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Characterization of the Transcriptional Elongation Factor ELL3 in B cells and Its Role in B-cell Lymphoma Proliferation and Survival

Lou-Ella M.m. Alexander
University of South Florida, reallouella@gmail.com

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by

Lou-Ella M. M. Alexander

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University of South Florida

Major Professor: Kenneth L. Wright, Ph.D.
Sheng Wei, M.D.
Shari A. Pilon-Thomas, Ph.D.
Mark G. Alexandrow, Ph.D.

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DEDICATION

This dissertation is dedicated to my mother, Jessica Alexander-Ogenia, and my father, Otmar Alexander, who provided me with the opportunity to get a great education abroad and taught me the importance of hard work, determination, patience and sacrifice to achieve one's dreams.

Mama i Tata, ma bai p'é i porfin ma logré!

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LIST OF ABBREVIATIONS

ABC DLBCL	Activated B-cell type Diffuse Large B cell Lymphoma
ADCC	Activation Dependent Cellular Cytotoxicity
Ag	Antigen
AID	Activation Induced Deaminase
Bach2	BTB and CNC homology 2
BAFF	B-cell Activating Factor
BCAP	B-cell Adaptor for Phosphoinositol 3 kinase
BCL1	B-cell Lymphoma 1
BCL2	B-cell Lymphoma 2
BCL6	B-cell Lymphoma 6
BCL10	B-cell CLL/Lymphoma 10
BCR	B-cell Receptor
bHLH	basic Helix-Loop-Helix
BL	Burkitt's Lymphoma
BLNK	B-cell Linker
BM	Bone Marrow
BRD4	Bromo Domain protein 4
BrdU	Bromodeoxyuridine
Btk	Bruton's tyrosine kinase
CARD11	Caspase Recruitment Domain containing protein 11
CDC	Complement Dependent Cytotoxicity
CBP	CREB binding protein
C_H	Constant region Heavy-chain locus
CHOP	Cyclophosphamide Hydroxydaunomycin Oncovin Prednisone
ChIP	Chromatin Immunoprecipitation
CIITA	Class II Major histocompatibility complex Transactivator
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CPSF	Cleavage and Polyadenylation Specific Factor
CSR	Class Switch Recombination
CstF	Cleavage stimulator Factor
CTD	C-terminal Domain
CTG	Cell Titer Glo
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DC	Dendritic Cell
D_H	Diversity region Heavy-chain locus
DH	Double Hit

DLBCL	Diffuse Large B Cell Lymphoma
DSIF	DRB-Sensitivity Inducing transcription Factor
DZ	Dark Zone
EAF	ELL Associated Factor
EBF	Early B-cell Factor
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraaceticacid
EGTA	Ethyleneglycoltetraaceticacid
ELL	Eleven-nineteen Lysine-rich Leukemia
ELP	Early Lymphoid Progenitor
ETP	Early T-cell Progenitor
FBS	Fetal Bovine Serum
FDC	Follicular Dendritic Cell
Flt3	Fms-related tyrosine kinase
Fo	Follicular
GC	Germinal Center
GC DLBCL	Germinal Center-derived Diffuse Large B Cell Lymphoma
GTF	General Transcription Factor
GRG4	Groucho Related Gene 4
HDAC	Histone Deacetylase
HEB	E-box-binding protein
HL	Hodgkin's Lymphoma
HSC	Hematopoietic Stem Cells
ID3	Inhibitor of DNA binding 3
Ig	Immunoglobulin
Ig_H	Immunoglobulin heavy chain
Ig_L	Immunoglobulin light chain
Ig_S	Immunoglobulin switch region
IKK	I Kappa B Kinase
INFβ	Interferon βeta
IP_3	Inositol-1,4,5-triPhosphate
IRF	Interferon Regulatory Factor
ITAM	Immunereceptor Tyrosine-based Activation Motif
J_H	Joining region Heavy-chain locus
KCl	potassium chloride
LEF1	Lymphoid Enhancer-binding Factor 1
LiCl	Lithium Cloride
LTRC	Long Term Repopulating Cell
LZ	Light Zone
MALT1	Mucosa Associated Lymphoid Tissue Lymphoma 1
MARE	Maf Recognition Element
MCL	Mantle Cell Lymphoma
MED	Mediator
MHC	Major Histocompatibility Complex
miRNA	micro RNA
MLL	Mixed Lineage Leukemia

MPP	Multi Potent Progenitor
mRNA	messenger RNA
NaCl	Sodium Chloride
NELF	Negative Elongation Factor
ncRNA	non-coding RNA
NHEJ	Non-Homologous End Joining
NHL	Non-Hodgkin's Lymphoma
NF-κB	NF kappa B
NEMO	NF kappa B Essential Modulator
NK	Natural Killer
NURD	Nucleosome Remodeling and Disruption complex
NP-40	Nonidet P-40
MZ	Marginal Zone
PAX5	Paired box 5
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
Pre-BCR	Pre-B Cell Receptor
PARP	Poly (ADP-Ribose) Polymerase
PAS	Poly (A) Signal
PI	Protease Inhibitor cocktail
PI3K	Phosphoinositide 3 kinase
PLCγ2	Phospholipase C gamma 2
PKCβ	Protein Kinase C Beta
Pol	RNA Polymerase
PRDM1	Positive Regulatory Domain binding factor 1
PRMT5	Protein arginine Methyltransferase 5
PVDF	polyvinylidene fluoride
RAG	Recombination Activating Gene
R-CHOP	Rituximab Cyclophosphamide Hydroxydaunomycin Oncovin Prednisone
rRNA	ribosomal RNA
RT	room temperature
SEC	Super Elongation Complex
SH2	Src Homology 2
shRNA	small hairpin RNA
siRNA	small interfering RNA
S1PR1	Sphingosine-1 Phosphate Receptor 1
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
SDS	Sodium Dodecyl Sulfate
SHM	Somatic Hyper Mutation
STRC	Short Term Repopulating Cell
SWI-SNF	Switching-defective Sucrose Non-Fermenting
TBP	TATA-box Binding Protein
TdT	Terminal deoxynucleotidyl Transferase
TF	Transcription Factor
TH	Triple Hit

TLR	Toll-like Receptor
tRNA	transfer RNA
TX-100	Triton X-100
V_H	Variable region Heavy-chain locus
WT	Wild type
qPCR	quantitative PCR
XBP1	X-box Binding Factor 1

ABSTRACT

The studies presented in this dissertation establish the dynamics of Eleven nineteen Lysine-rich leukemia (ELL) family of elongation factors during B cell differentiation and provide a description of ELL3 function in B cells.

The transition from a mature naïve B cells into an activated B cell is dependent on a large increase in transcriptional output, which is followed by focused expression on secreted immunoglobulin upon terminal differentiation into plasma cell. While ELL family members have previously been implicated in alternative splicing at the immunoglobulin heavy chain locus in plasma cells, their presence and function prior to differentiation is currently not known. However, the use of elongation factors has been implied by the finding of mostly paused RNA polymerase II in the genome of naïve B cells.

In the first study, the expression of transcriptional elongation factor ELL3 is shown to be restricted to activated B cells and B cell lymphomas. All three family members were characterized in B cell lymphoma cell lines, genome wide expression, microarray analysis and primary B cell stimulus. The expression of ELL3 was induced upon activation of B cells concurrently with family member ELL. In addition, the abundant expression of ELL3 was restricted to GC derived B cell lymphoma cell lines. While the expression of ELL is maintained, the expression of ELL3 is diminished and ELL2 is up-regulated in terminally differentiated plasma cells.

The expression of master regulator of terminal plasma cell differentiation PRDM1 was inverse correlated with that of ELL3. To further establish PRDM1s role in regulating the ELL family member dynamics, global binding was assessed in plasma cell lines. Chromatin immunoprecipitation followed by quantitative PCR was utilized to identify direct association of PRDM1 at exclusively the ELL3 loci. Ectopic expression of PRDM1 in B cells down regulated the expression of ELL3. Furthermore, two consensus PRDM1 binding sites were defined at the ELL3 loci, which mediate significant repression of the promoter activity. Collectively, these experiments indicate that PRDM1 mediates the switch from ELL3 in B cells to ELL2 in plasma cells.

The data presented in the final chapter aimed at defining a function for ELL3 in the cells that express it most abundantly, which are B cell lymphoma cell lines. Transient depletion of ELL3 in a Burkitt's lymphoma cell line resulted in a diminished proliferation rate due to a severe disruption of DNA replication and its regulators minichromosome maintenance proteins. Additionally, compromised cell division and mitotic regulators were observed along with increased DNA damage and cell death.

The data presented here demonstrate a key role for ELL3 in the proliferation and survival of B cell lymphomas and positions ELL3 as an attractive therapeutic target against B cell lymphoma's with a germinal center origin.

CHAPTER I:

GENERAL INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis is defined as the process by which all cellular components of the blood are formed and develops in the fetal liver or the adult bone marrow (BM) [1]. This process originates from a small population of pluripotent hematopoietic stem cells (HSCs) and is tightly regulated by transcription factors and cytokines [2]. Several models of hematopoietic development have been proposed. A schematic depiction is provided in **Figure 1.1**. It describes the hierarchical development of HSCs and involves several successive steps of commitment and terminal differentiation into lineage-restricted progenitors with progressively less self-renewal capability, resulting in the various mature blood cells [3, 4]. Two types of HSCs have been described. The first being long-term repopulating cells (LTRCs), which are capable of differentiating into all blood cells types (multi-potency) as well as the ability to self-replicate into progeny with similar potential (self-renewal) [5, 6]. Further commitment of the LTRCs results in generation of the second type of HSCs, the short-term re-populating cells (STRCs), which have limited ability of self-renewal and short-term multipotent abilities [6, 7]. Multipotent progenitors (MPPs) are the next developmental progenitor, which have full lineage pluripotency but no self-renewal potential [7, 8]. These MPPs further segregate into the dynamic oligopotent progenitors of respectively the myeloid and lymphoid lineages [9-12].

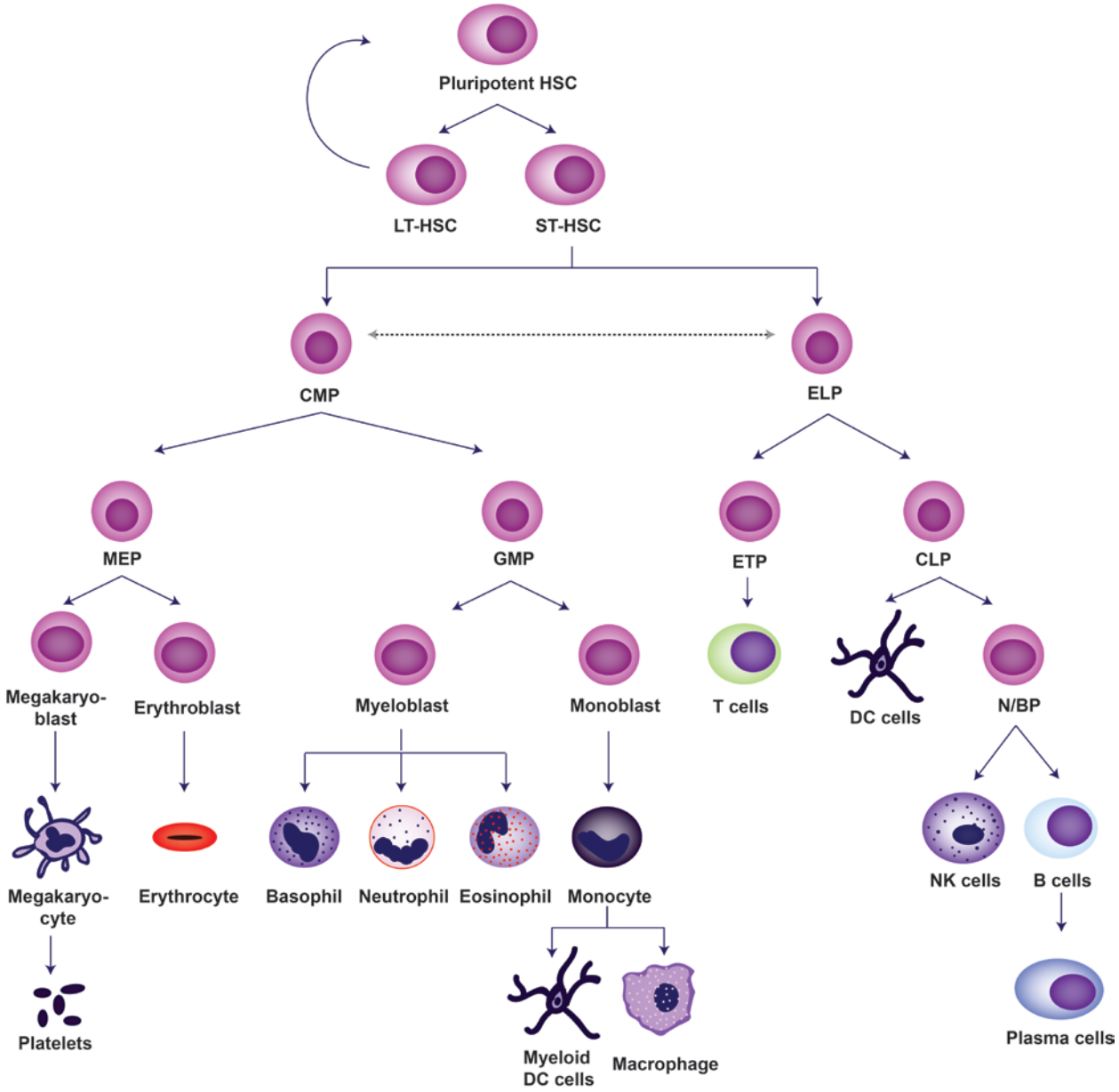

Figure 1.1 Human Hematopoiesis. All mature peripheral blood cells are derived from hematopoietic stem cells (HSCs) which reside in the bone marrow. Two types of HSCs are described with differing self renewal abilities, long term repopulating HSC (LT-HSC) and short term repopulating HSC (ST-HSC). Hematopoiesis is initiated from ST-HSC and through several differentiation steps is able to generate mature blood cells. Several models have been described. Dashed line indicates alternative differentiation route. CMP, common myeloid progenitor; ELP, early lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage; ETP, early T cell progenitor; CLP, common lymphoid progenitor; N/BP, NK-B cell progenitor.

Various alternative differentiation routes have been proposed for these oligopotent progenitors [13-15]. As of recently, these MPPs are reported to segregate into either the common myeloid progenitors (CMPs) or the early lymphoid progenitors (ELPs) [16]. The CMPs give rise to the mature cells of the erythroid (erythrocytes and thrombocytes) and myeloid cell lineages (mast cells, macrophages, dendritic cells (DCs) and various granular leukocytes) [13]. On the other hand, ELPs initiate the expression of recombination-activating gene 1 (RAG1) and RAG2, which sets the rearrangement at the immunoglobulin heavy chain (IgH) in motion [17]. They go on to further differentiate into the early T-cell-lineage progenitors (ETPs) or the common lymphoid progenitors (CLPs) in the bone marrow. CLPs are more lymphoid restricted and can generate B cells, DCs and natural killer (NK) cells [9-15]. This dissertation will further focus on B cells.

1.2 B cell Development

The generation of B cells involves several steps of differentiation that need to be successfully traversed to produce functionally competent cells. While the initial HSC developmental stages are shared by all hematopoietic cells, tight regulation by a hierarchy of transcription factors governs the final formation of the B cell lineage. These factors encompass both activating and silencing lineage-specific transcription factors (TF) that function cross-antagonistically and are modulated by signaling pathways [18, 19]. Advances in flow cytometry have made it possible to define the resulting cell surface phenotypes that distinguish the various differentiation steps during B cell development [20]. This section will exclusively describe the differentiation steps and the associated regulators that are necessary for the development into mature B cells. The process of B cell development can be divided into two phases, the antigen independent- and antigen dependent- phases [21].

1.2.1 Antigen-Independent Phase

The antigen independent phase is initiated with the **specification** stage. It is set in motion by microenvironmental cues and results in the resolution of the multipotent cell, as depicted in **Figure 1.2** [22, 23]. In humans, the HSC that initiates hematopoiesis is phenotypically characterized by the lack of mature lymphoid or myeloid lineage surface determinants (Lin^-), $CD34^+$, $CD38^-$, $CD90^+$($Thy1.1^+$), $CD45RA^-$ and $CD49f^+$ [24-39].

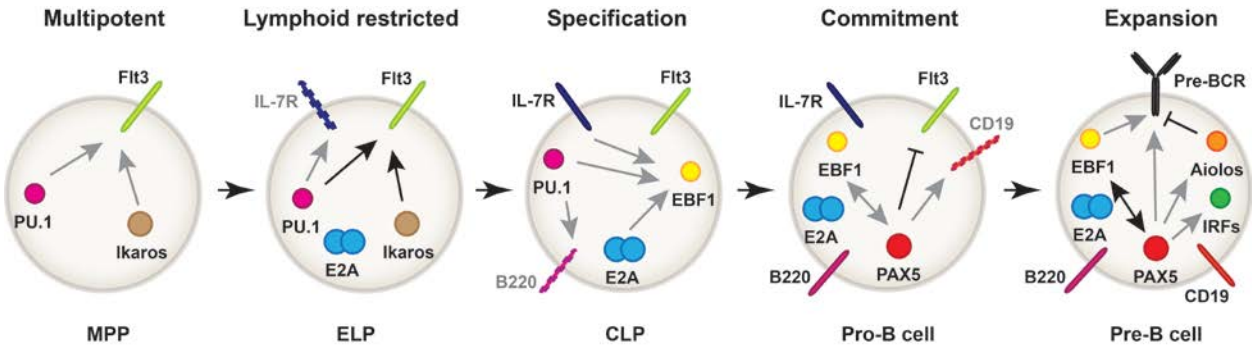

Figure 1.2 The Antigen-Independent Phase of B cell Development. Indicated are several successive differentiation steps from the multipotent progenitor (MPP), early lymphoid progenitor (ELP), common lymphoid progenitor (CLP), committed pro-B cell and pre-B cell. Key transcription factors and cell surface receptors are shown at each stage. Grey arrows indicate initiated signaling and dashed borders indicate initiated protein at each developmental stage. ↑ indicated up-regulation and ⊤ indicates inhibition of expression. IRFs are IRF4 and IRF8.

1.2.1.1 HSC to MPP Transition

The transition into the MPP is the first differentiation step of the HSC and is also where the first B cell lineage regulatory event is believed to occur. Differentiation into an MPP is characterized by the expression of the Fms-related tyrosine kinase 3 (Flt3/Flk2) on the cell surface and is associated with loss of self-renewal capacity as well as the myeloid lineage potential [8]. Inactivation of Flt3/Flk2 and deficiency of its ligand in murine models lead to a decrease of the B cell progenitor, CLP [40, 41]. Together these findings further highlight the

importance of Flt3/Flk2 in B cell specification. Two transcription factors have been implicated in the regulation of Flt3/Flk2, these include Ikaros and PU.1.

Ikaros is Kruppel-type zinc finger TF that was first discovered in a screen for regulators of T-lineage [42]. Alternative splicing of the Ikaros mRNA, results in a family of Ikaros isoforms with varying specificity and affinities [43, 44]. The Ikaros family is characterized by a C-terminal domain with two zinc fingers, which are utilized to associate into homo- and heterodimeric complexes [43, 45]. The N-terminal domains of Ikaros proteins contains between one to four zinc finger motifs. Three zinc finger motifs in the N-terminus are required for high-affinity DNA binding. Therefore only three isoforms (Ik-1, Ik-2 and Ik-3) have the ability to bind sequences with the GGGA core motif. The Ik-4 isoform with only two N-terminal zinc fingers has the ability to bind tandem recognition sites that share the recognition sequence. With either one (Ik-5 and Ik-7) or no (Ik-6 and Ik-8) zinc finger motifs, no high-affinity DNA engagement can be achieved [43]. In so, associations between isoforms can dramatically affect the DNA binding affinity and transcriptional activity of Ikaros [45]. The activity of Ikaros is rendered through assembly of higher order structures, which contain 10-12 Ikaros molecules as well as proteins of the nucleosome remodeling and disruption (NURD) complex (Mi-2β, Chd4, HDAC1, HDAC2, MTA2 and Rbp48/46) or switching-defective-sucrose non-fermenting (SWI-SNF) repressive complex by participating in nucleosome remodeling [46-49]. Mice homozygous for an Ikaros deletion lack lymphocytes and their earlier progenitors, while Ikaros null or Ikaros double negative hematopoietic progenitors are deficient in expression of Flt3/Flk2 [50, 51]. These findings suggest the participation of Ikaros during the hematopoietic lineage specification.

PU.1 is an Ets transcription factor that is required for differentiation of the MPP. Like Ikaros, PU.1 deletion in hematopoietic progenitors display reduced expression of Flt3/Flk2 and

IL-7R. Essentialy, these findings demonstrate that Ikaros and PU.1 function in parallel to regulate early lymphocyte differentiation [52].

1.2.1.2 MPP to ELP/CLP Differentiation

Transition into ELP/CLP is the next specification step and is characterized by the early expression of RAG 1/2, the terminal deoxynucleotidyl transferase (TdT), other enzymes involved in N-nucleotide insertion and initiation of rearrangement of the immunoglobulin heavy chain diversity (D_H) and joining (J_H) regions [16, 17]. In addition, IL7R is expressed on the ELP/CLP cell surface. While PU.1 was shown to directly regulate the expression of IL-7R α-chain, the activation of the Flt3/Flk2 also seems to promote its expression [52-54]. Simultaneous loss of Flt3/Flk2 and IL-7R results in absence of the B-cell lineage [55, 56]. However, IL-7R signaling appears to regulate early B cell development, since its stimulation alone is sufficient to induce further CLP differentiation and its loss causes reduction in CLP numbers in the BM [57-59]. These findings suggest that IL-7R signaling may regulate the activity and expression of the B cell determining TFs, E2A and EBF.

E2A is a member of the basic helix-loop-helix (bHLH) family of proteins. Differential splicing of the E2A gene generates two proteins products, E12 and E47, both of which are observed during early B cell development [60, 61]. E-box-binding protein (HEB) and E2-2 are additional family members which are also expressed in B cells. These proteins associate at the E-box sequence of CANNTG, hence they are referred to as E-box proteins [62]. Utilizing their C-terminal domain these proteins also have the ability to form homodimers (in B cells) or heterodimers (in T cells). Mutation of E2A in a mouse model mostly affects the B cell lineage [62-64]. Current understanding suggests that E2A expression is induced by IL-7R signaling [65].

E2A expression subsequently modulates the expression of the Early B-cell Factor (EBF) TF, RAG and rearrangement of Ig D_H-J_H regions [17]. E2A binding sites have been found in the intronic enhancer region of IgH as well as EBF promoter [63, 66]. Consequently targeted E2A deletion results in diminished levels of EBF, RAG and immunoglobulin rearrangement [64, 67]. In addition the ectopic expression of either E2A or EBF was shown to induce D_H-J_H rearrangement in a cell line model while its targeted deletion results in diminished levels of EBF and RAG gene expression [68, 69]. Together these findings indicate that E2A is a key player in establishing CLP transcription networks.

EBF is an atypical helix-loop-helix zinc finger protein with limited expression in the hematopoietic compartment within the B cell lineage [70]. Its expression is regulated by PU.1, E2A and IL7 signaling and is first detected in CLPs that express RAG1, RAG2 and rearranged D_H-J_H [53, 65]. EBF also activates the expression of genes that code for the early B cell lineage program which include MB-1 (CD79A/Igα), λ5 and VpreB genes [71-73]. Together the induction of E2A and EBF regulate the early B cell lineage gene expression.

BCL11A is kruppel zinc finger related protein that was originally identified as commonly translocated oncogene in various B cell malignancies. The expression of BCL11A is essential for the generation of specifically B cells. Hematopoietic progenitors that lack BCL11A are deficient in EBF, PAX5 and IL-7R [74-76].

1.2.1.3 CLP to Mature B cell Differentiation

The second stage termed **commitment**, is initiated by the expression of cell surface marker B220 by a subset of CLPs, also known as pro- B cells or CLP2s. Aided by stromal cell interactions and microenvironmental signals and cytokines, the pro B cells will undergo several

steps of division and differentiation aimed at commitment and maintenance of B cell fate [77, 78]. The TF that participates in this process is paired box 5 (PAX5).

PAX5 is a homeodomain protein that is expressed abundantly throughout the B cell lineage. The expression of *PAX5* is induced by EBF and is required for commitment, maintenance of the B cell fate and differentiation to the various B cell stages [67, 79]. PAX5 binds DNA through association of N-terminal paired-domain motif and recruits ets-family transcription factor 1 (Ets1) to the *MB-1* promoter, which with the help of E2A, EBF and RUNX1 is actively transcribed [79-82]. Additionally, B cell differentiation functions of PAX5 include regulation of EBF1, Ig heavy chain variable region (V_H) gene segment rearrangement, CD19, lymphoid-enhancer-binding factor 1 (LEF1), B-cell linker (BLINK), MB-1 (CD79A/Igα) and λ5 [83-85]. PAX5 also exerts repressive effects on genes that are not required for B-cell lineage through recruitment of the co-repressors of the Groucho family gene, groucho-related gene 4 (GRG4). This interaction requires PU.1, which also has the ability to recruit GRG4 [86, 87]. These genes include the M-CSF, IgH HS1,2 enhancer and the crucial T-cell pathway promoting factor, Notch [88, 89]. Recent gene profiling reported that PAX5 also activates additional genes that regulate various aspects of B cell differentiation. These include SpiB, Aiolos, LEF1, ID3, IRF4 and IRF8 [90]. These findings illustrate that PAX5 regulates a cascade that is essential for not only commitment to the B-cell lineage but also subsequent differentiation steps.

To generate the various B cell stages, the pro-B cells start by expressing CD19 and undergo rearrangement of the D_H to the joining J_H gene segments in the Ig heavy chain locus followed by variable region (V_H) to D_HJ_H rearrangement [91]. The process of VDJ recombination is imprecise and has a high probability of yielding non-productive reading frames. Failure of the

first attempt of $V_HD_HJ_H$ rearrangement, results in the rearrangement of the second chromosome. Failure of the second attempt results in apoptosis. Successful rearrangement results in the expression of μ heavy chain in large pre-BII cell stage. Subsequently, these cells silence the expression of RAG1 and RAG2 enzymes and express the rearranged IgH gene product on the cell surface. Together with the surrogate immunoglobulin light chains (IgLs), VpreB and λ5, and heterodimers of signaling molecules Igα (encoded by the *MB-1* gene) and Igβ (encoded by *B29* gene) this rearranged IgH product forms the pre-B-cell receptor (pre-BCR) [92]. Successful expression and signaling through the pre-BCR triggers silencing of surrogate light chain as well as the process of allelic exclusion, where the second allele of IgH is silenced [93, 94]. In addition, this stimulates a burst of proliferation of the large pre-BII cells, referred to as clonal expansion [95]. Further differentiation into small pre-BII cells results in down regulation of the surrogate light chain, sequestering of the productive μ chain in the endoplasmic reticulum, reactivation of Rag enzymes for rearrangement of the Ig light chain locus. The κ light chain is rearranged first. Failure to achieve a productive κ light chain on both chromosomes, results in rearrangement of the λ light chain [96]. Subsequently, the productive κ/λ light chain associates with the μ heavy chain and express the IgM and IgD BCR on the cell surface of the immature B cell [97]. At this stage the BCR also gets tested for auto-reactivity during the process of central tolerance. Those cells that show BCR reactivity to self antigens can either be rescued by a second round of immunoglobulin gene rearrangement during the receptor editing process, rendered inactive in the process of anergy or eliminated through apoptosis [98, 99]. Those immature clones that survive this step are exported out of the bone marrow to seed the peripheral organs like the spleen where they receive survival signals and undergo several additional

transitional steps to complete the first stage of development where they become fully mature but naïve B cells [100].

B cell receptor is a protein complex that is critical for the function of B cells. Other than the μ heavy chain and the K/λ light chain, the BCR also associates with the transmembrane Igα/Igβ heterodimer that contains the immunereceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail [101-103]. The BCR associates with cytoplasmic protein tyrosine kinase to mediate signaling. These include the Src family kinases Blk, Lyn and Fyn, Tec family kinase bruton's tyrosine kinase (Btk) and Syk tyrosine kinase [104, 105]. Linker molecules couple these receptor-associated kinases to downstream pathways through several functional domains, including Src homology (SH2) domain, proline-rich domains and several tyrosine-phosphorylation sites. In B cells, these molecules include the adaptor molecules BLNK and B cell adaptor for phosphoinositol 3-kinase (BCAP) [106, 107].

