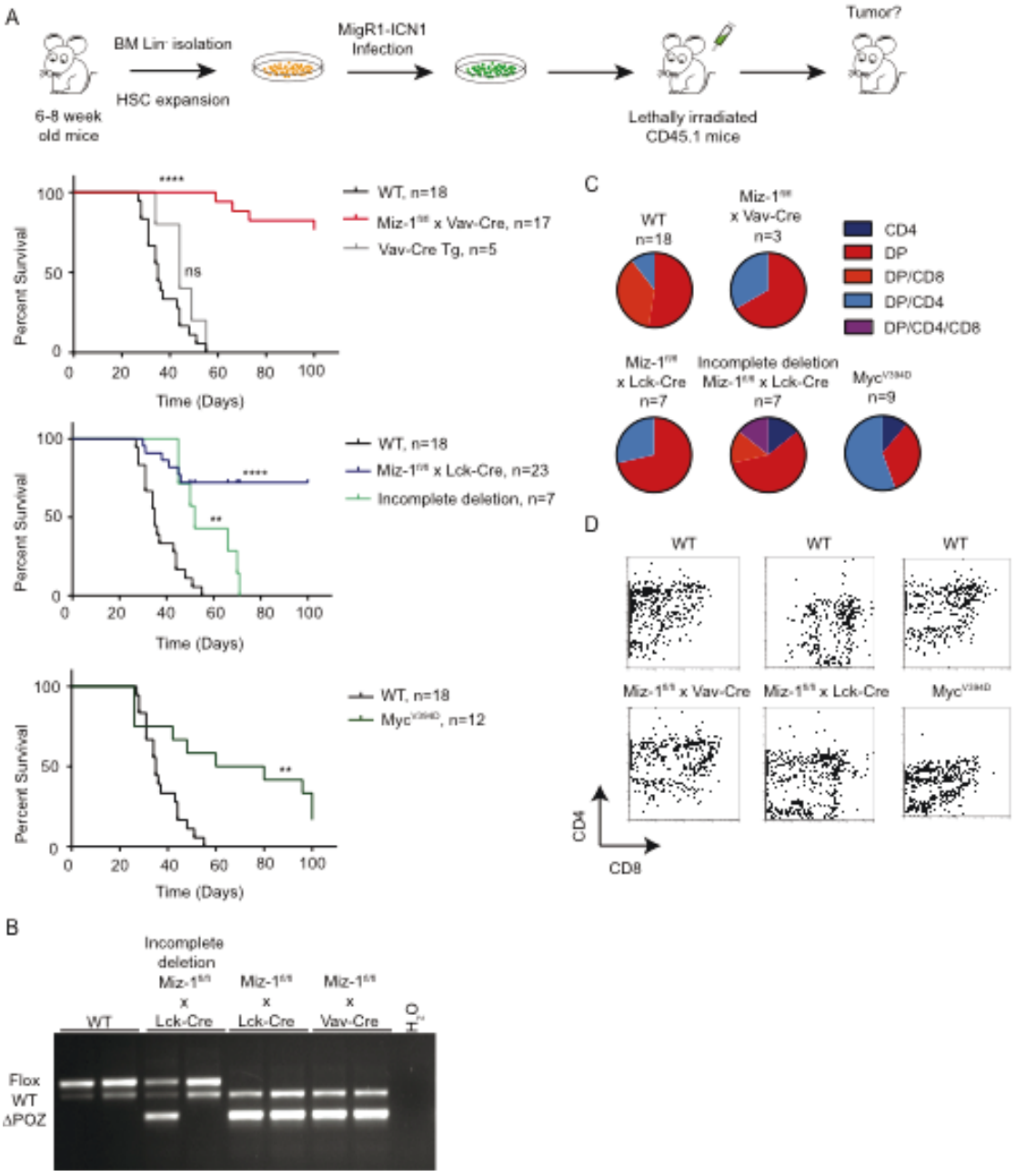


**Figure 3.1** Loss of Miz-1 increases the latency of Notch1-induced T-ALL. (A) Scheme of ICN1 leukemia induction. Kaplan-Meier survival curves of mice transplanted with ICN1-infected WT cells compared to Miz-$1^{fl/fl}$ x Vav-Cre and Vav-Cre Tg (top), Miz-$1^{fl/fl}$ x Lck-Cre (middle) and $Myc^{V394D}$ (bottom) cells. (B) PCR genotype analysis of the Miz-1 POZ domain in leukemic tissues. (C) Phenotype of T-ALL from (A) assessed by flow cytometry. (D) Representative flow cytometric analysis of leukemic spleen cells from (A).

## 3.3 Loss of p53 abrogates the effect of Miz-1 on T cell leukemogenesis

We have previously shown that Miz-1 controls p53 activation through the regulation of Rpl22 in DN3 pre-T cells (Rashkovan et al., 2014). It is well established that loss of p53 induces the development of a spectrum of malignancies in mice, however T cell lymphomas develop preferentially (Donehower et al., 1992). To test the link between Miz-1 and p53 in such a setting, we generated animals that had both deletions of the Miz-1 POZ domain and were p53-deficient. We found that Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$ mice developed malignancies with the same latency period as $p53^{-/-}$ mice (Figure 3.2A, top right graph). Furthermore, looking solely at T cell malignancies, we found that there was no significant difference in the survival of Miz-1/p53-deficient mice and single p53-deficient animals with a WT Miz-1 gene (Figure 3.2A, top left graph). We obtained the same results in Miz-$1^{fl/fl}$ x Lck-Cre x $p53^{-/-}$ mice compared to $p53^{-/-}$ mice (Figure 3.2A, bottom graphs). The T cell leukemia that developed in both sets of mice were also phenotypically similar (Figure 3.2B). There were no significant differences in dissemination of WBCs and blasts in the blood, as well as the size of the thymic tumors and the percentage of leukemic cells in the BM and spleen (Figure S3.3A-C). Furthermore, ICN1-mediated leukemia induction in mice deficient for p53 was not significantly different from mice deficient for both Miz-1 and p53 (Figure 3.2C). T-ALL from both sets of mice developed with a similar latency and 100% penetrance. The leukemia that developed in both sets of mice was phenotypically similar (Figure 3.2D) and did not display significant differences in the dissemination of WBCs and blasts in the blood (Figure S3.3D). The spleen size and cellularity as well as the percentages of $GFP^{+}$ cells in the thymus, BM, spleen and blood were similar between p53-deficient and p53/Miz-1 double deficient leukemia (Figure S3.3E,F). Taken together, these data underline a role of Miz-1

in regulating the p53 pathway during the development of T cell leukemia. These results also indicate that Miz-1 is no longer required for T cell lymphomagenesis once p53 is deleted.

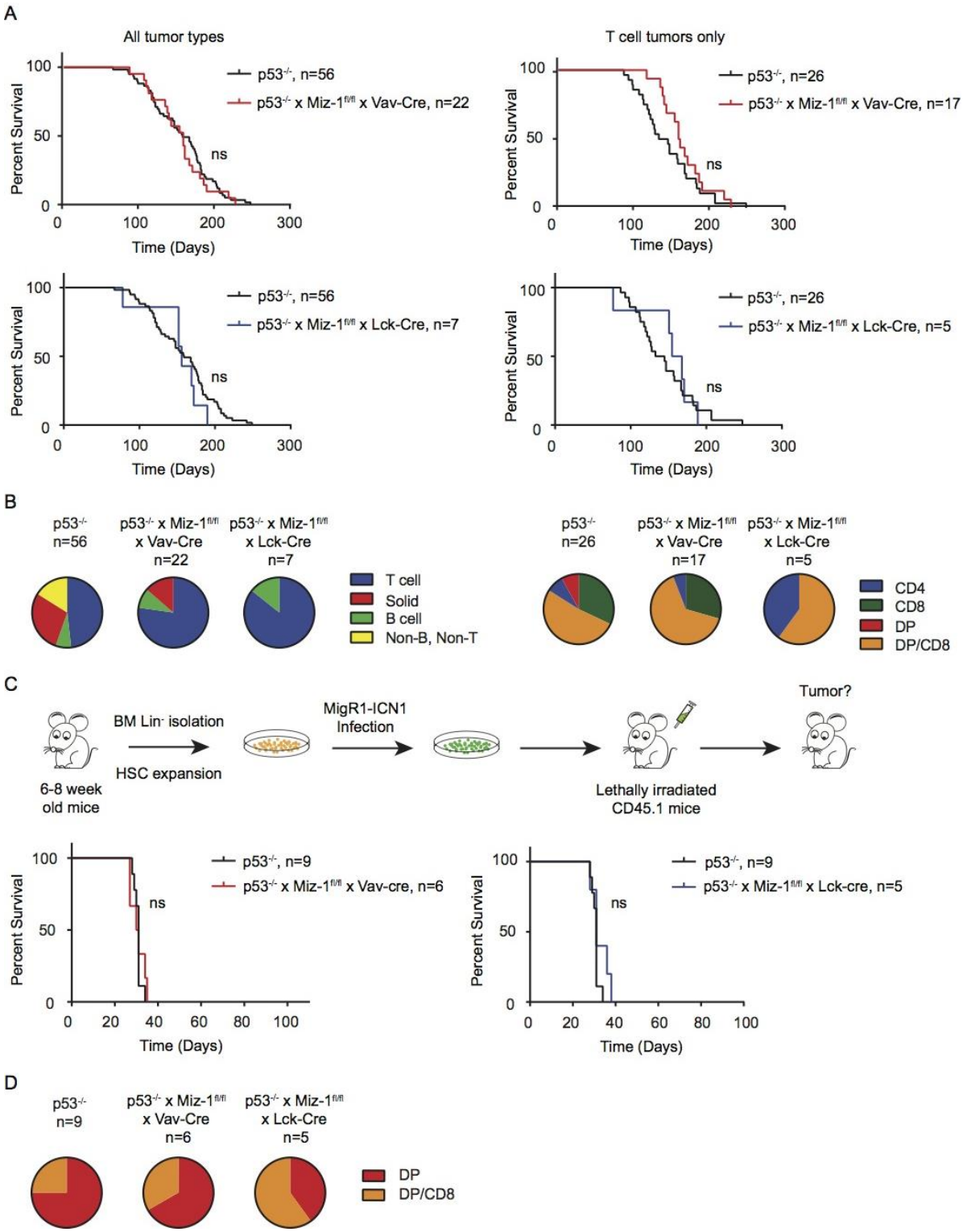


**Figure 3.2** Loss of Miz-1 does not affect the latency of p53-induced T-ALL. (A) Scheme of tumor induction using p53-deficient mice. Kaplan-Meier survival curve of $p53^{-/-}$ mice compared to $p53^{-/-}$ x Miz-$1^{fl/fl}$ x Vav-Cre (top) or $p53^{-/-}$ x Miz-$1^{fl/fl}$ x Lck-Cre

(bottom) mice for all tumor types (left) or T cell tumors only (right). (B) Phenotype of tumors from (A) assessed by flow cytometry. (C) Scheme of ICN1 leukemia induction and Kaplan-Meier survival curve of mice transplanted with ICN1-infected $p53^{-/-}$ cells compared to $p53^{-/-}$ x Miz-$1^{fl/fl}$ x Vav-Cre (left) or $p53^{-/-}$ x Miz-$1^{fl/fl}$ x Lck-Cre cells (right). (D) Phenotype of tumors from (C).

## 3.4 Miz-1-deficient leukemic T cells do not activate the p53 signaling pathway

As previously reported and confirmed here, loss of the Miz-1 POZ domain using a Vav-Cre recombinase leads to increased expression of p53 target genes in DN3 pre-T cells (Rashkovan et al., 2014) (Figure 3.3A). However, Miz-$1^{fl/fl}$ x Lck-Cre DN3 cells do not express abnormal levels of these target genes, possibly because deletion of the Miz-1 POZ domain occurs too late in T cell development for Miz-1 to regulate p53 (Figure 3.3A). Furthermore, abrogation of the Miz-1/c-Myc interaction also does not influence the expression of these p53 target genes, consistent with the fact that the regulation of p53 by Miz-1 is independent of c-Myc (Figure 3.3A). Miz-1-deficient leukemic cells, however, no longer showed upregulation of p53 target genes, since for instance the expression of p21, Bax and Puma in these cells relative to *Gapdh* expression was similar to the levels found in control leukemic cells (Figure 3.3B). This suggested that leukemia that develops from Miz-1-deficient cells have been selected to overcome the activation of the p53 pathway caused by the loss of Miz-1 in non-malignant cells. Based on these results, RNA-seq data from ICN1-induced WT, Miz-$1^{fl/fl}$ x Vav-Cre, Miz-$1^{fl/fl}$ x Lck-Cre and $Myc^{V394D}$ leukemic cells was subjected to Gene Set Enrichment Analysis (GSEA). Using this program, it is possible to determine the pathways that are up- or downregulated between two sets of leukemic samples based on even slight changes in gene expression (Subramanian et al., 2005). We found no significant differences in p53 target gene expression in Miz-1 deficient leukemic cells compared to leukemic cells that carried a WT Miz-1 allele (Figure 3.3C). This is in contrast to the upregulation of the

p53 signaling pathway observed when gene expression data from WT and Miz-1$^{fl/fl}$ x Vav-Cre normal non-malignant DN3 cells was subjected to GSEA (Figure 3.3C). Because we had previously found increased levels of p53 protein in Miz-1-deficient thymocytes compared to WT, we measured the levels of total p53 protein in the leukemic samples. However, consistent with a lack of increased signaling downstream of p53 in Miz-1-deficient leukemic cells, we found no significant differences in p53 protein expression (Figure 3.3D). We then measured the expression of *Rpl22* in ICN1-induced leukemic samples, as Miz-1 acts via activation of Rpl22 to regulate p53 in normal thymocytes. We found that the levels of *Rpl22* mRNA were still significantly decreased in Miz-1-deficient samples compared to WT samples, as in non-malignant DN3 cells (Figure 3.3E). These results indicate that, in the context of Notch1-induced T cell leukemia, loss of Miz-1 probably prevents leukemia development by increasing signaling via the p53 pathway, inducing apoptosis. However, some Miz-1-deficient leukemias are able to develop even when Rpl22 levels are low, indicating that there is probably a mechanism by which these leukemic cells are able to bypass the p53-mediated apoptosis normally induced by low levels of Rpl22.

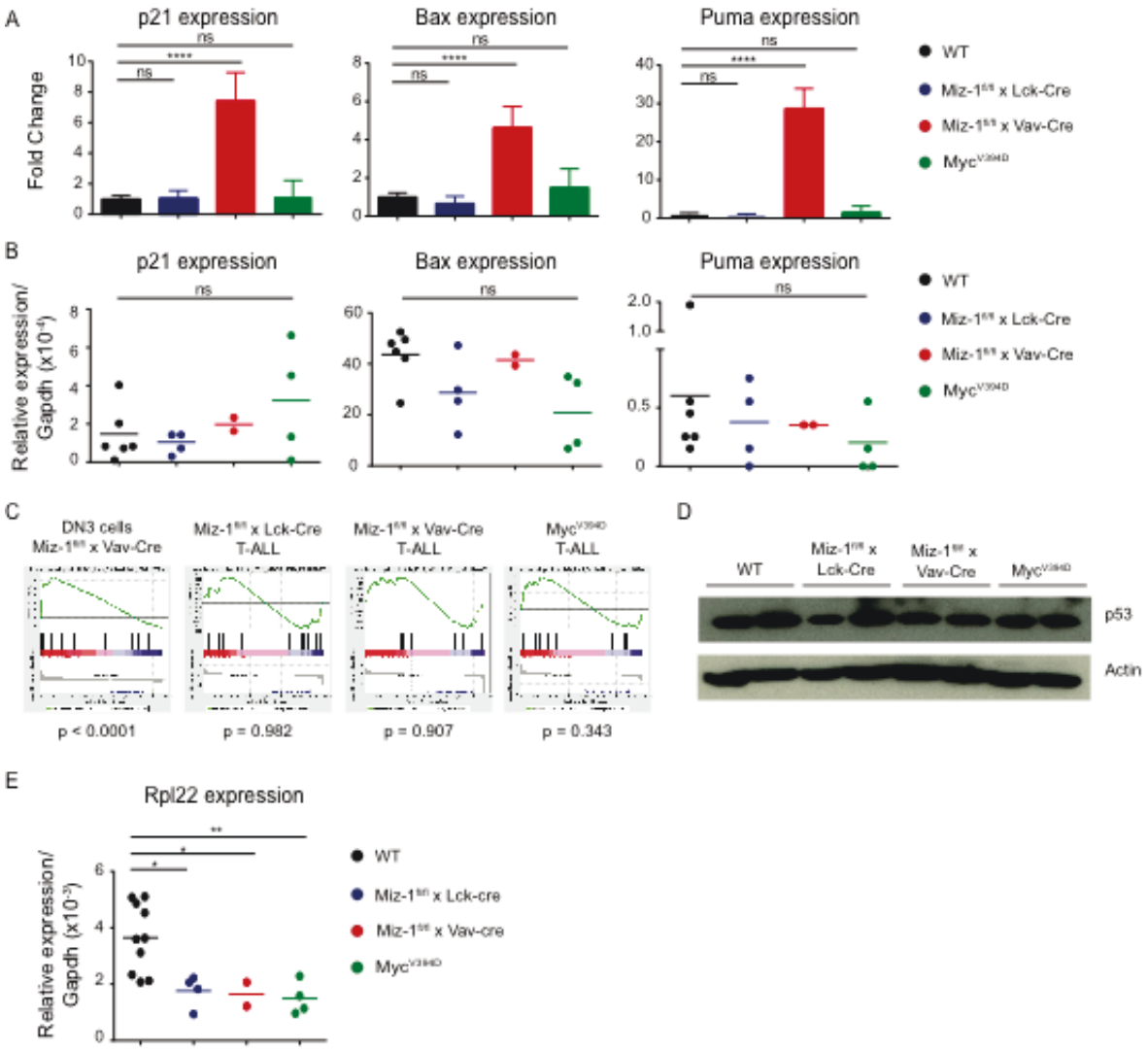


**Figure 3.3** The p53 pathway is not hyperactive in Miz-1-deficient T-ALL. (A) Expression of the p53 target genes p21, Bax and Puma in Miz-$1^{fl/fl}$ x Lck-Cre and Miz-$1^{fl/fl}$ x Vav-Cre DN3 cells compared to WT. Data are averaged from three independent experiments and are presented as mean fold change relative to *Gapdh* ± SD. p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA. (B) Expression of the p53 target genes p21, Bax and Puma in ICN1 samples of indicated genotypes. Data are presented as relative expression compared to *Gapdh.* p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA. (C) GSEA analysis for p53 pathway genes using array data from Miz-$1^{fl/fl}$ x Vav-Cre DN3 cells vs. WT DN3 cells (left). GSEA analysis for p53 pathways genes for RNA-seq data from indicated ICN1 samples compared to WT (right). (D) WB for p53 expression in two ICN1-induced WT, Miz-$1^{fl/fl}$ x Lck-Cre, Miz-$1^{fl/fl}$ x Vav-Cre and $Myc^{V394D}$ samples. Actin is used as a loading control. (E) *Rpl22* mRNA expression in indicated ICN1 sample phenotypes. Data are presented as relative value compared to *Gapdh*. p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA.

## 3.5 Miz-1-deficient ICN1 leukemic cells upregulate DNA synthesis and replication pathways

We next performed GSEA using RNA-Seq datasets from WT, Miz-1$^{fl/fl}$ x Vav-Cre, Miz-1$^{fl/fl}$ x Lck-Cre and Myc$^{V394D}$ leukemic cells induced by ICN1 overexpression to determine the pathways involved in leukemia initiation. We found upregulation of genes involved in DNA replication and repair as well as cell cycle regulation pathways (Table S3.1, Table S3.2). Most of these pathways are downregulated in normal Miz-1$^{fl/fl}$ x Vav-Cre DN3 cells compared to WT, but are significantly upregulated in Miz-1-deficient leukemic samples compared to WT and in Myc$^{V394D}$ leukemic cells compared to WT (Figure 3.4A,B). However, there are no significant differences in the expression of genes in these pathways between WT and Miz-1-deficient leukemic cells when p53 is inactivated (Figure 3.4B). Using the GSEA program, we looked at the expression of genes in the leading edge of DNA replication, repair and cell cycle regulation pathways. Genes in the leading edge of enriched gene sets are those that drive the pathway to be significantly up or downregulated in one sample compared to another. Furthermore, a gene that appears in the leading edge subsets of several GSEA gene sets is likely of higher significance for the mechanisms by which leukemic cells are selected in the absence of Miz-1 and thus indicate more closely the biological changes associated with it than genes that are in only a few of the leading edge subsets. Using this analysis, we found several groups of genes upregulated in Miz-1-deficient T-ALL samples compared to WT (Figure 3.4C). For instance, we found upregulation of MCM proteins, which are known to interact with c-Myc, as well as replication factor genes, such as RFC2-5, and DNA polymerase genes including POLD2 and POLD4. This indicates that, in order to overcome p53 activation, ICN1-induced Miz-1-deficient leukemic cells must upregulate genes involved in DNA replication, elongation DNA damage repair, activation of ATR, and the cell cycle.

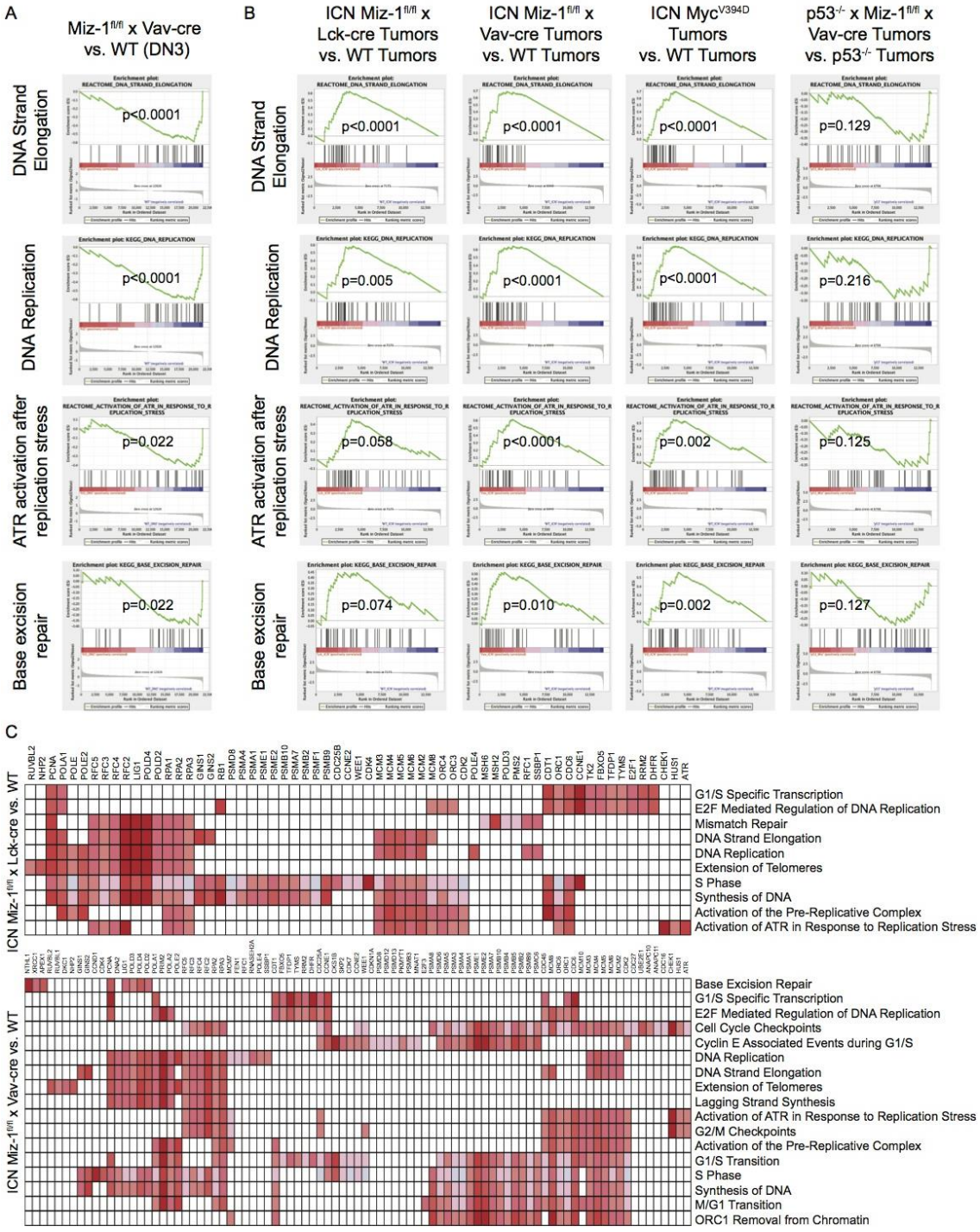


**Figure 3.4** Miz-1-deficient ICN1 T-ALL samples express increased DNA replication-associated genes. (A) GSEA analysis of Miz-1$^{fl/fl}$ x Vav-Cre DN3 cells compared to WT for genes involved in DNA strand elongation, DNA replication, ATR activation and Base Excision Repair. (B) GSEA analysis for genes involved in DNA strand elongation, DNA replication, ATR activation and Base Excision Repair in Miz-1$^{fl/fl}$ x

Lck-Cre ICN1 T-ALL, Miz-1$^{fl/fl}$ x Vav-Cre ICN1 T-ALL, Myc$^{V394D}$ ICN1 T-ALL and p53$^{-/-}$ x Miz-1$^{fl/fl}$ x Vav-Cre T-ALL compared to WT or p53$^{-/-}$ T-ALL, respectively. (C) Leading Edge Analysis for pathways involved in DNA replication, DNA damage repair and the cell cycle for Miz-1$^{fl/fl}$ x Lck-Cre ICN1 samples vs. WT (top) and Miz-1$^{fl/fl}$ x Vav-Cre samples vs. WT (bottom). Genes in red are in the leading edge of the gene sets analyzed and are therefore highly upregulated in Miz-1-deficient samples.

## 3.6 Loss of Miz-1 enhances signaling downstream of c-Myc

To rule out deficiencies in the Notch1 pathway contributing to the increased latency of Miz-1-deficient T-ALL, we looked at the activity of Notch1 in normal Miz-1$^{fl/fl}$ x Vav-Cre DN3 pre-T cells using a Notch1 reporter mouse (TNR) (Wu et al., 2007). Using these mice, we found no differences in signaling in the canonical Notch1 pathway in Miz-1$^{fl/fl}$ x Vav-Cre mice compared to WT (Figure S3.4A). Furthermore, we found that genes in the Notch signaling pathway or downstream of Notch1 activation are not significantly up or downregulated in Miz-1-deficient leukemic cells compared to WT (Figure S3.4B,C). These results indicate that signaling via Notch1 is intact in both normal Miz-1-deficient cells and Miz-1-deficient leukemic cells, and deficiencies in this pathway do not cause the observed increase in leukemia latency.

Since c-Myc is one of the primary targets of Notch1 signaling in the development of T-ALL and some functions of c-Myc require its interaction with Miz-1, we tested the expression of *c-Myc* mRNA in the ICN1 leukemic samples. Similar to the results obtained in normal DN3 pre-T cells, we found no significant differences in the level of *c-Myc* expression between Miz-1-deficient, Myc$^{V394D}$ and WT samples (Figure 3.5A). We also found that, while c-Myc protein levels are variable between T-ALL samples, there is no correlation between the genotype and the levels of c-Myc protein (Figure 3.5B). However, we found that c-Myc-regulated gene sets (i.e. gene sets containing c-Myc target genes) were highly enriched in RNA expression profiles from Miz-1-

deficient leukemic cells compared to controls, indicating that c-Myc target genes are upregulated in the absence of Miz-1 (Table S3.3). We also found enrichment of c-Myc gene sets in $Myc^{V394D}$ leukemic cells vs. controls, however these gene sets did not exclusively contain genes that are normally either activated or repressed by c-Myc.

Using previously published Miz-1 and c-Myc ChIP-seq data in mouse T cell lymphoma cells which overexpress c-Myc to identify Miz-1 and c-Myc target genes, we next looked at the expression of target genes of the Miz-1/c-Myc complex in each of the ICN1 leukemic samples compared to WT (Walz et al., 2014). Specifically, we looked at Miz-1/c-Myc co-regulated genes, which were upregulated in mutant T-ALL samples compared to WT because these genes should normally be targets of Miz-1/c-Myc-induced repression (Figure 3.5D). While only 4 genes were common to all mutant samples, 15 genes were significantly upregulated in Miz-1-deficient samples (Figure 3.5E). In this list of 15 genes, we found the paralog of Rpl22, *Rpl22l1*, whose expression is directly regulated by Rpl22, as well as *Kdm8*, a lysine histone demethylase which binds directly to the p53 DNA-binding domain (Huang et al., 2015; O'Leary et al., 2013). We saw no differences in the expression of these genes in normal DN3 cells between WT and Miz-1-deficient or $Myc^{V394D}$ samples (Figure 5E (left panel) & Figure 5F (left panel)). However, we found both *Rpl22l1* and *Kdm8* mRNA upregulated in Miz-1-deficient ICN1-induced T-ALL compared to WT, indicating that upregulation of these genes could be two mechanisms by which these tumors are able to bypass the upregulation of p53 normally found in Miz-1-deficient DN3 cells (Figure 5E (right panel) & Figure 5F (left panel)).

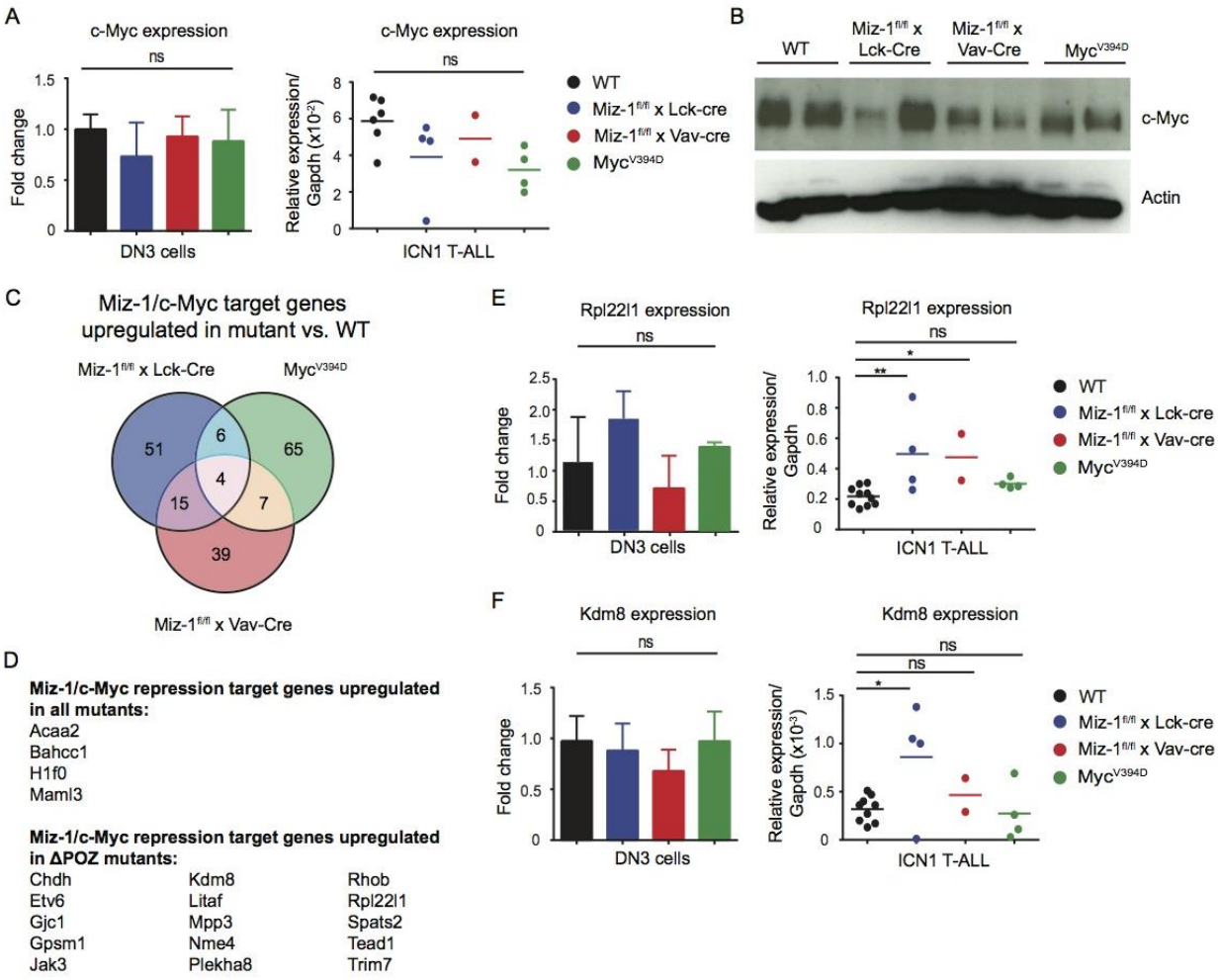


**Figure 3.5** Targets of c-Myc are upregulated in Miz-1-deficient ICN1 samples. (A) *c-Myc* mRNA expression in DN3 pre-T cells (left) and indicated ICN1 leukemia types (right). Data are presented as fold change relative to *Gapdh* (DN3 cells) or relative value compared to *Gapdh* (ICN1 T-ALL). p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA. (B) WB for c-Myc expression in two ICN1-induced WT, Miz-$1^{fl/fl}$ x Lck-Cre, Miz-$1^{fl/fl}$ x Vav-Cre and $Myc^{V394D}$ samples. Actin is used as a loading control. (C) Venn diagram indicating overlap of genes which are significantly ($p < 0.05$) upregulated in Miz-$1^{fl/fl}$ x Lck-Cre, Miz-$1^{fl/fl}$ x Vav-Cre and $Myc^{V394D}$ ICN1 samples compared to WT for targets of the Miz-1/c-Myc complex. (E) List of genes found in the overlaps from (D). (F) *Rpl22l1* mRNA expression in DN3 pre-T cells (left) and indicated ICN1 leukemia types (right). Data are presented as fold change relative to *Gapdh* (DN3 cells) or relative value compared to *Gapdh* (ICN1 T-ALL). p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA. (G) *Kdm8* mRNA expression in DN3 pre-T cells (left) and indicated ICN1 leukemia types (right). Data are presented as fold change relative to *Gapdh* (DN3 cells) or relative

value compared to *Gapdh* (ICN1 T-ALL). p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA.

## 3.7 Miz-1 is highly expressed in primary T-ALL samples compared to control samples

In order to determine whether Miz-1 could be a therapeutic target for T-ALL treatment, we analyzed the expression of Miz-1 in a cohort of healthy BM controls and T-ALL patients from stage 1 data from the Microarray Innovations in Leukemia (MILE) study (Haferlach et al., 2010; Kohlmann et al., 2008) (Figure 3.6A). Using this data, we found that Miz-1 mRNA is highly expressed in T-ALL patients compared to control BM samples. We then looked at the expression of Miz-1 in normal thymus samples compared to T-ALL samples obtained from the Quebec Leukemia Cell Bank, and common T-ALL cell lines, and found that Miz-1 expression is significantly higher in both primary T-ALL samples and T-ALL cell lines compared to normal thymus samples (Figure 3.6B). These data indicate that, while high expression of Miz-1 is not required for T-ALL formation, there are indeed some patients that do express high levels of Miz-1, who might benefit from therapy inhibiting the function of this transcription factor.

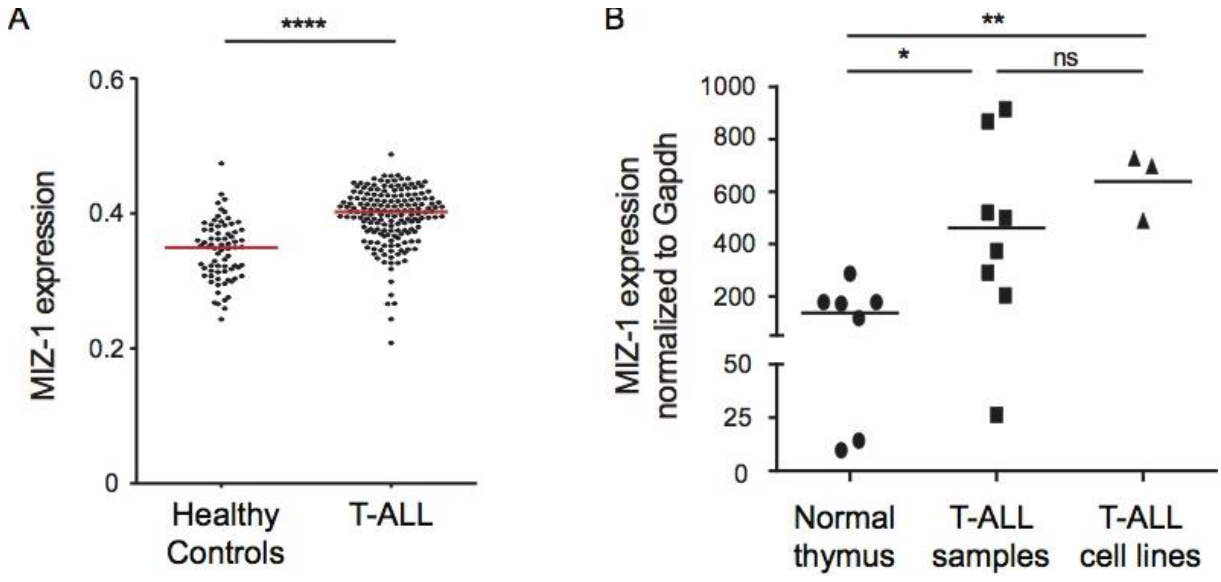


**Figure 3.6** Miz-1 is upregulated in some human T-ALL samples. (A) Miz-1 mRNA expression in healthy BM controls compared to T-ALL patients from the Microarray Innovations in Leukemia (MILE) study (GSE13159). Data are presented as normalized expression levels. p-value was calculated using a Students' t-Test. (B) Miz-1 mRNA expression in normal thymus samples, T-ALL patient samples and T-ALL cell lines. Data are presented as relative value compared to *Gapdh*. p-values were calculated using Dunnett's multiple comparison test following a one-way ANOVA.

# 4.0 Discussion

T cell leukemia is a hematopoietic malignancy that mainly affects children and young adults. While remission rates are high, current treatments are harsh and can have a harmful effect on the quality of life of those patients. It is therefore necessary to determine the effectors of T-ALL, which can be targeted by specific compounds in order to develop drugs that can be administered without the negative secondary effects of chemotherapy and radiation treatments. Here, we propose that Miz-1 could be an effector of T-ALL whose function could be targeted to decrease the toxicity of current therapies.

We have previously described a role of Miz-1 in early T cell development during β-selection, regulating p53 translation (Rashkovan et al., 2014; Saba et al., 2011a). This regulation happens during V(D)J recombination, where DNA double strand breaks arise, and where T cell transformation is thought to occur. Miz-1 is required for the development of several different types of cancer, including skin tumors and pancreatic tumors and the interaction between Miz-1 and c-Myc has been studied in the context of c-Myc-driven T cell lymphoma, but a role of Miz-1 in T cell leukemia has yet to be elucidated (Honnemann et al., 2012; Inoue et al., 2013; van Riggelen et al., 2010; Walz et al., 2014). We therefore decided to investigate the role of Miz-1 in the development of T cell leukemia using our conditional POZ domain-deficient mouse model. We also investigated the role of the Miz-1/c-Myc complex in the context of Notch1-induced T cell leukemia using the $Myc^{V394D}$ model. Here we provide evidence that loss of Miz-1 protects against the development of T-ALL by activating a p53-dependent cell death pathway.

Here, we show that bone marrow precursors from Miz-1-deficient mice are less efficient at inducing T-ALL when infected with a constitutively active Notch1. While we have previously determined that early T cell development is severely impaired when the Miz-1 POZ domain is deleted in bone marrow precursors, here we show that bypassing the earlier developmental defects by deletion of the Miz-1 POZ domain following β-selection also significantly impairs leukemia development. We also show that the interaction between Miz-1 and c-Myc is important for leukemia formation, as $Myc^{V394D}$ mice are significantly impaired in their ability to develop ICN1-induced T-ALL compared to WT. This is probably at least partially due to the fact that, in these mice, c-Myc, the major Notch1 target in T-ALL, is unable to induce cellular proliferation and other pro-leukemic functions as efficiently as cells containing WT c-Myc. Furthermore, similar to Miz-1-deficient leukemic cells, these cells upregulated genes involved in cell cycle progression, DNA replication, transcription elongation, and the DNA damage response, pathways which are known to be regulated by c-Myc (Dominguez-Sola and Gautier, 2014; Dominguez-Sola et al., 2007; Felsher et al., 2000; Lin et al., 2012; Vafa et al., 2002). Overexpression of c-Myc has also been shown to block double-stranded DNA repair, a process that we have found to be upregulated in Miz-1-deficient leukemia, as well as $Myc^{V394D}$ leukemia (Karlsson et al., 2003; Vafa et al., 2002). Loss of Miz-1 and loss of the interaction between Miz-1 and c-Myc prevents c-Myc from repressing target genes, leading to a global upregulation of c-Myc target genes in Miz-1-deficient T-ALL. However, while many c-Myc targets are upregulated in Miz-1-deficient leukemia and $Myc^{V394D}$ leukemia, we also find upregulation of genes which are targets of neither transcription factor, indicating that indirect effects of the loss of Miz-1 or disruption of the Miz-1/c-Myc complex on DNA replication also exist.

The interaction between Miz-1 and c-Myc has previously been examined using a mouse model in which either c-Myc or $Myc^{V394D}$ is overexpressed to induce T cell lymphoma (van Riggelen et al., 2010). The authors of this study found that $Myc^{V394D}$ is associated with increased senescence and activation of the TGFβ signaling pathway. Using our ICN1 model, which induces upregulation of either WT c-Myc or $Myc^{V394D}$, we do not detect deregulation of genes involved in senescence, nor in the TGFβ pathway by RNA-seq, suggesting that leukemic cells driven by Notch1 or by c-Myc are using different pathways to overcome a deficiency of forming c-Myc/Miz-1 complexes.

In normal DN3 cells, Miz-1 regulates the expression of Rpl22. Rpl22 then regulates the translation of p53, preventing cell death. In Miz-1 deficient DN3 pre T cells Rpl22 is decreased leading to elevated levels of p53 protein and activation of the p53 cell death pathway. Here, we show that like in Miz-1 deficient non-malignant DN3 pre T cells, Rpl22 levels are significantly decreased in Miz-$1^{fl/fl}$ x Vav-Cre, Miz-$1^{fl/fl}$ x Lck-Cre and $Myc^{V394D}$ ICN T-ALL compared to WT. However, the levels of p53 protein are similar between WT and Miz-1-deficient T-ALL samples. Moreover, loss of p53 completely abrogates the increased latency that occurs following loss of Miz-1 in ICN1-induced leukemia. This indicates that the ability of Miz-1 to induce T cell leukemia depends solely on the presence of p53. We therefore postulate that, in order for T cell tumors to develop in the absence of Miz-1, leukemia initiating cells must acquire the ability to overcome the activation of the p53 pathway that occurs upon loss of Miz-1 during early steps of normal T cell development. Our data suggest that activation of replicative stress (RS) pathways as well as the upregulation of genes which can regulate p53, including Rpl22l1 and Kdm8, are selected in Miz-1-deficient ICN1 leukemic cells. Cells transformed by oncogenes such as activated forms of Notch1 can display deregulated S-phase entry and as a consequence RS pathways are enhanced, because they can

stabilize replication forks and activate an intra-S-phase checkpoint in leukemic cells to delay mitosis until replication is completed. This would indicate that an anti-Miz-1 therapy could be combined with Chk1 kinase inhibitors, which can perturb replication fork stability and are already considered future drug candidates against several cancer types (Bryant et al., 2014a; Bryant et al., 2014b).

In this study, we show that loss of Miz-1 increases the latency of Notch1-induced T-ALL via the induction of p53. It has been shown that p53 is often not mutated in primary patient samples of T-ALL, and that mutation of this important tumor suppressor mainly occurs upon disease relapse (Hsiao et al., 1994). This makes activation of WT p53 an attractive therapeutic strategy. Small molecule inhibitors, which target the interaction between p53 and Mdm2, have been studied extensively and orally available analogs of nutlin, the first class of p53/Mdm2 inhibitors, have been tested in the clinic in patients with liposarcoma and acute leukemia (Ray-Coquard et al., 2012). However, adverse side effects were reported in these trials, including neutropenia and thrombocytopenia, prompting the development of a second generation of complex inhibitors with higher potency and selectivity (Vu et al., 2013). One possibility for further investigation would be the inhibition of p53 degradation using nutlins, combined with inhibition of Miz-1. This may provide an attractive way of both preventing degradation of p53 as well as increasing its translation in patients with T-ALL.

c-Myc is also an attractive target for drug development against T-ALL, because c-Myc amplification occurs in 30% of all human cancers and also in T-ALL through both Notch1 activation and chromosomal translocations (Gamberi et al., 1998; Nesbit et al., 1999). Many

inhibitors of c-Myc function and the c-Myc pathway have been developed in an attempt to target this oncogene (McKeown and Bradner, 2014). However, transcription factors are notoriously difficult to target, and inhibitors of c-Myc have mainly been designed to prevent the interaction with Max or to inhibit the BET family of bromodomains, including JQ1, to downregulate the c-Myc transcriptional program (Delmore et al., 2011; Frye, 2010; Meyer and Penn, 2008). Therefore, because Miz-1 is an important interaction partner of c-Myc, we propose that targeting Miz-1 could provide one alternative to attempting to target c-Myc directly.