Upon antigen encounter, BCR complexes localize into lipid rafts to form the immune synapse, where the interaction of several BCRs amplifies the signal [108]. Src family kinase associates with the phosphatase CD45 and remove phosphates from inhibitory tyrosine residues [109]. In addition, two main pathways of activation are induced. The first pathway results in the activation of phospholipase Cγ2 (PLCγ2) and Ras. The adaptor BLNK directly interacts with PLCγ2 and Btk, which allows for further activation of PLCγ2 [110-112]. BCAP is phosphorylated by Syk and Btk which generates binding sites for the phosphoinositide 3-kinase (PI3K) leading to additional phosphorylation of PLCγ2 and downstream targets [113, 114]. Subsequently, this activation produces the diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) further activates a number of downstream signaling pathways required for proliferation, survival or differentiation [108].

The second pathway activates nuclear factor κB (NFκB) through activation of Src family kinases and Tec family kinase Btk [115, 116]. Protein kinase C β (PKCβ) and I kappa B kinase (IKK) are also recruited into the lipid rafts [117, 118]. PKCβ phosphorylates several residues on caspase recruitment domain-containing protein 11 (CARD11) which controls its further association with B-cell CLL/lymphoma 10/ mucosa-associated lymphoid tissue Lymphoma (BCL10-MALT1) to form the CARD11-BCL10-MALT1 (CBM) complex. This trimolecular protein complex promotes NF-κB essential modulator (NEMO) polyubiquitination which triggers NFκB activation [119, 120].

1.2.2 Antigen-Dependent Phase

Following maturation, mature B cells exit the bone marrow and enter the circulation in order to migrate to secondary lymphoid tissues, e.g. spleen, lymph nodes, tonsils, peyer's patches and mucosal tissues, where they could encounter antigen (Ag). The antigenic signal triggers re-entry into the cell cycle and its absence leads to apoptosis [121]. Based on the B-cell receptor (BCR) signal strength, B cell activation will proceed either independent or dependent of thymus antigens presented by T_H cells, resulting in respectively marginal zone (MZ) B cells and follicular (Fo) B cells [122].

1.2.2.1 T-cell Independent Phase

Antigen encounter by MZ B cells most often occur in the spleen. MZ B cell activation results when antigen is captured, processed and presented in association with major histocompatibility complex (MHC) class II molecules and presented to T cells with the added delivery of co-stimulatory signals [123]. In addition these cells have the capacity to rapidly differentiate into

plasma cells and secrete IgM. However, MZ B cells are not able to generate memory B cells. Thus it is believed to generate a weaker humoral response [124]. While the exact mechanism for their development have not been fully elucidated, it is believed that Notch2, B-cell activating factor (BAFF), Toll-like receptor (TLR) and integrin mediated signaling for their retention in the marginal zone participate in their development [122, 125, 126]. Thus, MZ B cells are believed to be the first line of defense against blood borne pathogens.

1.2.2.2 T-cell Dependent Phase

The T-cell dependent phase is initiated by encounter of a naïve B cell with an exogenous antigen within a primary follicle [127]. Following the first signal of BCR crosslinking, B cells migrate to the border of the primary follicle which is a T- cell rich region. There, an interaction with the antigen-specific T helper cell (T_H) provides the second co-stimulatory signal of CD40L for their full activation [128, 129]. Those B cells that have not undergone an interaction in the follicle will get displaced to the periphery of the follicle which results in the formation of the mantle zone around the germinal center (GC) [130]. The subset of B cells with low affinity BCR, migrate to medullary chords where they differentiate into short-lived plasmablasts [131]. Finally, those B cells with the highest affinity for the Ag participate in forming the secondary follicle or GC towards the middle where a network of follicular dendritic cells (FDCs) is often found [132, 133]. The Fo B cells initiate rapid proliferation resulting in an increase of GC size. Within a week the GC is fully established and can be polarized into two compartments based on histological appearance, the dark zone and light zone [130, 134]. The dark zone (DZ) is an area that contained highly proliferative B cells (centroblasts) as well as T_{FH}, FDCs and macrophages, which is meant to generate a large repertoire of B cells [134]. This repertoire of cells underwent

several processes to improve antibody specificity and diversity. Subsequently, only those clones that have improved BCR affinity transition into the light zone (LZ) and get positively selected by FDCs (centrocytes) to undergo further differentiation into memory B cells and plasma cells [130].

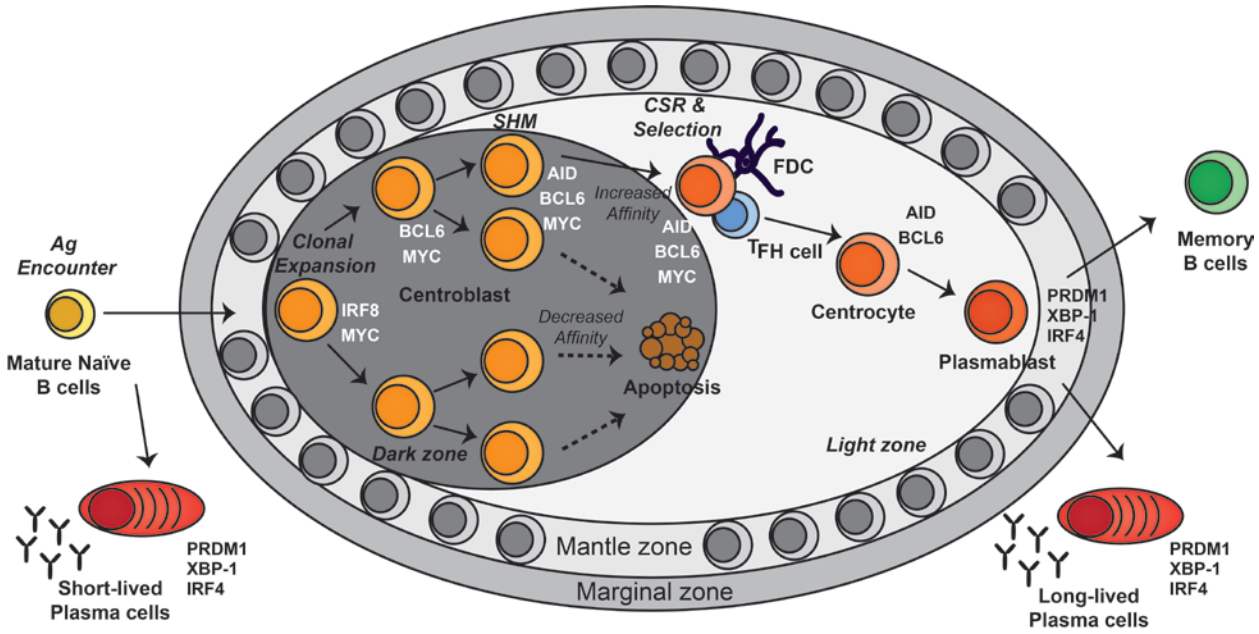

Figure 1.3 Germinal Center Reaction and Transcriptional Regulators. The various stages of GC B cell differentiation and the key regulatory factors at each stage. Antigen (Ag), Somatic Hypermutation (SHM), Class Switch Recombination (CSR), Follicular Dendritic Cell (FDC) and Follicluar T helper cell (TFH).

1.2.3 Antibody Diversity

Two key mechanisms are utilized during the GC reaction to generate antibody diversity and improved affinity, namely somatic hypermutation and class switch recombination. Both processes are mediated by the activation induced deaminase (AID) enzyme, whose functions will also be explained.

Somatic Hypermutation (SHM) involves the introduction of single base pair substitutions, deletions and insertions in the IgH and IgL chain by AID. The mutations accumulate in the

proliferating B cells and may either negatively or positively impact the BCR affinity. Those mutations that increase the affinity of BCR are further positively selected for expansion and survival, while those with decreased affinity will be negatively selected to undergo apoptosis [135].

Class Switch Recombination (CSR) involves the introduction of a DNA break at the DNA repetitive sequences at the switch (S) region upstream of the Ig heavy chain constant region (C_H) exon. Subsequently this region is attached to the S region in front of the subsequent C_H exon through the non-homologous end joining (NHEJ) repair mechanism. CSR is also mediated by AID. The breaks are subsequently repaired, which generates a cell surface Ig with a different C_H gene region. The Ig isotypes retain their specificity but have different effector functions [136, 137].

AID is the enzyme that is responsible for diversity in V-D-J as well as C_H genes. AID is a cytosine deaminase that enzymatically converts cytosine to uracil. dU mimics dT during replication and when mismatched with dG it triggers error-prone DNA repair. The expression of AID is tightly regulated since its functioning at non-Ig loci could cause mutation or translocations [135, 138].

1.2.4 Transcriptional Regulators in T-cell Dependent Phase

Several TF are known to regulate the GC process and subsequent differentiation into antibody secreting plasma cells **(Figure 1.3)**. These factors and their respective functions are described in the following section.

B-cell lymphoma 6 (BCL6) expression is abundant in GC B cells. Its expression is essential for initiating the GC reaction, since BCL6-deficient GC cells are unable to enter the follicle [139].

BCL6 regulates the expression of the chemokine receptor CXCR4 which is expressed in DZ B cells [140, 141]. BCL6 also has repressive functions on the sphingosine-1-phosphate receptor I (S1PR1) which is required for the confinement of B cells in the GC [142, 143].

MYC has critical role in regulating cell cycle progression, metabolism and telomere maintenance [144]. In GC MYC is essential in GC formation, as activated B cells with deleted MYC are unable to form GC [145]. *MYC* is known to be repressed by BCL6 in the rapidly dividing DZ B cells [146]. With the expression of both factors in the GC, dual expression of MYC and BCL6 appears to be temporary and precedes the BCL6 only stage [145]. Consistent with this idea, $MYC^{+}BCL6^{+}$ GC B cells express both the MYC target cyclin D2 (Ccnd2) in addition to the BCL6 target gene Ccnd3 which is a centroblast specific D-type cyclin [147, 148].

Interferon-regulatory factor (IRF) family members that participate in B cell development include IRF4 and IRF8. IRF8 is the first family member to be expressed in centroblasts and its expression is extinguished in plasma cells. Its expression is not exclusive to B cells and is also observed in macrophages, granulocytes and DCs. In B cells, IRF-8 binds directly to the regulatory regions of AID and BCL6 to activate their expression [149]. In addition, its functions have been shown to activate PRDM1 in mouse myeloid progenitor cells [150].

IRF4 is the second family member to be expressed and its expression is essential for the function and homeostasis of B and T cells [151]. Expression of IRF4 results in binding to IFN-stimulated response elements and repression of GC B cell marker genes such as *PAX5* and *BCL6*. Simultaneously, the up-regulation of plasma cell marker PRDM1 and XBP1 are observed with IRF4 induction [150]. Thus IRF family members are important for B cell development and differentiation.

X-box binding protein (XBP1) is a transcriptional activator that belongs to the CREB/ATF family [152]. Its expression is induced by interleukin-4 (IL-4) during differentiation whereas accumulation of misfolded proteins during immunoglobulin synthesis induces its post-transcriptional processing (splicing) into XBP1s. In addition XBP1 is involved in controlling the production of IL-6, which is essential for the survival of plasma cells [153]. Consequently, loss of XBP1 resulted in the absence of plasma cell TFs, IRF4 and PRDM1, plasma cell population and secreted Igs [152, 154]. XBP1 is also required for effective BCR signaling and a lack thereof resulted in aberrant expression of AID and S1R1 [152]. Its expression was induced by PRDM1s repressive function on PAX5 and was shown to initiate the IRF4 and PRDM1 feedback loop [152, 155]. In addition, PAX5 itself has repressive functions on XBP1 [153].

Positive Regulatory Domain I-Binding Factor 1 (PRDM1) is a kruppel type zinc finger containing TFs that was first characterized as a protein that bound the positive regulatory domain of human interferon β (INFβ). The expression of PRDM1 was induced upon viral induction and was shown to be involved in post-induction repression of IFNβ [156]. PRDM1 belongs to a family of TF that are characterized by the presence of positive regulatory (PR) domain. The PR domain is a derivative of SET domains and is believed to function as the protein binding interface for the regulation of chromatin-mediated gene expression [157]. PRDM1 contains five zinc finger motifs of the Kruppel type in tandem, two of which are utilized for DNA binding [158, 159]. PRDM1 also contains a proline rich region, which together with the zinc fingers is believed to mediate transcriptional repression through recruitment of co-repressor proteins that include, groucho proteins, histone deacetylase 1 and 2 (HDAC 1 and 2), methyl transferase G9a, LSD1 and protein arginine methyltransferase 5 (PRMT5) [160-164].

Also characteristic of PRDM family members is the expression of alternative protein products that differ in the presence of PR domain. Two isoforms of PRDM1 have been described. PRDM1α is the full length isoform, while PRDM1β lacks the N-terminal acidic region and has a disrupted PR domain [165]. The PRDM1β is observed in multiple myeloma and is suggested to induce resistance to chemotherapy in diffuse large B cell lymphoma [161, 166]. These mechanisms are believed to be mediated through hetero-dimerization of the isoforms, which can potentially alter their functionality.

In the B cell compartment, the expression of PRDM1 is observed in post GC B cells that have lost BCL6, plasmablasts and terminally differentiated plasma cells. These findings indicate the importance of PRDM1 for the commitment towards the plasma cell fate.

The expression of PRDM1 was shown to be induced through several stimuli and transcription factors. The stimuli include, BCR cross linking by anti-IgM, CD40 signaling, cytokine stimulus (IL-2, -5, -6, -10, and -21), toll-like receptor signaling (TLR4/TLR2) and cellular stress (unfolded protein response) [158, 167-172]. The transcription factors that mediate PRDM1 activation include IRF4, NF-κB, STAT3 and p53 [172-175].

The expression of PRDM1 is regulated by several B cell phenotype maintaining factors. These include PAX5 and BCL6. PAX5 is required for the expression of several B cell specific genes. Its functions also include direct association to *PRDM1* at a cis element located in exon 1 and suppressing its plasma cell inducing functions [176]. BCL6 exerts several mechanism of PRDM1 repression. The first includes BCL6 ability to inhibit the transcriptional of *AP-1* TFs [177]. Additionally, BCL6 can repress the *PRDM1* transcription through direct association to intron 3 of PRDM1 and recruitment of MTA3, a cell type specific subunit of the Mi-2/NuRD co-repressor complex [178]. The transcription repressor BTB and CNC homology 2 (Bach2) also

represses *PRDM1* through formation of a heterodimer with Mafk. A Maf recognition element (MARE) was identified 1.7kb upstream and within intron 5 of the *PRDM1* locus, both mediating repression of PRDM1. BCL6 was found to participate in the repression through association within intron 5 of PRDM1 [179, 180]. These factors may represent the mechanism utilized to keep PRDM1 at bay until differentiation.

PRDM1 is considered the master regulator of plasma cell differentiation and is known to extinguish a network of TF upon its induction in plasma cells **(Figure 1.4)**.

PAX5 is required for commitment to the B cell stage as well as during activation stages. It can function as an activator or repressor depending on its interaction with either co-repressors or positive regulators [181]. PAX5 is known to regulate V_H gene rearrangement, proliferation, isotype switching in GC B cells [83, 107, 182]. Its expression is maintained throughout B cell development until its down-regulation in plasma cells. PRDM1 mediates *PAX5* repression, in a site-dependent manner. This down-regulation is required for PRDM1s ability to drive differentiation of splenocytes into IgM secreting plasma cells. In addition, PRDM1 was sufficient to regulate PAX5 target genes CD19 and J chain but not XBP1 [155].

Class II Major Histocompatibility Complex Transactivator (CIITA) is an important transactivator of class II MHC genes. The activation domain of CIITA interacts with general transcription factors to induce transcription. This is achieved, by enhancing promoter clearance, transcription elongation and facilitating chromatin remodeling by recruiting histone acetyltransferase CREB binding protein (CBP). The CIITA locus contains four distinct promoters pI, pII, pIII and pIV with cell type specific activity [183]. In B cells, the pIII promoter is active and B cell TFs E47, PU.1, IRF4 and IRF8 are known to bind and synergistically activate its expression [184]. PRDM1s repressive function is achieved through direct interaction with the pIII promoter. The

PRDM1 binding site overlaps with that of IRF4 and blocks its activating functions. The repression of CIITA leads to the silencing of class II MHC genes [185].

Inhibitor of DNA binding (ID3) belongs to ID family of proteins containing a helix-loop-helix domain. The ID3 protein is missing the basic region adjacent to the HLH domain that is required for DNA binding. ID3 has the ability to associate with additional HLH proteins of MyoD, E12 and E47 and inhibit their DNA binding as homo- or heterodimers. As a result these proteins are not able to activate gene expression [186]. ID3 expression is abundant in proliferating B cells and is down modulated in differentiating cells [187]. PRDM1 represses ID3, which promotes the plasma cell phenotype [188].

BCL6 is required for GC B cell formation. PRDM1 and BCL6 can repress each other's expression as part of a feedback loop. Thus PRDM1 repressive functions results in repression of BCL6 target proteins [189].

MYC is well known factor that is critical for cell cycle regulation and proliferation. Its expression is abundant in dividing cells but minimal in quiescent or terminally differentiated cells. PRDM1 represses *c-myc*, however its loss alone is not sufficient to drive differentiation [190].

Spi-B is an Ets family protein with binding site sequences that resemble that of PU.1. Its expression is abundant in B cells and required for proper BCR signaling and maintenance of the GC, through repression of PRDM1 and XBP1 [191, 192]. Thus PRDM1s repression of *Spi-B*, releases cells from the B cell phenotype.

1.3 Lymphomas

Lymphomas belong to the hematological group of malignancies that arise as a result of abnormalities that occur during proliferation and differentiation of blood cells [193, 194]. The

American Leukemia & Lymphoma Society estimates that lymphomas are the largest type of hematological malignancies and comprises about 47%.

1.3.1 Lymphoma Statistics

Based on the World Health Organization, Lymphomas are broadly categorized into Hodgkin's lymphoma (HL) and Non-Hodgkin's lymphoma (NHL), based on the presence of Reed-Sternberg cells. Recently published data by the Centers for Disease Control for 2014, place NHL among the top 10 cancers among males and females in the United States. NHL represents a diverse group of diseases. The American Leukemia & Lymphoma Society indicates that 85% NHLs belong to the B-cell type, thus making B cell lymphomas the largest group of malignancies. NHL can be classified into two major categories that describe disease progression, namely aggressive and indolent types.

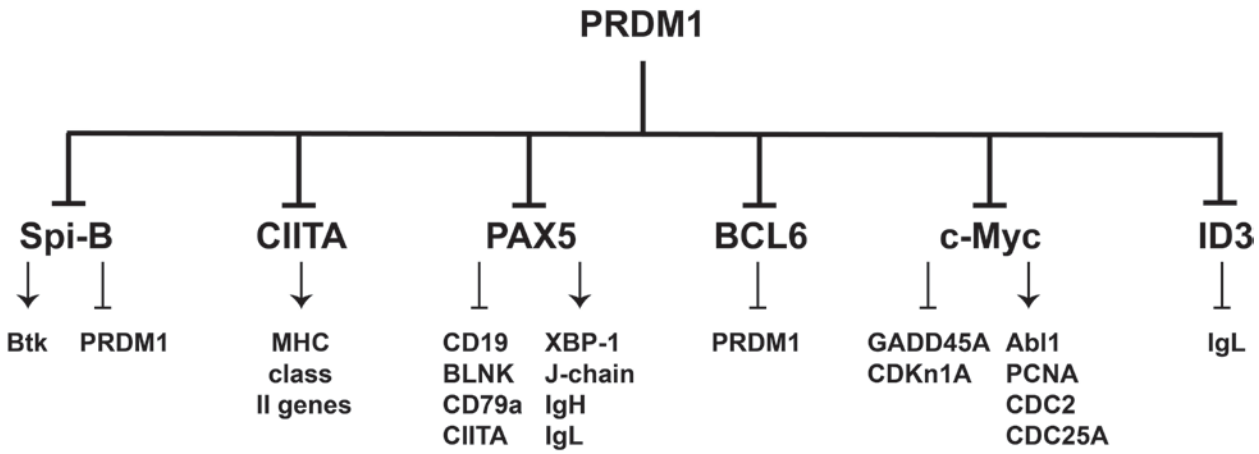

Figure 1.4 PRDM1 Targets in B cells. Schematic depiction of direct PRDM1 targets in B cells. Repression of these targets resulted in down-regulation of genes important for B cell functions as well as up-regulation of genes required for plasma cell differentiation.

1.3.2 B cell Lymphomas

B cell lymphomas can arise from the various stages of B cell development generating several B cell lymphoma subtypes. Therefore, each lymphoma subtype phenotypically resembles a B cell at a specific stage of differentiation. Thus, the normal B cell counterpart has often been termed the cell of origin of a B cell lymphoma [195]. However, this terminology has now been rebuked since the identification of lymphoma with an initiating oncogenic event occurring early during hematopoietic development followed by further differentiation [196].

1.3.2.1 Cause of B cell Lymphomas

B cells are particularly prone to malignant transformation. The same mechanisms that are used to generate antibody diversity are often involved in causing oncogenic mutations in non-Ig genes and chromosomal translocations of the Ig loci and a proto-oncogene. These mechanisms include V(D)J recombination, somatic hypermutation and isotype switching which share the attribute of generating double-strand DNA breaks in the Ig locus. Break points differ depending on the differentiation stage of the B cell. In malignant B cells, the translocation partner is transcriptionally deregulated due to its transposition into the Ig locus and becomes constitutively active. Examples include the cyclin involved in cell cycle control *BCL1*, apoptosis inhibitor *BCL2*, the major cell growth regulator *MYC* and GC factor BCL6 [197]. Somatic hypermutation may also cause mutations in non-Ig genes, examples include BCL6, death receptor CD95/Fas, PAX5, MYC, Ser/Thr kinase involved in cell proliferation PIM1, Rho/TFF [198-202].

In addition, there are also signaling and regulatory mechanisms that are co-opted from normal B cells and misused for survival of B cell lymphomas. These include:

Oncogenic signaling is often commandeered by the B cell lymphoma through gain-of-function mutations of activators, loss-of-function mutations of negative regulators or autocrine receptor activation. Examples include constitutive activation of NF-κB, oncogenic engagement of NF-κB by the CARD11 pathway, oncogenic BCR signaling and recurrent MYD88 [195].

Tumor suppressor and TF factor mutations are utilized by malignant B cells to sustain the phenotype and survival. A key tumor suppressor that is mutated in DLBCL is PRDM1 [203, 204]. Additional TF that are mutated include BCL6 and IRF4 [195].

Immune evasion is another mechanism utilized by B cell lymphomas and is accomplished through genetic lesions in genes necessary for immune recognition. Examples include *CIITA* gene fusions, β2M mutations and deletions and immunomodulatory cytokines and chemokines [195].

Epstein-Barr viral (EBV) infections can also malignantly transform B cells. EBV expresses several latency genes that compromise cell-signaling pathways to maintain B cell proliferation and inhibit differentiation [205].

1.3.2.2 B cell Lymphoma Classification

With the significant activity of somatic hypermutation and class switching during the GC, it is a major site of malignant transformation. **Figure 1.5** provides an overview of some lymphomas and the normal B cell type they resemble. This section describes some B cell lymphomas that will be described in this thesis and the various treatment options.

Mantle Cell Lymphoma (MCL) defined by four cytological variants, namely small cell variant, the MZ-like variant, pleomorphic variant and blastoid variant, with the latter two having the worst prognosis [206, 207]. Genetically MCL is defined by the t(11;14(q13:q32)

translocation which fuses the IgH enhancer to the Cyclin D1 (*CCND1*) [208]. Additional oncogenic mutations have been identified in the tumor suppressor genes, *ATM*, *CDKN2A*, *TP53*, and oncogenes, *MYC*, *SYK* and *BCL2* [209]. The treatment of MCL is determined by the age and fitness of the patient. Fit patients receive the more intense cytarabine-based treatment with autologous transplant.

-*Cytarabine* based treatment involves the combination of R-HyperCVAD with alternating Methotrexate/Cytarabine alternating with Rituximab high-dose Methotrexate/Cytarabine.

-*Rituximab* is an anti-CD20 antibody, which associates with cell surface CD20. The Fc portion is free to mediate antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), which subsequently causes cell death.

-*Cyclophosphamide* interferes with replication and transcription.

-*Vincristine* associates with tubulin and prevents cells from undergoing cytokinesis. As a result cells eventually undergo apoptosis.

Adriamycin (Doxorubicin hydrochloride) is an intercalating agent that sits in between DNA bases and damages DNA. In addition it also inhibits macromolecular biosynthesis.

-*Dexamethasone* is a corticosteroid which functions as an immune suppressant.

-*Methotrexate* inhibits DNA, RNA and protein synthesis.

-*Cytarabine* is an antimetabolic agent that interferes with the synthesis of DNA.

-*Autologous transplant* describes the process of stem cell collection from a patient, which is followed by chemotherapeutic treatment and/or radiation and subsequent transplant back into the patient to repopulate the patients' blood cells. Patients that are not able to tolerate the intensive approach can receive a variety of treatments. These include CHOP, Fludarabine and Cylophosphamide and Bendamustine.

-CHOP is a combination of several drugs, which include Cyclophosphamide, Hydroxydaunomycin, Oncovin (Vincristine) and Prednisone.

-*Prednisone* is a corticosteroid with anti-inflammatory abilities.

-*Fludarabine* inhibits DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase.

-*Bendamustine* is an additional alkylating agent.

For the frailer patient, less intensive therapeutic options include either Rituximab alone or in combination with Chlorambucil, Cladaribine or Thalidomide.

-*Chlorambucil* interferes with DNA replication and damaging DNA.

-*Cladaribine* inhibits enzyme adenosine deaminase, thus interferes DNA processing.

-*Thalidomide* is an immunomodulatory drug.

There is no standard treatment for relapse MCL. Thus an alternative immune-chemotherapeutic regimen is used [210]. Newer agents that are currently being tested on relapsed and refractory MCLs include Bortezomib, Temsirolimus, Lenalidomide and Ibrutinib.

-*Bortezomib* is a proteasome inhibitor.

-*Temsirolimus* is an mTOR inhibitor and interferes with protein synthesis, growth and survival of tumor cells.

-*Lenalidomide* is derivative of thalidomide and is an immunomodulator.

-*Ibrutinib* is chemotherapeutic drug that is characterized as a targeted therapy. Ibrutinib associates with the downstream BCR kinase BTK and inhibit its function [210].

Burkitt's Lymphoma (BL) is an aggressive B cell NHL that is defined by Ig and MYC translocation. This translocation alone is not sufficient for malignant transformation thus

additional mutations are thought to participate. Three distinct subtypes are recognized, namely endemic- (African), sporadic- and immunodeficiency-associated BL [211, 212]. Endemic is more prevalent in equatorial Africa [212]. The sporadic is a rare type. It is most common in younger individuals and accounts for 30% of pediatric lymphomas and less than 1% of adult NHL [213]. The immunodeficiency-associated type is prevalent among HIV infection [214]. Additional mutations observed include cyclin D3 (*CCND3*), *Bim*, *TP53*, *CDKN2A*, *p16*, *E2A* and *ID3*. Current treatment for BL, includes R-CHOP and results in significant myelosuppression as well as life threatening complications. Studies into less intense, targeted therapy are underway and are aimed at MYC or other contributing pathways [212].

Diffuse Large B Cell Lymphoma (DLBCL) is the most common type of NHL. Through gene expression profiling depicts the existence of two subtypes based on cell of origin. Namely, activated B Cell type (ABC) and GC-derived diffuse large B cell lymphoma. An additional third unclassified group has also been identified, which resembles both DLBCL and BL [215, 216]. The ABC-DLBCL and GC-DLBCL share the recurrent mutations in immune surveillance genes (B2M and CD58), chromatin modifying genes (MLL2/3, CREBBP and EP300), BCL6 protein activity (MEF2B) and cell cycle or apoptosis genes (FOXO1 and TP53).