Because Notch1 is activated in over 50% of T-ALL, it is probably the promising target for drug development to treat patients. Notch1 activation is dependent upon cleavage by the γ-secretase complex (Aster et al., 2008). Gamma-secretase inhibitors (GSI) are therefore the most prominent class of Notch-inactivating drugs on the market. The GSI Compound E has been shown to inhibit growth and induce apoptosis in T-ALL cell lines, while the GSI PF-03084014, which also induces apoptosis in several tumor types and a Phase I clinical trials for T-ALL has recently been completed with encouraging results (Papayannidis et al., 2015; Wei et al., 2010; Weng et al., 2003). However, most patients in clinical trials using GSIs show dose-limiting gastrointestinal toxicity, and GSIs are likely to have multiple off-target effects *in vivo* because they are not specific to the γ-secretase cleavage of Notch proteins (Riccio et al., 2008). It would therefore be preferable to use these inhibitors in combination with agents targeting other proteins acting to regulate T-ALL, in order to reduce the dose of GSI necessary to induce an anti-tumor response. We therefore propose that inhibition of Miz-1 along with Notch1 in T-ALL cases where both of these proteins are overexpressed, would downregulate the Notch1 pathway and reactivate the p53 pathway, possibly

leading to increased survival of T-ALL patients and decreased secondary effects normally induced by chemotherapy and radiation.

Our studies make use of a non-functional Miz-1, produced via deletion of the POZ domain. Normally, this domain forms tetramers on DNA for Miz-1 to exert its transcriptional activity (Stead et al., 2007; Stogios et al., 2010). A small molecule inhibitor has been found to selectively inhibit the activity of Bcl6 in diffuse large B cell lymphoma (DLBCL), which binds specifically to the Bcl6 POZ domain (Cerchietti et al., 2010). Because these domains are unique for each POZ domain-containing transcription factor, we propose that an inhibitor of Miz-1 tetramerization could be designed and could be used in the clinic to reactivate p53 in T-ALL patients and inhibit some functions of c-Myc. The use of targeted therapies such as this one could reduce the toxic side effects of radiation and chemotherapy suffered by patients following treatment.

# 5.0 Materials and Methods

**Human Samples**

Human thymus samples were obtained from Dr. Ellie Haddad at CHU Sainte-Justine. Frozen thymus tissue was transferred to RNA*later*-ICE (Ambion) and stored at -20$^{o}$C for 48 hours. The tissue was then homogenized and RNA extraction was performed using the RNEasy Mini Kit (Qiagen). Primary human T-ALL samples were obtained from Dr. Josée Hébert at the Quebec Leukemia Cell Bank. The use of human cells was approved by the IRCM ethics committee under the protocol number 2014-23.

**Mice**

Mice had been bred on C57BL/6 background for at least 10 generations and were maintained in Specific-Pathogen-Free Plus environment. Miz-1$^{\Delta POZ}$ and Myc$^{V394D}$ mice were previously described (Kosan et al., 2010). Trp53$^{-/-}$ mice were purchased from The Jackson Laboratory. TNR mice were obtained from Dr. Tannishtha Reya (Wu et al., 2007). The IRCM Animal Care Committee (ACC) has approved the animal protocols under which all animal experiments in this study have been performed (protocol 2013-01), and all animal experimental procedures were performed in compliance with the guidelines of the Canadian Council of Animal Care (www.ccac.ca).

**Flow Cytometry Antibodies**

All antibodies were from BD Bioscience except when indicated. DN thymic subsets were analyzed using CD25 (PC61.5 from eBioscience) and CD44 (IM7) plus lineage marker-negative cells (Lin$^-$) 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 PE. Normal thymi and spleens were analyzed for T cell subsets using CD4 (RM 4-5) and CD8α (53-6.7). Tumors were analyzed for phenotype using CD4 (RM 4-5), CD8α (53-6.7), B220 (RA3-6B2), CD19 (1D3), Gr-1 (RB6-8C5) and CD11b (Mac-1, M1/70). Antibody incubations were performed at 4$^o$C for 15 minutes in PBS buffer. Cells were analyzed with a LSR (Becton-Dickson). DN3 cell sorting was performed using a MoFlo cell sorter (Cytomation) or FACS Aria III (Becton-Dickson). Lineage$^-$ cell isolation was performed using a Miltenyi Kit (Lineage Depletion Kit - Mouse) on an AutoMACS machine (Miltenyi).

**RNA Isolation and Real-Time PCR**

For RNA isolation, cells were lysed in TRIzol reagent (Invitrogen) and cleaned using the RNEasy Mini Kit (Qiagen). RT-PCR was performed using Superscript II (Invitrogen). Real-time PCR was performed in triplicates on the ViiA7 using SYBR green reagent (Applied Biosystems) and indicated primers. For normal samples, the expression of the gene of interest was calculated relative to *Gapdh* mRNA ($\Delta C_T$) and is presented as fold induction relative to values obtained with the respective control (WT, set as 1-fold). For leukemia samples, the expression of the gene of interest was calculated relative to *Gapdh* mRNA ($\Delta C_T$) and is presented as relative expression over *Gapdh* for each sample. Primer sequences can be found in Table S5.1.

**Immunoblot Analysis**

For immunoblot analysis, cells were lysed in RIPA buffer containing protease inhibitors (complete Mini; Roche Diagnostics) on ice for 20 minutes and then sonicated for 10 seconds using a Branson digital sonifier. Immunoblotting was performed using anti-p53 (1C12, Cell Signaling), anti-c-Myc (N-262, Santa-Cruz Biotechnology) or anti-β-actin (AC-15, Sigma Aldrich).

**RNA Sequencing**

Assays were performed on sorted primary DN3 cells or leukemic cells as indicated. RNA was extracted using TRIzol reagent (Invitrogen) and cleaned using the RNEasy Mini Kit (Qiagen). For RNA-Seq, a biological triplicate of sequencing libraries were prepared from RNA extracts using the TruSeq Stranded mRNA kit from Illumina according to the manufacturer's instructions and sequenced using the TruSeq PE Clusterkit v3-cBot-HS on an Illumina HiSeq 2000 system. Sequencing reads were aligned to the mm10 genome using Tophat v2.0.10. Reads were processed

with Samtools and then mapped to Ensembl transcripts using HTSeq. Differential expression was tested using the DESeq R package (R Development Core Team, 2010). A genome coverage file was generated and scaled to reads per million reads (RPM) using Bedtools (Li et al., 2009; Quinlan and Hall, 2010).

**Tumorigenesis Assays**

MigR1-ICN1 virus was produced using Phoenix Eco cells. Briefly, MigR1-ICN1 was transfected into Phoenix Eco cells along with the packaging vector pCL-Eco using PureFection reagent (LV750A-1, System Biosciences). Supernatant was collected, filtered and concentrated using VivaSpin columns (GE Healthcare) 48 and 72 hours post-transfection. Viral titer was calculated using 3T3 cells.

For ICN1 transplantation, hematopoietic stem cells were isolated from the indicated mice using a lineage depletion kit (Miltenyi). Cells were expanded at 37ºC in MarrowMAX (Gibco) with 50ng/mL mSCF, 100ng/mL hFlt3L, 100ng/mL hIL-11 and 10ng/mL mIL-3 (Preprotech) for 48 hours. Virus was spun onto a RetroNectin-coated (Takara) plate, and cells were spun onto virus. 24 hours later, cells were analyzed by flow cytometry for GFP expression and 5,000 $GFP^+$ cells were injected intravenously with $2x10^5$ WT carrier cells into lethally irradiated CD45.1 recipient mice.

Blood analysis from leukemia mice was performed using an Advia 120 blood analyzer.

**Statistical Analysis**

Quantitative data are presented as mean ±SD and were analyzed using a one-way ANOVA or two-tailed student's *t* test. Survival curves were analyzed by log-rank Mantel-Cox test using GraphPad

Prism (GraphPad Software, La Jolla, CA, USA). A p-value ≤0.05 was indicated as statistically significant ( * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

## 6.0 Acknowledgements

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## 7.0 Author Contributions

M.R. and T.M. designed research; M.R., and J.F. performed research; M.R., C.J.B., C.V., and A.D. analyzed data; E.H, S.V. and J.H. provided reagents; and M.R. and T.M. wrote the paper.

# 8.0 Supplemental Information

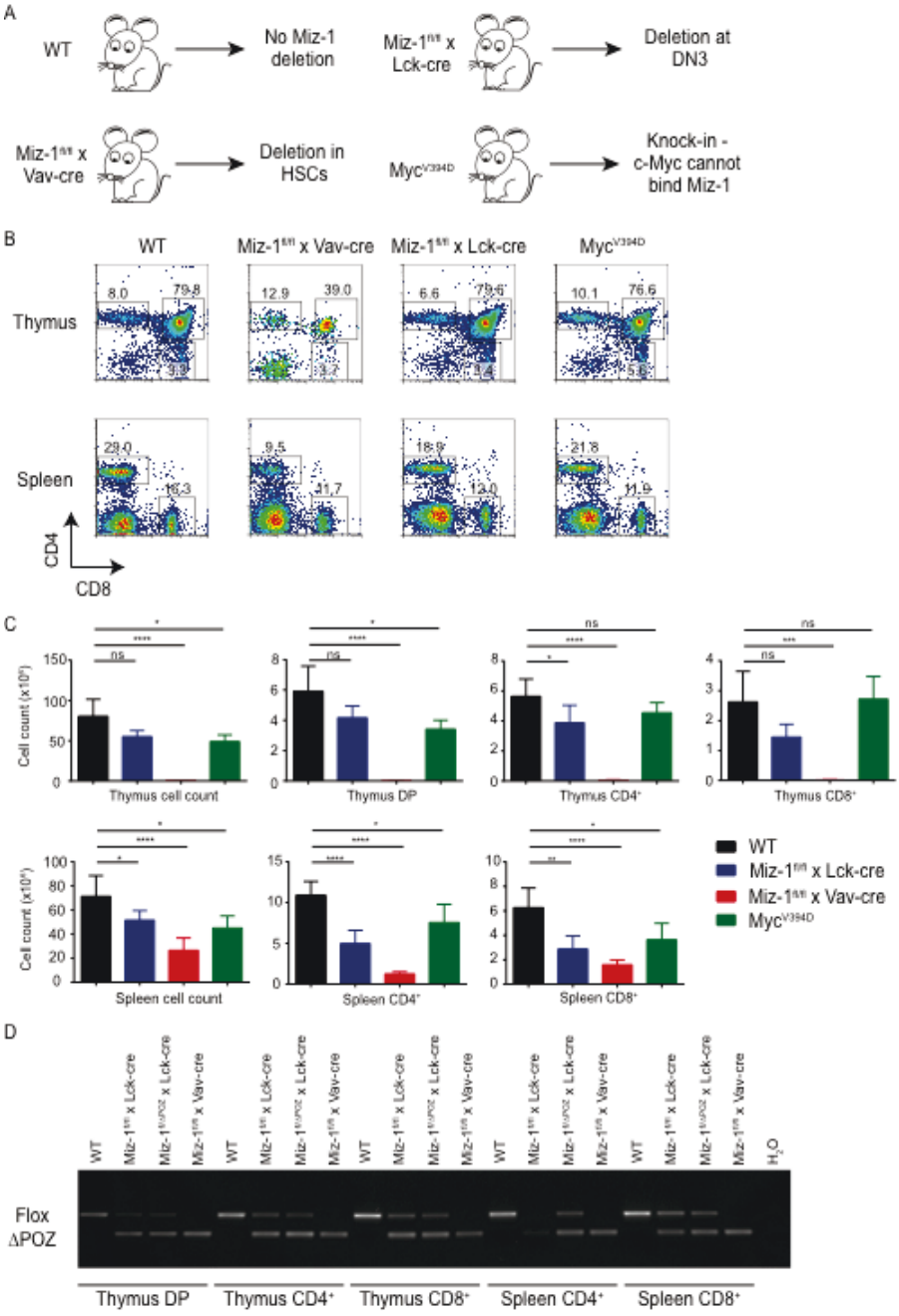


**Figure S3.1** Thymus and spleen T cell phenotypes of mutant mice. (A) Mice used in this study. (B) FACS analysis of thymus and spleen from 6-8 week-old WT, Miz-$1^{fl/fl}$ x Vav-Cre, Miz-$1^{fl/fl}$ x Lck-Cre and $Myc^{V394D}$ mice. FACS plots are a representative example of four independent experiments. (C) Absolute cell counts of thymic and splenic subsets from (B). Data are averaged from four independent experiments and are presented as mean ± SD.

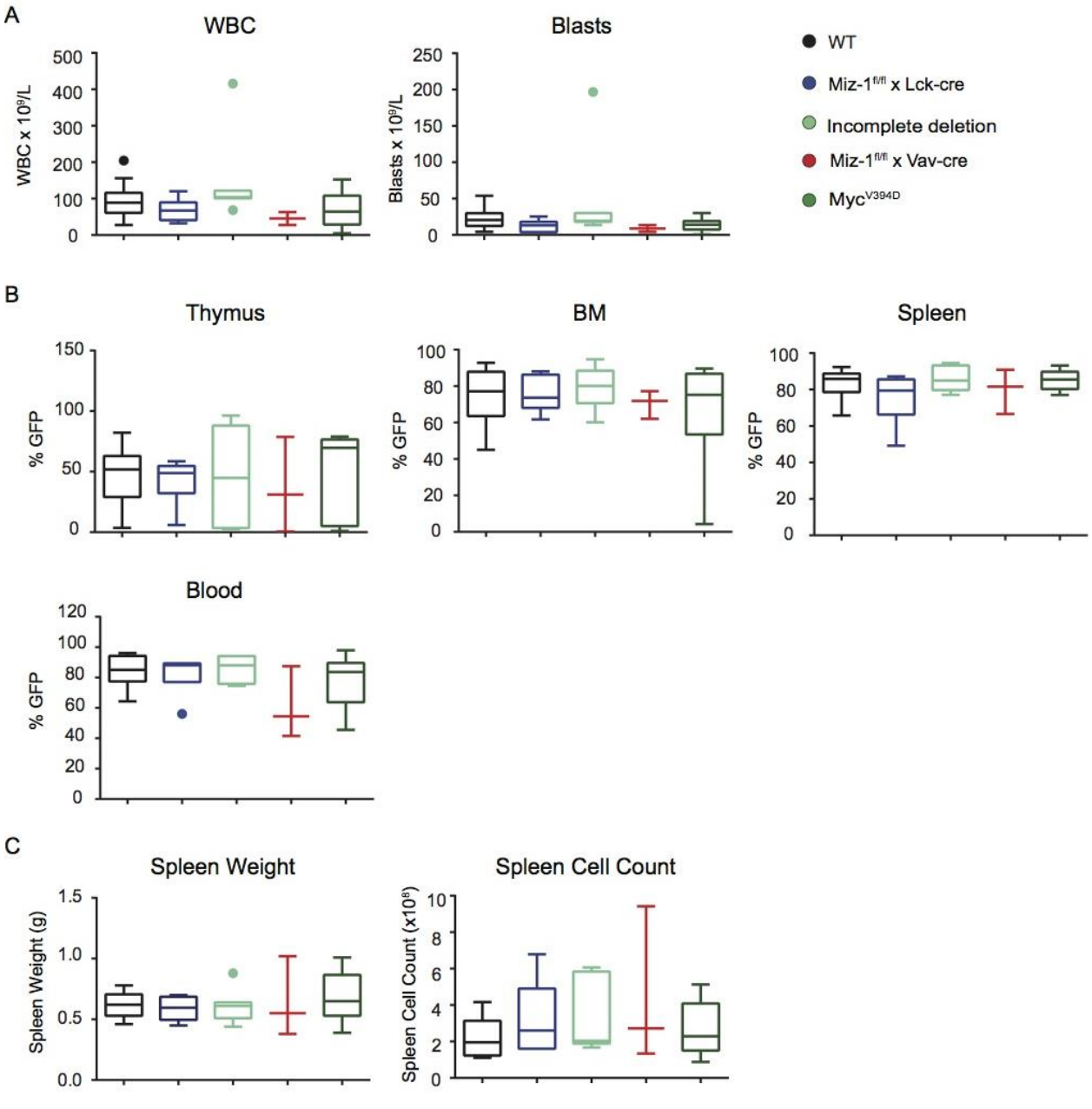


**Figure S3.2** Phenotypes of ICN1-induced leukemia. (A) WBC and Blast counts in the peripheral blood of ICN1 tumor mice. (B) Percentage of $GFP^+$ cells in the thymus, BM, spleen and blood of ICN1 tumor mice. (C) Spleen weight (in grams) and splenic cell count of ICN1 tumor mice.

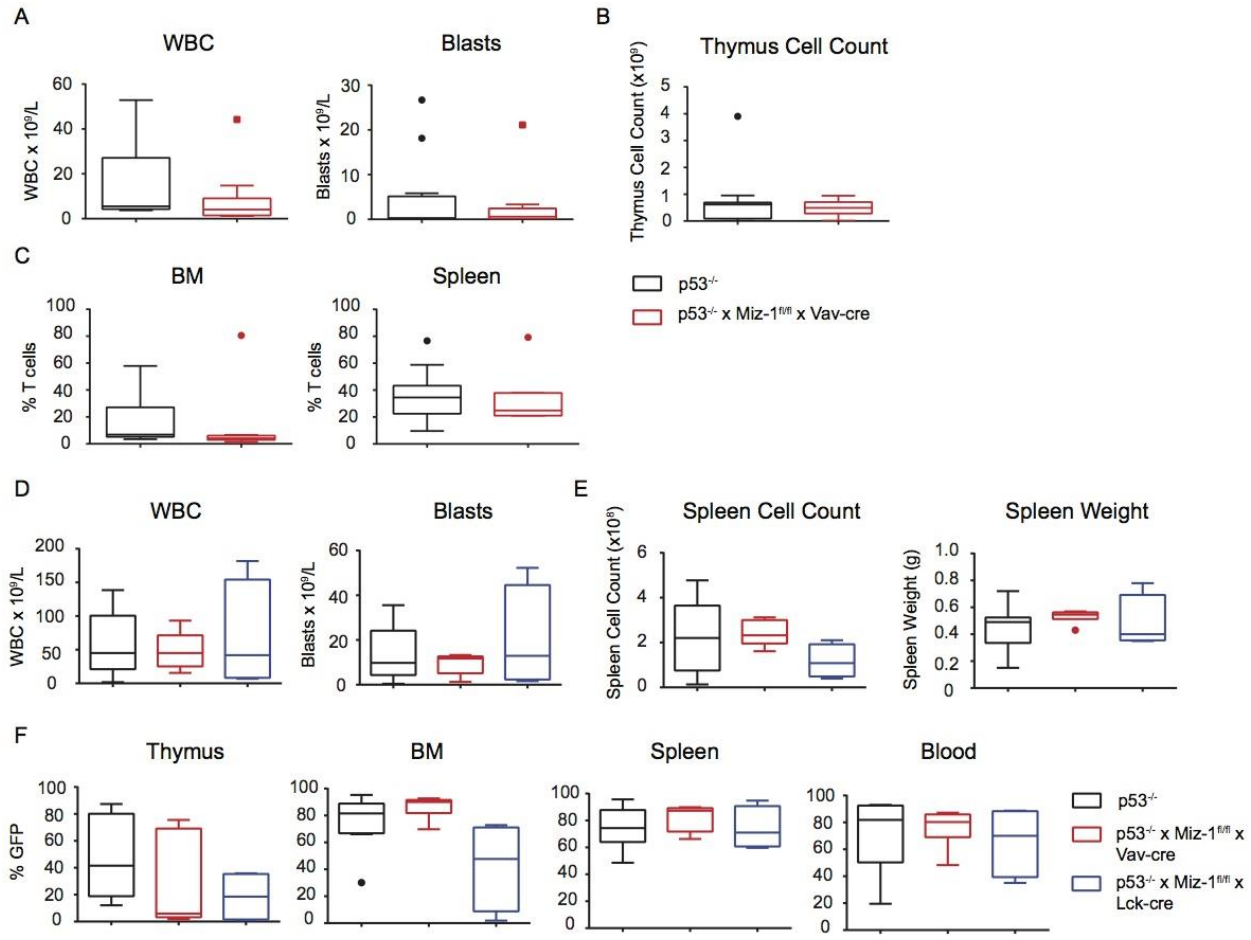


**Figure S3.3** Phenotypes of p53-induced tumors. (A) WBC and Blast counts in the peripheral blood of Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$ and $p53^{-/-}$ T cell tumor mice. (B) Thymic cell count of Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$ and $p53^{-/-}$ T cell tumor mice. (C) Percentage of T cells in the spleen and BM of Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$ and $p53^{-/-}$ T cell tumor mice. (D) WBC and Blast counts in the peripheral blood of Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$, Miz-$1^{fl/fl}$ x Lck-Cre x $p53^{-/-}$, and $p53^{-/-}$ ICN1 tumor mice. (E) Splenic cell count and weight (in grams) of Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$, Miz-$1^{fl/fl}$ x Lck-Cre x $p53^{-/-}$, and $p53^{-/-}$ ICN1 tumor mice. (F) Percentage of $GFP^{+}$ cells in the thymus, BM, spleen and blood of Miz-$1^{fl/fl}$ x Vav-Cre x $p53^{-/-}$, Miz-$1^{fl/fl}$ x Lck-Cre x $p53^{-/-}$, and $p53^{-/-}$ ICN1 tumor mice.