Differential mutational profiles among the different DLBCLs, are thought to contribute to their responsiveness to chemotherapy.

ABC-DLBCL depicts dependence on constitutive activation of BCR and NF-κB signaling pathways based on identified mutations in CD79A/B, CARD11, MYD88 and TNFAIP3. These mutations are thought to contribute to this subtypes poor response to R-CHOP regimens. It is more sensitive to Ibrutinib [217].

-*R-CHOP* is chemotherapy regimen that is a combination of drugs included in CHOP in addition to Rituximab.

GC-DLBCL also depicts dependence on constitutive activation of BCR and NF-κB signaling. The mutated genes differ from ABC-DLBCL and include EZH2, GNA13 and SGK1 genes. MYC rearrangement is associated with about 70% of GC-DLBCL. MYC rearrangements follow the double hit (DH) or triple hit (TH) model. The DH/TH model also have concurrent BCL2 (DH) and to a lesser extent also BCL6 (TH). The exact importance of MYC rearrangement with Ig genes or other partner genes for prognosis is currently unknown and is topic of investigation [217].

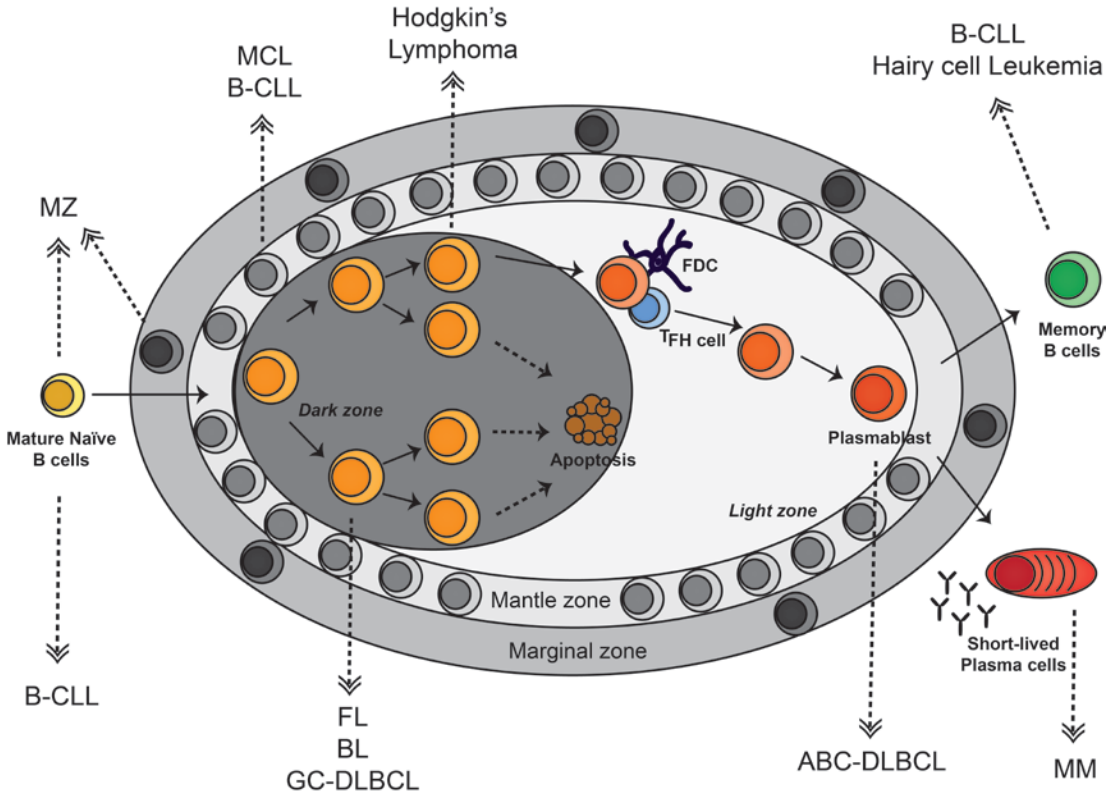

Figure 1.5 B cell Lymphomas and their Resemblance to Normal B cell Types. B cell lymphomas resemble B cells at various stages of B cell differentiation. Marginal Zone B cell Lymphomas (MZ), B-cell Chronic Lymphocytic Leukemia (B-CLL), Mantle Cell Lymphoma (MCL), Follicular Lymphoma (FL), Burkitt's Lymphoma (BL), Germinal Center-derived Diffuse Large B Cell Lymphoma (GC-DLBCL), Activated B Cell-type Diffuse Large B Cell Lymphoma (ABC-DLBCL), Multiple Myeloma (MM).

1.4 Transcription

In eukaryotes, transcription is performed by three possible multi-subunit RNA Polymerases (Pol), which include Pol I, Pol II and Pol III [218]. Each polymerase transcribes a different class of RNA. Pol I transcribes the 25S, 18S and 5.8S ribosomal RNA (rRNA). Pol II is responsible for the transcription of messenger RNA (mRNA), majority of microRNAs (miRNAs), non-coding RNAs (ncRNAs), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs). Pol III performs transcription of the short untranslated RNAs, 5S rRNA and transfer RNAs (tRNAs) [219-221]. To initiate transcription, all three polymerases associate with TATA box-binding protein (TBP) with either the general transcription factors (GTF) TFIIB, TFIIE and TFIIF (for Pol II) or proteins that are structurally and functionally related to GTF (for Pol I and Pol II) [218, 222]. Since this thesis will focus on transcriptional elongation factors that increase the catalytic rate of RNA Pol II transcription, the following sections will only discuss RNA Pol II in detail.

1.5 RNA Polymerase II Transcription

RNA Pol II transcription is a structured order of events that require transcription factor interactions and posttranslational modifications to allow for its proper progression. Transcription is divided into several distinct steps. It is initiated with the recruitment of Pol II to the promoter (pre-initiation complex assembly), assemble with the GTF (open complex formation) and initiate the transcript (initiation). These early events are often a main target for TF regulation and requires the recruitment of chromatin –remodeling complexes [223].

After initiation, Pol II departs from the promoter and engages in mRNA production (promoter clearance) [224]. Subsequently, efficient elongation requires that Pol II does not pause

or stall due to unusual DNA structures or DNA bound proteins [225, 226]. Co-transcriptionally the mRNA is also subjected to the maturation related processing mechanisms such as capping and splicing as well as the termination-coupled processes of mRNA poly-adenylation [227-230]. Following transcription termination, Pol II is recycled for a new round of transcription [231, 232]. Each step will be discussed in the following sections.

1.5.1 Transcriptional Initiation

The initiation of transcription is a tightly regulated process, where TF mostly exert their functions. Transcription is initiated with binding of sequence specific activators to enhancer elements and subsequent recruitment of general transcription factors and Pol II to the target gene core promoters **(Figure 1.6)** [233]. Target genes are categorized based on the presence of various core promoter elements. The TATA box containing promoters are among the most ancient and well understood [234-236]. The canonical 5'-TATAA-3' sequence is recognized and bound by the TFIID subunits, TBP as well as several TBP associated factors [237, 238]. Cooperatively, the adaptor complexes SAGA or Mediator (MED) are recruited to the un-phosphorylated C-terminal domain (CTD) of Pol II [239]. Their association facilitates their binding of additional GTFs [233]. TFIIB and TFIIA are the first to associate with TBP and recruit Pol II into the forming complex. TFIIB has additional functions in aligning and unwinding DNA as well as determining the directionality of transcription. Pol II is escorted to the complex by TFIIF after which TFIIE associates. This association is a prerequisite for the binding of TFIIH which completes the assembly of pre-initiation complex (PIC) [240-242]. MED also stimulates the TFIIH CTD kinase activity [239, 243, 244]. The Cdk7 subunit of TFIIH induces the key ATP-dependent switch from closed to open promoter complex, by hyper-phosphorylating the Serine 5 (Ser5) residue in

the CTD. The achievement of 15 base pairs (bp) of unwound promoter DNA forms the transcription bubble and formation of the first several phosphodiester bonds of the nascent RNA and subsequent transition into elongation [245, 246].

The gene specific activators are also responsible for recruiting histone modifying enzymes and chromatin remodeling factors to the chromatin for reorganization, which in turn permits Pol II transcription. The hallmark of an open permissive chromatin that is competent for transcription, involves the acetylation (H3K9, H3K14 and H4K16) and methylation ($H3K4me^2$ and $H3K4me^3$) of promoter proximal nucleosomes [247, 248]. Chromatin remodelers recognize these modifications and unwind the chromatin for transcription to begin [249].

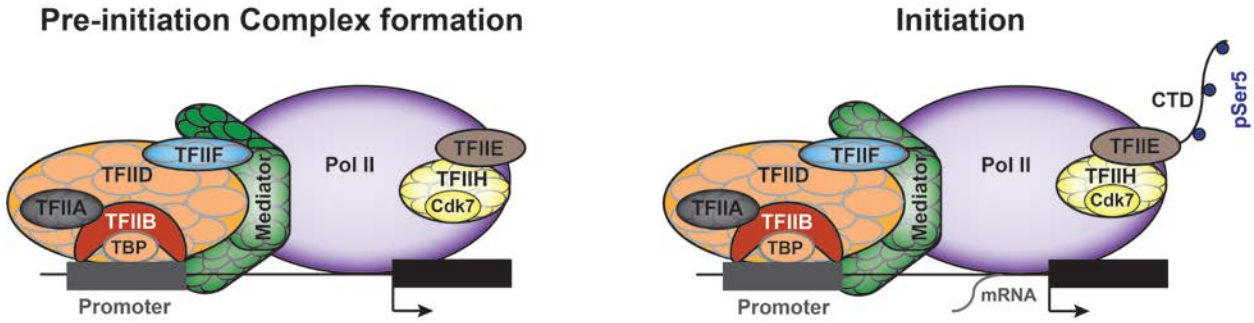

Figure 1.6 Pre-Initiation Complex Assembly and Initiation of Transcription. The recruitment of various general transcription factors is required for the assembly of the pre-initiation complex. The TFIID subunit, TATA binding protein, associates with the TATA box. Together with TFIIB, TFIIA and Mediator, these factors recruit the remaining complex components TFIIF, TFIIE which respectively recruit RNA polymerase II and TFIIH to the complex. The Cdk7 subunit of TFIIH, hyper-phosphorylates the C-terminal domain of RNA polymerase II at Ser5.

1.5.2 Transcriptional Elongation

Productive elongation by Pol II requires acquisition of structural changes to the initiating Pol II. As a result a whole gene is transcribed without Pol II disassociating from nascent RNA.

The steps required for transition into effective elongation are described in the following section **(Figure 1.7)**.

1.5.2.1 Promoter Clearance

Promoter clearance is the earliest step where Pol II disassociates from the promoter. This process is regulated by intrinsic factors and depends on the interaction of Pol II with the nascent RNA and sequences in the template DNA [250]. The process is initiated by forming the initial transcribing complex (ITC) during early transcription, which allows for abortive initiation [251]. Transcripts that are less than 5 nucleotide (nt) are unstable and results in abortive transcription. Transcripts that are 10 nt long favor promoter escape and coincides with the collapse of the transcription bubble and transition to the early elongation complex [251, 252].

1.5.2.2 Promoter Proximal Pausing

Promoter-proximal pausing is a state where Pol II pauses promoter-proximally. Such pausing is considered a rate-limiting step prior to transition into productive elongation [253]. The exact mechanism by which this pause occurs is not fully understood. However several ideas have been proposed. The first proposed mechanism reports that the pause is caused by transcript slippage and backtracking, due to the instability of the early elongation complex [254]. Minor backtracking of a few nt leads to transcriptional pausing that can be resolved by Pol II itself, while extensive slippage is thought to cause arrest that requires TFIIS induction of RNA nuclease activity [253, 255]. Alternatively site specific pausing, cis elements and nucleosome downstream of the transcription start site have been reported to cause transcriptional pausing [253, 256]. Transcriptional pausing has also been shown to facilitate capping of the nascent RNA [257]. For this process, capping enzymes were shown to associate to the CTD of Pol II,

suggesting that capping might be a prerequisite for overcoming the pause [258, 259]. A final mechanism involves the binding of negative elongation factor (NELF) and DRB sensitivity-inducing transcriptional factor (DSIF) that cooperate to induce transcriptional pausing [260-262].

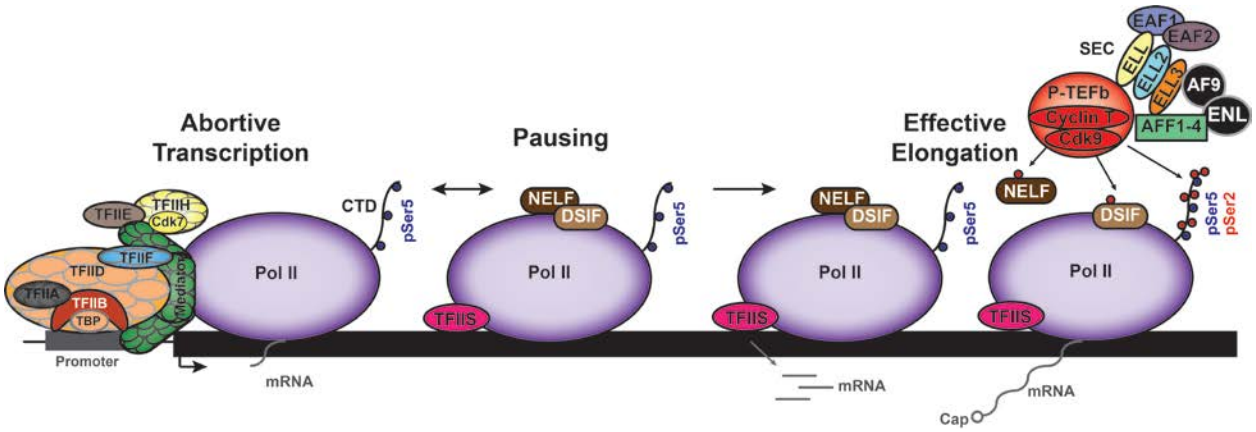

Figure 1.7 Abortive Transcription, Promoter Proximal Pausing and Effective Elongation. Pre-initiation complex assembly results in open complex formation, due to hyper-phosphorylation of the C-terminal domain on RNA polymerase at Serine 5. This process is not the most effective at transcription and may become paused due to cis elements or association of negative elongation factors. Stable pausing is thought to be relieved by TFIIS induced RNA polymerase II intrinsic nuclease activity. Association of the super elongation complex, results in hyper-phosphorylation of the C-terminal domain of RNA polymerase II by P-TEFb at Ser2 and effective elongation.

1.5.2.3 Effective Elongation

This step is characterized by the release of paused Pol II for productive elongation. Several factors are known to stimulate the activity of Pol II. The GTF, TFIIF, is essential for promoter clearance and paused states [263, 264]. For the backtracking model it was shown that arrest due to extensive backtracking could be relieved through cleavage of the extruding RNA with the help of TFIIS. TFIIS induces the intrinsic Pol II nuclease activity [265]. The inhibitory effects of DSIF and NELF on Pol II are relieved by the binding and phosphorylation of P-TEFb, which is a complex of Cdk9 and cyclin T. Cdk9 preferentially phosphorylates the DSIF component Spt5 and serine 2 (Ser2) in the CTD of Pol II [266-268]. Phosphorylated DSIF

maintains an association with Pol II, while NELF leaves the complex [269, 270]. At this stage DSIF has a positive effect on elongation [261, 271]. In addition, the Ser2 phosphorylated CTD recruits several elongation factors that assemble into a large macro molecular structure termed super elongation complex (SEC) [272]. Among these factors are Elongin, AFF family members (AFF1-4), YEATS domain containing protein family members (ENL or AF9) and Eleven-nineteen Lysine-rich Leukemia (ELL) family members (ELL, ELL2 and ELL3) and ELL associated factors (EAF1 and EAF2), which assemble on the RNA Pol II, increasing the catalytic rate of transcription allowing it to productively elongate [253, 263, 273-275].

1.5.3 Transcriptional Termination

Termination of transcription signals the processing of nascent RNA and the release of Pol II from the DNA [276, 277]. Majority of eukaryotic protein-coding genes have a conserved poly (A) signal (PAS), characterized by the 5'-AAUAAA-3' followed by a G/U-rich region. Pol II is thought to pause near the PAS site and recruit the polyadenylation factors cleavage and polyadenylation specific factor (CPSF), cleavage stimulatory factor (CstF) and the poly (A) polymerase [278-280]. A second, termination pathway is utilized for non-coding RNA transcripts. Their 3' ends are bound by the NRD1-NAB3-SEN1 pathway, which recruits exosome to an RNA substrate. In the absence of polyadenylation NRD1-NAB3-SEN1 pathway is thought to promote transcript degradation, while the presence of a polyadenylation blocks progressive degradation and is thought to be used for trimming sn/snoRNAs [281].

Following these processing mechanisms, the pre-mRNA undergoes further maturation and exported into the cytoplasm for translation. Subsequently, Pol II gets recycled for a subsequent round of transcription. Pol II is first de-phosphorylated and brought into the vicinity of the

initiation site through looping [232, 282]. TFIIB is responsible for the looping by binding the terminator and functioning as a scaffold for the promoter [283].

1.6 ELL Family Members

Transcriptional elongation factors of the ELL family are best known for the participation in the SEC. In humans, the ELL family consists of three family members which are characterized by their *in vitro* ability to increase the catalytic rate of Pol II transcription.

1.6.1 ELL

1.6.1.1 ELL Structure

Family member ELL was the first identified out of all family members. It is located on chromosome 19p13.1 and frequently undergoes translocation with the trithorax-like mixed lineage leukemia (MLL) gene on chromosome 11q23 in acute myeloid leukemias. The fusion protein is a combination of the N-terminal portion of the MLL with the C-terminal portion of ELL. Sequence analysis of the ELL portion showed similarity to the highly basic DNA binding domain of the poly (ADP-ribose) polymerase (PARP). Thus, it is thought that as part of the fusion protein, ELL is alters the specificity of MLL [274, 284]. A lysine rich region within the C-terminus is also required for the expression of AP-1 and c-Fos [263].

1.6.1.2 ELL Functions

ELL is the best functionally characterized MLL partner. Its elongation activation domain is located in the N-terminal domain and the C-terminus bears resemblance to the ZO-1 binding domain of Occludin [284]. The C-terminus has also been implicated in an association with p53 *in*

vitro and inhibits sequence specific trans-activation and sequence-independent trans-repression and p53 mediated-apoptosis [285]. The C-terminus of ELL was also implicated in regulation of cell growth and survival [286]. ELL also has roles prior to its assembly into the SEC. ELL was shown to also participate in transcriptional initiation and pause site entry. Its expression was critical for the stability of the pre-initiation complex. These effects roles are critical for transcriptional activity of rapidly induced genes [275].

1.6.2 ELL2

1.6.2.1 ELL2 Structure

The second family member to be identified was ELL2 based on sequence homology. ELL2 depicts 49% identity and 66% similarity to ELL. Homologous to ELL, structure-function studies localized ELL2s elongation activation domain to the N-terminal region between residues 7 and 353. Homology is also observed in the short lysine-rich region located between residue 443-474, and C-terminal region located between residues 516-640 [287]. The C-terminus included the lysine-rich region responsible for AP-1 and c-Fos expression [263].

1.6.2.2 Cell-type Specific Function of ELL2

ELL2 is also the only family member with known roles in the B-cell compartment. Its expression is exclusively observed in plasma cells, where it's responsible for loading the CstF-64 polyadenylation factor on Pol II. This association was dependent on the phosphorylation of Ser2 on Pol II. ELL2 activity enhanced the use of the weaker promoter-proximal poly(A) site and the non-consensus splice site in the secretory-specific exon of the immunoglobulin heavy chain

locus. Thus, ELL2 is required for secretion of immunoglobulins [288, 289]. The expression of ELL2 mRNA and its associated factor EAF2 is induced by IRF4 and PRDM1 [290-292].

1.6.3 ELL3

1.6.3.1 ELL3 Structure

ELL3 was the last family member to be identified. Similar to ELL2, ELL3 was identified based on sequence homology to ELL. ELL3 shows 50% similarity to both ELL and ELL2 and [293]. Both the N- terminal elongation activation domain and the C-terminal domain of ELL3 are highly homologous to ELL. ELL3 differs in that it is missing the central domain and the lysine-rich region in the C-terminal that is required for AP-1 and c-Fos [293].

1.6.3.2 Cell-type Specific Functions of ELL3

ELL3 was initially reported to be testis-specific. Recently, its ectopic expression was reported to mark enhancers in murine embryonic stem cells for future activation and stimulate differentiation and epithelial-mesenchymal transition [294, 295]. In breast cancer cell lines, its ectopic expression was reported to stimulate proliferation, drug resistance and cancer stem cell properties [296]. Finally, its ectopic expression was also shown to stabilize p53 [296, 297].

1.6.4 SEC-like Complexes

Our current understanding of SEC components stems from their regulatory role in developmental genes, heat-shock-inducible genes, proto-oncogenes, retrovirus transcript production and leukaemogenesis [274, 298-307]. SEC components were identified as common

MLL translocation partners in acute myeloid leukemia [273, 308]. These mechanisms have all depicted the use of P-TEFb with various combinations of SEC components.

P-TEFb kinase activity on the CTD of Pol II is required for productive elongation. This kinase activity is tightly regulated *in vivo* through formation of various complexes. The large majority of P-TEFb was reported to be inactive when sequestered in a complex with 7SK-RNA, MEPCE, LARP7 and HEXIM1 [309-312]. P-TEFb also complexed with the bromodomain protein 4 (BRD4). This formed an active complex that can phosphorylate the CTD of Pol II *in vitro* and activate specifically HIV transcription and not Tat-mediated transactivation [308, 313].

Elongation factor AFF4 is an essential component of SEC. Its expression is required for the assembly of the SEC. Two additional family members, AFF2 and AFF3, have been described with similar conserved domains [314]. An assessment of biochemical and molecular roles of AFF members reported that AFF1/AFF4 containing (SEC) -, AFF2 containing (SEC-L2) - and AFF3 containing (SEC-L3) -complexes are able to regulate distinct sets of genes. With SEC regulating rapidly induced genes [315].

Recently, ectopic expression of ELL family members depicted that ELL2 and ELL3 were co-expressed with ELL but not each other [273]. Thus, it is proposed that various elongation factor combinations within the SEC may alter gene target specificity or transcriptional output.

CHAPTER II:

MATERIALS & METHODS

2.1 Cell Lines and Reagents

The CA46, Raji, Namalwa, Ramos, Mino, Jeko-1, Maver-1, Z138, U266, NCI-H929, Jurkat and HEK-293T cell lines were purchased (ATCC, Manassas, VA). The 207 and 697 cell lines were provided by Dr. P.D. Burrows (University of Alabama Birmingham). The HBL2, Toledo, BJAB, OCI-Ly19, SU-DHL-4, and Pfeiffer cell lines were provided by Dr. J. Tao (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL). The U2932 cell line was provided by Dr. I.S. Lossos (Sylvester Comprehensive Cancer Center, Miami, FL) [316]. Cells were cultured in Hyclone RPMI 1640 or DMEM media supplemented with 10% FBS (GE Healthcare Life Sciences, Pittsburgh, PA) and 1% penicillin-streptomycin and for NCI-H929 only 55 µM 2-mercapotoethanol. All cells were cultured at 37°C in 5% CO_2.

2.2 Peripheral Blood Mononuclear Cell Isolation

To isolate peripheral blood mononuclear cells (PBMC), healthy human lymphocyte enriched peripheral blood was acquired (Florida Blood Services, Saint Petersburg, FL) and diluted with equal volume of cell culture sterile phosphate buffered saline (PBS). Every 10 ml diluted buffy coat mixture was layered drop-wise over 3 ml Ficoll-Paque Plus density gradient medium (GE Healthcare Life Sciences, Pittsburgh, PA) and subjected to gradient centrifugation

for 30 min at 829 x g, room temperature (RT) with no brake [317, 318]. Following centrifugation the buffy coat can be observed as being separated into four layers **(Figure 2.1)**. For each tube, the top layer was suctioned off, taking care not to disturb the second layer. The second PBMC layer was collected into a new 50 ml conical tube, taking care not to also collect the subsequent Ficoll-Paque Plus layer. The third and fourth layers were discarded. For each tube, the PBMC layer was subsequently washed five times by re-suspending in 50 ml RT PBS and centrifuging for 5 min at 829 x g at RT. For the last wash step, all donor tubes are combined into one prior to centrifugation. Following the last wash, the pellet was re-suspended in 10 ml separation buffer containing Ca^{2+} and Mg^{2+} free RT PBS containing 2% FBS and 1 mM EthyleneDiamineTetraaceticAcid (EDTA).

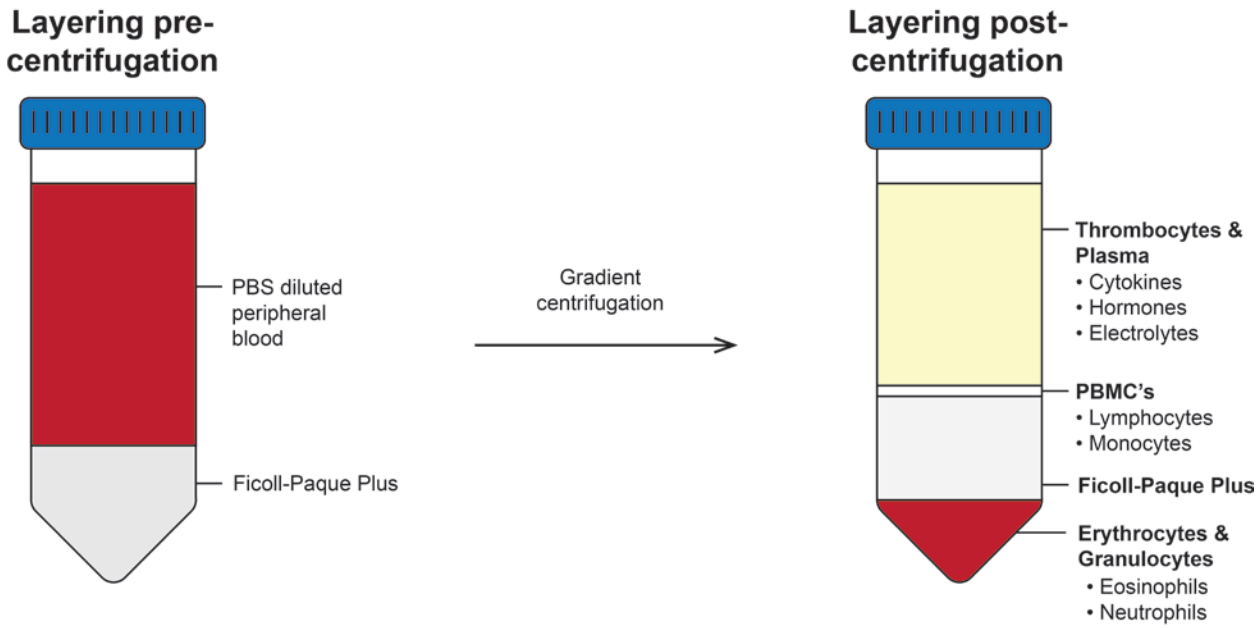

Figure 2.1 Schematic Depiction of Gradient Centrifugation Layering. Prior to gradient centrifugation, PBS diluted blood is layered on top of Ficoll-Paque Plus gradient medium taking care to protect the interface. Ficoll-Paque Plus (clear) is added first and the PBS diluted peripheral blood layered dropwise on top. After gradient centrifugation separation into four layers is observed. The top layer (transparent yellow) consists of thrombocytes and plasma and is discarded. The second layer (white) is the PBMC's layer is collected and used for lymphocyte isolation. The third layer (clear) is the Ficoll-Paque Plus will be discarded and not be collected with the PBMC layer as this could compromise PBMC viability. The fourth layer (red) will contain erythrocytes and granulocytes and will also be discarded.

2.3 Primary Naïve B cell Isolation

We utilized the EasySep Human Naïve B cell Enrichment kit strategy (STEMCELL Technologies Inc., Vancouver, Canada) described in **Figure 2.2** to perform negative selection under cell culture sterile conditions. To isolate naïve B-cells, a PBMC dilution was prepared in a 12 x 75 mm polystyrene round-bottom tube (Corning Inc., New York, NY) at $5x10^7$ cells/ml in a total volume of 1 or 2 ml. 50 µl Enrichment Cocktail was added per ml of PBMC dilution, mixed by pipetting up and down and incubated at RT for 10 min. To prepare the Magnetic Particles, they were vortexed for 30 sec. Subsequently, 250 µl of Magnetic Particles was added per ml of sample, mixed by pippeting up and down and incubated for 5 min at RT. The sample was topped up to 2.5 ml with separation buffer, mixed by gently pippeting up and down and 2-3 times. The polystyrene tube containing the mixture was placed into the EasySep Magnet without a lid and incubated for 5 min at RT. The magnet and tube were inverted in one continuous motion, pouring the enriched naïve cell suspension into a new conical tube. Cells were spun down at 298 x g for 5 min at RT and maintained in Hyclone RPMI 1640, supplemented with 10% FBS and 1% penicillin-streptomycin.

2.4 Cytokine Stimulation

In vitro activation and differentiation of primary B cells was adapted from previously described publication [171]. In a 24 well plate, cells were seeded at $1x10^6$ cells/ml and activated by adding 100 U/ml IL-2 and 100 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) or differentiated into plasma cells by adding 100 U/ml IL-2, 100 ng/ml IL-21, 5 µg/ml unlabeled goat anti-human IgM antibody (SouthernBioTech, Birmingham, AL), 10 ng/ml Histidine tagged CD40L and 10 µg/ml polyHistidine antibody (R&D Systems Inc., Minneapolis, MN). Both conditions were incubated

for 3 consecutive days at 37ºC days. 50 U/ml IL-2 and additional media was added to all conditions and incubated at 37 ºC for 2 days. All samples were harvested and washed with PBS prior to analysis.

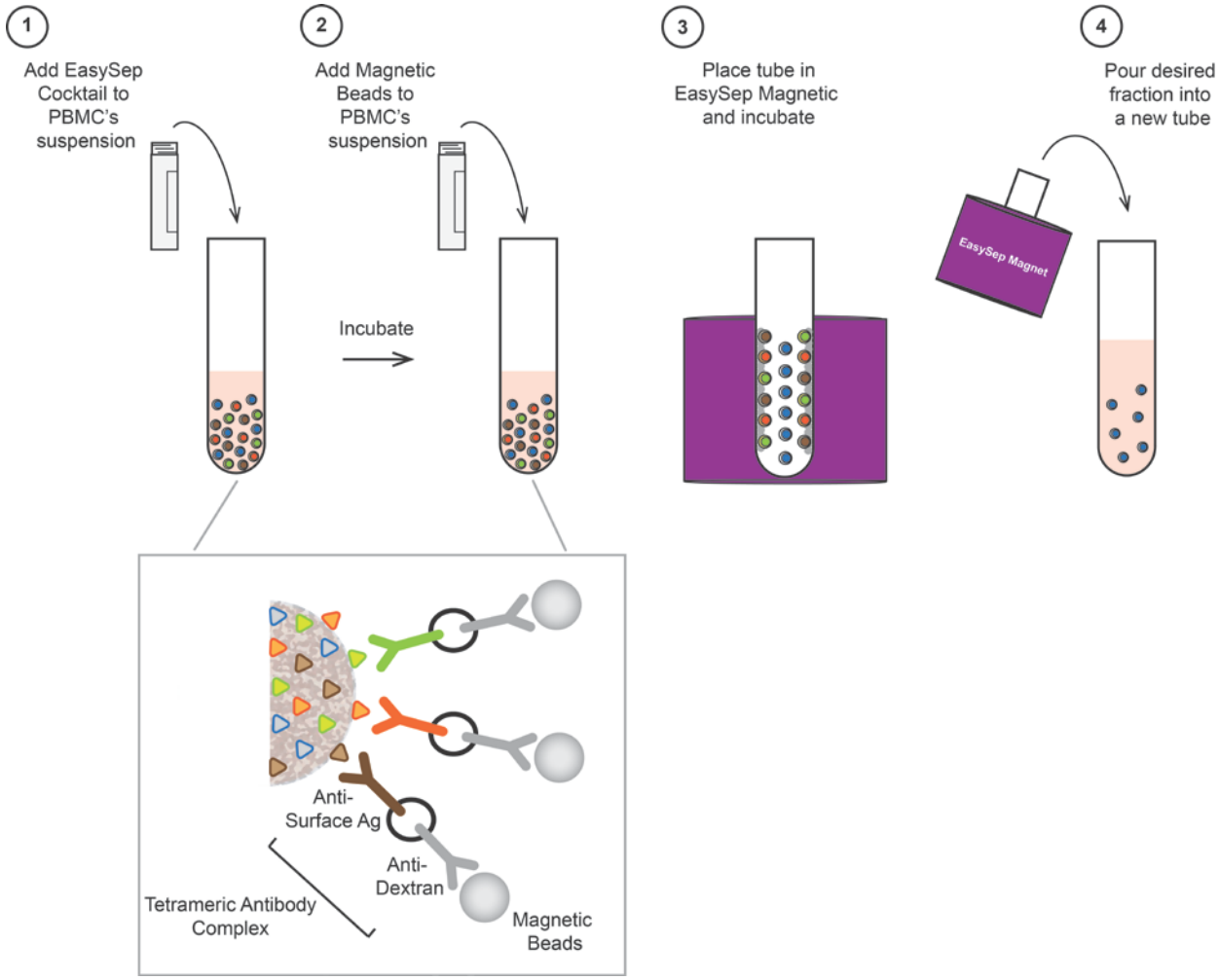

Figure 2.2 Schematic Representation of Primary Negative Selection Procedure. The EasySep Cocktail containing a combination of bispecific Tetrameric Antibody Complex (TAC) is mixed with PBMC suspension and incubated. During this process the anti- cell surface monoclonal antibody end of the TAC, with specificities to either CD2, CD3, CD14, CD16, CD36, CD43, CD56, CD66b, CD27 and Glycophorin A on human blood cells, is bound to their respective antigens. Subsequent addition of the EasySep Magnetic beads binds the anti-dextran end of the TAC and allows for magnetic retention of the captured cells through placement in the EasySep Magnet. The un-touched cell of interests is poured off into a new tube. Figure is adapted from STEMCELL Technologies product information sheet.

2.5 Chromatin Preparation

The preparation of chromatin was performed as described previously [167]. This involves the initial 10 min crosslinking of $2x10^7$ cells with 1% formaldehyde at RT with rotation. An excess of 0.125M Glycine was added to neutralize the reaction. Cells were then washed twice with ice cold PBS and resuspended at $4x10^6$ cells/ml in ice cold Triton X-100 (TX-100) Nonidet P-40 (NP-40) buffer containing 10 mM Tris (pH 8.1), 10 mM EDTA, 0.5M EthyleneGlycolTetraaceticAcid (EGTA), 0.25% TX-100, 0.5% NP-40, 1 mM PhenylMethylSulfonyl Fluoride (PMSF) and 0.5x Protease Inhibitor cocktail (PI). Cells were subsequently incubated for 10 min at 4ºC with rotation in 10ml ice cold salt-wash buffer containing 10 mM Tris (pH 8.1), 1 mM EDTA, 0.5 M EGTA, 200 mM Sodium Chloride (NaCl), 1 mM PMSF and 0.5x PI. Cells were lysed at $1x10^6$ cells/30µl by adding sonication buffer containing 10 mM Tris (pH 8.1), 1 mM EDTA, 0.5 M EGTA, 1% Sodium Dodecyl Sulfate (SDS), 1 mM PhenylMethylSulfonyl Fluoride (PMSF) and 1x Protease Inhibitor cocktail (PI). To obtain sheared chromatin of 100-600bp, lysates were sonicated using a water bath sonicator (Diagenode Inc., Denville, NJ).

2.6 Chromatin Immunoprecipitation (ChIP)

ChIP was performed using the equivalent of $2x10^6$ cells and 5µg of normal rabbit IgG (EMD Millipore, Billerica, MA) or PRDM1 (C14A4) rabbit mAb (Cell Signaling Technology, Beverly, MA). Immunoprecipitated chromatin was sequentially washed with low salt wash buffer (20 mM Tris (pH 8.1), 2 mM EDTA, 150 mM NaCl, 0.1% SDS and 1% TX-100), high salt wash (20 mM Tris (pH 8.1), 2 mM EDTA, 500 mM NaCl, 0.1% SDS and 1% TX-100) and Lithium Cloride (LiCl) wash buffer (10 mM Tris (pH 8.1), 250 mM LiCl, 1% NP-40, 1% sodium

deoxycholic acid and 1 mM EDTA. DNA was eluted using the elution buffer (10 mM Tris (pH 8.1), 1% SDS, 1 mM EDTA) and de-crosslinked by incubating with 312 mM NaCl at 65ºC for 4 h. The immunoprecipitated DNA was treated with RNase at 37ºC and proteinase K (Roche, Indianapolis, IN) for 1 h at 45ºC. Qiagen PCR spin columns were used to purify the DNA.

2.7 ChIP-Sequencing and Data Processing

For ChIP-Sequencing, at least ten PRDM1-enriched DNA or input were pooled for the U266 and NCI-H929 cell lines. Sequencing was performed by the Molecular Genomics Core Facility at the H. Lee Moffitt Cancer Center & Research Institute. 50 ng of PRDM1-enriched or input DNA was fragmented to 300 nt DNA fragments using a Covaris M220 Focused-ultrasonicator (Covaris, Inc., Woburn, MA) and then used to generate sequencing libraries using the Illumina TruSeq Library Preparation Kit according to manufacturer protocol (Illumina, Inc., San Diego, CA). The size and quality of the library was evaluated using the Agilent BioAnalyzer, and the library was quantified by qPCR. Each enriched DNA library was sequenced on an Illumina HiScan SQ sequencer to generate approximately 15 million 50-base paired-end reads. The raw sequence data were de-multiplexed using the Illumina CASAVA 1.8.2 software and the reads were aligned using BowTie [319]. PRDM1 binding sites were identified using the MACS v1.4 peak-finding software and enriched for 50 or more mapped reads (peaks) located within 10 kb of a promoter, within a gene and within 2 kb of the 3'UTR and a False Discovery Rate of less than 5% [320]. Data is deposited in GEO database under the experiment number GSE102360.

2.8 Direct ChIP

For direct-ChIP, PRDM1-enriched or input DNA were analyzed by qPCR using primers described ***Table I***. Primers to HLA-DRA promoter was used as negative control for specificity. C_t values for each sample were linearized and the percentage over input calculated.

Table I Primer sequences for ChIP. Primer sequences used to detect bound promoter sequences of immunoprecipitated proteins. *-Primers were designed by the authors.*

ELL3 ChIP	FWD: TTTAGGCCACGAGGTGAGA REV: GGCAGCAGTGAAAGTTGG

2.9 Microarray Data Analysis

Microarray expression data derived from flow sorted primary naïve B cells, GC B cells, plasma cells, and memory B cells were obtained from Gene Expression Omnibus (GEO) experiment GSE12366. Three replicates were available per B-cell subtype. ELL, ELL2, ELL3, and PRDM1 relative probe expressions were available and represented as an average; error bars represent standard deviation (SD) [321].

Microarray expression data derived from flow sorted primary splenic B cells and bone marrow plasma cells from the C57BL6/J mice were obtained from GEO experiment GSE39916. Three replicates were available per cell type and represented individually [289].

RNA-sequencing data derived adoptive-transfer experiment were obtained from GEO experiment GSE70294. Briefly, these samples are derived from purified splenic $B220^+$ B cells from $CD45.1^+$ C57BL/6J donor mice were labeled with Cell Titer Violet and adoptively transferred into CD45.2 µMT host mice. Host mice were challenged with LPS 1 d after adoptive cell transfer. At 3 d after challenge, cells were sorted based on CTV dilution, representing divisions 0, 1, 3, 5 and those that divided 8 times. In addition, populations that divided at least 8 times were sorted into $CD138^-$ (8^-) and $CD138^+$ (8^+) [322].

Microarray expression data derived from transfection of two human donor GC B cells with either pcDNA3.1-PRDM1α or empty pcDNA3.1 control were obtained from GEO experiment GSE27670 [323]. Expression was assessed based on one probe and represented as fold change over control condition for each donor.

2.10 Immunoblotting

Immunoblotting was performed as described previously [324]. Cell pellets were washed once with PBS and lysed at $0.5x10^6$/25µl in RIPA buffer containing 150mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 50mM Tris pH 7.2, 2 mM EDTA, 1mM PMSF and 1x PI. The equivalent of $0.5x10^6$ cells was resolved per well on 8% SDS-PAGE gels. For chemiluminescent detection, proteins were transferred onto polyvinylidene fluoride (PVDF) (EMD Millipore, Billerica, MA) and blocked by incubation in 5% skim milk in PBS solution (137 mM NaCl, 2.7 mM potassium chloride (KCl), 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.0)) with 0.05% Tween-20 (0.05% PBST). For fluorescent detection, proteins were transferred onto nitrocellulose membrane (GE healthcare Life Sciences, Pittsburgh, PA) and blocked by incubation in 5% skim milk in TBST solution (Tris (pH 7.5), 5M sodium chloride and Tween-20) with 0.05% Tween-20 (0.05% TBST) for 1 h at RT with gentle shaking. Primary antibodies were diluted in 0.5% skim milk in 0.05% PBST or 0.05% TBST overnight at 4°C with rotation. Detection antibodies include: purified ELL3 mouse pAb (#H000080237-B02P lot WuLz 08310, -B01P lot E1172, 08295 WuLz; 1:300; Abnova, Taipei city, Taiwan), purified ELL2 rabbit pAb (#A302-505A; 1:10,000; Bethyl Laboratories Inc., Montgomery, TX), ELL rabbit pAb (#51044-1-AP)1:800; Proteintech Group, Chicago, IL), β-actin mouse mAb (AC-15; 1:12,000; Sigma Aldrich, St. Louis, MO), GAPDH mouse mAb (B7; 1:5000; Santa Cruz, Biotechnology Inc., Dallas, TX),

purified MCM4 chicken mAb and MCM5 rabbit mAb (gifted by Mark G. Alexandrow, Ph.D.) [325]. PRDM1 rabbit mAb, PARP rabbit mAb (46D11), Phospho-Histone H2A.X (Ser139) rabbit mAb (#2577), Cleaved Caspase-3 (Asp175) rabbit mAb (#9661), Cyclin B1 (V152) mouse mAb, Phospho-Cyclin B1 (Ser133) rabbit mAb (9E3) and HA-Tag (C29F4) rabbit mAb (1:1000; Cell Signaling Technology, Danvers, MA). PVDF membranes were washed three times by rotating in 0.05% PBST for 8 min at RT and nitrocellulose membranes were washed 4x by rotating in 0.05% TBST for 10 min at RT. Secondary antibodies were diluted in 0.5% skim milk in 0.05% PBST or 0.05% TBST for 2 h at RT with rotation. Horse radish peroxidase conjugated secondary antibodies used were anti-mouse (1:12,000) or anti-rabbit (1:2000) (GE healthcare Life Sciences, Pittsburgh, PA) as appropriate. Visualization was done with ECL substrate (Promega, Madison, WI) or Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA). Membranes were exposed to HyBlot ES autoradiography film (Denville Scientific, Holliston, MA) for empirically determined exposure times prior to film development. IRDye conjugated secondary antibodies used were goat anti-mouse IRDye®800CW (926-32210) or goat anti-rabbit IRDye®680RD (926-68071) (1:15.000). Fluorescence was detected using the Odyssey® Fc Imaging System and visualized using Image Studio™ software (LI-COR Biotechnologies, Lincoln, NE).

2.11 RNA Isolation and Quantitative mRNA Analysis

RNA was isolated from cells using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA) and first strand cDNA was synthesized from 1 ug of RNA using the qScript cDNA synthesis Kit (Quanta Biosciences Inc., Gaithersburg, MD). The cDNA sample was diluted one to eleven with filter sterilized purified water. Each 10 µl qPCR reaction contained

3µl of the diluted cDNA sample, 200 µM of each forward and reverse primers, and PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD). Reactions were run in duplicate using the CFX 96 thermal Cycler for 40 cycles at the annealing temperature appropriate for the specified primer set and analyzed with CFX Manager (Bio-Rad Laboratories, Hercules, CA). Expression data was analyzed using the ΔΔ threshold cycle (C_t) method, with normalization to 18S or GAPDH [326]. Prior to use, primer quality was verified for the presence of a single curve on melt curve, and efficiencies between 90-110%. Primer sequences and annealing temperatures are described in ***Table II***.

2.12 DNA Constructs

2.12.1 ELL3 Promoter Constructs

A fragment of the human ELL3 promoter -587 to +343 nucleotides relative to the transcription start site was PCR cloned from human genomic DNA into pCR 2.1 (Invitrogen Life technologies, Grand Island, NY) using specific primers described in ***Table III***. The fragment was subcloned into pGL3-basic (Promega, Madison, MI) using the XhoI and KpnI restriction sites to generate pGL3-ELL3-WT. Two mutant constructs were generated using the PCR-mediated overlap extension method [327]. pGL3-ELL3-Mut I eliminates the -239 to -229 PRDM1 site, substituting 5'-AACTTTCACTG-3' with 5'-A**gagc**TCACTG-3' and creating a novel SacI site. pGL3-ELL3-Mut II eliminates the +14 to +24 PRDM1 site, substituting 5'-AGCTTTCACTT-3' to 5'-AGC**gg**T**ac**CTT-3', and creating a novel KpnI site. pGL3-ELL3-Mut I & II was created through SacII-XhoI restriction subcloning from the single mutant constructs. Primers are described in ***Table III***. All clones were verified by DNA sequencing.

Table II Primer sequences for gene expression by qPCR. Primer sequences were designed to span exons with the aim of exclusively detecting mRNA transcripts of the gene of interest. - *Primers were designed by the authors, + Primers purchased from realtimeprimers.com.*

Primer	**Sequence (5'→3')**	**Reference**
18S	FWD: CGGCTACCACATCCAAGGAAGG REV: CCCGCTCCCAAGATCCAACTAC	[324]
ELL	FWD: CTGGGCAAGGTTCAGTT REV: CACTCGCCAAGTTGATGG	–
ELL2	FWD: AGAGTCTCCTGAGTGGTTCGTC REV: AAAGGCCAAGATGTCCAAGA	–
ELL3	FWD: ACCTGACTGAAGATGCCAGA REV: ACTGTCCTTGGTTGCTTGC	–
PRDM1α	FWD: TACATACCAAAGGGCACACG REV: TGAAGCTCCCCTCTGGAATA	[328]
BZLF1	FWD: CGCCTCCTGTTGAAGCAGAT REV:AAATTTAAGAGATCCTCGTGTAAAACATC	[329]
BMRF1	FWD: CAACACCGCACTGGAGAG REV:GCCTGCTTCACTTTCTTGG	[330]
BLLF1	FWD: ACTCATTATCACACGAACGG REV: ATCCAGTTGTATTCAAGGTAGG	[331]
MYC	FWD: GGAACGAGCTAAAACGGAGCT REV: GGCCTTTTCATTGTTTTCCAACT	+
BCL6	FWD: CACCATCCCTTTTTGAAGTG REV: AACGCGGTAATGCAGTTTAG	+
PAX5	FWD: TGGAGGATCCAAACCAAAGG REV: GGCAAACATGGTGGGATTTT	–
Membrane bound IgM	FWD: GTGTCCGAAGAGGAATGGAA REV:GTTCTCAAAGCCCTCCTCGT	–
Secreted IgM	FWD: GTGTCCGAAGAGGAATGGAA REV: ATGACCAGGGACACGTTGTA	–

2.12.2 ELL3-overexpression Construct

To create an ELL3 expression plasmid, ELL3 cDNA was PCR amplified from the Raji cell line and cloned into pCR2.1. Primers are described in ***Table III***. The KpnI/EcoRV fragment encoding ELL3 was subcloned into pcDNA3.1 with an HA tag at the amino-terminus by replacement of the KpnI/PmeI fragment of the previously described pCDNA3.1-HA-PRDα construct [164]. The construct was confirmed through sequencing. 1 µg of the final pcDNA3.1-HA-ELL3 construct was transfected for 48 hrs into HEK-293T cells using the FuGENE 6

transfection reagent according to the manufacturer protocol (Promega, Madison, WI) and appropriate protein expression was confirmed by immunoblot detection with both an HA and an ELL3 specific antibodies.

Table III Primer sequences for mutation of PRDM1 binding sites in ELL3 promoter. Primer sequences were designed to clone the 930 nt WT ELL3 promoter or introduce mutation in the form of a restriction enzyme site within the each PRDM1 recognition sequence. *-Primers were designed by the authors.*

ELL3 promoter cloning	FWD: GGGATTGTGCAGGTCCA REV: CCTCTGTTCAGGGTTTGGTT
ELL3 PRDM1 Mut I	FWD: GTGACAGCCAGAGCTCACTGCTGCC REV: GGCAGCAGTGAGCTCTGGCTGTCAC
ELL3 PRDM1 Mut II	FWD: GCAGGTTCAGCGGTACCTTAGAGACAGC REV: GCTGTCTCTAAGGTACCGCTGAACCTGC
ELL3 cDNA cloning	FWD: TGCACTCGAACTCGTCGC REV: GCACAGTGCCCATACCCTAA

2.12.3 ELL3 shRNA Constructs

For the knockdown of ELL3 expression two independent small hairpin RNA (shRNA) vectors targeting different regions of the ELL3 mRNA MISSSION® TRC2 pLKO.5-puro ELL3shRNA (TRCN0000289149; ELL3sh-1), MISSSION® TRC2 pLKO.5-puro ELL3shRNA (TRCN0000296220; ELL3sh-2) or a control MISSSION® TRC2 pLKO.5-puro Non-Mammalian control shRNA (SHC202; NTsh; Sigma Aldrich, St. Louis, MO) were acquired.

2.12.4 ELL3 mCherry-shRNA Constructs

To facilitate flow cytometric identification of shRNA transduced cells, a second set of shRNA vectors were generated which maintained identical ELL3 targeting sequences but included the mCherry gene. To create these mCherry tagged shRNA constructs, we utilized the commercially purchased MISSSION® TRC2 pLKO.5-puro Non-Mammalian shRNA,

MISSSION® TRC2 pLKO.5-puro ELL3shRNA-1, MISSSION® TRC2 pLKO.5-puro ELL3shRNA2 (Sigma Aldrich, St. Louis, MO) and pLVmCherry (Addgene) constructs. The puromycin gene was removed from the commercially purchased MISSION shRNA plasmids through a MluI and BamHI restriction, while the mCherry insert was obtained through a SalI and BamHI digest of pLVmCherry. Overhangs on both fragments were filled in with klenow. The mCherry insert was ligated to the MISSION vector fragment and the resulting pLKO.5-mCherry Non-Mammalian shRNA (NTsh), pLKO.5-mCherry ELL3shRNA-1 (ELL3sh-1) and pLKO.5-mCherry ELL3shRNA-2 (ELL3sh-2) screened with PstI and BamHI to confirm insert orientation.

2.13 Luciferase Reporter Assay

Luciferase reporter transfections and analysis were done as previously reported [167]. Transfections were performed using 20.5 µg total plasmid into $1x10^7$ cells by electroporation at 250V, 1070µF in 300µl RPMI1640 using the Bio-Rad Gene Pulser II. This total includes 15 µg of luciferase reporter promoter, 5 µg of pcDNA3.1-PRDM1α overexpression construct or control pcDNA3.1 and 0.5 µg of the pRL-TK Firefly Renilla internal control. Cells were cultured for 48 h at $1x10^6$ cells/ml and lysed with 500 µl passive lysis buffer. Luciferase activity measured according to the Dual Luciferase kit manufacturer protocol (Promega, Madison, WI) and data was analyzed by normalizing Firefly luciferase activity to Renilla luciferase activity.

2.14 siRNA-Mediated Knockdown

Knockdown of ELL3 expression by small interfering RNA (siRNA) was done using a predesigned cocktail of 4 ELL3 specific siRNAs (Accell siRNA SMARTpool E-014601-00-

0005) and the non-targeting control siRNA (Accell siRNA D-001910-01-50) (GE Dharmacon, Lafayette, CO). Cells were plated at $5x10^5$/ml in serum free Accell siRNA Delivery media on a 24 well plate. A total of 1µM of siRNA was added to each well and incubated. At 24 h, fresh Accell siRNA Delivery media and 2% FBS was added to all wells and incubated for an additional 24 h. At 48 h, 1µM siRNA was added and incubated for 24 h. At 72 h, 10% FBS was added and incubated for 24 h. Transient knockdown was established at 96 h.

2.15 Production of Lentiviral shRNA Particles

Lentiviral particles were produced in HEK-293T cells using shRNA vectors and 3rd generation lentiviral packaging construct mixture (Applied Biological Materials Inc., Richmond, Canada) using the jetPRIME transfection reagent (Polyplus transfection, Illkirch, France). The lentiviral supernatant was harvested at 48 and 72 h post-transfection, clarified by centrifugation at 1200xg at 4°C for 5 min and filtration through a 0.45 µm PVDF membrane (EMD Millipore, Billerica, MA), concentrated by ultra-centrifugation for 2 hrs at 95000xg, 4°C in a SW32Ti rotor and viral particle pellet resuspended overnight.

2.16 Lentiviral shRNA-Mediated Knockdown

The $5x10^7$ cells/ml were plated on a 96 well plate and transduced by a 2 h spinfection at 1500xg, RT in the presence of 1 µg polybrene (Merck Millipore, Billerica, MA). Each well was transferred to a 24well plate, incubated for 48 h and expanded into larger culture flasks. Transient knockdown was established within 5 days.

2.17 CellTiter-Glo (CTG) Luminiscent Cell Viabilty Assay

To establish proliferation curves, all conditions were seeded at $1x10^4$ cells/ml and only wild type (WT) was counted by trypan blue for 5 consecutive days. Luminescence of all cultures was measured daily in triplicate using the CTG Luminescent Cell Viability substrate (Promega, Madison, WI) according to manufacturer protocol and the Cytation 3 Cell Imaging Multi-Mode reader (BioTek instruments, Inc., Winooski, VT). A daily value of signal/cell was calculated based on WT condition. This value was used to calculate the number of cells in each condition.

2.18 Bromodeoxyuridine (BrdU) Incorporation

To assess if cells are replicating DNA in S-phase, exponentially growing cells shRNA transduced conditions at 5 days post transduction were incubated with 10 µM Bromodeoxyuridne (BrdU) for 30 min at $2x10^5$ cells/ml. Four biological replicates were generated over an 8 h period at 2 h intervals for two independent experiments. At each time point samples were collected, washed, fixed according to manufacturer protocol and stored at 4ºC until ready for staining (BrdU Flow kit; BD Biosciences, San Jose, CA). All conditions were stained simultaneously with anti-Brdu-FITC and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Sigma Aldrich, St. Louis, MO) according to manufacturer's protocol (BrdU Flow kit; BD Biosciences, San Jose, CA). Flow cytometric detection was performed at the Flow Cytometry Core Facility at the H. Lee Moffitt Cancer Center & Research Institute using the LSRII (BD Biosciences, San Jose, CA). The distribution of the cell cycle was assessed in $mCherry^+DAPI^+$ cells using ModFit (Verity Software House, Topsham, ME) and incorporated BrdU levels was assessed in $mCherry^+DAPI^+FITC^+$ cells using FlowJo (FlowJo, LLC, Ashland, OR).

2.19 Microscopy

For cell size studies mCherry-shRNA transduced cells were plated on a glass bottom 12-well plate (MatTek, Ashland, MA) at $5x10^5$ cells/ml. Imaging was performed at the Analytic Microscopy Core Facility at the H. Lee Moffitt Cancer Center & Research Institute on a Zeiss inverted microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) for mCherry and phase at 20x magnification. 3 wells were plated per condition and 4 images were taken per well. Image studio software (LICOR, Inc, Lincoln, NE) was used to determine the cell area of all cells in each image. Cell areas were categorized using Excel analysis toolpak (Microsoft, Redmond, WA).

For time-lapse imaging studies mCherry-shRNA transduced cells were plated on a 6-well flat bottom plate at $2x10^5$ cells/ml. The plate was placed in Evos Onstage Incubator set at 37ºC and 20%O_2 and imaged every 5 min for 24 h on Evos Auto FL Cell Imaging System (Thermo Fisher Scientific Inc., Waltham, MA). All images were taken at 20x magnification using the RFP filter and phase.