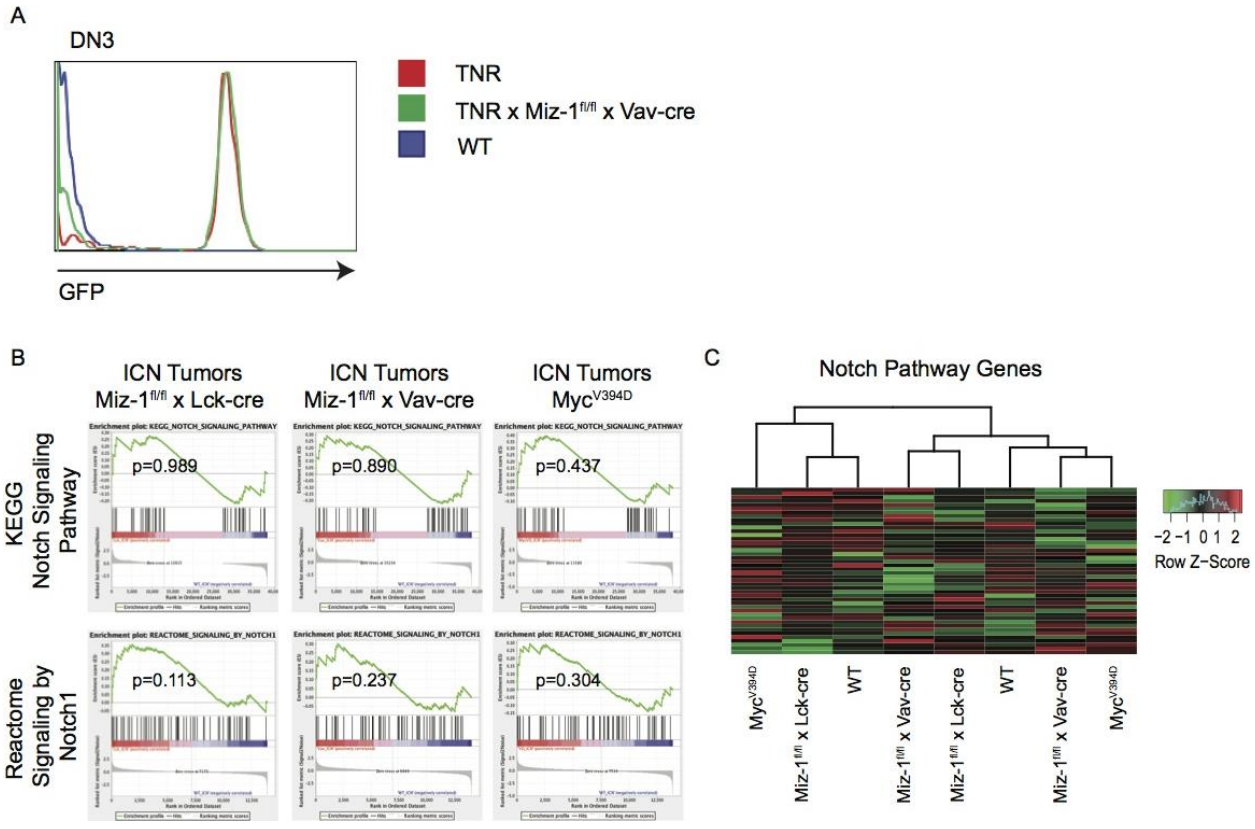


**Figure S3.4** Signaling downstream of Notch1 does not depend on Miz-1 expression. (A) FACS analysis of GFP expression in DN3 cells from the thymus of WT, TNR Tg or TNR Tg x Miz-1$^{fl/fl}$ x Vav-Cre mice. FACS plot is a representative example of three independent experiments. (B) GSEA analysis for KEGG Notch Signaling pathway (top) or Reactome Signaling by Notch1 (bottom) genes for RNA-seq data from indicated ICN1 tumors compared to WT. (C) Heatmap of genes from KEGG Notch Signaling pathway data set in (B).

**Table S3.1** Selected KEGG pathways upregulated in mutant ICN1-induced tumors vs. WT (NES > 1.5, $p < 0.05$).

| Genotype vs. WT | Geneset | NES | p-value | q-value |
|---|---|---|---|---|
| Miz-1$^{fl/fl}$ x Lck-Cre | CELL_ADHESION_MOLECULES_CAMS | 1.86 | 0.000 | 0.041 |
| | DNA_REPLICATION | 1.86 | 0.005 | 0.029 |
| | PATHWAYS_IN_CANCER | 1.66 | 0.000 | 0.130 |
| | HEMATOPOIETIC_CELL_LINEAGE | 1.66 | 0.000 | 0.115 |
| | LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION | 1.66 | 0.007 | 0.102 |
| | TGF_BETA_SIGNALING_PATHWAY | 1.65 | 0.002 | 0.110 |
| | ADHERENS_JUNCTION | 1.62 | 0.005 | 0.132 |
| | MAPK_SIGNALING_PATHWAY | 1.57 | 0.000 | 0.137 |
| | MISMATCH_REPAIR | 1.56 | 0.032 | 0.132 |
| | TIGHT_JUNCTION | 1.54 | 0.003 | 0.149 |
| | JAK_STAT_SIGNALING_PATHWAY | 1.52 | 0.012 | 0.171 |
| | CALCIUM_SIGNALING_PATHWAY | 1.50 | 0.009 | 0.190 |
| Miz-1$^{fl/fl}$ x Vav-Cre | DNA_REPLICATION | 2.16 | 0.000 | 0.002 |
| | HEMATOPOIETIC_CELL_LINEAGE | 2.10 | 0.000 | 0.001 |
| | OXIDATIVE_PHOSPHORYLATION | 2.04 | 0.000 | 0.002 |
| | VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION | 1.99 | 0.000 | 0.003 |
| | RIBOSOME | 1.96 | 0.000 | 0.003 |
| | PYRIMIDINE_METABOLISM | 1.87 | 0.000 | 0.011 |
| | MISMATCH_REPAIR | 1.87 | 0.000 | 0.008 |
| | PURINE_METABOLISM | 1.77 | 0.000 | 0.022 |
| | ECM_RECEPTOR_INTERACTION | 1.77 | 0.000 | 0.021 |
| | BASE_EXCISION_REPAIR | 1.69 | 0.007 | 0.042 |
| | RNA_POLYMERASE | 1.59 | 0.019 | 0.080 |
| | CITRATE_CYCLE_TCA_CYCLE | 1.58 | 0.043 | 0.082 |
| | GLUTATHIONE_METABOLISM | 1.57 | 0.015 | 0.090 |
| | CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION | 1.56 | 0.002 | 0.088 |
| Myc$^{V394D}$ | LYSOSOME | 2.16 | 0.000 | 0.002 |
| | DNA_REPLICATION | 2.02 | 0.000 | 0.009 |
| | LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION | 1.94 | 0.000 | 0.008 |
| | ABC_TRANSPORTERS | 1.86 | 0.000 | 0.017 |
| | ADHERENS_JUNCTION | 1.86 | 0.000 | 0.014 |
| | REGULATION_OF_ACTIN_CYTOSKELETION | 1.83 | 0.000 | 0.013 |
| | CALCIUM_SIGNALING_PATHWAY | 1.83 | 0.000 | 0.012 |
| | BASE_EXCISION_REPAIR | 1.79 | 0.000 | 0.016 |
| | PPAR_SIGNALING_PATHWAY | 1.76 | 0.002 | 0.022 |
| | ERBB_SIGNALING_PATHWAY | 1.75 | 0.000 | 0.020 |
| | GAP_JUNCTION | 1.75 | 0.002 | 0.020 |
| | PATHWAYS_IN_CANCER | 1.71 | 0.002 | 0.023 |
| | FATTY_ACID_METABOLISM | 1.70 | 0.000 | 0.027 |
| | MISMATCH_REPAIR | 1.62 | 0.017 | 0.048 |
| | PEROXISOME | 1.60 | 0.002 | 0.053 |
| | BIOSYNTHESIS_OF_UNSATURATED_FATTY_ACIDS | 1.57 | 0.028 | 0.061 |
| | FOCAL_ADHESION | 1.56 | 0.004 | 0.061 |
| | VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION | 1.55 | 0.008 | 0.063 |
| | PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM | 1.53 | 0.007 | 0.071 |

**Table S3.2** Selected Reactome pathways upregulated in mutant ICN1-induced tumors vs WT (NES > 1.5, p < 0.05).

| Genotype vs. WT | Geneset | NES | p-value | q-value |
|---|---|---|---|---|
| Miz-1$^{fl/fl}$ x Lck-Cre | DNA_STRAND_ELONGATION | 1.90 | 0.000 | 0.049 |
| | CELL_CELL_COMMUNICATION | 1.86 | 0.002 | 0.066 |
| | SIGNALING_BY_HIPPO | 1.86 | 0.005 | 0.054 |
| | POTASSIUM_CHANNELS | 1.73 | 0.004 | 0.114 |
| | CELL_JUNCTION_ORGANIZATION | 1.72 | 0.009 | 0.114 |
| | APOPTOTIC_CLEAVAGE_OF_CELLULAR_PROTEINS | 1.65 | 0.012 | 0.178 |
| | LAGGING_STRAND_SYNTHESIS | 1.63 | 0.018 | 0.172 |
| | CELL_CELL_JUNCTION_ORGANIZATION | 1.59 | 0.023 | 0.236 |
| | EXTENSION_OF_TELOMERES | 1.57 | 0.017 | 0.245 |
| | G1_S_SPECIFIC_TRANSCRIPTION | 1.55 | 0.024 | 0.278 |
| | APOPTOTIC_EXECUTION_PHASE | 1.55 | 0.027 | 0.269 |
| | TGF_BETA_RECEPTOR_SIGNALING_ACTIVATES_SMADS | 1.55 | 0.025 | 0.273 |
| | ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX | 1.54 | 0.037 | 0.275 |
| | GPCR_DOWNSTREAM_SIGNALING | 1.53 | 0.001 | 0.261 |
| | SYNTHESIS_OF_DNA | 1.50 | 0.008 | 0.315 |
| | S_PHASE | 1.50 | 0.013 | 0.313 |
| Miz-1$^{fl/fl}$ x Vav-Cre | DNA_STRAND_ELONGATION | 2.24 | 0.000 | 0.000 |
| | RESPIRATORY_ELECTRON_TRANSPORT | 2.22 | 0.000 | 0.000 |
| | S_PHASE | 2.21 | 0.000 | 0.000 |
| | SYNTHESIS_OF_DNA | 2.16 | 0.000 | 0.000 |
| | EXTENSION_OF_TELOMERES | 2.14 | 0.000 | 0.001 |
| | ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX | 2.03 | 0.000 | 0.005 |
| | ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS | 2.03 | 0.000 | 0.005 |
| | G1_S_TRANSITION | 1.96 | 0.000 | 0.008 |
| | 3_UTR_MEDIATED_TRANSLATIONAL_REGULATION | 1.94 | 0.000 | 0.009 |
| | LAGGING_STRAND_SYNTHESIS | 1.94 | 0.000 | 0.009 |
| | M_G1_TRANSITION | 1.91 | 0.000 | 0.013 |
| | TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT | 1.88 | 0.000 | 0.015 |
| | PROCESSIVE_SYNTHESIS_ON_THE_LAGGING_STRAND | 1.87 | 0.000 | 0.015 |
| | TELOMERE_MAINTENANCE | 1.84 | 0.000 | 0.020 |
| | METABOLISM_OF_NUCLEOTIDES | 1.84 | 0.000 | 0.019 |
| | PEPTIDE_CHAIN_ELONGATION | 1.84 | 0.000 | 0.019 |
| | G2_M_CHECKPOINTS | 1.81 | 0.002 | 0.023 |
| | METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES | 1.81 | 0.000 | 0.023 |
| | MITOTIC_G1_G1_S_PHASES | 1.77 | 0.000 | 0.033 |
| | G1_S_SPECIFIC_TRANSCRIPTION | 1.76 | 0.009 | 0.036 |
| | TRANSLATION | 1.75 | 0.000 | 0.040 |
| | CYTOSOLIC_TRNA_AMINOACYLATION | 1.73 | 0.006 | 0.045 |
| | RNA_POL_III_TRANSCRIPTION_TERMINATION | 1.73 | 0.008 | 0.044 |
| | MITOCHONDRIAL_PROTEIN_IMPORT | 1.72 | 0.002 | 0.046 |
| | ORC1_REMOVAL_FROM_CHROMATIN | 1.71 | 0.000 | 0.050 |
| | ASSEMBLY_OF_THE_PRE_REPLICATIVE_COMPLEX | 1.71 | 0.002 | 0.048 |
| | CELL_CYCLE_CHECKPOINTS | 1.69 | 0.000 | 0.053 |
| | INTEGRIN_CELL_SURFACE_INTERACTIONS | 1.69 | 0.007 | 0.053 |
| | CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION | 1.66 | 0.006 | 0.064 |
| | CITRIC_ACID_CYCLE_TCA_CYCLE | 1.64 | 0.012 | 0.069 |
| | E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION | 1.63 | 0.015 | 0.076 |
| | P53_INDEPENDENT_G1_S_DNA_DAMAGE_CHECKPOINT | 1.61 | 0.021 | 0.084 |
| | RNA_POL_III_CHAIN_ELONGATION | 1.59 | 0.023 | 0.098 |
| | CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX | 1.56 | 0.009 | 0.107 |

| | | | | |
|---|---|---|---|---|
| | DOWNREGULATION_OF_TGF_BETA_RECEPTOR_SIGNALING | 1.56 | 0.037 | 0.106 |
| | REGULATION_OF_APOPTOSIS | 1.56 | 0.011 | 0.106 |
| | PROTEIN_G_ACTIVATION | 1.55 | 0.036 | 0.106 |
| | CHEMOKINE_RECEPTORS_BIND_CHEMOKINES | 1.54 | 0.027 | 0.116 |
| | CDK_MEDIATED_PHOSPHORYLATION_AND_REMOVAL_OF_CDC6 | 1.53 | 0.020 | 0.120 |
| $Myc^{V394D}$ | DNA_STRAND_ELONGATION | 2.22 | 0.000 | 0.000 |
| | ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX | 2.01 | 0.000 | 0.016 |
| | E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION | 1.94 | 0.000 | 0.020 |
| | LAGGING_STRAND_SYNTHESIS | 1.89 | 0.000 | 0.033 |
| | G1_S_SPECIFIC_TRANSCRIPTION | 1.89 | 0.002 | 0.028 |
| | SYNTHESIS_OF_DNA | 1.87 | 0.000 | 0.029 |
| | ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS | 1.79 | 0.006 | 0.050 |
| | S_PHASE | 1.76 | 0.000 | 0.065 |
| | PROCESSIVE_SYNTHESIS_ON_THE_LAGGING_STRAND | 1.76 | 0.011 | 0.065 |
| | PHOSPHOLIPASE_C_MEDIATED_CASCADE | 1.73 | 0.002 | 0.071 |
| | LIPOPROTEIN_METABOLISM | 1.72 | 0.006 | 0.080 |
| | PLC_BETA_MEDIATED_EVENTS | 1.71 | 0.002 | 0.078 |
| | PI3K_EVENTS_IN_ERBB2_SIGNALING | 1.70 | 0.006 | 0.087 |
| | PI_3K_CASCADE | 1.68 | 0.007 | 0.095 |
| | BIOLOGICAL_OXIDATIONS | 1.68 | 0.002 | 0.091 |
| | G2_M_CHECKPOINTS | 1.67 | 0.006 | 0.092 |
| | G1_S_TRANSITION | 1.67 | 0.000 | 0.089 |
| | MITOTIC_G1_G1_S_PHASES | 1.63 | 0.002 | 0.098 |
| | PI3K_CASCADE | 1.63 | 0.000 | 0.102 |
| | SIGNALING_BY_ERBB2 | 1.59 | 0.005 | 0.108 |
| | BASE_EXCISION_REPAIR | 1.55 | 0.037 | 0.133 |
| | METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS | 1.52 | 0.000 | 0.141 |
| | EXTENSION_OF_TELOMERES | 1.52 | 0.038 | 0.139 |
| | DNA_REPAIR | 1.52 | 0.006 | 0.137 |

**Table S3.3** c-Myc-regulated pathways upregulated in mutant ICN1-induced tumors vs. WT ($p < 0.05$).

| Genotype vs. WT | Geneset | NES | p-value | q-value |
|---|---|---|---|---|
| Miz-1$^{fl/fl}$ x Lck-cre | YU_MYC_TARGETS_DN | 1.65 | 0.004 | 0.189 |
| | DANG_MYC_TARGETS_UP | 1.47 | 0.013 | 0.276 |
| | ALFANO_MYC_TARGETS | 1.41 | 0.008 | 0.321 |
| | DANG_REGULATED_BY_MYC_DN | 1.34 | 0.020 | 0.363 |
| Miz-1$^{fl/fl}$ x Vav-cre | DANG_MYC_TARGETS_UP | 2.32 | 0.000 | 0.000 |
| | SCHUHMACHER_MYC_TARGETS_UP | 2.12 | 0.000 | 0.003 |
| | SCHLOSSER_MYC_TARGETS_AND_SERUM_RESPONSE_DN | 1.81 | 0.000 | 0.037 |
| | COLLER_MYC_TARGETS_UP | 1.70 | 0.011 | 0.065 |
| | CAIRO_PML_TARGETS_BOUND_BY_MYC_UP | 1.69 | 0.017 | 0.070 |
| | SCHLOSSER_MYC_AND_SERUM_RESPONSE_SYNERGY | 1.66 | 0.011 | 0.077 |
| | PID_MYC_ACTIVPATHWAY | 1.64 | 0.000 | 0.085 |
| | KIM_MYC_AMPLIFICATION_TARGETS_UP | 1.58 | 0.000 | 0.113 |
| | ALFANO_MYC_TARGETS | 1.53 | 0.002 | 0.138 |
| | SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM | 1.52 | 0.007 | 0.144 |
| | BENPORATH_MYC_TARGETS_WITH_EBOX | 1.42 | 0.010 | 0.200 |
| | DANG_BOUND_BY_MYC | 1.35 | 0.000 | 0.246 |
| | FERNANDEZ_BOUND_BY_MYC | 1.35 | 0.024 | 0.249 |
| Myc$^{V394D}$ | CEBALLOS_TARGETS_OF_TP53_AND_MYC_DN | 1.52 | 0.037 | 0.102 |
| | ACOSTA_PROLIFERATION_INDEPENDENT_MYC_TARGETS_UP | 1.51 | 0.026 | 0.103 |
| | DANG_REGULATED_BY_MYC_DN | 1.51 | 0.002 | 0.105 |
| | LEE_LIVER_CANCER_MYC_DN | 1.48 | 0.042 | 0.117 |
| | LEE_LIVER_CANCER_MYC_UP | 1.46 | 0.037 | 0.128 |
| | ALFANO_MYC_TARGETS | 1.37 | 0.017 | 0.176 |

**Table S5.1** qPCR Primer Sequences

| Primer | Sequence | Reference |
|---|---|---|
| Cdkn1a (p21) F | AGATCCACAGCGATATCCAGAC | (Li et al., 2012) |
| Cdkn1a (p21) R | ACCGAAGAGACAACGGCACACT | |
| Puma (Bbc3) F | ACGACCTCAACGCGCAGTACG | (Li et al., 2012) |
| Puma (Bbc3) R | GAGGAGTCCCATGAAGAGATTG | |
| Bax F | CAGGATGCGTCCACCAAGAA | (Li et al., 2012) |
| Bax R | AGTCCGTGTCCACGTCAGCA | |
| Gapdh F | TTCCGTGTTCCTACCCCCAATG | (Kosan et al., 2010) |
| Gapdh R | GGAGTTGCTGTTGAAGTCGCAG | |
| c-Myc F | TTTGTCTATTTGGGGACAGTGTT | (Wolf et al., 2013) |
| c-Myc R | CATCGTCGTGGCTGTCTG | |
| Rpl22 F | AGGTGCCTTTCTCCAAAAGGTATT | (Rashkovan et al., 2014) |
| Rpl22 R | AAACCACCGGTTTTGTTCCT | |
| MIZ-1 F (human | GTGTGATGTGCGGTAAGGC | This study |
| MIZ-1 R (human) | TGGACTGGACGAATCTCTTGC | |
| GAPDH F (human) | CTGGGCTACACTGAGCACC | This study |
| GAPDH R (human) | AAGTGGTCGTTGAGGGCAATG | |

# CHAPTER V

# General Discussion & Perspectives

# 1.0 Summary

At the start of my Ph.D., our lab determined that Miz-1 is essential during early stages of lymphocyte development (Kosan et al., 2010; Saba et al., 2011a; Saba et al., 2011b). Specifically, at the ETP and pre-pro-B cell stages of T and B cell development respectively, Miz-1 was found to regulate signaling via the IL-7 receptor, playing a crucial role in early lymphocyte survival. Furthermore, both T and B cell development were impaired during V(D)J recombination in Miz-1-deficient cells. Because we had previously found upregulation of p53 target genes and increased cell death specifically in DN3 pre-T cells, I hypothesized that Miz-1 regulates the p53 pathway during V(D)J recombination. Because p53 mRNA is not upregulated in these cells, I also hypothesized that the regulation of the pathway by Miz-1 must either be via direct binding to p53 target gene promoters, or via regulation of the levels of the p53 protein. I found that reduction of p53 in Miz-1-deficient pro-B and pre-T cells allows the cells to undergo V(D)J recombination without premature cell death and rescues the blockage in the development of these cells. Additionally, I showed that, while Miz-1 does not bind directly to the promoters of p53 target genes, it does bind to and activate the Rpl22 promoter. Furthermore, in the absence of Miz-1, and thus Rpl22, translation of p53 is increased, leading to increased production of p53 protein and high levels of apoptosis **(Chapter II**, Figure 1**)**.

Previous studies have shown that loss of Miz-1 is favorable during skin carcinogenesis and in neuroblastoma. Because I showed that Miz-1 regulates the levels of p53, a tumor suppressor, at the DN3 β-selection checkpoint, where T cell transformation is thought to occur, I hypothesized that loss of Miz-1 would be advantageous in preventing T-ALL onset. Using the ICN1 mouse model of T-ALL induction, I found that loss of Miz-1 increases the latency of tumor development. Furthermore, I found that this delay is completely abrogated when p53 is non-functional, indicating

that Miz-1 regulates the p53 pathway during T cell transformation as well as during T cell development. I also determined that this striking effect on tumor latency is partially due to the interaction of Miz-1 with c-Myc, as $Myc^{V394D}$ mice are also impaired in their ability to induce T-ALL. Using this model, I found that Miz-1-deficient tumors could only develop when genes involved in DNA synthesis, replication and cell cycle progression were upregulated, overcoming p53-induced apoptosis (**Chapter III**, Figure 2).

I have therefore been able to show that Miz-1 is an essential regulator of the p53 pathway during both T cell development and transformation.