2.20 Statistical Analyses

Two-tailed paired *t*-test was used for statistical analyses; p values less than 0.05 was considered significant. The *p-values* were indicated as following: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.00001$. The calculations were performed in Microsoft Office Excel (Microsoft, Redmond, WA).

CHAPTER III:

EXPRESSION PATTERN OF ELL FAMILY MEMBERS IN THE B-CELL COMPARTMENT

3.1 Introduction

Antigen encounter by a fully matured naïve B cell activates a network of transcription factors, with both activating and repressive functions, that regulate activated B cell processes and differentiation into antibody secreting plasma cells. Studies of the GC reaction have significantly enhanced our understanding of these transcription networks. During the activation stage TF factors, BCL6, cMYC, PAX5, SPI-B and ID3 are known to regulate the functions of clonal expansion, somatic hypermutation and class switching [332]. The differentiation into antibody secreting plasma cells is known to be regulated by the transcriptional repressor PRDM1. Through direct binding and repression of the activated B cell TF promoter sequences, PRDM1 is thought to extinguish the activated B cell TF network [188]. However what remains to be elucidated are those factors that participate in the genome amplification that is observed with activation of a naïve B cells [333].

The genome of a naïve B cells is poised for rapid activation. These quiescent cells or G_0 cells have about 90% of their promoters loaded with paused RNA pol II but have un-melted promoters which only support basal transcription. Activation of resting lymphocytes induces the expression of TFIIH complex which include the expression of the XPB and XPD helicases which

are involved in promoter unwinding and extension of the open complex [334]. What is currently not known is what factors participate in releasing the paused RNA Pol II in naïve B cells.

The RNA Pol II pausing is thought to be caused by the association of the negative elongation factors, NELF and DSIF, which associate with the RNA Pol II and inhibit progression of transcription. Assembly of several transcriptional elongation factors into a complex termed the SEC is believed to overcome this inhibition [267]. In general, the SEC is believed to contain PTEF-B and several frequent translocation partners of the MLL gene. Among these factors are the ELL family of transcriptional elongation factors [272]. Three family members; ELL, ELL2 and ELL3 have been identified [284, 287, 293]. Recently, cell-type specific expression and function has been reported in terminally differentiated plasma cells. There ELL2 was shown to affect splicing of the immunoglobulin heavy chain locus to generate secreted immunoglobulin [288, 335]. Whether all ELL family members participate in the process of B cell activation and differentiation is currently not known.

To establish an expression pattern for ELL family members in the B cell compartment, cell line models were utilized to characterize the presence of ELL family members at various stages of B cell development. Subsequently, we validated the ELL family expression pattern through re-analysis of genome wide expression data and cytokine stimulus on primary human and mouse B cell development stages. Finally we established PRDM1s role in regulating these factors. This data presented herein describes the ELL family dynamic during the major stages of B cell differentiation.

3.2 Results

3.2.1 ELL3 and ELL2 are Differentially Expressed in the B cell Compartment

Of the ELL family members, ELL2 was the first with known expressions in the B cell compartment. Comparison of ELL2 levels in primary activated B cells and cell line models, depict that it was abundantly expressed in primary plasma cells and cell line models where it contributes to secretion of immunoglobulin [288]. However, the presence of the other ELL family members has not been described previously in B cell compartment. Given the potential significance of ELL family members in B cell function, we assessed the mRNA levels of all three ELL family members in cell line models **(Figure 3.1)**.

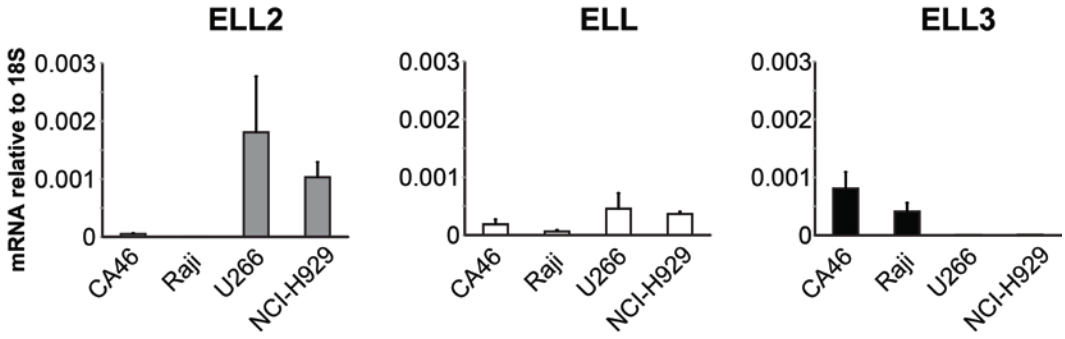

Figure 3.1 Differential Expression of ELL Family Members in Human B-cell Lymphoma cell Lines. The relative mRNA quantitation of the three ELL family members (ELL, ELL2 and ELL3) in two BL (CA46 amd Raji) and two MM (U266 and NCI-H929) cell lines. Data is the average of 3 independent qRT-PCR experiments; errors bars represent standard deviation (SD). Data is presented in *Alexander et al.* [336].

Consistent with previous findings, ELL2 was highly expressed in the two Multiple Myeloma (MM) plasma cell lines, U266 and NCI-H929, but not the two Burkitt's Lymphoma (BL) B cell lines, CA46 and Raji. Both BL and MM cell lines depicted minimal but detectable levels of family member ELL. In contrast, ELL3 expression was most abundantly observed in the two BL cell lines but not the MM cell lines. Together these findings provided the first suggestion that all ELL family members are present but differentially expressed in B cell compartment.

BL and MM cell lines have long been utilized as models for studies of B cells and plasma cells. However we believed it was important to also validate these findings in normal primary cells. A microarray data set on cell sorted human tonsillar B cell sub-populations was previously published by Longo et al. [321]. Re-analysis of this microarray data set for the expression of ELL family members depicted the selective expression of ELL2 mRNA in the terminally differentiated plasma cell population **(Figure 3.2)**. Consistent with its known role in plasma cell differentiation, PRDM1 was also exclusively detected in these plasma cells [188, 337]. In contrast, ELL3 mRNA was highly expressed in primary GC B cells and to a lesser extent in the naïve and memory B cells. Similar to cell lines, the expression of ELL mRNA was minimal across the B-cell compartment. Together these findings indicated that the expression of ELL family members is selective in the B cell compartment. Specifically in humans, ELL3 is restricted to B cells while ELL2 is selectively expressed in plasma cells.

A previous study of mRNA processing in plasma cells, also demonstrated selective ELL2 expression in the murine MCP11 plasmacytoma cell line and plasma cells [289]. In addition they demonstrated that comparatively, ELL3 expression was exclusive to the A20 GC B cell line but not the MCP11 cell line which suggested that the ELL family expression dynamics are conserved in mice. To assess if murine B cells and plasma cells depict a similar pattern of ELL family member expression, a sequencing data set performed on murine splenic B cells and bone marrow plasma cells from 3 donors was re-analysed. Consistent with our findings in primary human B cells, ELL3 was most abundantly detected in the splenic B cells, while ELL levels were minimal but relatively equal between the two cell types **(Figure 3.3)**. These findings indicate that the dynamics of ELL family members are highly conserved between human and mouse.

3.2.2 Abundant ELL3 Protein Expression in B cells

We next investigated if the ELL3 mRNA data was reflected at the protein level. Several ELL3 reactive antibodies are commercially available, however only a minimal number validated. We established their specificity through depletion of ELL3 in the CA46 BL cell line with a siRNA cocktail. The ELL3 B02P antibody recognized a protein band of the expected 60kD molecular weight in the non-transduced and control cells, which was eliminated in the ELL3-depleted cells **(Figure 3.4)**.

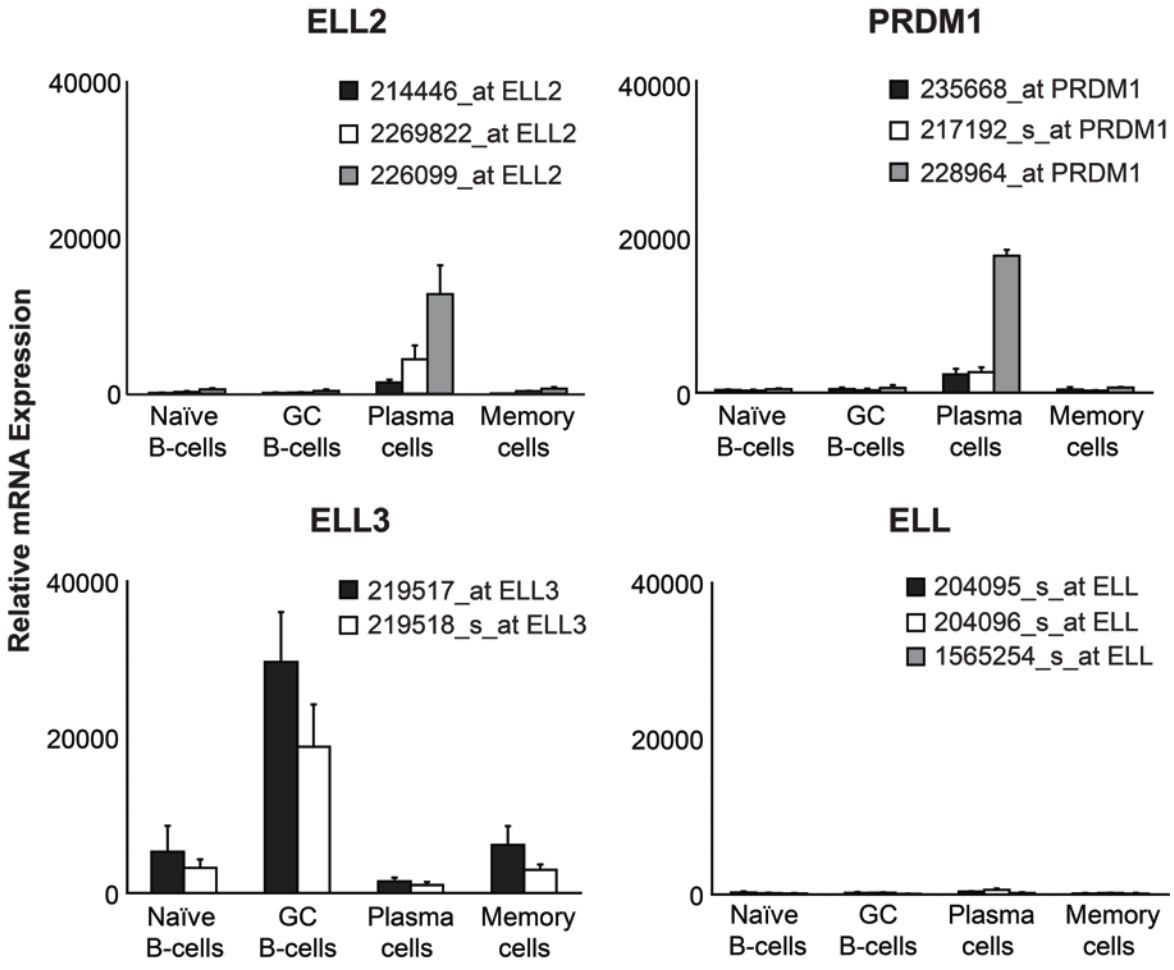

Figure 3.2 ELL Family Member Expression in Primary Human Tonsillar B cell subpopulations. Expression levels of all ELL family members were obtained from GSE12366 [321]. Depicted values are an average of 3 microarray hybridizations on cell sorted primary human B-cell subpopulations; error bars represent SD. Probe ID number is indicated in each graph. Data is presented in *Alexander et al.* [336].

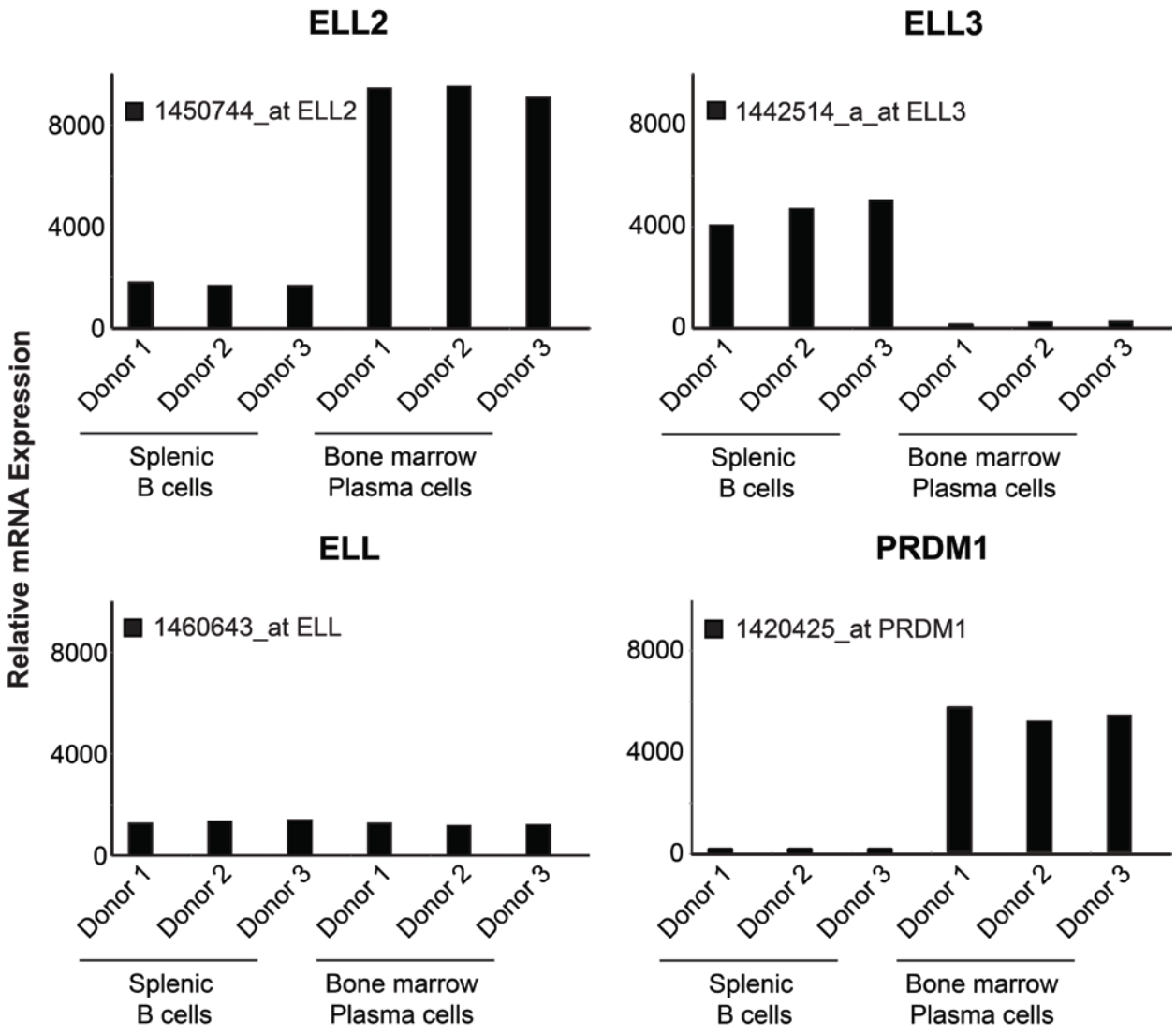

Figure 3.3 ELL Family Member Expression in Primary Murine B cell Subpopulations. Expression levels of ELL family members and PRDM1 in murine splenic B cells and bone marrow plasma cells. Date was obtained from GSE39916 [289]. The relative mRNA levels from three independent are presented and the probe ID number is indicated in each graph.

We obtained similar results with two distinct shRNAs in the Namalwa BL line **(Figure 3.4)**. In addition, two additional protein bands of unknown origin (50kD and 37kD) were consistently detected but not affected by ELL3 targeting shRNA in both cell lines. An ELL3 over-expression construct containing an HA-tag was generated and over-expressed in HEK-293T cells. Our analysis indicates that this construct is also expressed as a protein product at

approximately 60kD by the B02P antibody and a second ELL3 antibody, B01P **(Figure 3.5)**. Consistent with our findings at mRNA level, both ELL3 antibodies specifically recognized ELL3 in the BL cell lines, CA46 and Raji but not the MM cell lines **(Figure 3.5)**. These findings further confirmed that the ELL3 protein expression is observed in B cells prior to differentiation.

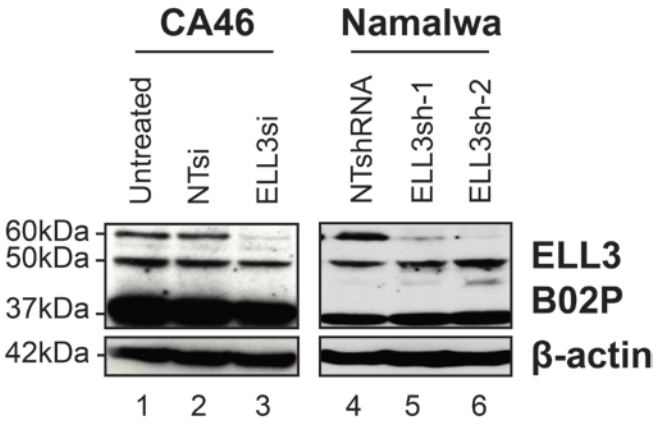

Figure 3.4 Validation of ELL3 Antibodies. Immunoblot of ELL3 after depletion in two BL cell lines confirms antibody specificity. CA46 cells were treated for 4 days with either control (NTsi) or a cocktail of 4 ELL3-targeting siRNAs (ELL3si). Namalwa cells were transduced for 5 days with either control (NTsh) or one of two independent ELL3-targeting shRNAs (ELL3sh-1 and -2). B02P is ELL3 antibody lot. Data is presented in *Alexander et al.* [336].

3.2.3 ELL3 Expression is Primarily Restricted to Mature and Activated B cells

With the expression of ELL family members observed at different stages of B cell differentiation, we set out to determine at what B cell differentiation stage each of their expression is first established. We profiled the expression of all ELL family members in a panel of B cell lines that resemble the different stages of activation or differentiation **(Figure 3.6A and B)**. ELL3 protein expression is robust in 8 of the 10 cell lines that represent the GC, including all of the BL lines and 3 of 5 Diffuse Large B cell Lymphoma (DLBCL) lines. Similarly, we found that ELL3 mRNA levels are the highest in these B cell lines. Low expression of ELL3 was observed in the Mantle Cell Lymphoma (MCL) naïve B cell line, Jeko-1, and to a lesser extent in the Mino MCL cell line.

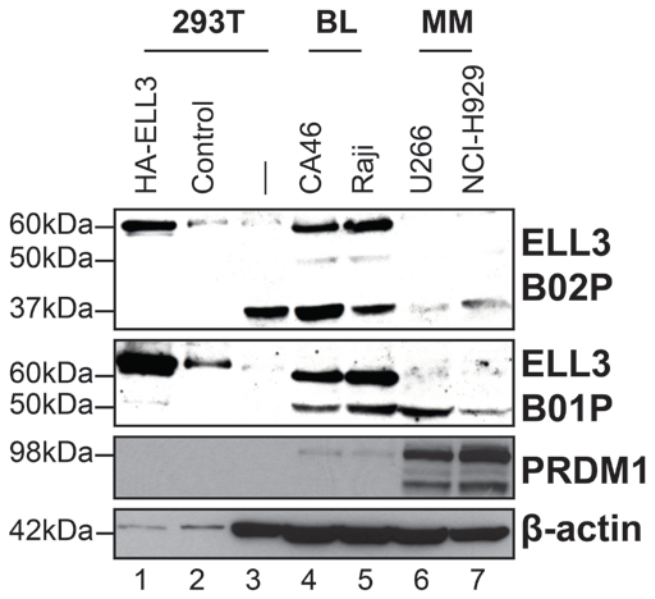

Figure 3.5 ELL3 Protein Levels in Lymphoma and Myeloma Cell Line Models. ELL3 immunoblot using two independent antibodies detects expression in BL lines but not MM lines. Cell lines are indicated above each lane. Whole cell lysates from $0.5x10^6$ cells were used for each except HEK-293T lysate which was diluted 1:50. B02P and B01P are two different ELL3 antibody lots. Data is presented in *Alexander et al.* [336].

The MM cell lines have undetectable levels of ELL3 protein and mRNA. ELL protein and mRNA were at similar levels in most cell lines, but displayed comparatively low protein expression in the B cell Precursor Acute Lymphoblastic Leukemia (BCP-ALL) premature B cell line, 697, and low mRNA in the DLBCL line Toledo. The ubiquitous expression of ELL is consistent with prior findings that ELL is crucial for assembling the SEC and the little elongation complex (LEC) [275, 338]. As expected, ELL2 protein and mRNA levels were most abundant in MM cell lines. However, we also observed ELL2 protein expression in one MCL and one DLBCL cell line. These results in immortalized cell lines broadly suggest that ELL expression is ubiquitous, while the family members ELL3 and ELL2 predominate at respectively GC activated B cells and terminally differentiated plasma cells. In addition, these findings imply that during B cell differentiation ELL3 expression is switched for ELL2.

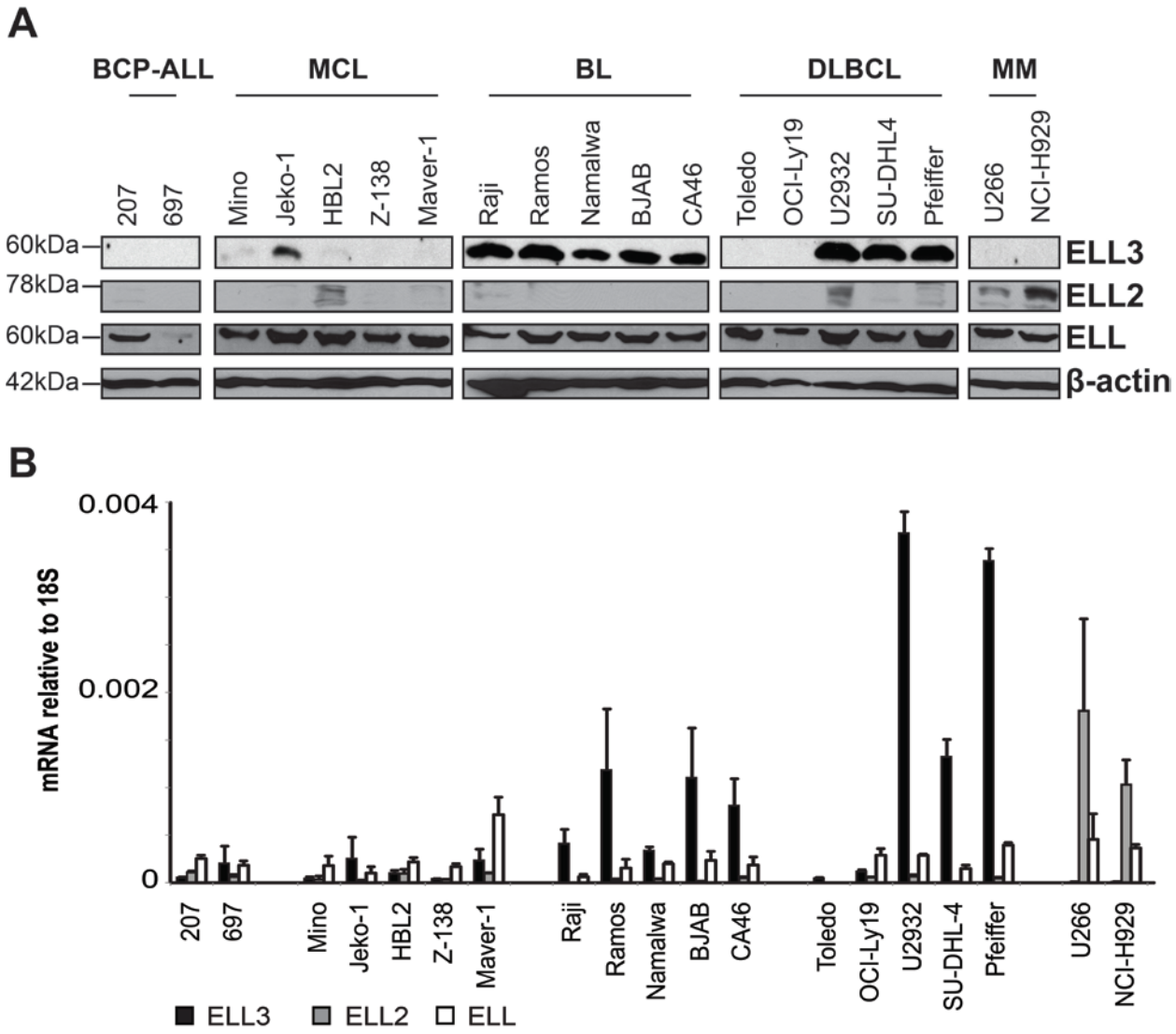

Figure 3.6 Differential Expression of ELL Family Members in B cell Line Models. A. Immunoblot of ELL family members in B cell lymphoma cell lines. Cell line labels are BCP-ALL (B cell Precursor Acute Lymphoblastic Leukemia) MCL (Mantle Cell Lymphoma), BL (Burkitts' Lymphoma), DLBCL (Diffuse Large B Cell Lymphoma) and MM (Multiple Myeloma). **B.** Relative mRNA expression profile of ELL family members across human B cell lines as described in figure 3A. Data represents the average of 3 independent experiments; errors bars represent SD. Data is presented in *Alexander et al.* and *Alexander et al.* [336, 339].

3.2.4 ELL3 is Switched for ELL2 Upon Plasma cell Differentiation

To assess if the ELL family dynamics are present during normal B cell differentiation, we assessed ELL family member protein expression during *ex vivo* primary B cell activation and

differentiation. Peripheral naïve B cells from healthy human donors had undetectable levels of the ELL family members **(Figure 3.7)**. Upon B cell activation, extensive ELL3 up-regulation as well as modest ELL expression was observed, while ELL2 levels remained minimal. In contrast, primary B cells stimulated to differentiate into plasma cells had minimal ELL3, while ELL2 was up-regulated. Additionally, ELL expression further increased upon differentiation. Robust up-regulation of PRDM1 in this population confirmed differentiation toward the plasma cell phenotype **(Figure 3.7).** In support of this observation, we re-analyzed the global gene expression data from Barwick et al. on *in vivo* LPS stimulated adoptively transferred mouse B cells that were cell sorted by cell division and CD138 surface expression **(Figure 3.8)** [322]. As expected, ELL3 levels predominated during early $CD138^-$ B cell divisions. ELL3 expression diminished as B cells progressed towards the plasma cell phenotype and gained the expression of PRDM1. Maximum extinction of ELL3 was observed in the $CD138^+$ plasma cells. Furthermore, ELL2 expression was induced in plasma cells. Indicating that as in humans, murine B cells exhibit a switch from ELL3 to ELL2 during plasma cell differentiation. In addition, these findings suggest that family members ELL3 and ELL2 may have distinct roles within the B cell compartment.

3.2.5 PRDM1 Directly Associates and Represses the ELL3 Promoter

Terminal plasma cell differentiation is driven by transcriptional reprogramming mediated by the transcriptional repressor PRDM1 [337]. Our lab and others have previously demonstrated that PRDM1 directly suppresses components of activated B cells, suggesting that PRDM1 might have a key role in regulating ELL family expression [188, 340, 341]. In **Figure 3.2, 3.3, 3.5, 3.7 and 3.8**, the expression of PRDM1 is shown to coincide with loss of ELL3 expression. To directly assess the functional role of PRDM1, we re-analyzed microarray expression data of

human tonsilar GC B cells transfected with a PRDM1 expression plasmid which we previously used to demonstrate a role for PRDM1 in Epstein Barr Virus (EBV) pathogenesis [323]. ELL3 expression was reduced by 19.1% and 38.5% in two independent donors upon expression of PRDM1 **(Table IV)**. This effect on ELL3 paralleled the impact on well characterized targets of PRDM1, which include CIITA and BCL6. We utilized ChIP-Seq in the U266 MM cell line for PRDM1 associations and identified a total of 574 association peaks. A predominant PRDM1 association was centered 180 base pairs upstream of the ELL3 transcription start site **(Figure 3.9A)**. We validated PRDM1 association by direct ChIP in two PRDM1-positive MM cell lines **(Figure 3.9B)**.