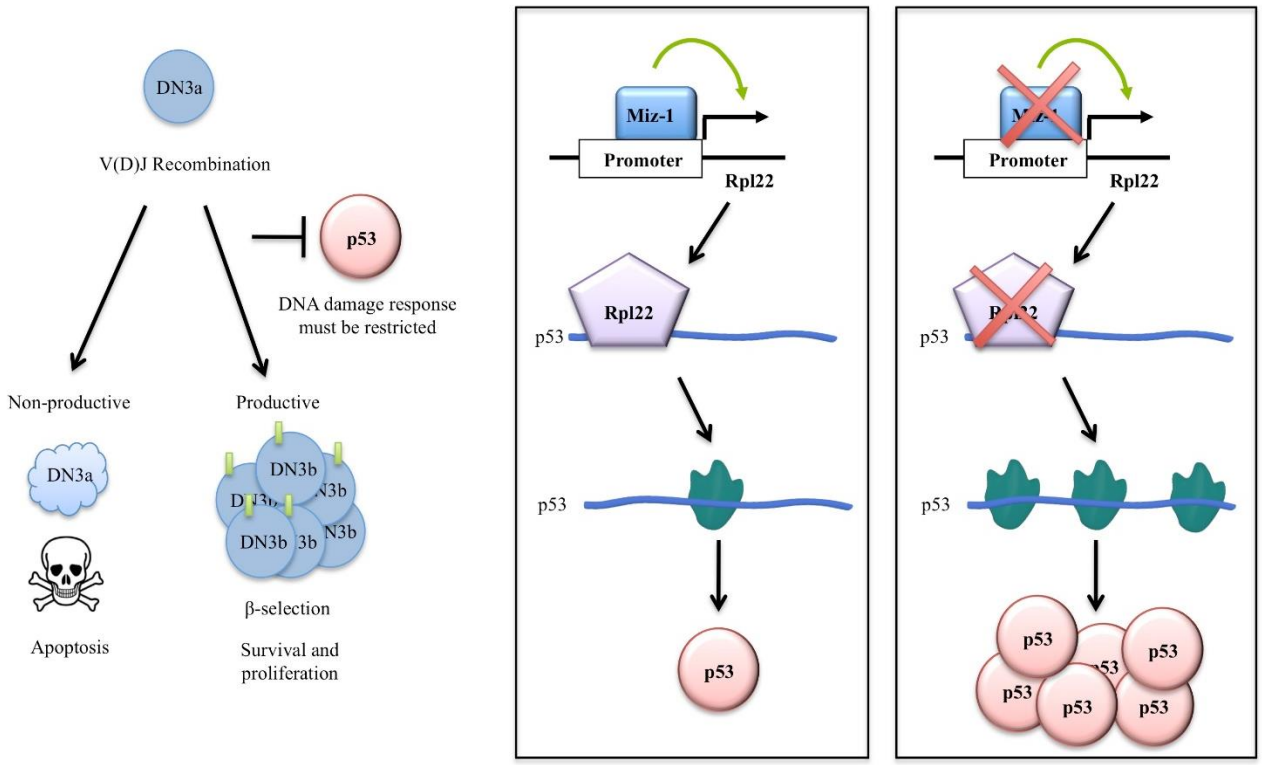


**Figure 1** Proposed model for Miz-1 regulation of p53 during V(D)J recombination. During β-selection, recombination of the TCRβ chain can be non-productive, leading to apoptosis of the DN3a cell, or it can be productive, leading to survival and proliferation of a DN3b cell. If recombination is productive, p53 and the DNA damage response must be restricted in order for the cell to survive (left panel). We propose that Miz-1 binds to the promoter of Rpl22 to activate transcription. Rpl22 then binds directly to p53 mRNA, leading to low levels of translation of p53 and therefore low levels of p53 protein (middle panel). Loss of Miz-1, therefore, leads to loss of Rpl22, high levels of p53 translation and therefore high levels of p53 protein (right panel).

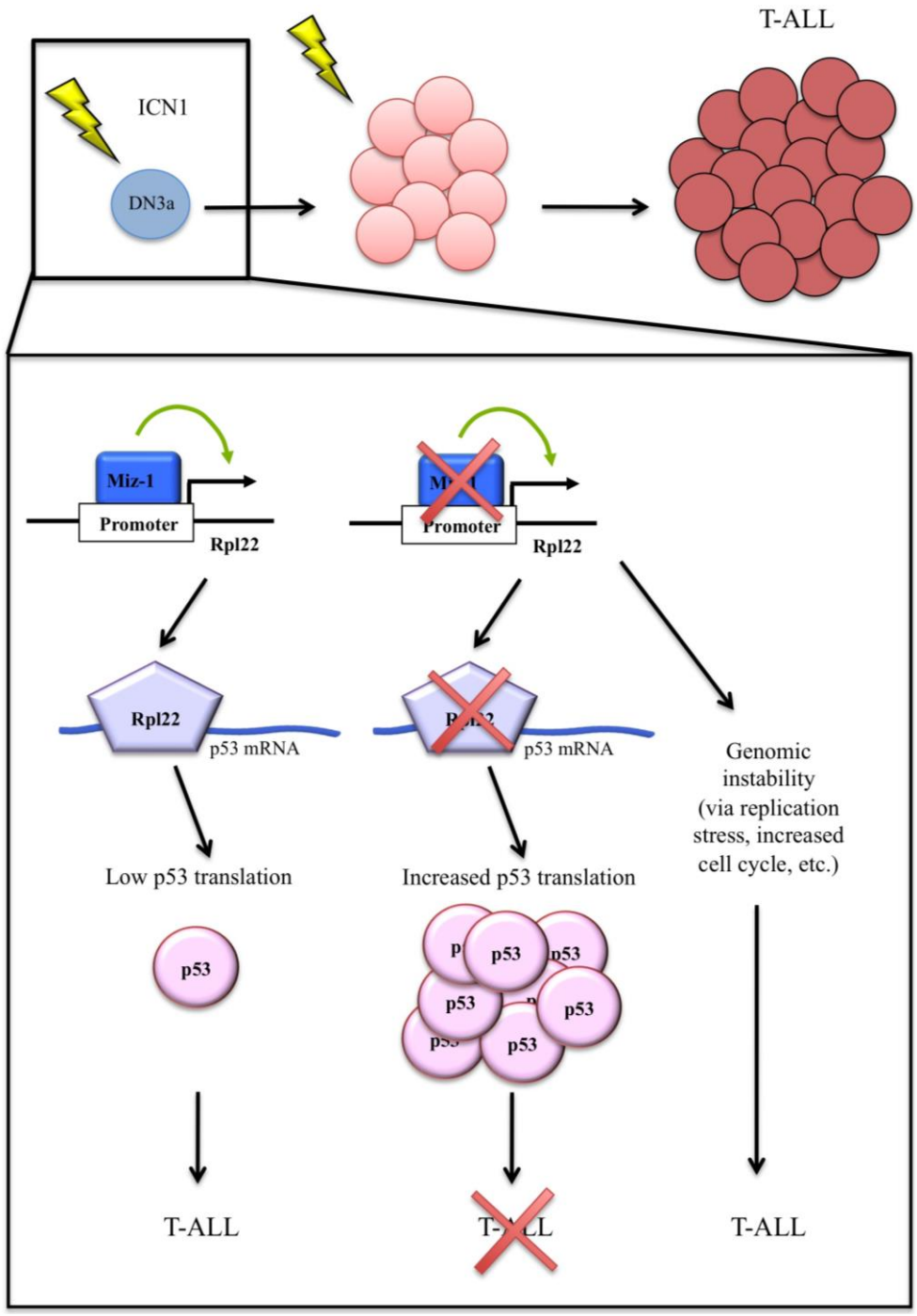


**Figure 2** Proposed model for Miz-1 regulation of p53 during the development of ICN1-induced T-ALL. During the initiation of T-ALL, Miz-1 regulates the expression of Rpl22, which regulates the expression of p53. Loss of Miz-1 leads to a loss of Rpl22 and high levels of p53 protein. This induces apoptosis and T-ALL cannot occur. However, if high levels of genomic instability are induced in the absence of Miz-1 (via replication stress or increased cell cycle progression, T-ALL can be initiated.

## 2.0 Functions of p53 at the β-selection checkpoint

p53 is involved in the regulation of many pathways following DNA damage and other cellular stresses. In Chapter II, I determined that the main effect of the increased p53 protein levels during β-selection is to increase the levels of apoptosis. However, while complete loss of p53 rescues the developmental block in Miz-1-deficient thymocytes, a mutant p53 (p53K117R), which cannot activate the pro-apoptotic p53 target genes Bax and Puma, does not exhibit a comparable rescue. While it has been suggested that the main function of p53 during V(D)J recombination is to prevent cell death due to DNA double strand breaks (Guidos et al., 1996), it is clear that, at least in the models used in my studies, p53 regulates other pathways during this process.

We have already shown that the DN3 developmental defect is not caused by the upregulation of $p21^{CIP1}$ (Saba et al., 2011a). Cells undergoing V(D)J recombination usually arrest in $G_1$ and p53-mediated cell cycle arrest at $G_1$ is a result of $p21^{CIP}$ induction (Godfrey et al., 1994; Lin and Desiderio, 1995; Pearse et al., 1989). However, cell cycle arrest at $G_2$ during V(D)J recombination has also been reported, and p53-mediated $G_2$ arrest is mainly mediated by 14-3-3σ (Hermeking et al., 1997). A role of this particular 14-3-3 isoform in T cell development has yet to be elucidated, but it is possible that, because other 14-3-3 proteins are essential for T cell development and function, 14-3-3σ is essential as well. It is also possible that $p21^{CIP1}$ is not the only p53-induced mediator of cell cycle arrest, and this unknown protein may play a role in the block in T cell development that occurs in Miz-1-deficient mice.

As discussed in Chapter I, p53 can play a dual role in the induction of autophagy. Importantly, autophagy can be activated by signaling pathways downstream of the pre-TCR, including via the MAPK and the PI(3)K pathways, indicating that autophagy might play an essential role in cell survival following β-selection (reviewed by Sridharan et al., 2011). It is therefore possible that

loss of Miz-1 not only induces p53-mediated apoptosis, but also induces p53-mediated induction of autophagy. Moreover, as Miz-1 itself has been shown to regulate autophagy, the induction of autophagy following β-selection may also be independent of p53 activation in Miz-1-deficient DN3 cells (Wolf et al., 2013). While direct autophagic targets of Miz-1 have not specifically been implicated in T cell development or function, many essential regulators of autophagy are necessary for these processes (Arsov et al., 2011; Arsov et al., 2008; Willinger and Flavell, 2012). Further experimentation will need to be performed in order to determine whether autophagy is inhibited in Miz-1-deficient DN3 pre-T cells contributing to their inability to pass through β-selection. If this is the case, it would also be important to determine whether this is due to defective activation of Miz-1 autophagy targets, activation of p53-dependent mediators of autophagy, or both.

## 3.0 c-Myc-dependent functions of Miz-1

When Miz-1 was first discovered, it was thought that the most important functions of this transcription factor were performed in collaboration with c-Myc. Our lab and others have shown that in mice, the functions of Miz-1 that are most critical are those which occur independently of its interaction with c-Myc. These functions include induction of autophagy in neuronal cells and regulation of IL-7 signaling during early B and T cell development (Kosan et al., 2010; Saba et al., 2011b; Wolf et al., 2013). However, in Chapter II, using the $Myc^{V394D}$ mouse model, I showed that the interaction between Miz-1 and c-Myc is essential for the proliferation of β-selected cells, at the DN-to-DP transition. Furthermore, I showed that the role of this interaction at this point in T cell development is to upregulate the c-Myc target genes CD71 and CD98, which are key metabolic genes involved in growth and differentiation of DN4 cells. It is interesting, however, that these $Myc^{V394D}$ knock-in mice exhibit no defects in lymphocyte development under normal

conditions, as I have shown in Chapter III (Figure S3.1). For example, in order to observe the defective DN-to-DP transition, it was necessary to cross these mice into a Rag-1-deficient background and stimulate the cells by αCD3 injection. Moreover, induction of T-ALL by overexpression of Notch1 in these mice is significantly delayed compared to WT, as I have shown in Chapter III. These results indicate that the Miz-1/c-Myc complex may only play a role in gene regulation under conditions of cellular stress, which may account for the inability of our lab to detect Miz-1 binding at the promoters of its "traditional" target genes, *Cdkn1a* and *Cdkn2b*, in normal, unstressed lymphocytes.

## 4.0 The c-Myc/Miz-1 complex in leukemia and lymphoma

In normal T cells, Miz-1 activates Rpl22, which regulates p53 protein levels. I have shown that this regulation also takes place during T cell transformation. However, while Miz-1-deficient cells express high levels of p53, some T cell tumors are able to develop under these conditions. Based on the RNA-seq results from our ICN1-induced T-ALL samples, we propose that in order for a Miz-1-deficient leukemia to develop, the cells must have high levels of genomic instability, possibly caused by DNA replication stress or increased progression through the cell cycle.

I have also found that Miz-1 is able to regulate the initiation of T cell leukemia other than those induced by overexpression of Notch1. Using the Moloney Murine Leukemia Virus (MoMuLV) model of T-ALL induction, I have shown that loss of Miz-1significantly increases the latency of retroviral-induced T cell leukemia, and significantly increases leukemia incidence (Appendix II). However, the mechanisms behind this regulation of T-ALL induction are not the same as those responsible for ICN1-mediated T-ALL. I show that Miz-1-deficient MoMuLV tumors express high levels of ribosome and translation-associated genes, and not DNA replication

stress genes (Appendix III). Therefore, while the increased latency and decreased T-ALL incidence of Miz-1-deficient MoMuLV-induced leukemia is probably also caused by overexpression of p53, the mechanisms by which these cells overcome this increased apoptosis are not the same as in the ICN1-induced leukemic samples. This indicates that, depending on the method with which T-ALL is induced, Miz-1-deficient cells may find different ways to overcome the activation of p53 that occurs following loss of Miz-1 during normal T cell development.

While many of the experiments I performed in Chapter II were focused on pre-T cells, based on the partial rescue of B cell development after loss of p53, it is possible to infer that similar regulation of p53 by Miz-1 occurs during V(D)J recombination in both lymphocyte populations. Therefore, since Miz-1 acts on p53 translation, we can infer a role of Miz-1 in B cell lymphomagenesis as well. In fact, loss of Miz-1 in mice overexpressing c-Myc under a B cell-specific promoter (EμMyc mice) leads to the development of B cell lymphoma at a significantly slower rate than those expressing a functional Miz-1 (Ross, J. et al., unpublished data). However, further experimentation will be required to determine whether or not this is due to increased levels of p53. There are, however, other possible mechanisms to explain the increased tumor latency in these mice. ChIP-seq experiments show that Miz-1 binds to the Ras promoter, probably leading to activation of Ras transcription. Loss of Miz-1, therefore, could suppress signaling via the Ras/Raf/MEK pathway, which has been shown to inhibit Myc-induced B cell lymphomagenesis (Gramling and Eischen, 2012). Moreover, direct targets of the Miz-1/c-Myc complex include cell cycle regulators and deregulation of these genes could also play a role in the increased latency of c-Myc-dependent B cell lymphoma in Miz-1-deficient mice.

## 4.1 Targeting Miz-1 in Cancer

The mouse models described in my experiments utilize a conditional deletion of the POZ domain, whereby a non-functional Miz-1 is produced. Loss of the POZ domain prevents dimerization and tetramerization, which are necessary for Miz-1 transactivation of target genes (Stead et al., 2007). This makes Miz-1 a possible target for treatment of T-ALL, in conjunction with other therapies. Another BTB-POZ domain transcription factor, Bcl6, has been implicated in the development of DLBCL (Qi et al., 2000). Moreover, a small-molecule inhibitor targeting the Bcl6 POZ domain has been identified, and kills DLBCL cells both *in vitro* and *in vivo* (Cerchietti et al., 2010). It is therefore imaginable that a small-molecule inhibitor of the Miz-1 POZ domain could be used to selectively kill T-ALL cells overexpressing Miz-1, possibly by reactivating the translation of WT p53. Using a small-molecule inhibitor specific to one transcription factor in conjunction with other drugs could eventually lead to less toxic, more targeted therapies for T-ALL patients, who are most often children and suffer from therapy-induced side effects later in life (Armstrong et al., 2009; Feig, 2001; Mertens et al., 2001; Moller et al., 2001; Neglia et al., 2001).

# 5.0 Miz-1 and the Germinal Center reaction

As discussed in Chapter I, Miz-1 interacts with the BTB-POZ domain transcription factor Bcl6, which is the "master regulator" of both GC B cells and Tfh cells, the main components of the GC reaction. A role of Miz-1 in the GC reaction would therefore not be surprising.

While the exact role of Bcl6 in Tfh development has not yet been elucidated, it is clear that this transcription factor is necessary for the GC reaction (Hatzi et al., 2015; Yu et al., 2009). Because an interaction between Miz-1 and Bcl6 has been described in GC B cells, it is possible

that these transcription factors also interact in Tfh cells. Furthermore, because Miz-1 is essential for normal T cell development in the thymus, it would not be surprising to hypothesize a role of Miz-1 in Tfh development and function in the periphery. However, preliminary results in Miz-$1^{\Delta POZ}$ peripheral T cells indicate that loss of the Miz-1 POZ domain does not affect the initiation of a GC reaction, nor does it affect the function of Tfh cells within this reaction (Rashkovan, M. and Ross, J., unpublished data). Specific mutation of the Bcl6 POZ domain leads to loss of GC B cells, but results are contradictory with regards to the role of the Bcl6 BTB-POZ domain in Tfh differentiation and function (Huang et al., 2013; Nance et al., 2015b). Interestingly, however, mutation of the Bcl6 repression domain, a domain which is not found in Miz-1, prevents the recruitment of the co-repressor MTA3 to target genes involved in Tfh cell biology (Nance et al., 2015a). These results suggest that Miz-1 may not play a role in Tfh cell biology, even though it interacts with Bcl6 in GC B cells and is structurally similar to Bcl6.

A role of Miz-1 has already been reported in GC B cells. As described in Chapter I, Miz-1 and Bcl6 bind to the $p21^{CIP1}$ and Bcl2 promoters in these cells (Phan et al., 2005; Saito et al., 2009). It has yet to be determined, however, if Miz-1 has any Bcl6-independent functions in GC B cell differentiation and function. Preliminary results indicate that, unlike in Tfh cells, loss of Miz-1 in mature B cells may be detrimental to GC B cell development. In fact, Miz-$1^{\Delta POZ}$ peripheral B cells are less efficient at initiating a GC reaction (Rashkovan, M. and Ross, J., unpublished data). Further experiments are necessary, however, in order to determine the exact functions of Miz-1 in these cells.

# 6.0 The future of Miz-1 mouse models

Deletions of either the complete Miz-1 protein or the POZ domain specifically in the entire mouse lead to embryonic lethality (Adhikary et al., 2003; Kosan et al., 2010). It is for this reason that the conditional POZ domain mutant mouse model was developed. However, while this mouse model has proven to be a useful tool in uncovering the functions of Miz-1 in many different cell types, it remains necessary to determine the effect of conditional deletion of the entire Miz-1 protein on these functions. Several technologies could be used to engineer this mouse model, including CRISPR-Cas9-mediated destruction of the two transcription start sites in the Miz-1 gene, or the knock-in of a floxed Miz-1 allele, containing loxP sites at both the 5' and 3' ends of the gene. Experiments using these mice would, for example, allow our lab to determine whether the roles we have uncovered of Miz-1 in lymphocyte development are dependent on the presence of the entire protein or on the POZ domain alone. For example, I would hypothesize that activation of the Rpl22 promoter by Miz-1 as described in Chapter II is POZ domain dependent, and Miz-1 probably recruits co-activators to this gene via the POZ domain. However, because the DN-to-DP transition appears to depend on the interaction between Miz-1 and c-Myc which occurs between zinc fingers 12 and 13 on Miz-1, I would hypothesize that loss of the entire Miz-1 protein would have a more severe effect on this transition than loss of the POZ domain alone. A complete conditional Miz-1 knock-out would therefore most likely have lymphocyte defects similar to the Miz-1$^{\Delta POZ}$ mouse, combined with the defects of the Myc$^{V394D}$ mouse.

# 7.0 Concluding remarks

Lymphocyte development is a complex process that requires the coordinated expression of many transcription factors. These transcription factor networks must be tightly controlled in order to prevent leukemic transformation. Based on the two studies presented in this thesis as well as work previously published by our lab, I propose a model in which Miz-1 is necessary to control the activation of p53 in lymphocyte development and leukemic transformation. The findings presented here could be used to develop therapies targeting Miz-1 in T-ALL patients, in order to reduce the toxicity of the treatments given to these individuals.

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# Miz-1 regulates translation of *Trp53* via ribosomal protein L22 in cells undergoing V(D)J recombination

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**To be effective, the adaptive immune response requires a large repertoire of antigen receptors, which are generated through V(D)J recombination in lymphoid precursors. These precursors must be protected from DNA damage-induced cell death, however, because V(D)J recombination generates double-strand breaks and may activate p53. Here we show that the BTB/POZ domain protein Miz-1 restricts p53-dependent induction of apoptosis in both pro-B and DN3a pre-T cells that actively rearrange antigen receptor genes. Miz-1 exerts this function by directly activating the gene for ribosomal protein L22 (Rpl22), which binds to p53 mRNA and negatively regulates its translation. This mechanism limits p53 expression levels and thus contains its apoptosis-inducing functions in lymphocytes, precisely at differentiation stages in which V(D)J recombination occurs.**

Miz-1 | p53 | V(D)J recombination | Rpl22

The development of T lymphocytes starts with early thymic progenitors (ETPs) that first enter the thymus after transiting through the bloodstream from the bone marrow. These progenitor cells differentiate through four $CD4^-CD8^-$ double-negative stages of development (DN1–4) before becoming first $CD4^+CD8^+$ double-positive (DP) cells and then either $CD4^+$ or $CD8^+$ single positive T cells. The four DN subsets are differentiated based on their expression of the cell surface markers CD44 and CD25. Cells at the DN3 stage ($CD44^-CD25^+$) are fully committed to the T cell lineage and require signaling through cytokine receptors and Notch1 to survive (1). This DN3 population can be further subdivided into DN3a and DN3b based on their size and CD27 surface expression.

Importantly, DN3a cells ($FSC^{lo}CD27^{lo}$) actively rearrange the genes encoding the T cell receptor β (TCRβ) chain through V(D)J recombination (1–3). Those DN3a cells that do not productively rearrange the TCRβ locus on both alleles are eliminated by apoptosis. In contrast, cells that productively rearrange their TCRβ chain genes are selected and become DN3b cells ($FSC^{hi}CD27^{hi}$), which express a pre-T cell receptor (pre-TCR), a heterodimer between the TCRβ chain and a pTα chain. DN3b cells grow in size, owing in part to increased metabolic activity (4), and give rise to the ($CD44^-CD25^-$) DN4 subset, which in turn rapidly expands to produce $CD4^+CD8^+$ DP cells.