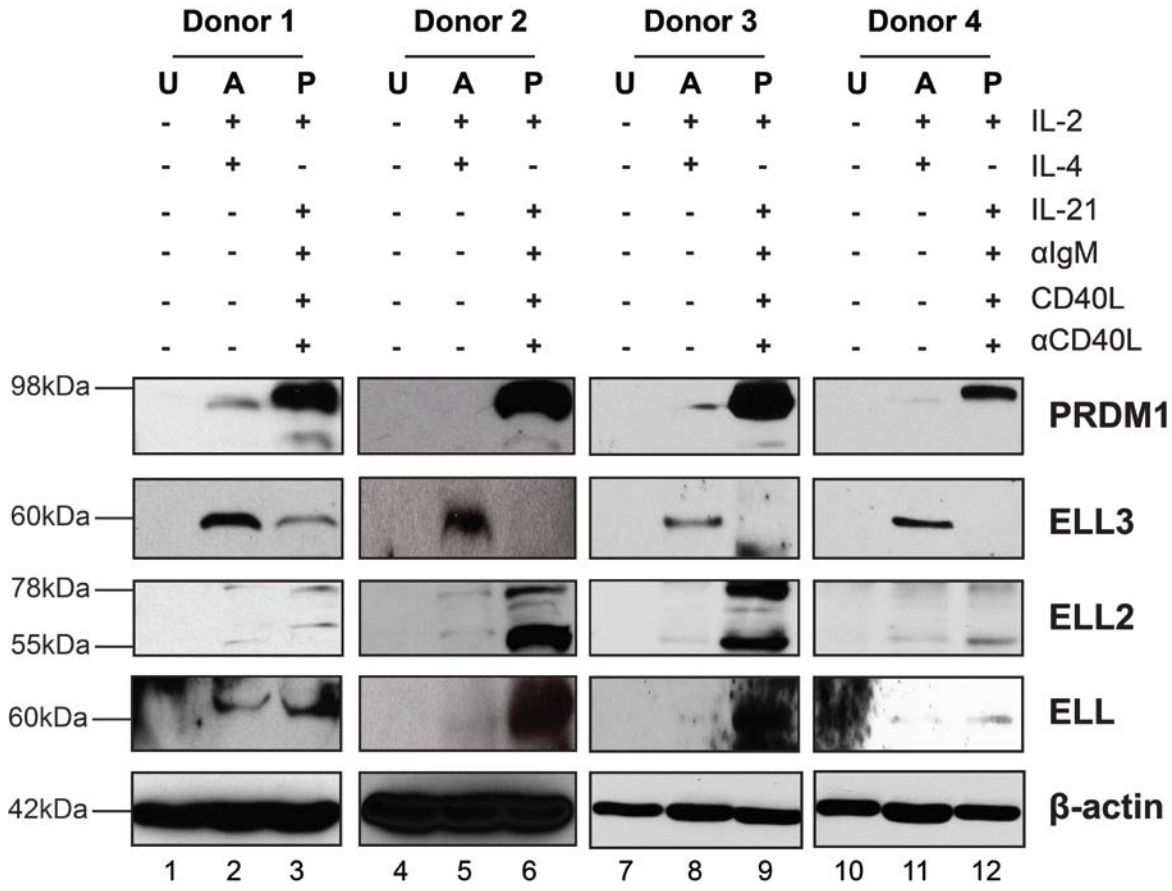

Figure 3.7 ELL Family Member Expression in *in vitro* Stimulus of Human Primary Naïve B cells. Immunoblot of ELL family members and PRDM1 in human primary naïve B cells un-stimulated (U), stimulate into activated B cells (A), and stimulated into plasmablasts (P). Data is from 4 representative healthy human donors. Data is presented in *Alexander et al.* [336].

We identified two consensus PRDM1 binding sites at the ELL3 proximal promoter region, termed site I (-239 to -229) and site II (+14 to +24). To assess the effect of PRDM1 on ELL3 promoter activity, a DNA fragment containing both sites was cloned into a luciferase-reporter construct **(Figure 3.10A)**.

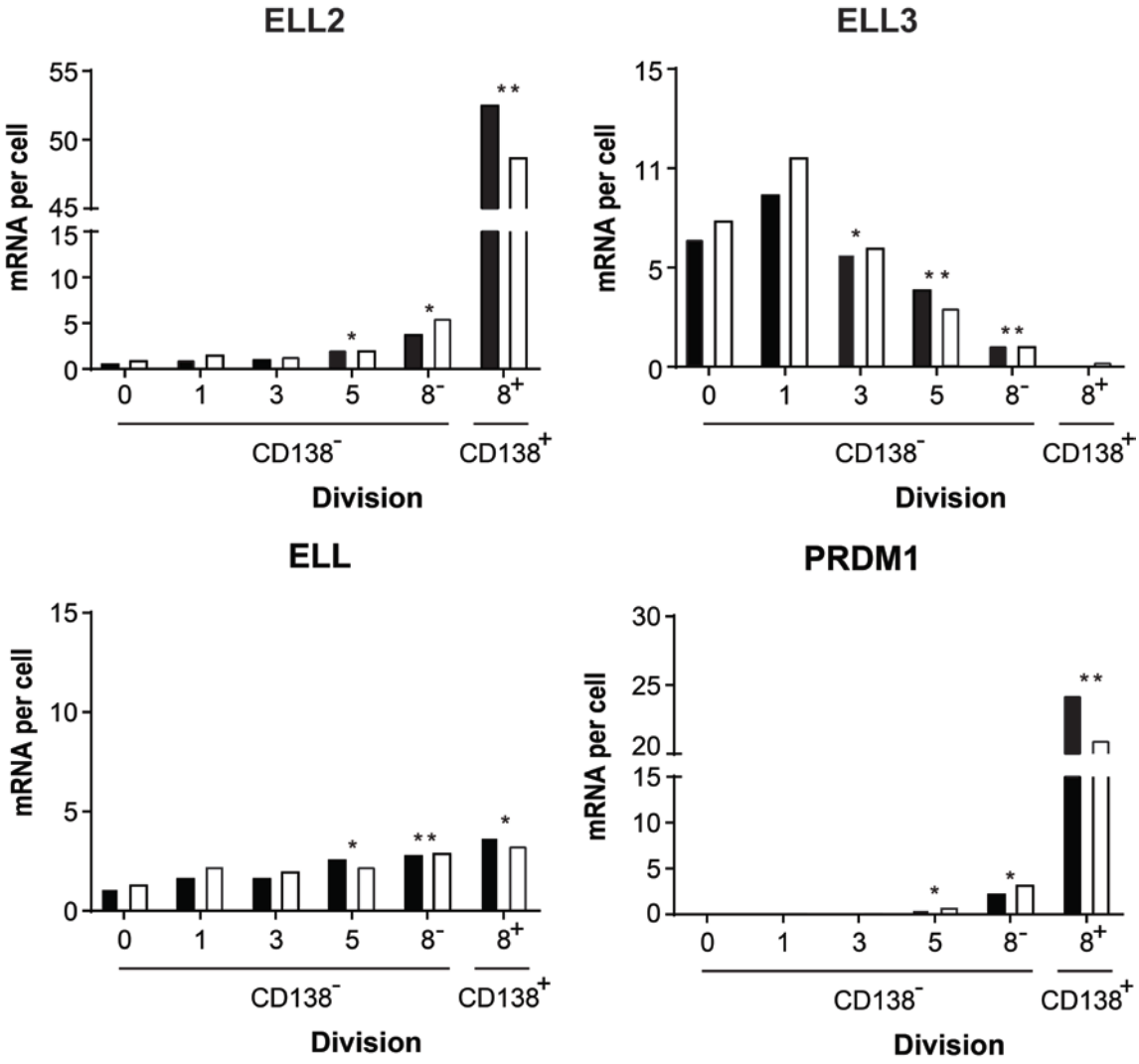

Figure 3.8 ELL Family Member Expression in *ex vivo* Stimulated Murine Primary B cells. Expression of ELL family members and PRDM1 mRNA in LPS stimulated murine B cells that are sorted by cell division and CD138 expression. Data was from GSE70294 [322] and presented as mRNA copies per cell. The X-axis represents the number of cell divisions. CD138 positivity is indicated by the (+) and represents the fully differentiated plasma cells. Data represents one experiment with biological duplicates. *p<0.05, **p<0.01 (*two-tailed t-test*). Data is presented in *Alexander et al.*[339].

When transfected into Raji BL cells the ELL3 promoter demonstrated a high level of basal activity that was repressed by approximately 50% when co-transfected with a PRDM1 expression construct **(Figure 3.10B)**.We generated two additional ELL3 promoter constructs, with mutated site I or site II. ELL3 promoter activity was not significantly altered when either of the two potential PRDM1 binding sites was mutated independently. Finally, we generated promoter with both site I and site II mutated. However simultaneous mutation of both sites eliminated the repressive activity of PRDM1 without altering the basal promoter activity. These results indicate that the ELL3 promoter is suppressed by direct PRDM1 binding and each site is sufficient to mediated transcriptional repression.

3.3 Discussion

The data presented here provides a description of the ELL family expression dynamics during the B cell differentiation processes. ELL3 is up-regulated in conjunction with ELL upon activation of B cells and switched to the expression of ELL2 and ELL upon terminal plasma cell differentiation. Such timed expression could have implications for the current understanding of molecular mechanism that are involved in the B cell immune response and are co-opted by lymphomas for their survival.

Assessment of ELL family member expression in human B cell lymphoma model cell lines suggested for the first time that all ELL family members are expressed with different dynamics during B cell differentiation. Of all three, ELL2 is the only family member with reported functions in the B cell compartment. Its expression was induced in plasma cells, where it enhances polyadenylation and exon skipping at the immunoglobulin heavy chain locus resulting in the use of the weaker promoter-proximal poly(A) site and generation of secreted

immunoglobulin pre-mRNA. In addition, ELL2 is required for the loading of the CstF-64 polyadenylation factor on RNA Pol II on μ- and γ-gene segments and is indispensible for immunoglobulin heavy chain mRNA processing [288]. Endogenous ELL3 expression was previously reported in the testis [293] and amongst differentially expressed genes with predicted mRNA splicing abilities during mouse B cell differentiation [289].Finally, ELL was observed in both B cells and plasma cells, which concurs with its previously reported requirement for transcription [275]. Our analysis of global gene expression data sets on primary B cell and plasma cells from both human and mouse illustrate that the ELL family dynamics are not caused by immortalization or malignant transformation, but is certainly conserved between normal human and mouse B cells.

Table IV Changes in micro array signal intensity following ectopic expression of PRDM1 in murine tonsillar GC B cells. *Expression levels of ELL3 and the known PRDM1-silenced genes in purified human tonsillar GC B cells transfected with either a PRDM1 expression construct (pcDNA3.1-PRDM1α) or an empty vector (pcDNA3.1). The microarray data set source is GSE27670 [323]. Depicted values are fold change over control of from 2 independent donors. Probe ID number is indicated in each graph. Data is presented in Alexander et al. [336].*

Probe ID	**Gene symbol**	**Donor 1**		**Donor 2**	
		+pcDNA3.1	+pcDNA3.1-PRDM1α	+pcDNA3.1	+pcDNA3.1-PRDM1α
217192	*PRDM1*	64.8	7095.7	44.8	3364.0
219518	*ELL3*	3462.6	2802.4	2782.3	1712.3
203140	*BCL6*	3144.1	2261.8	3632.7	2312.2
205101	*CIITA*	490.5	400.5	642.7	110.5

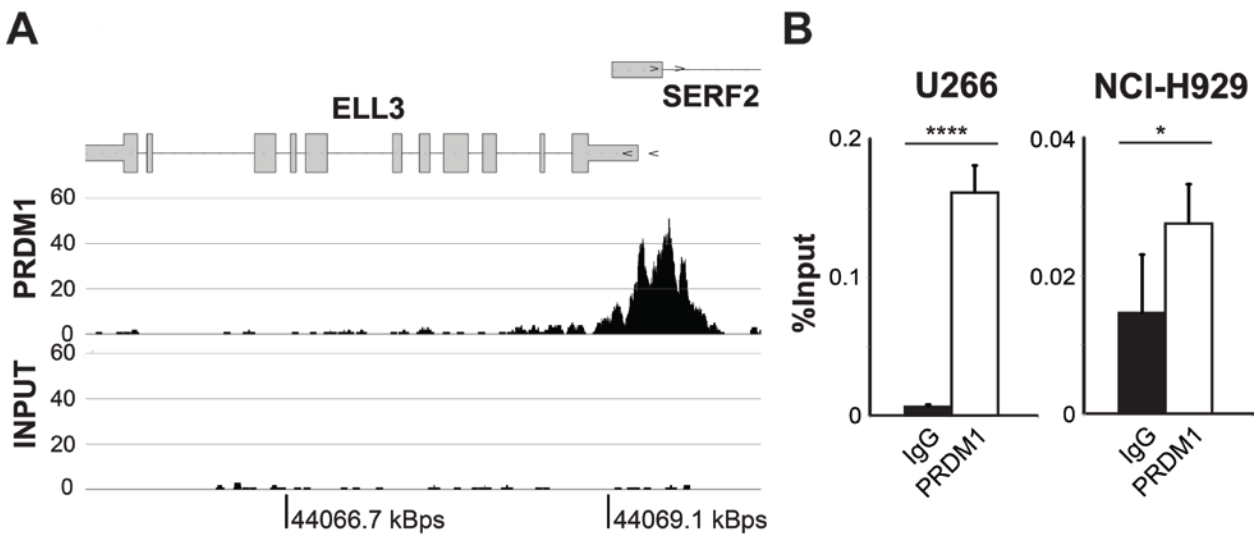

Figure 3.9 PRDM1 Association at the ELL3 Locus. A. Schematic depiction of PRDM1 ChIP-Seq reads at the ELL3 locus in the U266 MM cell line. **B.** ChIP-qPCR assessment of PRDM1 binding at ELL3 in MM lines. Data is represented as the average of at least 4 independent experiments. Data represents the average of 6 independent experiments; error bars represent SD.*p<0.05, ****p<0.0001 (*two-tailed t-test*). Data is presented in *Alexander et al.* [336].

The inclusion of global expression data set on cell sorted primary human B cell subtypes in our studies, provided a first look at their levels in naïve and memory B cells and suggested that the expression of ELL3 is established through GC activation and switched for ELL2 upon terminal plasma cell differentiation. Through utilization of physiologically relevant combination of cytokines, we demonstrate that this ELL family member dynamic holds true at the protein level during B cell differentiation. Our studies utilized the two previously reported cytokine cocktails of IL-2 and IL-4 for activation and IL-2, IL-21, α-IgM and CD40L for differentiation, which generates the two major stages of GC reaction in parallel [171]. While eliminating any issues with timing, these independent stimuli also represent each stage well. IL-2 and IL-4 stimulus was shown to not express much PRDM1 mRNA transcripts and no ability to generate terminally differentiated plasma cells [171]. These findings are consistent with proliferation and differentiation promoting abilities of IL-2 and the inhibitory functions of IL-4 on B cell

activation [342, 343]. The IL-2, IL-21, α-IgM and CD40L stimuli is believed to provide robust differentiation signals. α-IgM and CD40L are believed to provide the Ag and T cell signal, IL-21 was required for the expression of PRDM1, AID and class switch recombination, while IL-2 was shown to have an enhancing effect on the stimulus [171].

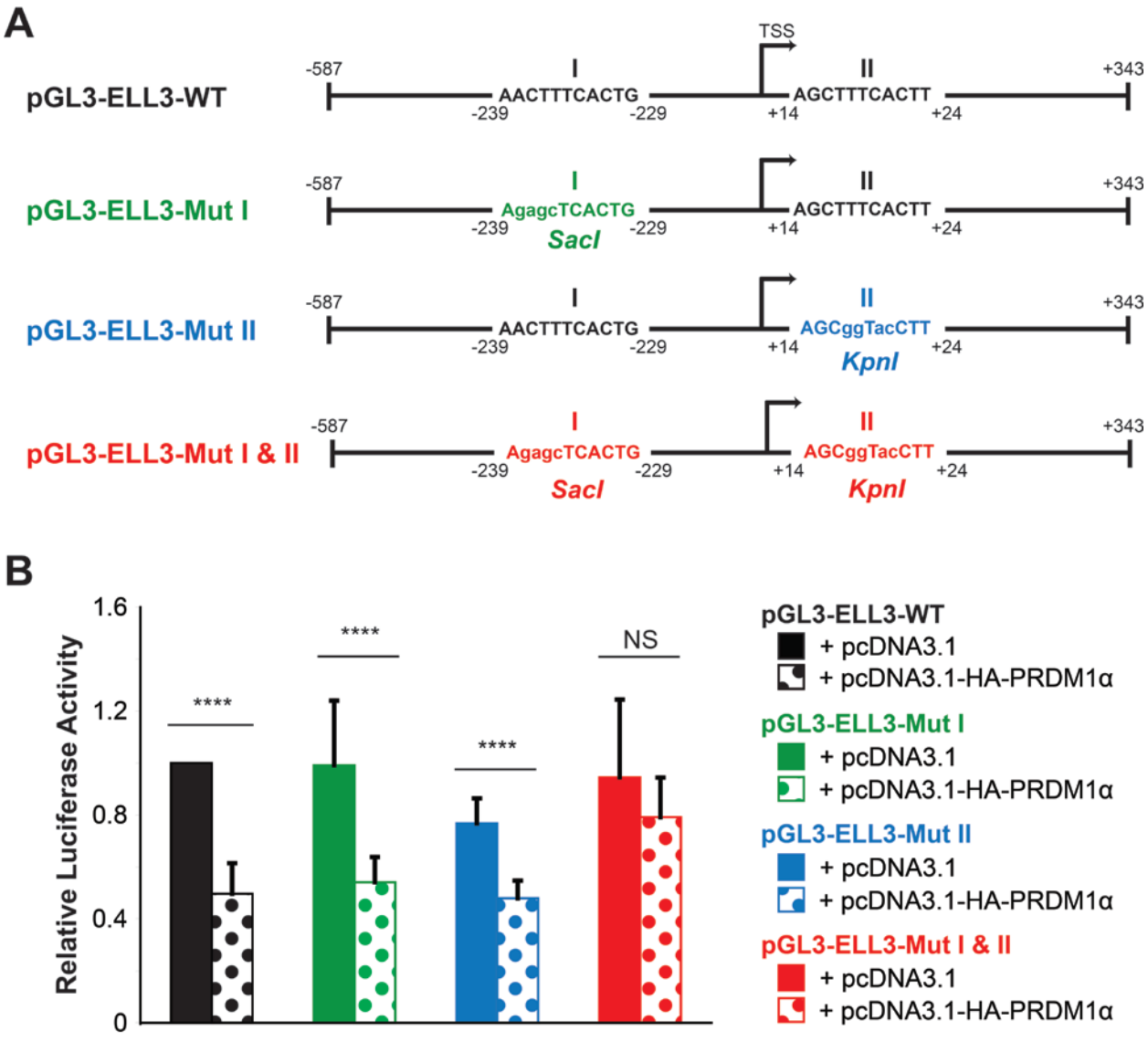

Figure 3.10 PRDM1 Mediated Direct Repression of ELL3 Promoter. A. Schematic depiction of the cloned 930 nt ELL3 promoter constructs. The two PRDM1 sites are indicated with I and II. **B.** Promoter activity of ELL3 promoter constructs co-transfected with either control or PRDM1 over-expression vector. Data represents the average of 6 independent experiments; error bars represent SD. ****p<0.0001 (*two-tailed t-test*). Data is presented in *Alexander et al.* [336].

Thus, the stimulation of freshly isolated naïve B cells *ex vivo* mimics two key stages of GC reaction and provides insights into the establishment of ELL family member expression during B cell differentiation.

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The expression of ELL3 is observed in the presence of various additional protein products. In addition the expression of the full length ELL3 at 60kDa, both stimulus of primary

B cells and cell line depict the expression of smaller protein products that are responsive to the two ELL3 antibodies. These findings resemble the previous report of family member ELL2 displaying the existence of isoforms, through the post-translational processing of cleavage as well as translation initiation sites in a region that is highly conserved across many species [288]. Cleavage and alternate translation initiation sites are also present and highly conserved in various species of ELL3, suggesting that the smaller ELL3 protein products may correspond to alternative isoforms of ELL3 with possible important functions.

The ELL3 locus undergoes PRDM1 mediated regulation during B cell differentiation. PRDM1-mediated repression of factors that participate in the functional activation of immune cells is well known. Its repressive functions have been described in NK cells, T cells, dendritic cells and plasma cells [324, 341, 344]. For a long time the list of direct PRDM1 targets included the transcription factor *BCL6*, *c-myc*, *PAX5*, *CIITA*, *Spi-B*, *ID3*, *LMO2* and *HGAL* [188, 340, 345]. Depletion of each one of these genes individually resulted in the partial appearance of the plasma cell phenotype or the loss of B cell specific functions [84, 188, 190, 346]. The identified peak in our global assessment of PRDM1 binding in the MM cell line encompassed two MAGYGAAAGYK binding sites that conferred significant repressive activity, similar to the previously reported repressive activity on PRDM1 targets [340, 345]. Through ChIP we confirmed direct association of PRDM1 at the ELL3 locus indicating that its part of the transcription factor network extinguished by PRDM1 during the process of differentiation.

ELL3 levels characterize GC derived B cell lymphoma cell lines. Most genetic lesions that are associated with lymphomagenesis are the result of aberrancies during the immunoglobulin rearrangement process; these include translocations and somatic mutations [347, 348]. However insights from B cell malignancies are revealing the presence of pathway

dependencies in a large fraction of malignancies. These include oncogenic mutant signaling, -modifications affecting immune recognition, over-expressed or modified transcriptional regulators and epigenetic deregulation [195]. Abundant ELL3 expression is observed in BL and a portion of DLBCL cell lines that contain a variety of genetic lesions. Consistent with this finding, both BL and a portion of DLBCL share features of normal GC B-cells [349]. Together these findings indicate that ELL3 expression is restricted to GC derived B cell lymphomas and may be a transcriptional regulator these lymphomas may depend on.

CHAPTER IV:

CHARACTERIZATION OF ELL3 FUNCTION IN B-CELL LYMPHOMA CELL LINES

4.1 Introduction

Transcriptional elongation factors are best known for their assembly into the SEC complex where they participate in the release of paused RNA Pol II which allows for productive elongation of the mRNA transcript [350]. Of the ELLs, family member ELL was cloned from MLL cells. The C-terminal of ELL was found to be a fusion partner with the H3K4 methylase *MLL* [284]. Family members ELL2 and ELL3 were subsequently cloned based on their homology to ELL. Structurally ELL family members are characterized by the presence of a N-terminal elongation domain, the central domain and the C-terminal occluding homology domain. At respectively 602 and 633 amino acids, ELL and ELL2 are the largest family members and contain all three domains. The smallest family member ELL3 is 397 amino acids long and differs in that it's missing a portion of the central domain.

Along with elongation, the N-terminus of ELL family members are also able to undergo protein-protein associations. The N-terminus is responsible for binding of polymerase associated factor which recruits the mRNA polyadenylation factors [351]. The elongation activator, elongation associated factor 1 (EAF1) was also found to associate with this domain, which is essential for ELLs malignant transformative abilities [352].

The central domain in ELL and ELL2 is rich in both hydrophobic (proline and leucine) and hydrophilic (glutamate and lysine) amino acids residues which are suggested to promote protein associations [284, 287, 293].

The C-terminus is highly conserved amongst the ELL family members and is highly homologous to the integral plasma membrane protein, occludin. The occluding-like domain forms a highly positively charged surface which is proposed to facilitate protein-protein interactions [353]. EAF2 was also shown to associate with the C-terminus [354]. The C-terminus of ELL2 was also shown to contain a Siah1 ubiquitination site which controls the protein degradation. Association of the AF4 elongation factor, blocks Siah1 binding and enhances ELL2 stability [355]. Finally, p53 was found to directly associate with this domain, inhibits its activity and cause immortalization of myeloid progenitors [356, 357]. ELL3 identified p53 interaction in mouse stem cells are believed to be mediated through the C-terminal interaction [297].

The differences amongst these proteins suggest that they may have unique interactions and functions. This idea is further supported by the finding of different SECs with specific gene specificities [315]. The involvement of ELL family members in cell proliferation and survival has previously been demonstrated when ELL is over expressed in 293T cells [286]. In the B-cell compartment ELL2 was shown to drive alternative splicing at the Ig locus [288]. Its functions have been implicated in the testis, epithelial-mesenchymal transition and marking future gene activation in mouse embryonic cells [294-297]. A function for ELL3 in B cells is currently unknown. To establish the function of ELL3 in BL cell line cells, its levels were transiently depleted and the phenotypical and morphological outcomes recorded.

4.2 Results

4.2.1 Loss of ELL3 Does Not Induce Differentiation

To assess ELL3 function, we transiently depleted ELL3 in Namalwa BL cells using two independent. Depletion of ELL3 protein and mRNA expression was highly efficient as was confirmed by immunoblot and mRNA quantitation **(Figure 4.1)**. In addition, the expression of ELL and ELL2 was also assessed. Both ELL and ELL2 appeared unchanged at protein and mRNA level, indicating that loss of ELL3 was not accompanied by compensatory up-regulation of ELL or ELL2 **(Figure 4.1)**.

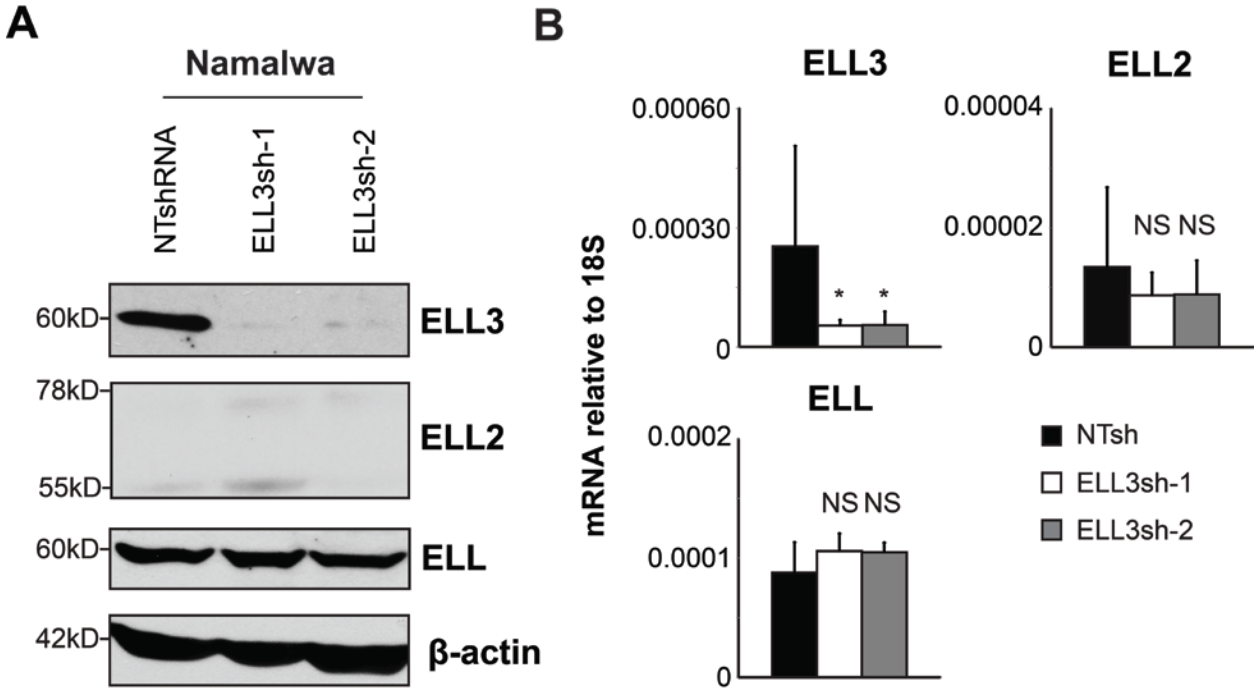

Figure 4.1 Transient ELL3 Depletion Did Not Affect Levels of ELL2 and ELL. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2 for 5 days. **A.** Immunoblot of ELL3, ELL2 and ELL. The equivalent of $0.5x10^6$ whole cell lysates were assayed. **B.** The relative mRNA quantitation of all ELL family members after ELL3 depletion. Data is presented as the average of 5 independent experiments; errors bars represent SD. *$p<0.05$; NS is not significant (*two-tailed t-test*). Data is presented in *Alexander et al.* [336].