Because V(D)J recombination involves DNA double-strand breaks, it has been speculated that the activity of p53 as a DNA damage response factor must be contained and regulated during this process, to prevent DN3a cells from prematurely undergoing apoptosis (5, 6). V(D)J recombination occurs in DN3a cells when they are in G1, and a checkpoint might exist at this stage that specifically protects against DNA damage-induced p53-mediated apoptosis. Similar to pre-T cells, early B cell precursors in the bone marrow, so-called "pro-B" cells, begin to rearrange the genes encoding the Ig heavy chain, also through V(D)J recombination (7). Thus, a similar mechanism as for pre-T cells might also exist for pro-B cells at this stage to protect them from DNA damage-induced p53-mediated apoptosis (8). As for pre-T cells, pro-B cells that have productively rearranged the IgH locus become pre-B cells and express a pre-B cell receptor (pre-BCR) on their surface, which consists of the Ig heavy chain and two surrogate light chains (9). Those pro-B cells that have not productively rearranged the Ig heavy chain genes die by apoptosis, as is the case for DN3a cells that fail to productively rearrange their TCRβ chain genes, mainly because the pre-TCR– and pre-BCR–mediated proliferation and survival signals are lacking.

Myc-interacting zinc finger protein 1 (Miz-1) is an 87-kDa protein that was first described as an interaction partner for the proto-oncoprotein c-Myc (10). It contains 13 $C_2H_2$ type zinc finger domains at its C terminus and a BTB/POZ domain at its N terminus. This POZ domain is required for Miz-1 function, because it mediates the formation of homotetramers and interactions with various partner proteins (11). In addition, loss of this domain renders Miz-1 incapable of stably binding to chromatin (12). Among the validated Miz-1 target genes are those that encode the negative cell cycle regulators Cdkn2b (p15) (12, 13) and Cdkn1a (p21) (14, 15). Furthermore, a number of upstream factors regulate Miz-1 activity, including Bcl-6 (15, 16), which is itself a POZ domain transcription factor that binds directly to Miz-1, the DNA damage sensor protein TopBP1 (14), the E3 ubiquitin ligase HectH9 (17, 18), and the proto-oncoprotein Akt (10, 19). However, it also has been shown that recruitment of the c-Myc/Max complex by Miz-1 inhibits transcriptional

## Significance

**V(D)J recombination occurs in lymphoid precursors to enable their maturation, but also induces DNA damage. Thus, it has been proposed that the activity of the tumor suppressor and gatekeeper protein p53 must be controlled during this process to prevent premature induction of apoptosis. In this study, we show that the transcription factor Miz-1 can exert such a function. Miz-1 activates expression of the ribosomal protein Rpl22, which in turn controls the translation of p53 specifically in lymphoid precursors. We propose that this Miz-1–Rpl22–p53 pathway prevents p53 from inducing cell death as a response to V(D)J recombination in lymphoid precursors from both the T-lineage and the B-lineage.**

Author contributions: M.R. and T.M. designed research; M.R., J.R., M.G., and C.K. performed research; W.-K.S. and W.G. contributed new reagents/analytic tools; M.R. and C.V. analyzed data; and M.R. and T.M. wrote the paper.

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activation, very likely by interfering with the formation of an activating Miz-1/p300 complex (12).

We have previously described a Miz-1–deficient mouse, in which the POZ domain of Miz-1 is conditionally deleted specifically in hematopoietic cells (Miz-1$^{flox/flox}$ × Vav-Cre; hereafter referred to as Miz-1$^{\Delta POZ}$ animals). This model has allowed us to identify a role of Miz-1 in the regulation of IL-7 receptor signaling in early steps of T cell and B cell development (20, 21). We also have demonstrated that Miz-1 regulates expression of p53 effector genes in DN3a cells undergoing pre-TCR selection to generate DN3b cells (22). Although Miz-1 does not affect V(D)J recombination, Miz-1–deficient DN3a cells die and exhibit increased transcriptional activation of a subset of p53 target genes known for inducing apoptosis (22).

In this study, we present evidence of a novel mechanism that controls p53 activity in pre-T and pro-B cells when V(D)J recombination occurs. Our results indicate that in these cells, Miz-1 activates transcription of the gene encoding ribosomal protein L22 (Rpl22), a component of the 60S ribosomal subunit. Because our data indicate that Rpl22 binds directly to p53 mRNA and negatively controls its translation, we propose that Miz-1 controls p53 protein expression levels via Rpl22 at stages of lymphoid development when p53 action must be contained.

## Results

**Ablation of *Trp53* Restores the Development of Miz-1–Deficient αβ-Lineage Pre-T and Pro-B Cells.** To determine whether the developmental block and increased apoptosis seen in Miz-1–deficient pre-T cells and pro-B cells are related to increased p53 signaling, we generated p53-deficient Miz-1$^{\Delta POZ}$ mice. We found significant increases in both the percentage and absolute number of DN3b and DN4 cells in Miz-1$^{\Delta POZ}$ × Trp53$^{-/-}$ mice, as well as almost completely restored thymic cellularity, compared with Miz-1$^{\Delta POZ}$ mice (Fig. 1 *A* and *B*). Deletion of p53 also partially restored the ability of Miz-1–deficient DN3 pre-T cells to transition to the DN4 stage in vitro on an OP9-DL4 stromal layer, whereas proliferation of these cells after DN4 remains impaired (Fig. S1*A*). Miz-1$^{\Delta POZ}$ DN3a pre-T cells have been shown to exhibit increased apoptosis and deficient progression through the cell cycle (22). The increase in apoptosis in Miz-1$^{\Delta POZ}$ DN3a pre-T cells is completely abolished in Miz-1$^{\Delta POZ}$ × Trp53$^{-/-}$ mice, as shown by annexin V staining (Fig. 1 *C* and *D*). Furthermore, ablation of p53 rescues the ability of Miz-1–deficient DN3 pre-T cells to progress through the cell cycle, as shown by both propidium iodide and BrdU stainings (Fig. S1 *B* and *C*). The development of Miz-1–deficient pro-B cells was rescued in Miz-1$^{\Delta POZ}$ × Trp53$^{-/-}$ mice as well, as indicated by the threefold increase in the percentage of pro-B cells expressing CD19 on the surface and the threefold increase in the absolute number of pre-B cells in the bone marrow (Fig. 2 *A* and *B*). These results suggest that the developmental defects in Miz-1$^{\Delta POZ}$ pre-T and pro-B cells are linked to both p53-mediated cell cycle arrest and apoptotic cell death.

**The Developmental Arrest in Miz-1–Deficient Pre-T Cells and Pro-B Cells Is Associated with Induction of *Puma* and *Bax*.** Ablation of p53 in Miz-1$^{\Delta POZ}$ DN3 pre-T cells and pro-B cells restored the overexpression of p53 target genes *Cdkn1a* (p21), *Bax*, and *Bbc3* (*Puma*) to WT levels (Figs. 3*A* and 4*A*). We had previously shown that deletion of *Cdkn1a* fails to rescue the transition from DN3a to DN3b (22), which excludes the possibility that p53-mediated cell cycle arrest through activation of *Cdkn1a* (p21) is responsible for this differentiation block. Thus, we attempted to restore pre-T cell development by preventing activation of the proapoptotic p53 targets *Puma* and *Bax*, using p53$^{K117R}$ knock-in mice in which p53 is specifically rendered unable to activate transcription of these genes (23–25). Lymphocyte development in Miz-1–deficient p53$^{K117R}$ (Miz-1$^{\Delta POZ}$ × p53$^{K117R}$) mice is partially restored, as indicated by the decreased percentage of total DN cells compared with the Miz-1–deficient thymus (Fig. 3*B*). Furthermore, there was a fivefold decrease in the percentage of DN3a cells undergoing apoptosis compared with Miz-1$^{\Delta POZ}$ DN3a cells, as shown by annexin V staining (Fig. 3*B*). In addition, total thymic cellularity and absolute numbers of DN3b cells were increased in Miz-1$^{\Delta POZ}$ × p53$^{K117R}$ mice compared with Miz-1$^{\Delta POZ}$ mice (Fig. 3*C*). The development of pro-B cells was also partially restored in Miz-1$^{\Delta POZ}$ × p53$^{K117R}$ mice compared with Miz-1$^{\Delta POZ}$ mice, as indicated by the twofold increase in the percentage of pre-B cells expressing CD19 on the surface and the absolute number of pre-B cells in the bone marrow of Miz-1$^{\Delta POZ}$ × p53$^{K117R}$ mice (Fig. 4 *B* and *C*).

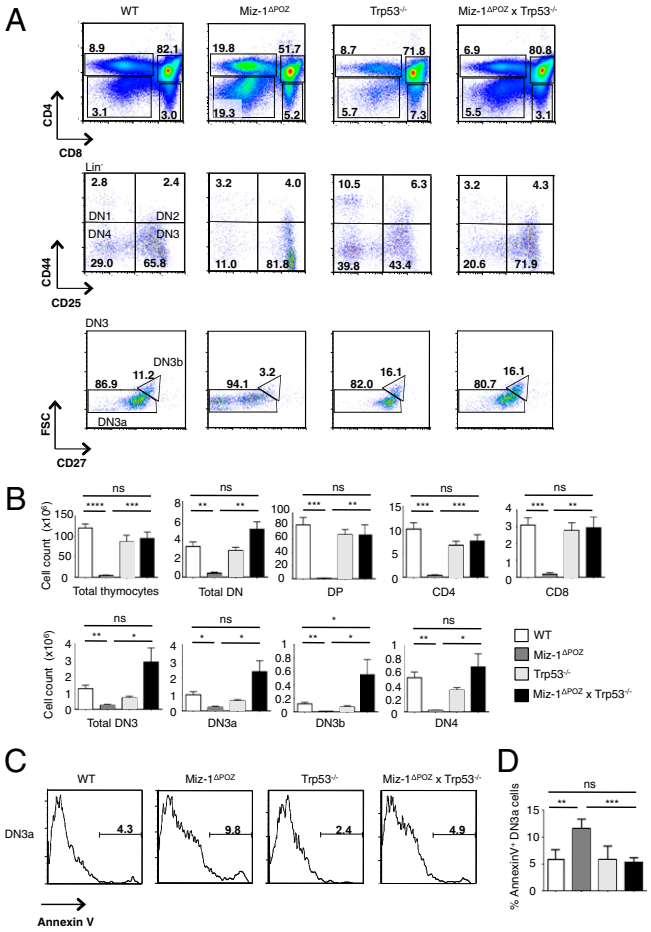


**Fig. 1.** Deletion of p53 restores pre-T cell development in Miz-1$^{\Delta POZ}$ mice. (*A*) FACS analysis of thymic subsets from WT, Miz-1$^{\Delta POZ}$, Trp53$^{-/-}$, and Miz-1$^{\Delta POZ}$ × Trp53$^{-/-}$ mice. FACS plots are a representative example of four independent experiments. (*B*) Absolute cell counts of thymocyte subsets from *A*. Data are averaged from four independent experiments and are presented as mean ± SD. (*C*) FACS analysis of annexin V staining on DN3 pre-T cells. FACS plots are a representative example of three independent experiments. (*D*) Quantification of percentage of annexin V$^{+}$ cells from staining in *C*. Data are averaged from three independent experiments and are presented as mean ± SD.

**Miz-1–Deficient DN3 Cells Progress to DN4 in the Absence of V(D)J Recombination.** To determine whether V(D)J recombination-induced DNA damage might be responsible for the p53-mediated differentiation block of Miz-1–deficient pre-T cells at the DN3a stage, we crossed Miz-1$^{\Delta POZ}$ mice with *Rag1*$^{-/-}$ mice that are defective in rearranging TCR or Ig genes. We injected these *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$ mice with αCD3 antibodies to mimic pre-TCR signaling, and then followed the differentiation of DN3

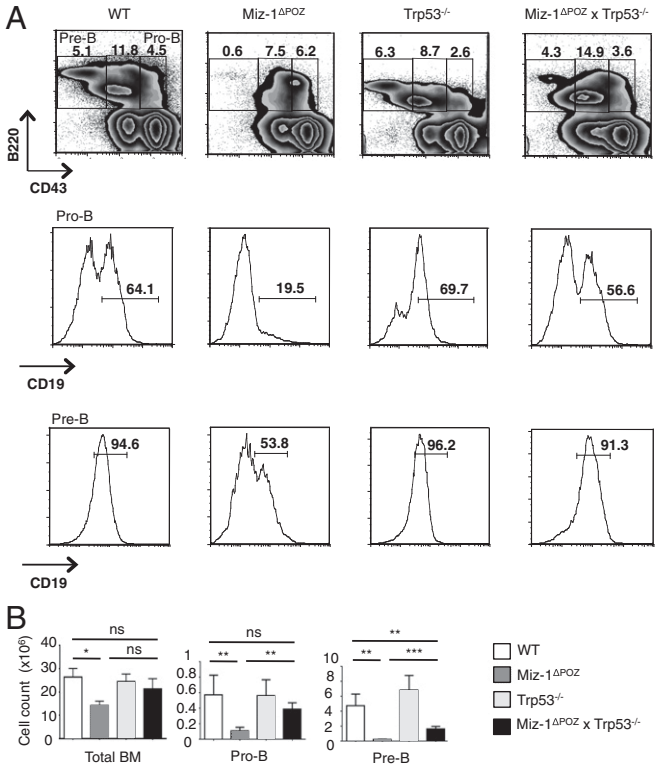


**Fig. 2.** Deletion of p53 restores early steps of B cell development in Miz-$1^{\Delta POZ}$ mice. (*A*) FACS analysis of B cell subsets from BM of WT, Miz-$1^{\Delta POZ}$, $Trp53^{-/-}$, and Miz-$1^{\Delta POZ}$ × $Trp53^{-/-}$ mice. FACS plots are a representative example of four independent experiments. (*B*) Absolute cell counts of B cell subsets from *A*. Data are averaged from four independent experiments and are presented as mean ± SD.

cells into the DN4 and DP stages as described previously (26). Whereas αCD3 stimulation had no effect on Miz-1–deficient pre-T cells with a functional *Rag1* gene (Fig. S2), the ablation of *Rag1* in Miz-$1^{\Delta POZ}$ mice clearly allowed a full differentiation of Miz-1–deficient DN3 cells to DN4 cells (Fig. 5*A*); however, although $Rag1^{-/-}$ thymocytes were able to further differentiate into DP cells at 72 h after αCD3 injection, $Rag1^{-/-}$ × Miz-$1^{\Delta POZ}$ thymocytes were blocked at the DN4 stage (Fig. 5*A*) and were unable to proliferate to the same extent as $Rag1^{-/-}$ thymocytes (Fig. 5*B*).

Because c-Myc is a Miz-1 cofactor and has been identified as a mediator of the DN-to-DP transition, we tested whether the ability of c-Myc to bind to Miz-1 is critical for this function. For this test, we used $Myc^{V394D}$ knock-in mice, in which c-Myc is unable to interact with Miz-1 owing to a mutation in its helix–loop–helix domain, but can still dimerize with Max (14, 27). As expected, stimulation of both $Rag1^{-/-}$ × $Myc^{V394D}$ and $Rag1^{-/-}$ thymocytes with αCD3 led to full differentiation of DN3 cells to the DN4 stage (Fig. 5*C*). However, although both $Rag1^{-/-}$ and $Rag1^{-/-}$ × $Myc^{V394D}$ thymocytes were able to differentiate into DP cells at 72 h after injection, this transition was less efficient in the $Rag1^{-/-}$ × $Myc^{V394D}$ animals, as indicated by the lower poststimulation absolute cell counts and DP cell percentages in these mice (Fig. 5 *C* and *D*). Two c-Myc effectors, CD71 and CD98, which are highly expressed pre-TCR and post-TCR selection, were not up-regulated on the surface of αCD3-stimulated $Rag1^{-/-}$ × Miz-$1^{\Delta POZ}$ T cells (Fig. 5*E*). Furthermore, both CD71 and CD98 were less efficiently up-regulated on the surface of $Rag1^{-/-}$ × $Myc^{V394D}$ T cells stimulated with αCD3 compared with $Rag1^{-/-}$-stimulated T cells (Fig. 5*F*). These results indicate that in the absence of V(D)J recombination, Miz-1–deficient thymocytes regain the ability to pass through pre-TCR selection and give rise to DN4 cells, but still are unable to differentiate further to become DP thymocytes, because they likely require a functional Miz-1/c-Myc complex for this step.

**Miz-1 Binds to the Promoter of *Rpl22* and Regulates Its Expression in DN3 Pre-T cells and Pro-B Cells.** ChIP-seq experiments in P6D4 cells, a DN3 pre-T cell line, and 70Z/3 cells, a pre-B cell line, showed that Miz-1 does not bind to the promoters of p53 target genes, such as *Cdkn1a*, *Bax*, or *Puma*, in these cells, although they are deregulated in Miz-$1^{\Delta POZ}$ cells (Figs. S3*A* and S4*A*). P53 target genes are actively transcribed in these cells, however, as demonstrated by the presence of the histone activation marks in the ChIP-seq experiment and active transcription in the RNA-seq experiment performed in the same cells. The *Vamp4* gene promoter contains a Miz-1–binding site and has been shown to be a bona fide Miz-1 target (28), and is used as a control. The data from the Miz-1 ChIP-seq experiments were confirmed by

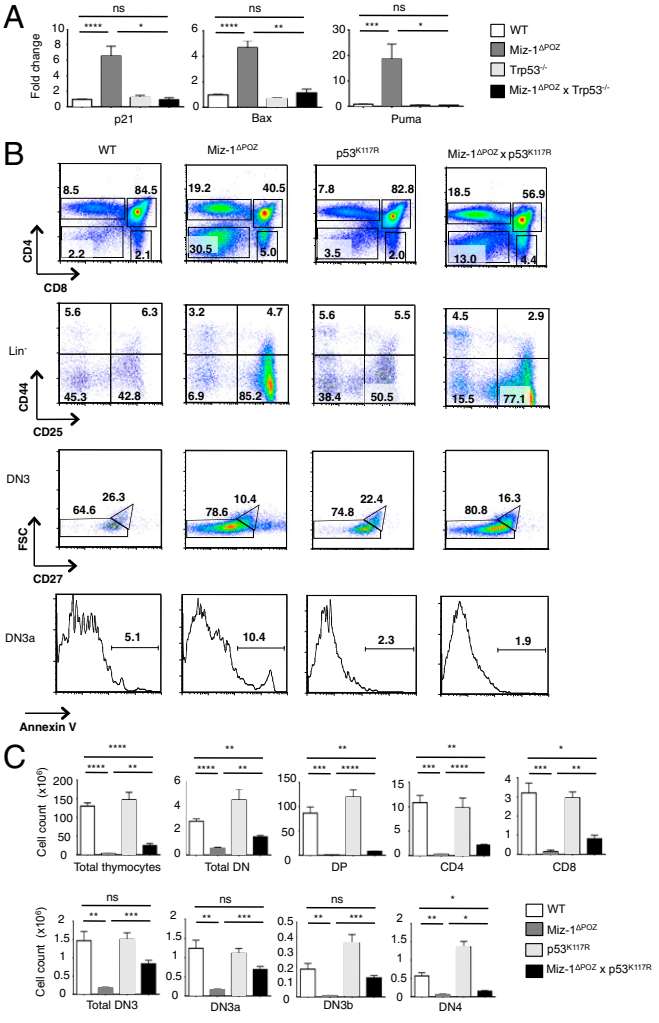


**Fig. 3.** The developmental arrest in Miz-1–deficient thymocytes is associated with the overexpression of several p53 effectors. (*A*) Analysis of p53 target gene expression by RT-qPCR in sorted DN3 pre-T cells from WT, Miz-$1^{\Delta POZ}$, $Trp53^{-/-}$, and Miz-$1^{\Delta POZ}$ × $Trp53^{-/-}$ mice. Data represent average fold change over *Gapdh* from three independent experiments and are presented as mean ± SD. (*B*) FACS analysis of thymic subsets from WT, Miz-$1^{\Delta POZ}$, $p53^{K117R}$, and Miz-$1^{\Delta POZ}$ × $p53^{K117R}$ mice. FACS plots are a representative example of three independent experiments. (*C*) Absolute cell counts of thymocyte subsets from *B*. Data are averaged from three independent experiments and are presented as mean ± SD.

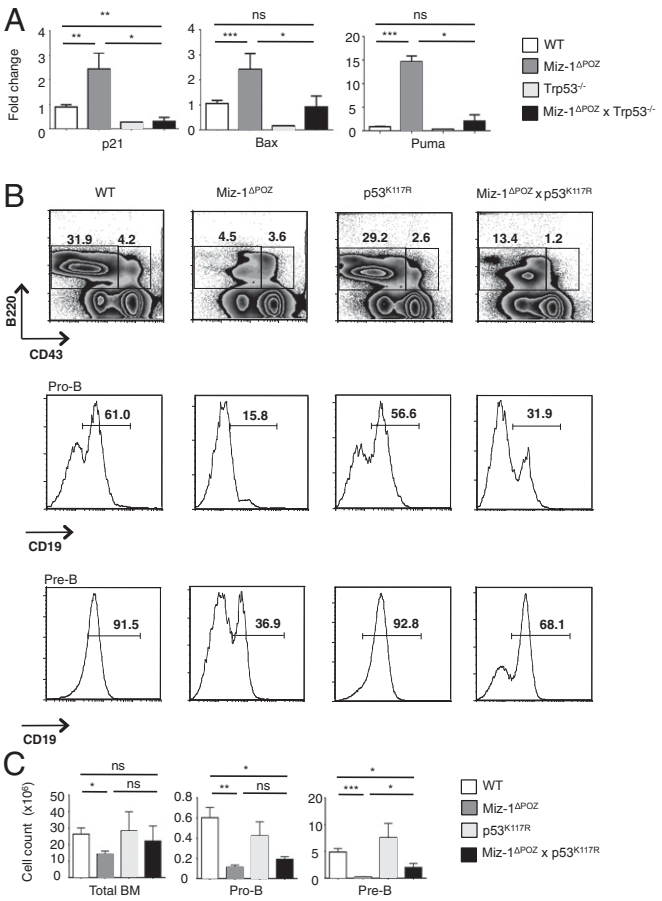


**Fig. 4.** The developmental arrest in Miz-1–deficient pre-B cells is associated with several p53 effectors. (*A*) Analysis of p53 target gene expression by RT-qPCR in sorted pro-B cells from WT, Miz-1$^{\Delta POZ}$, Trp53$^{-/-}$, and Miz-1$^{\Delta POZ}$ × Trp53$^{-/-}$ mice. Data represent average fold change over *Gapdh* from two independent experiments and are presented as mean ± SD. (*B*) FACS analysis of B cell subsets from BM of WT, Miz-1$^{\Delta POZ}$, p53$^{K117R}$, and Miz-1$^{\Delta POZ}$ × p53$^{K117R}$ mice. FACS plots are a representative example of three independent experiments. (*C*) Absolute cell counts of B cell subsets from *B*. Data are averaged from three independent experiments and are presented as mean ± SD.