With the expression of ELL3 in B cells preceding that of ELL2 in plasma cell, we hypothesized that loss of ELL3 may induce differentiation. To assess differentiation the expression of the master regulator of plasma cell differentiation PRDM1 was profiled. Unexpectedly, ELL3-depletion resulted in an increase of PRDM1 both at protein and mRNA level in **(Figure 4.2)**.

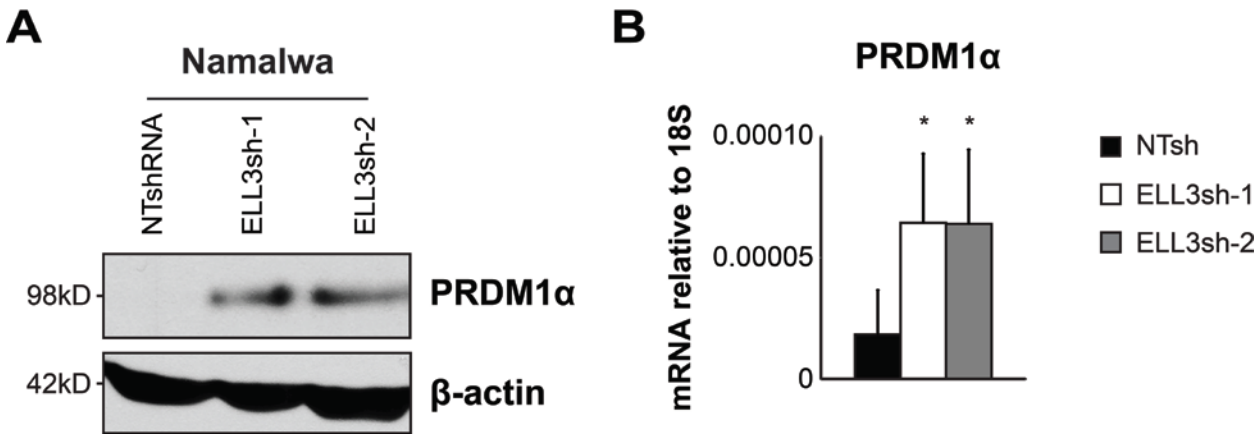

Figure 4.2 PRDM1 Up-regulation with ELL3 Depletion. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2 for 5 days. **A.** Immunoblot of PRDM1. The equivalent of $0.5x10^6$ whole cell lysates were assayed. **B.** The relative mRNA quantitation of PRDM1 after ELL3 depletion. Data is presented as the average of 5 independent experiments; errors bars represent SD. *p<0.05; NS is not significant (*two-tailed t-test*). Data is presented in *Alexander et al.*[339].

PRDM1 is known for its ability to repress a network of transcription factors that are required to maintain the B cell phenotype. Extinguishing this phenotype is also required for full differentiation into plasma cells [188]. To assess if PRDM1 repressed the expression of these TFs and resulted in terminal differentiation of plasma cells, we profiled transcript levels of B cell factors and immunoglobulin in both control and ELL3-depleted conditions. Despite the induction of PRDM1 we did not observed any changes in B cell factors, which include BCL6, PAX5 and MYC nor did we observe any induction of immunoglobulin secretion **(Figure 4.3)**. These findings indicate that ELL3 depletion did not cause any detectable changes in differentiation state.

PRDM1 has also been implicated in reactivation of EBV out of latent state [358]. To establish if the induced PRDM1 caused reactivation of latent EBV into lytic replication, we assessed the transcript levels of the immediate early- (BZLF1), early- (BMRF1) and late- (BLLF1) lytic replication factors in control and ELL3-depleted conditions **(Figure 4.4)**. All EBV lytic replication genes remained un-affected indicating no change in EBV status in the EBV positive Namalwa cells. Together these findings indicate that pathways are not contributing to the following phenotypic changes.

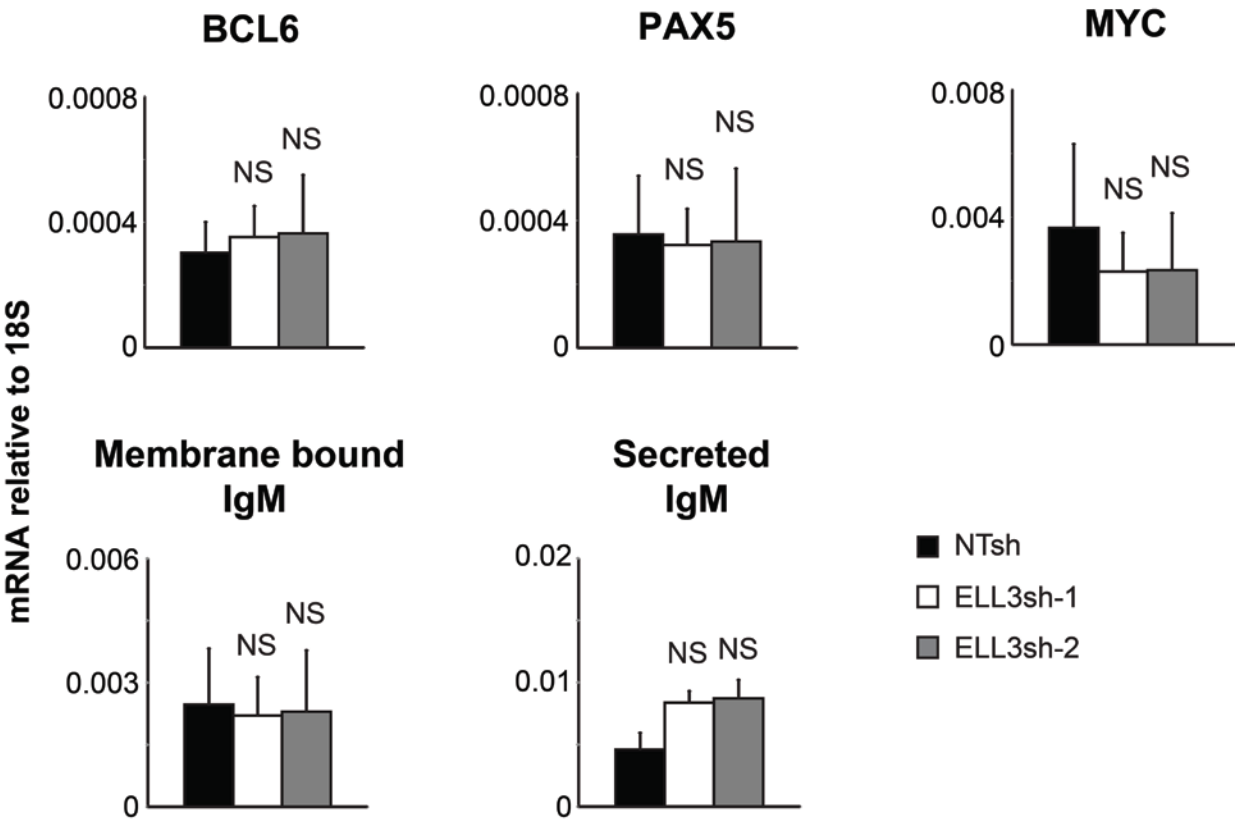

Figure 4.3 ELL3-depletion Did Not Cause Differentiation of B cells. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2 for 5 days. The relative mRNA were quantitation of the B cell factors, BCL6, PAX5 and MYC, and plasma cell factors membrane bound and secreted IgM. Data is presented as the average of 5 independent experiments; errors bars represent SD. *p<0.05; NS is not significant (*two-tailed t-test*). Data is presented in *Alexander et al.*[339].

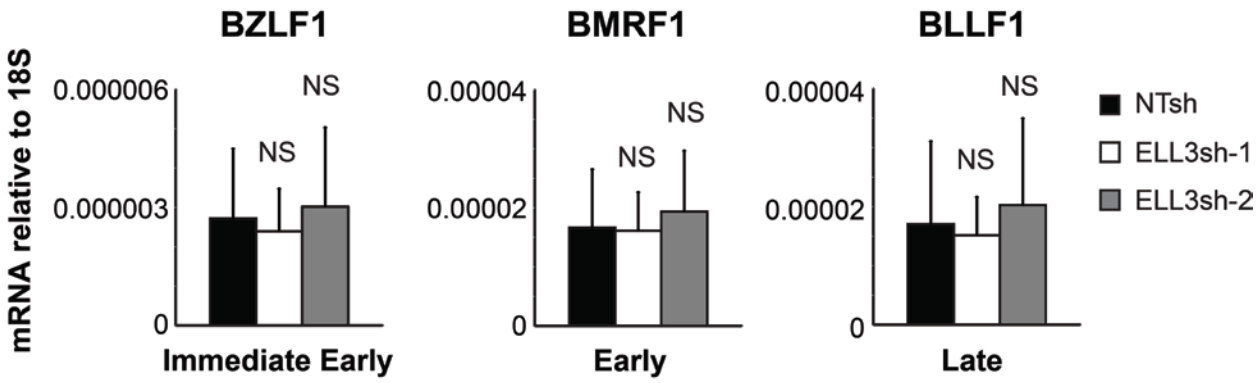

Figure 4.4 ELL3-depletion Did Not Reactivate EBV Lytic Replication. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2 for 5 days. The relative mRNA quantitation of BZLF1, BMRF1 and BLLF1. Data is presented as the average of 5 independent experiments; errors bars represent SD. *p<0.05; NS is not significant (*two-tailed t-test*). Data is presented in *Alexander et al.*[339].

4.2.2 ELL3 is Necessary for Proliferation and Cell Cycle Progression

Remarkably, we noted less cell growth upon transient ELL3 depletion in BL cell lines **(Figure 4.5)** and were unable to establish stable cell lines in the absence of ELL3. Similar results were observed in the Raji BL cell line **(data not shown)**. This finding suggests that ELL3 depletion may compromise cell cycle progression or viability.

To assess the effects of ELL3 depletion on cell cycle progression, total DNA content was assessed in the mCherry-shRNA containing cells by flow cytometry. The total DNA content in control and ELL3-depleted conditions is displayed in DNA histograms **(Figure 4.6A)**. In comparison to control, ELL3-depleted conditions displayed an altered cell cycle distribution. This prompted further investigation of the cell cycle distribution. Based on the total DAPI levels, we assessed the percentage of cells at each stage of the cell cycle. Our findings indicate that approximately 42% of control cells were in G0/G1 stage of the cell cycle. This percentage was significantly elevated to approximately 75% in ELL3 depleted conditions (black bars) **(Figure 4.6B)**. Furthermore, the S-phase population diminished in ELL3-depleted cells to 24% (ELL3sh-

1) and 23% (ELL3sh-2), compared to 52% in control cells (white bars). Finally, the G2/M population peaked at 12.6% in control cells while remaining below 4% in ELL3-depleted conditions (grey bars). Together, these findings indicated that ELL3 depletion caused an accumulation of cells in G0/G1 and suggested that they are unable to enter S-phase and replicate DNA.

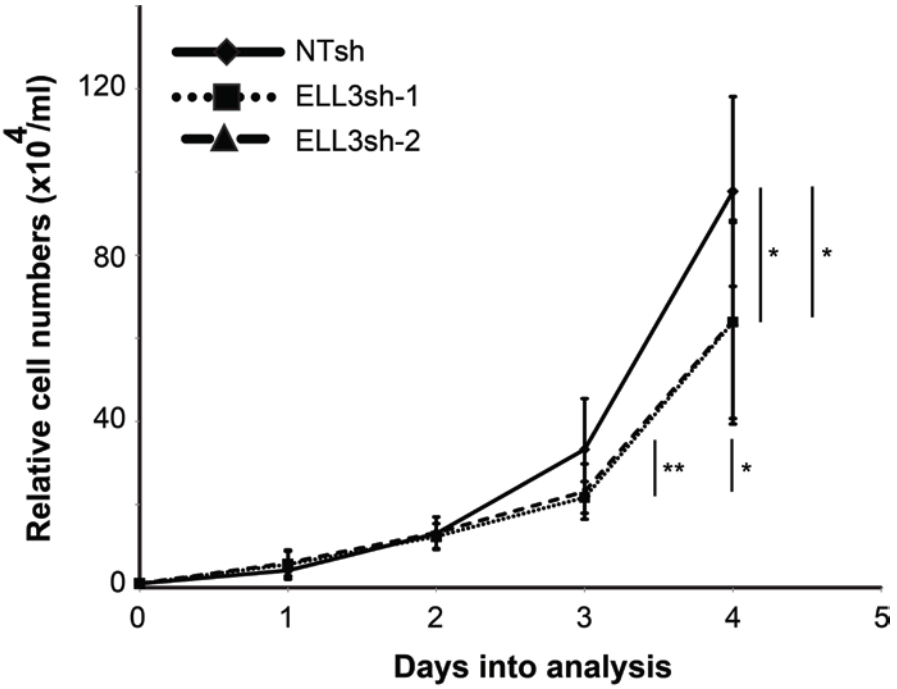

Figure 4.5 ELL3-depletion Compromised Cell Viability. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2. Starting at day 5 post transduction, cell viability was assessed through measurement of intercellular ATP content every 24 h for 4 consecutive days. Data is the average of 6 independent experiments. Errors bars represent SD in percentage. *p<0.05, **p<0.01 (*two-tailed t-test*). Data not presented previously.

To assess active DNA replication we pulse labeled control and ELL3-depleted conditions with Bromodeoxyuridine (BrdU), which only gets incorporated into newly synthesized DNA. The additional staining with DAPI allowed for distinction of the various cell cycle stages. Comparatively, ELL3-depleted conditions displayed less cells with incorporated BrdU **(Figure 4.7A)**. We quantified the cells in S-phase and determined that only 20% of ELL3-depleted cells

incorporated BrdU compared to approximately 50% of the control cells, indicating that DNA replication was compromised in ELL3-depleted cells **(Figure 4.7B)**. In combination, these findings suggest that ELL3-depleted cells arrest in G0/G1 and are limited in the ability to replicate DNA.

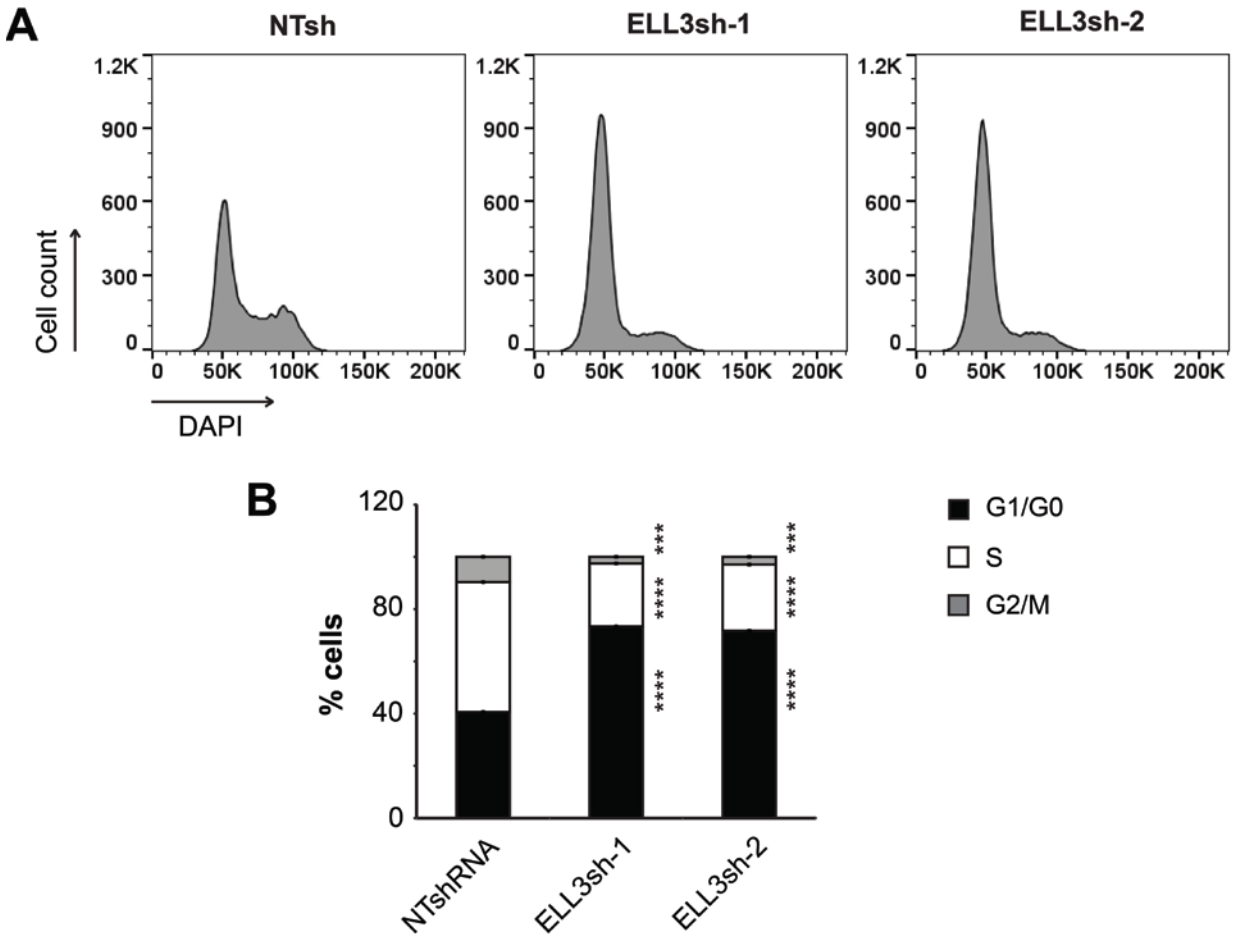

Figure 4.6 ELL3-depletion Compromised Cell Cycle Progression. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2. **A.** Representative total DNA content profile after ELL3 depletion measured by DAPI staining and flow cytometry. **B.** Cell cycle distribution after ELL3 depletion. Cells stained with DAPI for DNA content and gated on mCherry^{+} as marker of shRNA transduced cells. 150,000 cells were modeled with ModFit. Depicted are percentages at each stage of the cell cycle. All assays were performed on day 5 post-transduction. Errors bars represent SD in percentage. ***p<0.001, ****p<0.00001 (*two-tailed t-test*). Data is presented in *Alexander et al.* [336].

4.2.3 Loss of ELL3 Compromised S-phase Regulators

DNA replication is governed in part by the heterohexameric Mini Chromosome Maintenance (MCM) proteins. Six MCM proteins, MCM 2-6, are known to participate within this helicase complex and are critical for DNA replication [359]. An assessment of their expression in a previously published microarray data set on cell sorted human tonsillar B cell sub-populations by Longo et al., illustrated that their expression peaked in the highly proliferative GC B cell subtypes **(Figure 4.8)** [321].

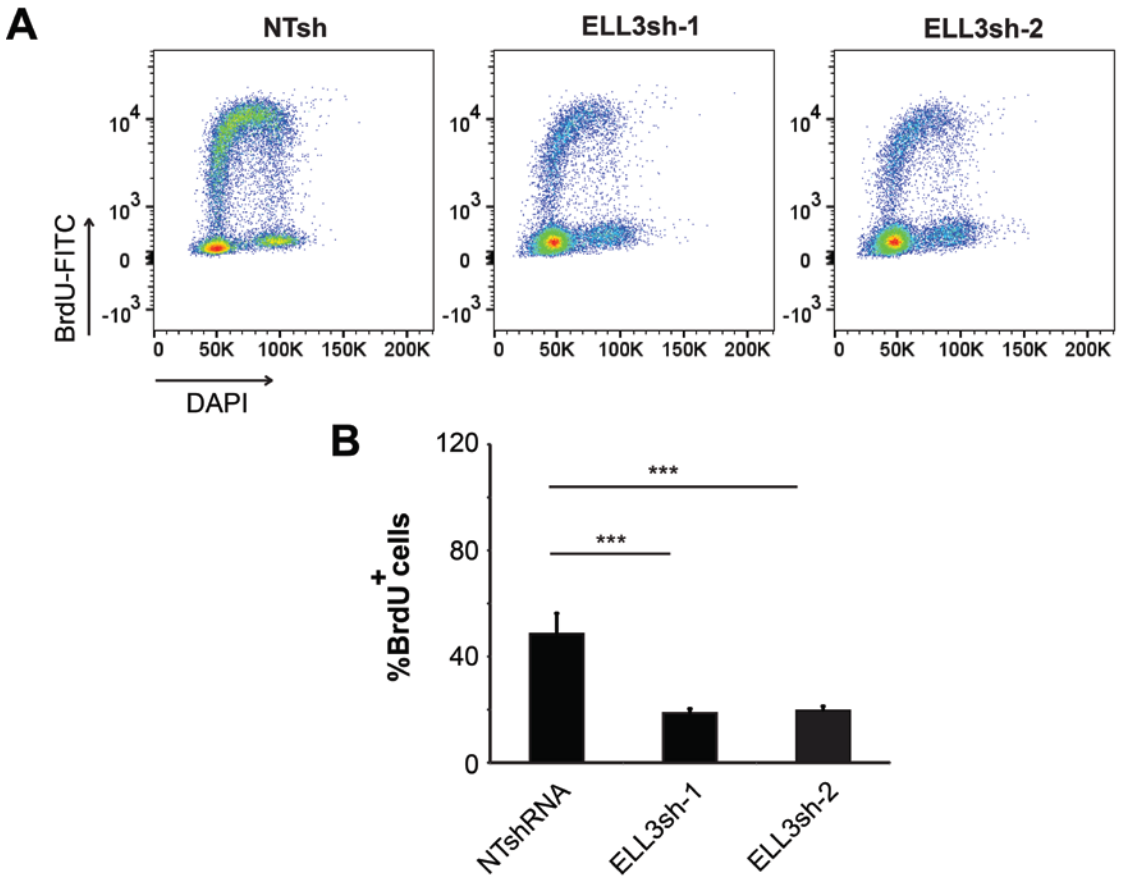

Figure 4.7 ELL3-depletion Compromised DNA Replication. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2. **A.** Representative dual color fluorescence density plot of DNA-DAPI and BrdU-FITC from cells pulsed with BrdU after ELL3 depletion. **B**. DNA replication detected by BrdU pulse labeling (30 min) and modeled for at least 190,000 cells. Data is depicted as percentage of total mCherry^{+} cells. Data are from 2 biological independent experiments with 4 replicates each. All assays were performed on day 5 post-transduction. Errors bars represent SD in percentage. ***p<0.001, ****p<0.00001 (*two-tailed t-test*). Data is presented in *Alexander et al.* [336].

MCM proteins have high affinity for one another within the MCM helicase complex and depletion of one is sufficient to destabilize the complex and halt replication [360, 361]. Following ELL3 depletion we assessed the expression of MCM proteins. Expression of MCM4 and MCM5 were dramatically reduced in ELL3-depleted cells **(Figure 4.9)**.

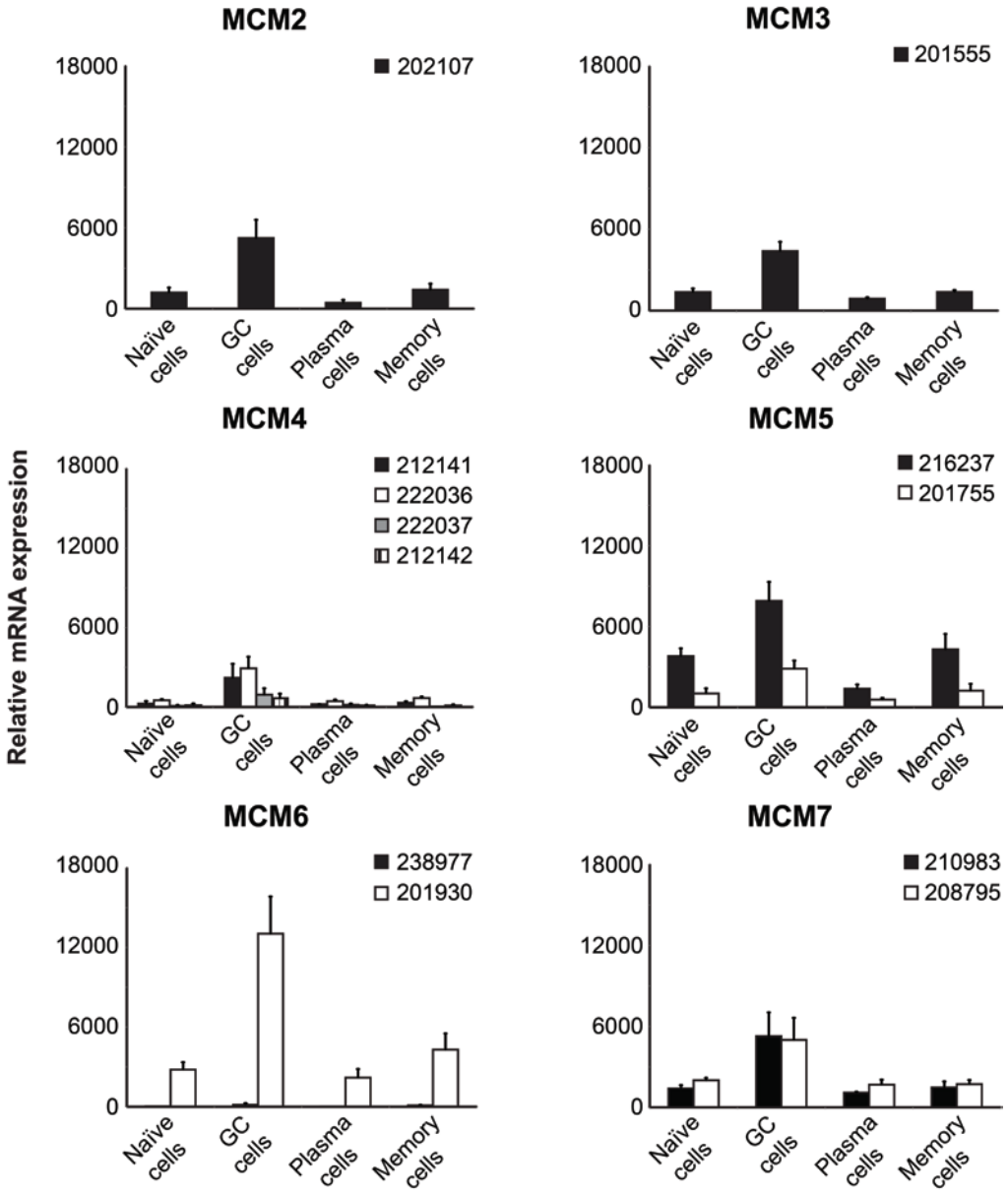

Figure 4.8 MCM Proteins in Primary Human Tonsillar B cell Subpopulations. Expression levels of all MCM helicase proteins were obtained from GSE12366 [321]. Depicted values are an average of 3 microarray hybridizations on cell sorted primary human B-cell subpopulations; error bars represent SD. Probe ID number is indicated in each graph.

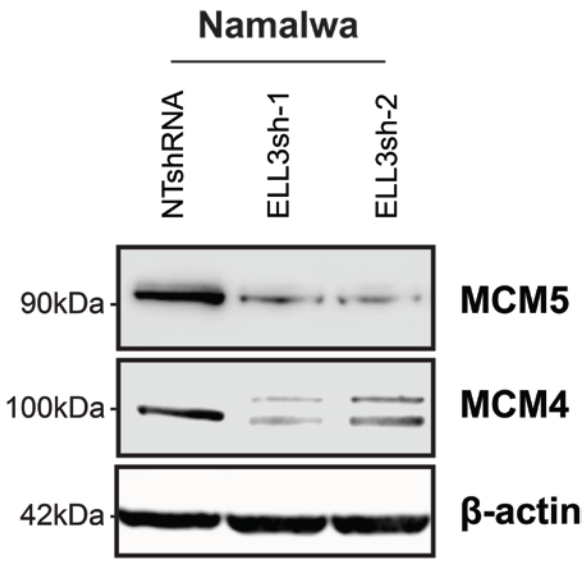

Figure 4.9 ELL3-depletion Compromised Helicase Components. Namalwa cells were transduced with either NTsh, ELL3sh-1, or ELL3sh-2 for 5 days. **A.** Immunoblot of MCM4 and MCM5. Data is representative of 3 independent experiments.