ChIP-quantitative PCR (qPCR) with P6D4 cells (Fig. S3*B*) and with sorted primary WT DN3 pre-T cells (Fig. S3*C*), as well as with 70Z/3 cells (Fig. S4*B*). These results suggest that the expression of the proapoptotic p53 target genes *Puma* and *Bax*, which are partially responsible for the developmental block in Miz-1$^{\Delta POZ}$ DN3 pre-T cells and pro-B cells (Figs. 3 and 4), is not directly regulated by Miz-1.

The analysis of a microarray dataset from Miz-1–deficient DN3 pre-T cells (22) identified *Rpl22* as the most down-regulated gene in these cells compared with WT DN3 pre-T cell controls. Both DN3 pre-T cells and CD19$^{+}$ pro-B cells were sorted from WT and Miz-1$^{\Delta POZ}$ littermates, and a significantly down-regulated *Rpl22* mRNA expression level was confirmed in both cell types (Fig. 6*A*). Furthermore, ChIP-seq data from P6D4 and 70Z/3 cells indicated that Miz-1 occupies the promoter of *Rpl22*. A combination of the presence of active histone marks (ChIP-seq) and high levels of mRNA (RNA-seq) indicated that this gene is actively transcribed in both P6D4 pre-T cells and 70Z/3 pre-B cells (Fig. 6*B*). Miz-1 binding to the *Rpl22* promoter was validated by ChIP-qPCR in P6D4 cells, sorted WT DN3 pre-T cells, and 70Z/3 cells (Fig. 6*C*) using different primer pairs (Fig. 6*B*, primer pairs 2 and 3), indicating that *Rpl22* is a direct Miz-1 target gene.

To confirm activation of the *Rpl22* promoter by Miz-1, we cotransfected 293T cells with the human *Rpl22* promoter fused to luciferase and increasing amounts of human Miz-1. We found that increasing amounts of Miz-1 led to increased activation of the *Rpl22* promoter (Fig. 6*D*). This suggests that Miz-1 not only binds to the *Rpl22* promoter in DN3 pre-T cells and pre-B cells, but also favors transcriptional activation of this gene.

We previously showed that overexpression of Bcl2 in Miz-1$^{\Delta POZ}$ mice (Miz-1$^{\Delta POZ}$ × Bcl2 Tg) rescues the apoptosis of Miz-1–deficient ETPs and partially rescues total thymic cellularity, but has no effect on the developmental block of Miz-1–deficient DN3 pre-T cells (21). Because Miz-1$^{\Delta POZ}$ × Bcl2 Tg mice have increased numbers of thymocytes compared with Miz-1$^{\Delta POZ}$ mice, we used them to test the effect of Miz-1 deficiency on the expression levels of p53 protein. Thymocyte extracts from Miz-1$^{\Delta POZ}$ × Bcl2 Tg mice showed increased p53 protein levels compared with Bcl2 Tg mice (Fig. 6*E*). This is consistent with

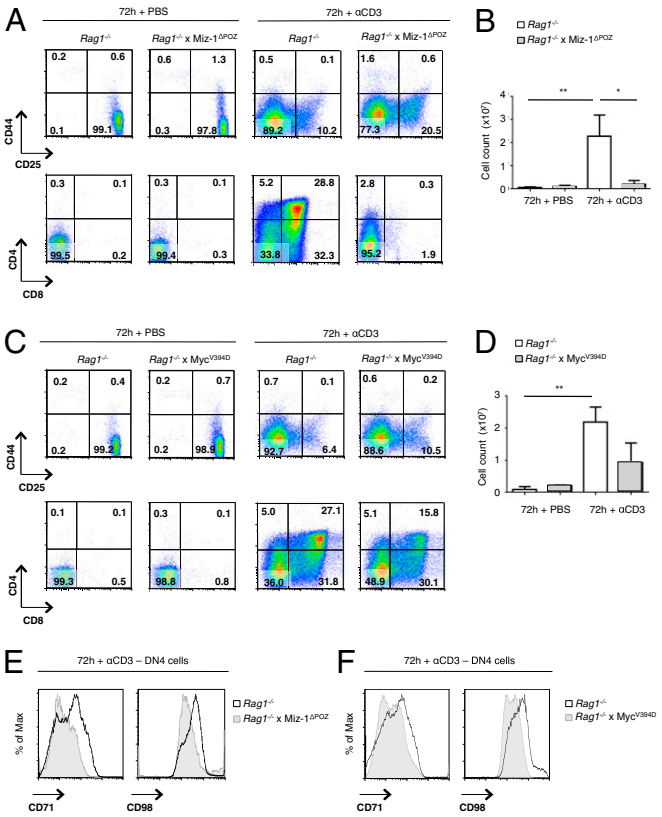


**Fig. 5.** Mitogenic stimulation of *Rag1*$^{-/-}$, *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$, and *Rag1*$^{-/-}$ × Myc$^{V394D}$ KI pre-T cells. (*A*) FACS analysis of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$ mice at 72 h after injection with PBS or αCD3 antibodies. FACS plots are a representative example of three independent experiments. (*B*) Absolute cell counts of total thymocytes from *A*. Data are averaged from three independent experiments and are presented as mean ± SD. (*C*) FACS analysis of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ × Myc$^{V394D}$ KI mice at 72 h after injection with PBS or αCD3. FACS plots are a representative example of three independent experiments. (*D*) Absolute cell counts of total thymocytes from *B*. Data are averaged from three independent experiments and are presented as mean ± SD. (*E*) FACS analysis of CD71 and CD98 on *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$ thymocytes at 72 h after injection with αCD3. FACS plots are a representative example of three independent experiments. (*F*) FACS analysis of CD71 and CD98 on *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ × Myc$^{V394D}$ thymocytes at 72 h after injection with αCD3. FACS plots are a representative example of two independent experiments.

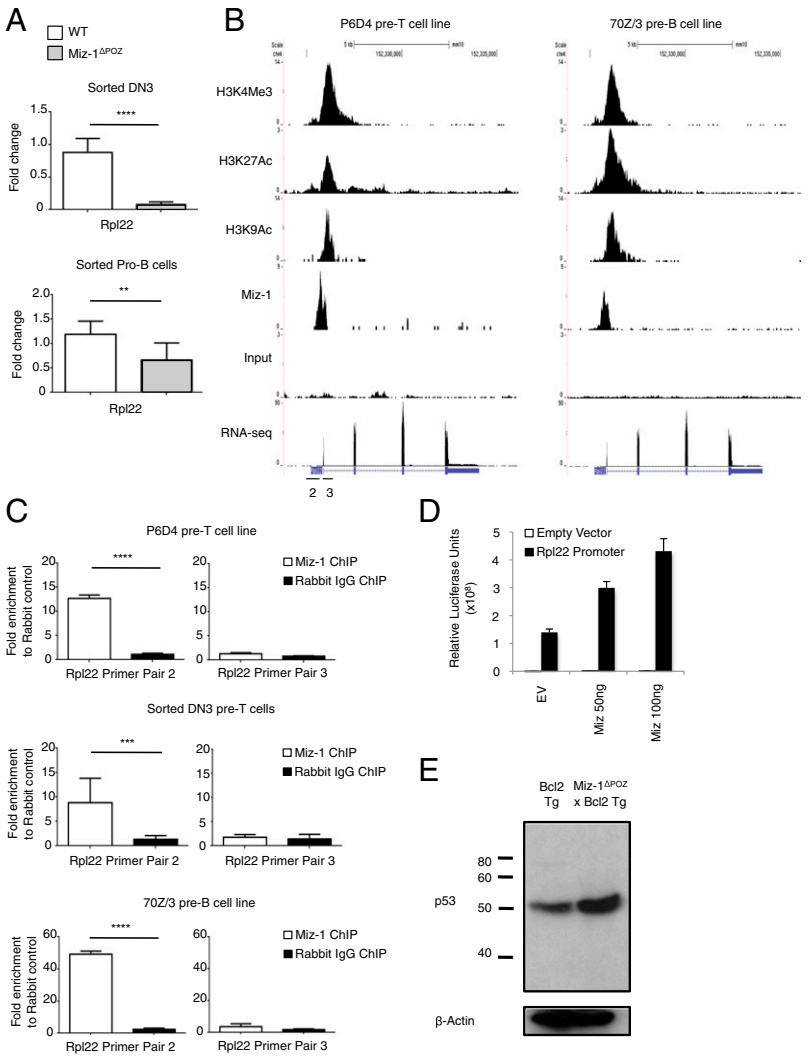


**Fig. 6.** Miz-1 regulates the expression of *Rpl22* in DN3 pre-T cells and pro-B cells. (*A*) *Rpl22* mRNA expression was assessed in sorted DN3 pre-T cells (*Upper*) and sorted pro-B cells (*Lower*) from WT and Miz-1$^{\Delta POZ}$ mice. Data are averaged from three independent experiments and are presented as mean ± SD. (*B*) ChIP-seq experiments for Miz-1 and histone activation marks from P6D4 murine pre-T cells (*Right*) and 70Z/3 murine pre-B cells (*Left*). Shown is the *Rpl22* locus. Scale is in number of RPM. Primer pairs were designed in the promoter (2) or first exon (3) of *Rpl22* to determine Miz-1 binding by ChIP. (*C*) ChIP-qPCR experiments to determine binding of Miz-1 to the promoter of *Rpl22* in P6D4 murine pre-T cells (*Top*), sorted WT DN3 pre-T cells (*Middle*), and 70Z/3 murine pre-B cells (*Bottom*). Graphs show fold enrichment of anti–Miz-1 ChIP over rabbit IgG control ChIP. Data represent as average fold change ± SD from at least three independent experiments. (*D*) 293T cells were transfected with the human *Rpl22* promoter fused to luciferase and pcDNA3.1 empty vector (EV) or pcDNA3.1 with human Miz-1 in varying concentrations. Data were normalized for transfection using β-galactosidase. Data are presented as average relative luciferase units ± SD and are representative of three independent experiments. (*E*) Whole protein extracts from total thymus of Bcl2 Tg or Miz-1$^{\Delta POZ}$ × Bcl2 Tg mice were evaluated for p53 expression by Western blot analysis. Data are representative of at least three independent experiments.

a previous report that loss of *Rpl22* correlates with an increased synthesis of p53 protein (29). Furthermore, this suggests that loss of Miz-1 leads to down-regulation of *Rpl22*, which increases the expression of p53 protein in DN3 pre-T cells.

To determine whether overexpression of Rpl22 could rescue the phenotype of Miz-1$^{\Delta POZ}$ pre-T cells, we infected DN3a cells from WT and Miz-1$^{\Delta POZ}$ mice with a retrovirus expressing Rpl22. Overexpression of Rpl22 partially restored the ability of these cells to survive and differentiate into DN4 and cells in vitro on OP9-DL4 stromal cells after 4 d (Fig. 7*A*); however, although the absolute numbers of both live cells and GFP$^{+}$ DN4 cells could be clearly restored on forced expression of Rpl22 (Fig. 7*B*), the cells were still unable to proliferate and differentiate past the DN4 stage into DP cells (Fig. 7*A*). Owing to the limited number of DN4 cells produced on the OP9-DL4 stromal layer in this experiment, it was not possible to measure the effect of Rpl22 overexpression on the levels of p53 protein; such an experiment will be necessary to further support direct links among Miz-1, Rpl22, and p53 protein levels.

**Rpl22 Binds to p53 mRNA to Regulate Translation.** To test whether Miz-1 deficiency affects the stability of p53 mRNA, we sorted DN3 pre-T cells from WT and Miz-1$^{\Delta POZ}$ littermates and treated them with actinomycin D, an inhibitor of RNA polymerase II, over 4 h (Fig. 8*A*). The degradation of p53 mRNA over this time period is comparable in both WT and Miz-1–deficient cells, indicating that the increase in p53 protein is not related to increased stability of p53 mRNA. To determine whether loss of Miz-1 leads to increased incorporation of p53 mRNA into actively translating ribosomes, we again used Miz-1$^{\Delta POZ}$ × Bcl2 Tg

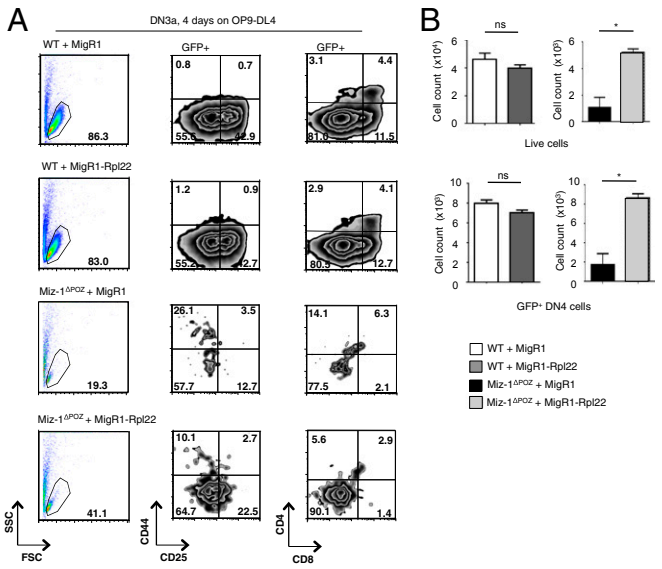


**Fig. 7.** Overexpression of Rpl22 in Miz-1–deficient DN3a cells partially restores survival and differentiation. (*A*) Flow cytometry analysis of sorted WT and Miz-1$^{\Delta POZ}$ DN3a cells infected with MigR1 or MigR1-Rpl22 after 4 d of coculture on OP9-DL4. Indicated graphs are gated on a GFP$^+$ population. Data are representative of two independent experiments. (*B*) Absolute total live cell number (*Upper*) and total GFP$^+$DN4 cell number (*Lower*) from OP9-DL4 experiments in *A*.

mice, because the low number of thymocytes in Miz-1$^{\Delta POZ}$ animals limits experimentation. Thymocytes from Bcl2 Tg and Miz-1$^{\Delta POZ}$ × Bcl2 Tg mice were isolated, and lysates were sedimented through sucrose gradients. RNA from the fractionated gradients was extracted and analyzed by RT-qPCR for the presence of p53 mRNA. In Miz-1–deficient samples, the percentage of p53 mRNA in polysome fractions (fractions 8–12) was significantly increased compared with WT controls (Fig. 8*B*). Because the percentage of *β-actin* mRNA in polysomes is not significantly different in Miz-1–deficient and WT thymocytes (Fig. 8*C*), a global shift in translation in Miz-1–deficient cells can be ruled out, suggesting that Miz-1 is required to specifically regulate p53 protein levels by controlling the incorporation of p53 mRNA into actively translating ribosomes, likely via Rpl22.

To determine whether the increased translation of p53 is related to direct interaction of p53 mRNA with Rpl22 protein, we performed RNA immunoprecipitation (RIP) in P6D4 pre-T cells. Using this technique, we found a 25-fold increase in the amount of p53 mRNA bound by the Rpl22 protein compared with the rabbit IgG control (Fig. 8*D*). Rpl22 has been shown to bind the mRNA of its paralog, *Rpl22l1* (30), which served as a positive control for the Rpl22 RIP. We found a 10-fold increase in the amount of *Rpl22l1* mRNA bound by Rpl22 compared with the rabbit IgG control (Fig. 8*D*), confirming that both *Rpl22l1* and p53 mRNA are bound by Rpl22. We also assessed the presence of *β-actin* mRNA in the RIP and found that although translation of this gene is not significantly different in Miz-1–deficient thymocytes compared with WT, it is bound by Rpl22.

## Discussion

V(D)J recombination is necessary to rearrange TCR or Ig gene segments and to ensure generation of a large repertoire of antigen receptors. T and B lymphocytes, which carry one antigen-specific TCR or Ig at their cell surface, require such a repertoire to ensure recognition of a very large spectrum of antigens. The process of V(D)J recombination itself must be tightly regulated, however, and must be coordinated with DNA replication and mitosis to avoid genomic damage. This is achieved in part by linking V(D)J recombination to cell cycle progression by periodic phosphorylation and destruction of the Rag-2 recombinase that breaks and rejoins Ig or TCR gene segments, with the effect of allowing V(D)J recombination only in cells at G1 (31). In addition, however, it has been proposed that a DNA damage response pathway that may be initiated as a consequence of the double-strand breaks occurring during V(D)J recombination must be controlled to avoid the induction of p53-mediated apoptosis (5, 6). Here we provide evidence that the BTB/POZ domain transcription factor Miz-1 exerts such a function in both T- and B-lineage cells by restricting the expression of p53 through the inhibition of its translation via the ribosomal protein Rpl22 specifically in cells that undergo V(D)J recombination.

Miz-1–deficient pre-T cells express all of the necessary components to properly undergo pre-TCR selection (22), but are blocked at the DN3 stage and do not give rise to significant numbers of DN4 cells. In addition, DN3a cells lacking a functional Miz-1 show increased rates of apoptosis, and several experiments conducted during the present study suggest that this differentiation block and the increased cell death rate in DN3 pre-T cells is mediated by an overactive p53. First, as shown previously and confirmed here, various direct, known p53 target genes, including *Cdkn1a*, *Bax*, and *Puma*, are up-regulated in Miz-1–deficient cells. Second, deletion of p53 restores not only the numbers of DN3 pre-T cells, but also proper expression levels of *Cdkn1a*, *Bax*, and *Puma* and overall thymic cellularity, indicating full rescue of T cell differentiation. In addition, p53 deletion also partially restores the numbers of pre-B cells that otherwise do not develop from pro-B cells in Miz-1–deficient mice. Although there is a complete rescue of T cell differentiation in the thymus, loss of p53 expression only partially restores B cell development in Miz-1–deficient mice. This may be related to the fact that the previously reported defect in IL7 signaling has a stronger impact on B cell precursors than on T cell precursors (20). The fact that both pre-T cells and pro-B cells are affected by Miz-1 ablation and can regain their differentiation ability after concomitant p53 deletion precisely at stages in which V(D)J recombination occurs supports the hypothesis that this function of Miz-1 represents a general mechanism controlling potentially damaging consequences of V(D)J recombination regardless of cell lineage.

Although p53 affects both cell cycle progression and apoptosis, our data suggest that the proapoptotic p53 effectors *Puma* (*Bbc3*) and *Bax* are mainly responsible for the developmental block of Miz-1–deficient pre-T and pro-B cells. This suggestion is supported by our observation that Miz-1$^{\Delta POZ}$ mice that carry a p53$^{K117R}$ allele, in which p53 has specifically lost its ability to activate expression of both *Puma* and *Bax*, exhibited noticeable rescue of their pre-T and pro-B cell developmental defects. Although loss of the acetylation site at K117 in mouse p53 completely abolishes p53-mediated apoptosis, it has no effect on p53 translation or expression levels and does not affect p53-dependent cell cycle arrest or senescence (23–25). Moreover, total thymic cellularity is only partially restored in these mice, as is the percentage of DN3b pre-T cells. Furthermore, in the bone marrow B cell compartment, absolute numbers of pre-B cells are partially restored. This indicates that other functions of p53 also may be partially responsible for the pre-T cell and pro-B cell differentiation block in Miz-1–deficient mice.

Our findings are consistent with the hypothesis that Miz-1 is involved in regulating a p53-mediated response initiated by V(D)J-induced DNA damage in pre-T or pro-B cells. Further evidence supporting this contention is provided by our finding that Miz-1–deficient DN3 pre-T cells fully develop into DN4 cells in the absence of V(D)J recombination. Indeed, our experiments with *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$ mice showed a massive accumulation

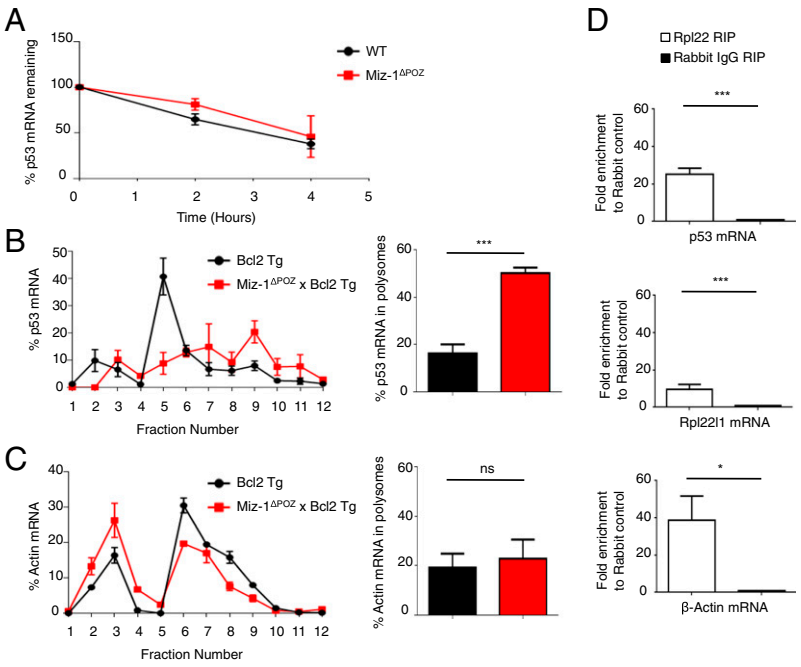


**Fig. 8.** Rpl22 regulates the translation of p53. (*A*) Sorted DN3 cells from WT or Miz-1$^{\Delta POZ}$ mice were treated with 5 μg/mL actinomycin D and harvested at the indicated time points. The percentage of p53 mRNA remaining at each time point was assessed by RT-qPCR and normalized to *Gapdh*. Data are averaged from three independent experiments and are presented as mean ± SD. (*B*) (*Left*) Total thymic extracts from Bcl2 Tg and Miz-1$^{\Delta POZ}$ × Bcl2 Tg mice were sedimented through a sucrose gradient and fractionated. qRT-PCR was performed to measure p53 mRNA in the fractions collected. Data are presented as the percentage of p53 mRNA in each fraction. The graph is representative of three independent experiments. (*Right*) Quantification of the percentage of p53 mRNA in polysomes (fractions 8–12) from left. Data are averaged from three independent experiments and are presented as mean ± SD. (*C*) (*Left*) Total thymic extracts from Bcl2 Tg and Miz-1$^{\Delta POZ}$ × Bcl2 Tg mice were sedimented on a sucrose gradient and fractionated. qRT-PCR was performed for *β-actin* on the fractions collected. Data are presented as the percentage of *β-actin* mRNA in each fraction. The graph is representative of three independent experiments. (*Right*) Quantification of percentage of *β-actin* mRNA in polysomes (fractions 8–12) from the left panel. Data are averaged from three independent experiments and are presented as mean ± SD. (*D*) RNA-IP of Rpl22 in P6D4 pre-T cells. The graph shows fold enrichment of anti-Rpl22 RIP over rabbit IgG control RIP. *Rpl22l1* mRNA served as a positive control for Rpl22 RIP. *β-actin* mRNA is also enriched in anti-Rpl22 RIP. Data represent average fold change ± SD from three independent experiments.

of DN4 cells on CD3 receptor stimulation, to the same extent as seen in *Rag1*$^{-/-}$ mice that express functional Miz-1; however, at this stage, the role of Miz-1 might not be limited to regulating the p53 pathway, because Miz-1$^{\Delta POZ}$ × Trp53$^{-/-}$ DN3 pre-T cells progress to DN4, but these DN4 cells are still less efficient than WT DN4 in their ability to proliferate and further differentiate to DP cells in vitro. In addition, although *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$ mice efficiently generate DN4 cells on αCD3 stimulation, they do not differentiate into DP cells.

Because the Miz-1 binding partner c-Myc is necessary for the transition of DN4 to DP (32), and because the *c-Myc* gene is activated by pre-TCR signaling, we reasoned that the interaction of c-Myc with Miz-1 might be required for an efficient DN4-to-DP transition. This idea is supported by data from *Rag1*$^{-/-}$ × Myc$^{V394D}$ mice, which when stimulated with αCD3 antibodies were less efficient in producing DP cells than *Rag1*$^{-/-}$ mice with a functional c-Myc protein that still binds to Miz-1. In addition, CD71 and CD98, key metabolic genes up-regulated at the DN-to-DP transition and direct targets of c-Myc (33, 34), are not up-regulated on *Rag1*$^{-/-}$ × Miz-1$^{\Delta POZ}$ DN4 cells after mitogenic stimulation. Thus, we propose that, in contrast to the differentiation of DN3 cells to DN4, the DN4-to-DP transition represents a step requiring a functional c-Myc/Miz-1 complex. This is likely necessary for the activation of key metabolic pathways to support cell growth and differentiation of DN4 cells to generate a large number of DP thymocytes.

Although p53 mediates effectors involved in both cell cycle progression and regulation of apoptosis, it is unlikely that V(D)J recombination leads to p53-induced cell cycle arrest, given that the dissociation between DNA replication and V(D)J recombination is ensured by cell cycle stage-specific expression of Rag-2. We have found increased expression of *Cdkn1a* (p21), which can arrest cell cycle progression and is a p53 target gene, in Miz-1–deficient pre-T and pro-B cells; however, we previously showed that deletion of *Cdkn1a* in Miz-1–deficient cells did not rescue the defects caused by ablation of Miz-1, indicating that this part of the p53 pathway, although affected by Miz-1–dependent regulation of p53, is not responsible for the phenotype seen in Miz-1–deficient pre-T and pro-B cells. Thus, we propose that p53 can be activated to induce apoptosis in response to the DNA strand breaks that occur during V(D)J recombination, and that this activation of p53 is at least partially mediated through a mechanism involving Miz-1 and its effector, the ribosomal protein Rpl22. However, it is likely that additional mechanisms for activating p53 (e.g., through posttranslational mechanisms such as phosphorylation or mechanisms that do not involve p53) are also affected by Miz-1 and also play an important role at this critical point in pre-T cell and pro-B cell differentiation. For instance, it is possible that Rpl22 also acts on other proteins, not only via p53, and that forced expression of Rpl22 alone might not be sufficient to completely restore physiological p53 expression levels in Miz-1–deficient cells. Further experiments are needed to clarify the nature of these additional mechanisms and their link to Miz-1.

The regulation of p53 can occur at many levels. P53 expression is most commonly regulated at the posttranslational level, and ribosomal proteins have been shown to play a role in this regulation. Rpl5 (35), Rpl11 (36, 37), Rpl23 (38, 39), and Rpl26 (40) interact directly with the p53 E3 ubiquitin ligase Mdm2 and inhibit its activity, leading indirectly to enhanced stability of p53

under conditions of ribosomal biogenesis stress. Furthermore, Rpl26 has been shown to bind to both the 5′ and 3′ UTRs of p53 mRNA to increase its translation and induction after DNA damage, whereas nucleolin binds specifically to the p53 5′ UTR after DNA damage (41, 42). Our results indicate that, unlike other ribosomal proteins that have been shown to either activate p53 translation or act as Mdm2 antagonists under physiological conditions, Rpl22 directly suppresses p53 translation in a cell type- and developmental stage-specific manner, namely in cells undergoing V(D)J recombination. This finding agrees with previous reports that Rpl22-deficient mice exhibit no defects in global translation, but are defective specifically in regulation of p53 expression during pro-B cell and pre-T cell development (29, 30, 43). The data presented here strongly suggest that this regulation occurs through Miz-1, and that Miz-1 is a direct upstream regulator of the *Rpl22* gene. Furthermore, the data presented here provide evidence for a previously unidentified regulation of p53 translation by a ribosomal protein, in that Rpl22 would be the only example of such a protein to repress the translation of p53 under physiological conditions, by decreasing its incorporation in actively translating ribosomes. In addition, the results presented here indicate that Rpl22 may bind numerous mRNA transcripts, including Rpl22l1 and β-actin, as part of its function in the ribosomal complex. However, based on our polysome fractionation experiment, the interaction between Rpl22 and p53 mRNA increases the incorporation of this mRNA specifically into polysomes and not other mRNA transcripts. How this specificity is achieved requires further investigation.

The expression of p53 must be tightly regulated to promote survival of cells that rearrange the TCRβ or IgH loci. These cells have to break and join DNA ends, a signal that normally initiates a p53-dependent DNA damage response. Our data support a function of Miz-1 that, via Rpl22, acts as a regulator of the translation of p53 in those cells at risk for elimination by p53-dependent cell death because they are undergoing V(D)J recombination.

## Materials and Methods

**Mice.** Mice had been bred on C57BL/6 background for at least 10 generations and were maintained in a specific-pathogen-free plus environment. Miz-$1^{\Delta POZ}$ mice have been described previously (20). $Trp53^{-/-}$ mice were purchased from Jackson Laboratory. $p53^{K117R}$ mice were provided by Dr. Wei Gu (23). The Institut de recherches cliniques de Montréal Animal Care Committee approved the animal protocols under which all animal experiments in this study were performed (protocol 2013–01), and all animal experimental procedures were performed in compliance with the guidelines of the Canadian Council of Animal Care (www.ccac.ca).

**Flow Cytometry Antibodies and Cell Lines.** OP9-DL4/OP9 cultures and P6D4 SCID.adh murine thymic lymphoma and 70Z/3 pre-B cells were used, as described previously (20, 21). All antibodies were purchased from BD Bioscience except when indicated otherwise. DN thymic subsets were analyzed using CD25 (PC61.5; eBioscience) and CD44 (IM7) plus lineage marker-negative cells ($Lin^-$) 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; eBioscience), Pan-NK (DX5), and TCRγδ (GL3), followed by streptavidin-PerCPCy5.5 or PE. The same staining was performed with CD27 (LG.3A10) to differentiate between DN3a and DN3b subsets. Pre-B and pro-B cells were analyzed using B220 (RA3-6B2), CD43 (S7), and CD19 (1D3). Antibody incubations were performed at 4 °C for 20 min in PBS buffer. Cells were analyzed with a BD Biosciences LSR cell analyzer. Cell sorting was performed with a Cytomation MoFlo cell sorter.

**Cell Cycle and Cell Death Analysis.** Cell cycle analysis was performed on sorted DN3 and DN4 cells. Cells were sorted directly into modified Krishan buffer (0.1% sodium citrate, 0.3% Nonidet P-40) containing 0.05 mg/mL propidium iodide (PI) and 0.02 mg/mL RNase and analyzed after 30 min of incubation on a BD FACSCalibur. For bromodeoxyuridine (BrdU) analysis, mice were injected i.p. with 100 mg/kg BrdU in PBS and killed after 16 h. Staining was performed using a FITC BrdU Kit (BD Pharmingen) according to the manufacturer's instructions. Apoptosis rates were measured by annexin V staining (Annexin V-FITC Apoptosis Detection Kit; BD Pharmingen).

**RNA Isolation and Real-Time PCR.** For RNA isolation, cells were FACS-sorted directly into RLT buffer with β-mercaptoethanol (RNEasy Micro Kit; Qiagen). RT-PCR was performed using SuperScript II (Invitrogen). Real-time PCR was performed in triplicate on the ViiA7 using SYBR Green reagent (Applied Biosystems) and indicated primers. The expression of the gene of interest was calculated relative to Gapdh mRNA ($\Delta C_T$) and is presented as fold induction relative to values obtained with the respective control (set as 1-fold). Primer sequences are provided in Table S1.

**Polysome Fractionation.** Polysome fractionation was performed as described previously (44). In brief, single-cell suspensions of $10 \times 10^6$ cells from total thymus were treated with cycloheximide (100 μg/mL) for 15 min at 37 °C. Cells were washed in cold PBS containing 100 μg/mL cycloheximide and then lysed in ice-cold hypotonic lysis buffer (5 mM Tris·HCl pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM KCl, 1× protease inhibitor mixture (complete Mini; Roche Diagnostics), and 400 U/mL RNaseIN (Promega)). The lysates were immediately adjusted to 100 μg/mL cycloheximide, 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate. Cell extracts were centrifuged for 5 min at 20,817 × *g*, after which the supernatants were collected and loaded onto a 10–50% sucrose gradient. Gradients were placed in a Beckman SW40Ti rotor and centrifuged at 266,000 × *g* for 2 h at 4 °C. Fractions were collected (12 1-mL fractions) using a tube piercer system (Brandel) and a fraction collector. Samples were incubated with proteinase K, and RNA was extracted using phenol/chloroform. RT-PCR was performed using SuperScript II (Invitrogen). Real-time PCR was performed in triplicates on the ViiA7 using SYBR Green reagent (Applied Biosystems) and indicated primers.

**RNA Immunoprecipitation.** Single-cell suspensions of $10 \times 10^6$ P6D4 cells were treated with cycloheximide (100 μg/mL) for 15 min at 37 °C. Cells were washed in cold PBS containing 100 μg/mL cycloheximide and then lysed in ice-cold hypotonic lysis buffer [5 mM Tris·HCl pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM KCl, 1 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 100 μg/mL cycloheximide, 1× protease inhibitor mixture (Complete Mini; Roche Diagnostics), and 400 U/mL RNaseIN (Promega)] for 10 min. Cell extracts were centrifuged for 10 min at 20,817 × *g*, and the supernatants were collected and precleared with 10 μL of Pprotein A/G Dynabeads (Life Technologies) for 30 min. Immunoprecipitation was performed using 10 μg of anti-Rpl22 (H-106; Santa Cruz Biotechnology) or rabbit control IgG antibodies (Santa Cruz Biotechnology). Washes were performed using RIP wash buffer (50 mM Tris pH 7.5, 300 mM KCl, 12 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, and 200 μg/mL cycloheximide). RNA was extracted using the Qiagen RNEasy Mini Kit, followed by RT-PCR and qPCR.

**Immunoblot Analysis.** For immunoblot analysis, cells were lysed in RIPA buffer containing protease inhibitors (complete Mini; Roche Diagnostics) on ice for 20 min and then sonicated for 10 min in a water bath (Branson 5510). Immunoblotting was performed using anti-p53 (1C12; Cell Signaling), or anti-β-actin (AC-15; Sigma-Aldrich).

**ChIP Sequencing and RNA Sequencing.** Assays were performed on sorted primary DN3 cells, P6D4 SCID.adh cells, or 70Z/3 cellsas indicated. Cells were fixed with 1% formaldehyde. Cell lysis was performed using the following buffers: for total cell lysis, 5 mM Pipes pH 8, 85 mM KCl, 0.5% Nonidet P-40, 1× protease inhibitor mixture, and 1 mM PMSF; for nuclear lysis, 50 mM Tris, 10 mM EDTA, 1% SDS, 1× protease inhibitor mixture, and 1 mM PMSF. After sonication (Covaris E220 sonicator), immunoprecipitation was performed using Protein A/G Dynabeads (Life Technologies) and 10 μg of rabbit anti-Miz-1 (H-190; Santa Cruz Biotechnology), H3K4Me3 (Abcam), H3K27Ac (Abcam), H3K9Ac (Millipore), or rabbit control IgG antibody (Santa Cruz Biotechnology). The primers used in the ChIP-qPCR experiments are listed in Table S2.

For ChIP-Seq experiments, sequencing libraries were prepared from immunoprecipitated chromatin using an Illumina TruSeq DNA Kit in accordance with the manufacturer's instructions, and sequenced using the TruSeq PE Clusterkit v3-cBot-HS on an Illumina HiSEq 2000 system. Sequencing reads were aligned to the mm10 genome using Bowtie2 v2.10 (45). Reads were processed and duplicates were removed using Samtools, and a genome coverage file was generated and scaled to reads per million reads (RPM) obtained for each sample using Bedtools (46, 47).

For RNA-Seq, a biological triplicate of sequencing libraries was prepared from RNA extracts using the Illumina TruSeq Stranded mRNA Kit according to the manufacturer's instructions, and sequenced using the TruSeq PE Clusterkit v3-cBot-HS on an Illumina HiSEq 2000 system. Sequencing reads

were aligned to the mm10 genome using Tophat v2.0.10. Reads were processed with Samtools and then mapped to Ensembl transcripts using HTSeq. Differential expression was tested using the DESeq R package (R Coding Team) (48). A genome coverage file was generated and scaled to RPM using Bedtools (46, 47) (GEO accession no. GSE57694).

**Actinomycin D Treatment.** DN3 cells were FACS-sorted into serum-free Opti-MEM medium (Gibco) and rested at 37 °C for 30 min. Cells were then treated with 5 μg/mL actinomycin D at 37 °C and lysed in RLT buffer with β-mercaptoethanol. RNA was extracted using the Qiagen RNEasy Mini Kit, followed by RT-PCR and qPCR.

***Rpl22* Reporter Assay.** 293T cells were transfected with 250 ng of human *Rpl22* promoter fused to luciferase (Switchgear Genomics), β-galactosidase, and either pcDNA3.1 alone or with 0.5 or 1 μg of human Miz-1 in pcDNA3.1. Cells were lysed after 48 h and analyzed for *Renilla* luciferase expression and β-galactosidase expression for normalization, using LightSwitch Luciferase Assay Reagent (Switchgear Genomics) and ONPG, respectively.

**Anti-CD3 Injections.** Four- to 6-wk-old Miz-1$^{\Delta POZ}$ × Rag1$^{-/-}$, MycV394D × Rag1$^{-/-}$, and Rag1$^{-/-}$ mice were injected i.p. with 50 μg of anti-CD3 (145-2C11) per mouse or with PBS alone (32). Mice were killed at the indicated times and analyzed by flow cytometry.

**Retroviral Transfection.** Rpl22 was cloned into the MigR1 vector using the following primers: 5′-CGACTCGAGATGGCGCCTGTGAAAAAGCTTG-3′ and 5′-CGAGAATTCTTAATCCTCGTCTTCCTCCTCCTC-3′. MigR1 and MigR1-Rpl22 were generated using Phoenix Eco cells. DN3a cells were sorted and resuspended in viral supernatant in the presence of 8 μg/mL polybrene. Cells were centrifuged at 515 × *g* for 90 min. Media was changed at 4 h after infection, and cells were plated onto OP9-DL4 stromal cells with 5 ng/mL Flt3L and 1 ng/mL IL-7 for 4 d.

**Statistical Analysis.** Quantitative data are presented as mean ± SD and were analyzed using one-way ANOVA or the two-tailed Student *t* test. A *P* value ≤0.05 was considered to indicate statistical significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

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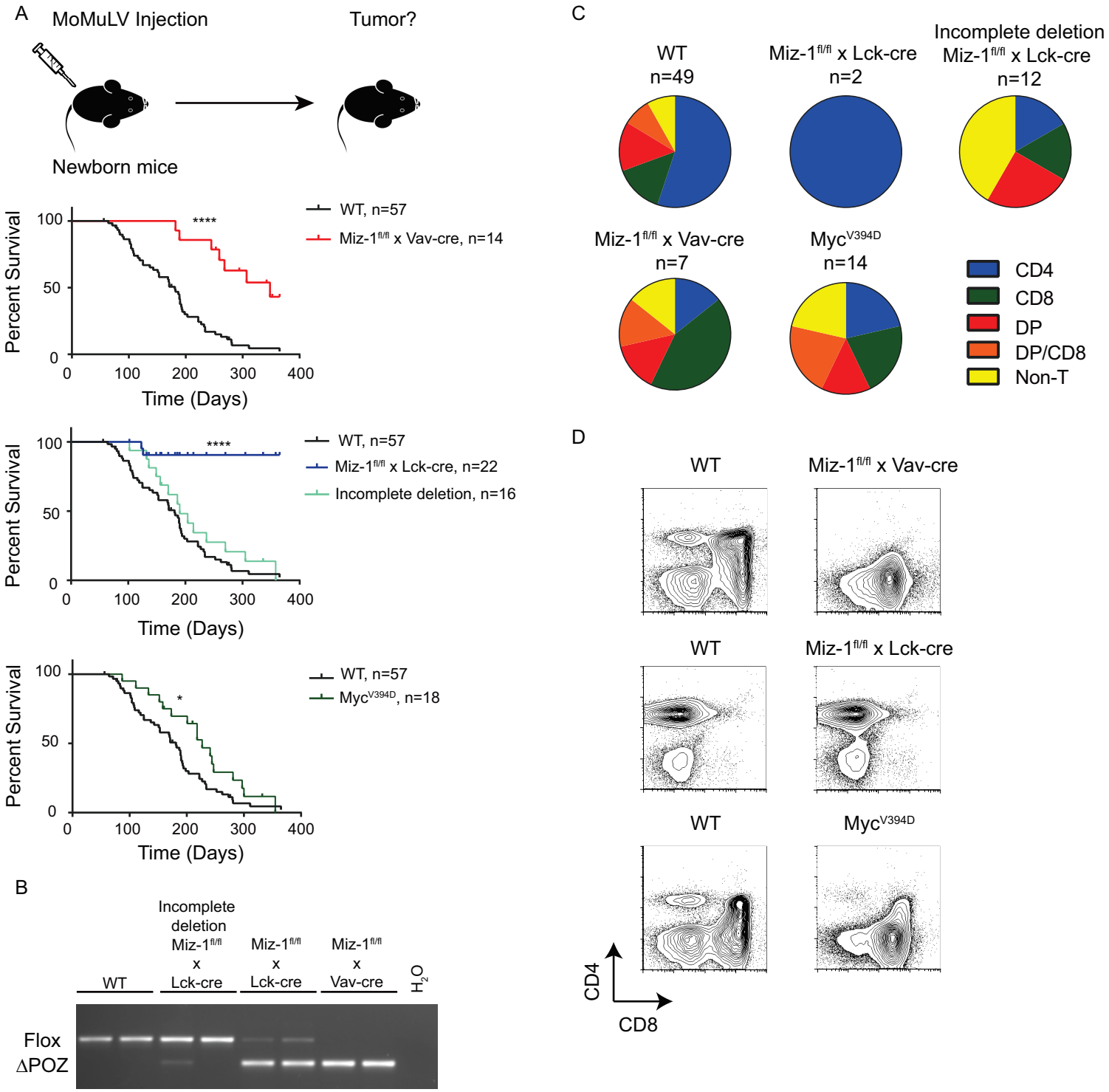
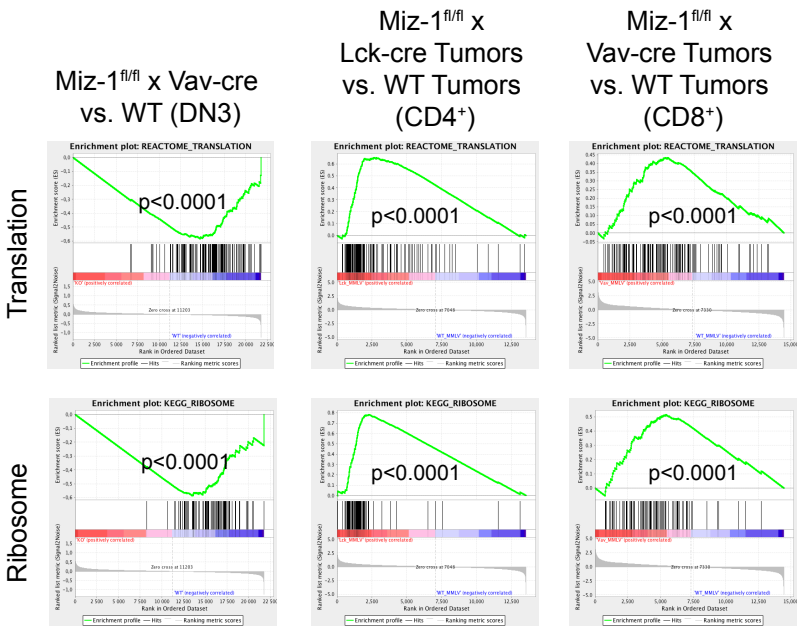



Loss of Miz-1 increases the latency of MoMuLV-induced T-ALL. (A) Scheme of MoMuLV tumor induction. Kaplan-Meier survival curves of WT mice compared to Miz-1$^{fl/fl}$ x Vav-Cre (top), Miz-1$^{fl/fl}$ x Lck-Cre (middle) and Myc$^{V394D}$ (bottom) mice. (B) PCR genotype analysis of the Miz-1 POZ domain in tumor tissues. (C) Phenotype of tumors from (A) assessed by flow cytometry. (D) Flow cytometric analysis of splenic tumors from (A).

A




Miz-1-deficient MoMuLV samples express increased translation and ribosome-associated genes. (A) GSEA plots for Miz-1$^{fl/fl}$ x Vav-Cre DN3 pre-T cells compared to WT (left), Miz-1$^{fl/fl}$ x Lck-Cre CD4$^{+}$ MoMuLV samples compared to WT (middle) and Miz-1$^{fl/fl}$ x Vav-Cre CD8$^{+}$ samples compared to WT (right) for translation (top) and ribosome (bottom) associated genes.