4.2.4 ELL3-depletion Resulted in DNA Damage and Morphological Aberrations

A diminished DNA replication and cell cycle progression suggest that the integrity of the genome is compromised [362]. To assess the presence of DNA damage we assayed the levels of a commonly used DNA damage marker phosphorylated H2AX (γH2AX) in both control and ELL3-depleted conditions. Our findings illustrate the marginal expression of γH2AX in control cells. Comparatively, ELL3-depleted conditions depict elevated levels of γH2AX. The presence of DNA damage suggests that the activation of p53 checkpoint mechanism [362]. Interestingly, control cells depict abundant p53 levels, which remain unchanged in ELL3-depleted samples. Together these findings indicate that the increase in DNA damage was not accompanied by an increase of p53 **(Figure 4.10)**.

Additionally, ELL3-depleted conditions underwent morphological changes consistent with loss of genomic integrity. These changes were first apparent in our flow cytometric analyses of the cell cycle. The flow cytometric detection of the forward scatter (FSC) and side scatter

(SSC) parameters depicted an increase in both size and granularity upon depletion of ELL3 **(Figure 4.11A)**. We visualized the cell size changes through fluorescent microscopy and confirmed the presence of cell size increase in ELL3 depleted conditions **(Figure 4.11B)**. To quantify the cell size changes observed upon ELL3 depletion, the sizes of all cells in an image were measured images and categorized into cell size categories (bins). Our measurements indicate that while 90% of control cells were in the smallest cell size category of <175 μM^2. Comparatively only 48% and 52% of ELL3-depleted populations were within this category **(Figure 4.11C)**. Overall, ELL3-depleted conditions had a significantly higher representation within the 176-350 μM^2 size category, at 40% (ELL3sh-1) and 39% (ELL3sh-2), compared to only 9% of control cells. In addition, while only 0.3% of the control cells were larger than 350 μM^2, 10-12% of the ELL3-depleted cells were found in size ranges, representing cells more than double the normal cell volume.

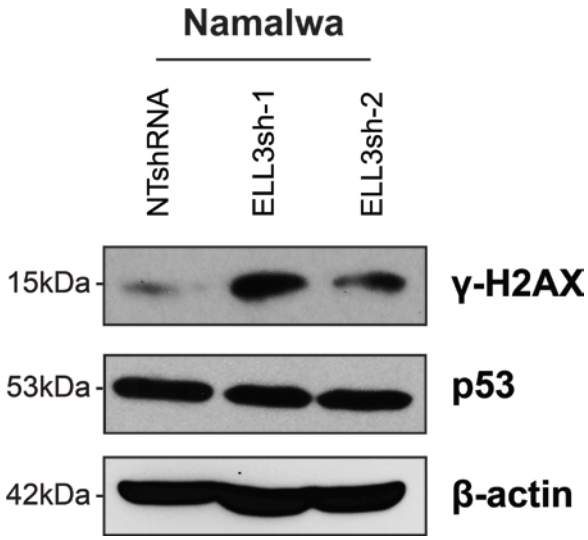

Figure 4.10 ELL3-depletion Resulted in DNA Damage. Namalwa cells were transduced with NTsh, ELL3sh-1, or ELL3sh-2 and assayed at day 5 post-transduction. **A.** Immunoblot of Ser139 phosphorylated H2AX and total p53. Data depicts representative images of 3 independent experiments. Data is presented in *Alexander et al.* [336].

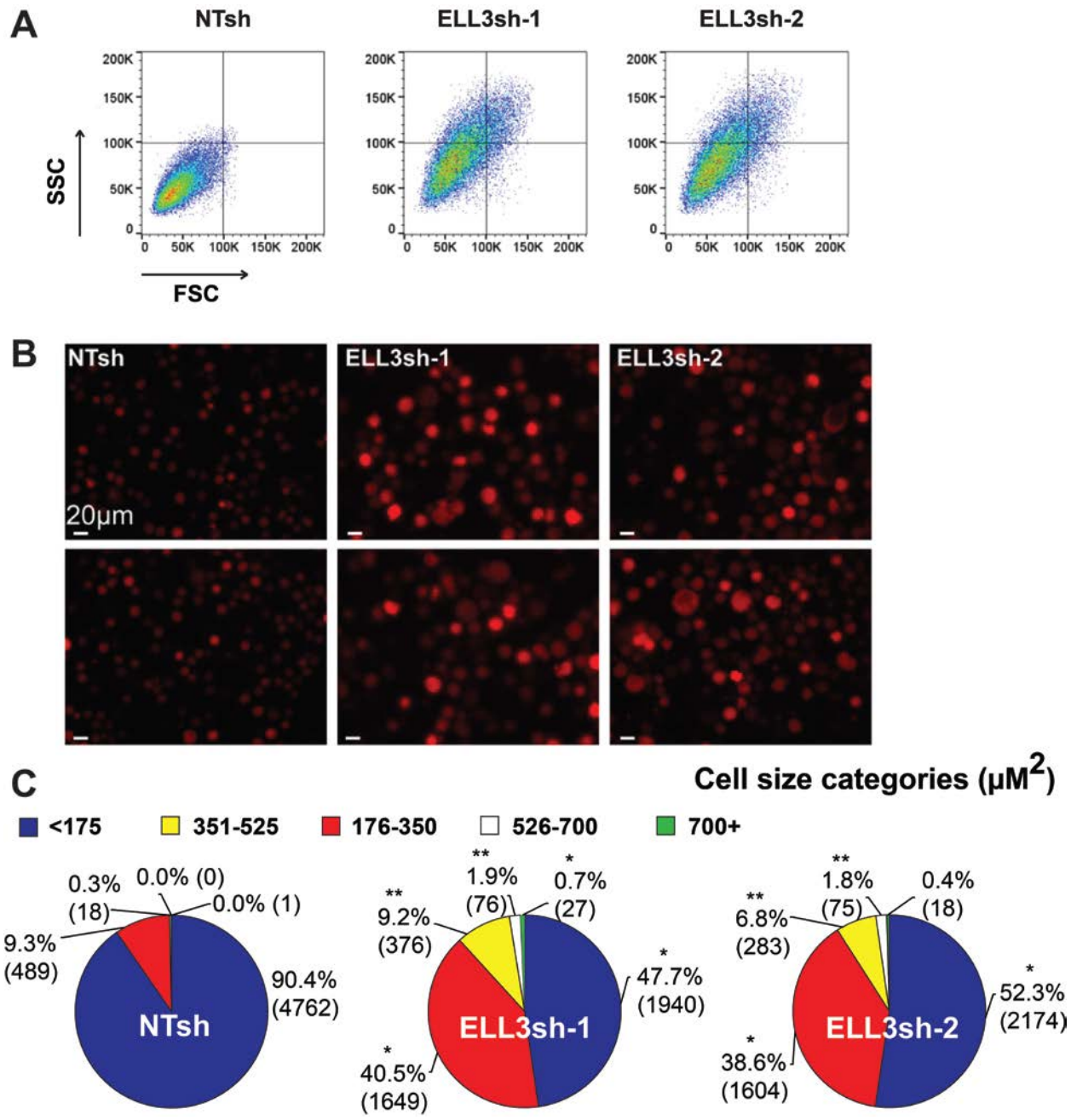

Figure 4.11 ELL3-depletion Resulted in Morphological Changes. Namalwa cells were transduced with NTsh, ELL3sh-1, or ELL3sh-2. **A.** Cell size and granularity were assessed at day 5 post transduction by flow cytometric detection of Forward Scatter (FSC) and Side Scatter (SSC) signals in mCherry^{+} cells. Data is representative of 2 independent experiments. **B.** Imaging of mCherry^{+} shRNA transduced cells at day 6 post transduction indicates alterations in cell size. Two representative images are shown per condition. C. Quantitation of cell area was determined from at least 4000 mCherry^{+} imaged cells per condition and categorized into 5 size bins. Data is presented as the percentage of total cells per cell size category across 3 independent experiments. Total number of cells indicated in parentheses. *p<0.05, **p<0.01 (*two-tailed t-test*). Data is presented in *Alexander et al.* [336].

Time lapse imaging allowed for further microscopic observation of the enlarged cells over time. While the control condition mostly depicts short term enlargement prior to cytokinesis, ELL3-depleted conditions exhibit mitotic aberrations. These aberrations include cells that over time fail to divide (column 3-4), increase in nuclear size (column 5-6), display multiple nuclei (column 7-8) and have aberrant cytokinesis (column 9-10) **(Figure 4.12)**. The presence of these mitotic aberrations suggests that the loss of ELL3 also perturbs cell growth and division.

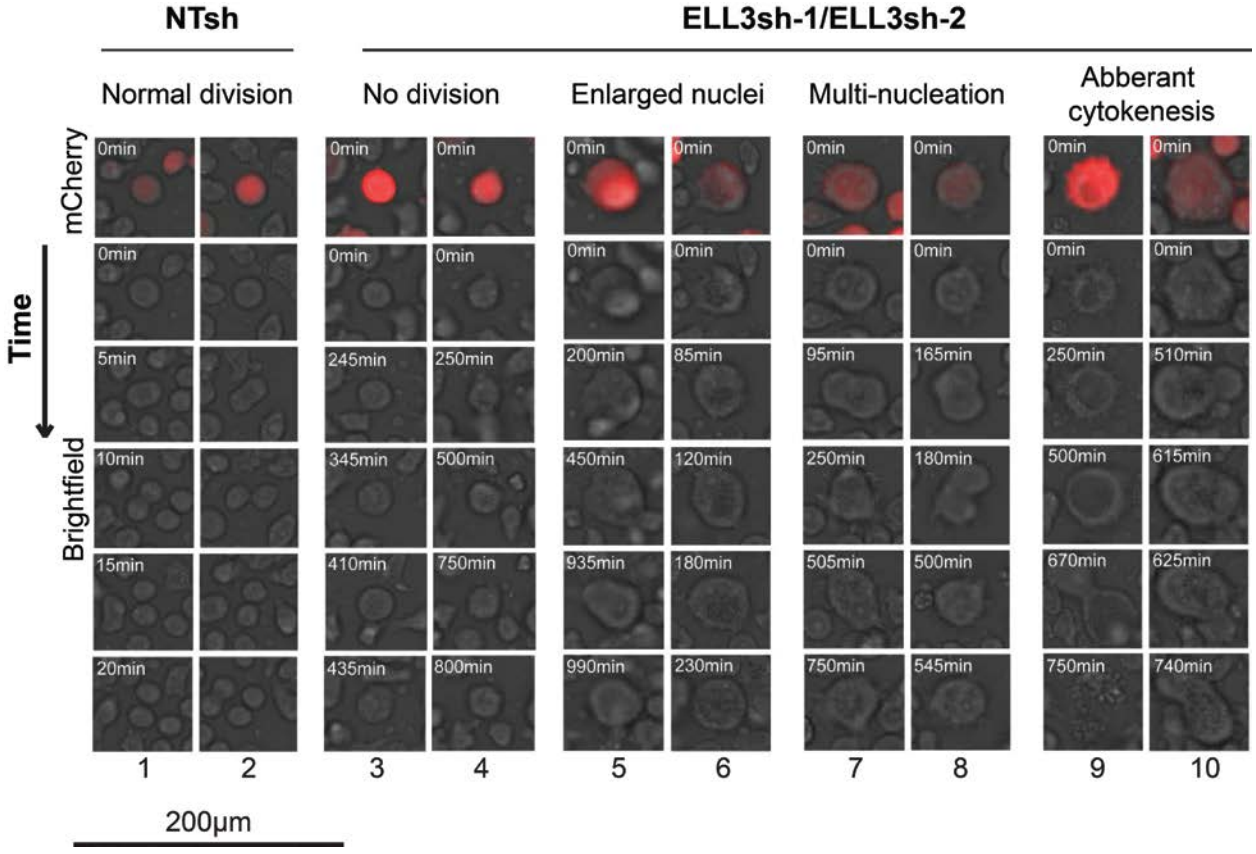

Figure 4.12 ELL3-depletion Presented as Various Morphological Aberrations. Namalwa cells were transduced with NTsh, ELL3sh-1, or ELL3sh-2. **A.** At day 6 post transduction, cells were subjected to time lapse imaging. Images were taken every 5 min. over 24 h. Data depicts representative images of the control and ELL3-depleted cells. The mCherry fluorescence signal (red) was used to identify shRNA transduced cells. Subsequent images are from the same cell but only imaged with phase to facilitate observation of morphological changes. Time of acquisition is indicated in each image. Data is presented in *Alexander et al.* [339].

The M-phase promoting factor (MPF) Cyclin B1-CDK1 complex is the key initiator of mitosis. In its absence mitotic aberrations are commonly observed [363, 364]. Based on the findings of mitotic aberrations, we assessed if ELL3-depletion affected the expression of the MPF subunit, cyclin B. As expected, ELL3 depletion caused a diminishment of both total and phosphorylated Cyclin B1 (pCyclinB1) levels **(Figure 4.13)**.

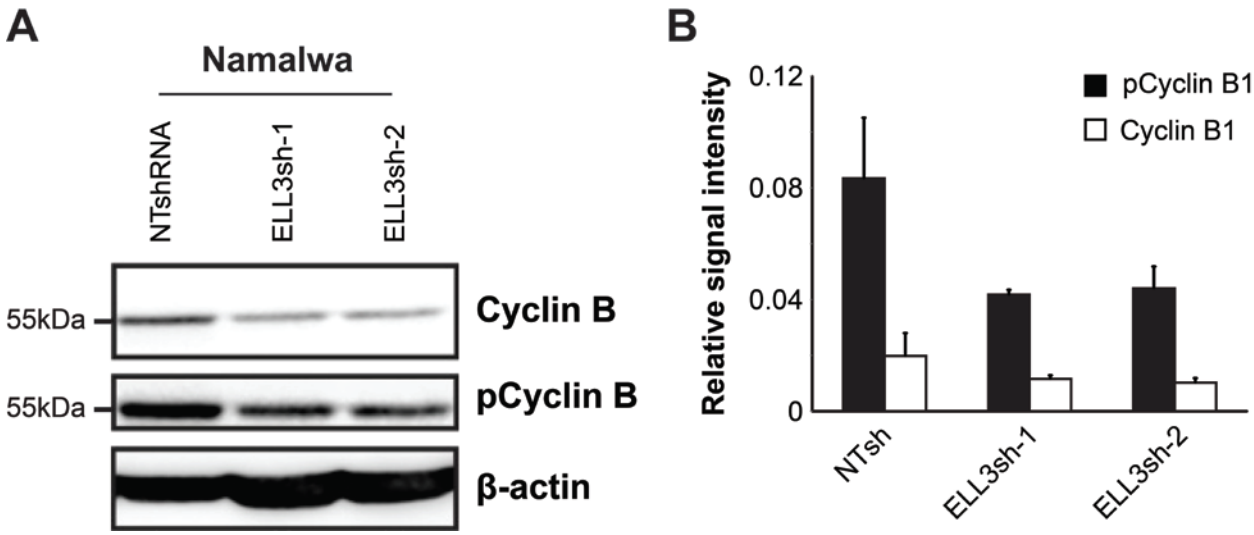

Figure 4.13 ELL3-depletion Resulted in Loss of Mitotic Regulators. Namalwa cells were transduced with NTsh, ELL3sh-1, or ELL3sh-2 for 5 days **A.** Immunoblot of Cyclin B1 and Ser133 phosphorylated Cyclin B1 levels. **B.** Fluorescence intensity of Cyclin B1 and pCyclin B1 was quantified and normalized to β-actin. Bar graph represents average fluorescence intensity of 3 independent experiments. Errors bars represent SD in percentage. Data is presented in *Alexander et al.* [336].

The Cyclin B1-CDK1 complex also has well established role in regulating cell viability through survivin-mediated regulation of apoptosis [365, 366]. Given the depleted levels of Cyclin B1, we assessed if the apoptotic pathway was induced. Both ELL3-depleted conditions displayed higher levels of cleaved caspase-3 and cleavage of its target, poly ADP ribose polymerase (PARP), indicating activation of apoptosis **(Figure 4.14)**. Collectively, these findings indicate that depletion of ELL3 compromises mitotic regulators resulting in mitotic disarray and loss of survival.

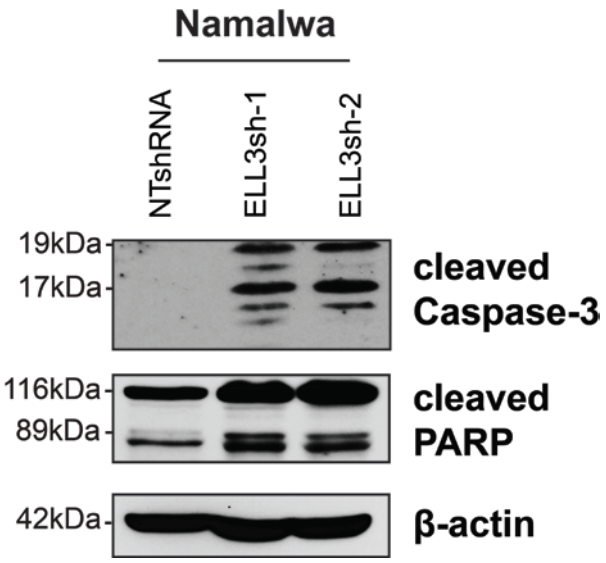

Figure 4.14 ELL3-depletion Resulted in Induction of Apoptotic Cell Death. Namalwa cells were transduced with NTsh, ELL3sh-1, or ELL3sh-2 for 5 days. Immunoblot of cleaved paspase-3, and its substrate , poly ADP ribose polymerase (PARP). Data depicts representative images of 3 independent experiments. All assessments were done at day 5 post transduction unless otherwise stated. Data is presented in *Alexander et al.* [336].

4.3 Discussion

The experiments performed in this chapter are aimed at determining the functional role of ELL3 in B cell lymphoma cell line cells. We identified that loss of ELL3 resulted in diminished ability to proliferate caused by compromised S-phase and its regulators, MCM proteins. Additionally, loss of ELL3 caused morphological changes and loss of survival.

B cell lymphoma cell lines served as a model to study the function of ELL3. The studies presented in this chapter depict for the first time that endogenous ELL3 levels could successfully be modulated to show profound effects on cell proliferation. Previous studies in a breast cancer cell line and embryonic kidney cells utilized ectopic expression to establish their involvement in proliferation [286, 296]. Similarly, the transient depletion of endogenous ELL3 at day 5 in BL cell line depicts significant diminishment in their proliferative capacity. Proliferation is essential in generating a large and diverse B cell repertoire and is a characteristic that is overly active in B

cell lymphomas [367]. The overactive proliferation is facilitated by well described alterations, mutations and translocations of various proliferation and cell cycle regulatory factors [195]. Our findings illustrate that ELL3 depletion results in the loss of the MCM DNA helicase proteins and M-phase promoting factor Cyclin B, suggesting that these factors are regulated by ELL3. ELL family members have been implicated in two types of functions, which include SEC-mediated transcriptional elongation and physiological functions as a result of protein-protein interactions. The physiological functions are thought to be mediated by the C-terminus which is highly conserved among ELL family members [284, 287, 293]. Our attempts at immunoprecipitating protein or chromatin with the current polyclonal antibodies for ELL3 have been unsuccessful. Thus it is currently unknown if ELL3 directly associates with either the MCM or Cyclin B proteins or transcripts. The generation of a new highly specific ELL3 antibody will aid in elucidating its binding partners and sites. Particularly in the case of the helicase MCM proteins, direct binding is critical. The helicase complex consists of six MCM proteins, which include MCM2, 3, 4, 5, 6 and 7. MCMs have high affinity for one another within the MCM helicase complex and depletion of one is sufficient to destabilize the complex and halt replication [360, 361]. Thus, it is currently unknown if all MCMs are affected by the depletion of ELL3 or if ELL3 has specificity towards one MCM complex member. We assessed the genome wide effects of stable ELL3 depletion in Raji cells. The single preliminary data set illustrated that ELL3 depletion compromised the levels of MCM5 transcript, which we also observed by qPCR. A most recent repeat in the Namalwa cell lines depicted that all helicase MCMs transcripts are diminished with ELL3 depletion. However without the association data, we are currently unsure whether these findings are the cause or effect of some of the proliferation defects that we

observe. Future studies should reveal if ELL3 regulates these factors transcriptionally or through physiological association.

The effect of ELL3 on these factors suggests that it's important for normal B cells. Our attempts at depleting ELL3 in primary B cells utilizing shRNA have been unsuccessful and may be caused by low transducability of primary B cells. Mild stimulation of quiescent lymphocytes appears to improve transducability and may be an option for further depletion studies [368-372]. The additional alternatives of siRNA or a mouse models, would require some significant financial investment, but may prove to be valuable and accessible tools to further study the role of ELL3 in B cell biology.

Depletion of ELL3 is not sufficient for differentiation of B cell lymphoma cell line cells. The depletion of ELL3 in BL cell line surprisingly resulted in up-regulation of PRDM1. As a master regulator of differentiation this finding suggests the occurrence of differentiation. Consistent with known PRDM1 targets, loss of ELL3 alone was not sufficient to cause differentiation into plasma cells, as is demonstrated by lack of ELL2, secreted IgM and levels of activated B cell markers expression [84, 190, 346]. Alternatively, PRDM1 was shown to participate in autoregulatory negative-feedback loops, with its targets BCL6, PAX5 and Spi-B [176, 192, 341, 373]. In those cases, direct binding of these factors was observed at *PRDM1* promoter. To date ELL3 binding to promoters could not be determined due to unavailability of an ELL3 antibody that is usable for ChIP or IP. The development of a new ELL3 specific antibody in combination with a PRDM1 reporter promoter would allow for further assessment of the possible feedback loop mechanism.

ELL family members do not participate in compensatory mechanisms in B cell lymphoma cell line cells. With a high degree of conservation observed amongst the ELL family

members, overlapping functions are not too farfetched [284, 287, 293]. Our depletion of endogenous ELL3 at 5 days did cause any change in the expression of family members ELL or ELL2, indicating that there is no compensation occurring among the family members and that their functions may not be interchangeable.

ELL3 is required to maintain survival of B cell lymphoma cell line cells. In our studies, transient ELL3 depletion in the BL cell lines resulted in induction of apoptotic cell death as indicated by the cleavage of caspase 3 and its target PARP. Particularly a prolonged S-phase is believed to cause DNA damage and may cause induction of apoptotic mechanisms [374]. Consistent with this notion, we observe a correlation of diminished DNA replication with accumulation of H2AX and induction of apoptotic cell death. These mechanisms are known to occur through induction of p53 [374]. ELL3 has previously been reported to stabilize p53 in breast cancer cell lines [297]. The presence of such a mechanism in BL cell lines and GC activated B cells may imply that other than transcriptional functions, ELL3 may also have physiological functions. The presence of a similar mechanism in BL remains to be determined. Like many malignancies, BL cell lines are known to have mutated p53, which allows them to circumvent death mechanisms [367]. A previous publication reported that the Namalwa cell line contained a p53 mutation in the DNA binding domain, R248Q mutation [375, 376]. We observed stable levels of p53 in control conditions which remained unchanged with ELL3 depletion, suggesting that induction of death mechanisms may not be through p53.

Catastrophic effects on the cell cycle may be a mechanism that is at a play upon ELL3 depletion. Catastrophic cell death is characterized by aberrant mitotic events, similar to our morphological findings that result in the induction of death mechanisms. These effects are commonly observed in malignant cells which have multiple cell cycle regulators deregulated

[377]. It is well known that B-cell lymphomas have several compromised cell cycle regulators [195]. Thus, we hypothesize that the depletion of ELL3 may induce death mechanism termed mitotic catastrophe. With the depletion of ELL3, we observe that helicase proteins MCM4 and MCM5 are diminished and consequently DNA replication abilities are compromised. In addition, we observe that ELL3-depleted cells attempt and fail to successfully complete mitosis in the absence of mitotic regulator Cyclin B. Cell cycle checkpoints serve to arrest cell cycle in the presence of incomplete DNA replication or sufficient cell cycle regulators. However, it is apparent that ELL3-depleted cells still progress through the cell cycle despite these issues. These findings are consistent with failure of the cell cycle checkpoints. As a result, we hypothesize that ELL3-depleted cells end up in a catastrophic state, where cells with insufficient replicated DNA attempt to divide into two daughter cells without the sufficient quantity of DNA and regulators for proper execution. Thus, explain some of the mitotic aberrancies and induction of death mechanisms. Overall ELL3s profound effects on cell survival position it as a favorable molecular target for the development of anti-tumor therapeutics.

CHAPTER V:

DISCUSSION AND SCIENTIFIC SIGNIFICANCE

The studies presented in this dissertation establish an expression pattern for ELL family members in normal B cells as well as malignant B-cell lines, in addition to characterizing the function of ELL3 in B-cell lymphoma cell lines. ELL family members are transcriptional elongation factors that function to increase the catalytic rate of transcription. This function is executed through assembly of the super elongation complex containing various combinations of additional elongation factors, including other ELL family members. Several elongation factors that participate within the super elongation complex were first described as common translocation partners of the multiple lineage leukemia (MLL) gene which results in the oncogenic release of the paused RNA pol II at the developmentally regulated genes. Prior to the studies presented in this dissertation, ELL2 was the only family member with described functions in the B cell compartment. Its functions regulate alternative splicing at the immunoglobulin heavy chain locus in terminally differentiated plasma cells [288, 289, 335]. Independently, family member and ELL and ELL3 have been implicated in the proliferation and survival of malignant cells [286, 296].

ELL3 is up-regulated coordinately with activation of B cells. The expression of ELL3 is limited in normal human tissues with predominant presence in lymph nodes and spleen [378]. Healthy human peripheral naïve B cells do not depict expression of any ELL family members.

This finding is consistent with naive B cells supporting only basal transcription [333]. The necessity of elongation factors is further highlighted by the finding of paused RNA Pol II in naïve lymphocytes and the rapid increase in the transcriptional output that occurs upon their activation [334, 379]. As a transcriptional elongation factor that is up-regulated with activation of B cells, ELL3 is positioned as a key participant in the release of paused RNA Pol II. To date no direct studies into transcriptional activation of ELL3 have been reported. However a study into the binding sites of EBF1 in murine B cells reveals in vivo association at the ELL3 loci and a correlation of EBF1 and ELL3 transcripts levels [70, 380]. With its known roles in lineage specification and activation of B cells, it is possible that EBF1 may serve as a pioneer factor to establish future ELL3 expression during B cell activation.

The expression of ELL3 precedes that of ELL2. Our cytokine mediated differentiation of human naïve B cells depicts a lack of ELL3 expression with a significant induction of ELL2. ELL2 was previously reported to alter mRNA splicing of the immunoglobulin locus, which is necessary to generate secreted immunoglobulins [288, 289, 335]. The abundance of ELL3 in the mechanism that directly precedes terminal plasma cell differentiation is consistent with a notion that its expression precedes that of ELL2.

ELL is co-expressed with either ELL3 or ELL2. ELL family members were initially described to simultaneously function as components of the SEC [253]. The findings described in this dissertation illustrate that ELL3 and ELL2 are not co-expressed in the B cell compartment and thus are unlikely to function within the same complex. The expression of ELL3 in activated B cells and ELL2 in plasma cells suggests the assembly of SEC complexes with distinct constituents. Such complexes, named SEC-like complexes, have been previously reported to contain P-TEFb with various combinations of the transcriptional elongation factors of the AFF

family, YEATS domain containing protein family members, and ELL family members [315]. Although our analysis depicts a non-concurrent expression of ELL2 and ELL3, both are observed in the presence of ELL upon cytokine stimulus. Our findings, replicate a previous observation where all three proteins are over-expressed concurrently, SEC-like complexes could be isolated containing ELL with either ELL3 or ELL2 but not both [273]. The presence of additional SEC elongation factors is yet to be determined in each of the proposed SEC-like complexes. Their identification is critical as SEC-like complex constituents are thought to render target specificity [275]. Based on these findings we postulate that differential usage of either ELL3 or ELL2 within SEC-like complexes may contribute to target specificity during transcription in the B cell compartment. Additionally, ELL3 was shown to preferentially bind transcription enhancers in murine embryonic stem cells prior to ELL2 to establish promoter occupancy allowing subsequent ELL2/SEC transcription program [294]. It is currently unknown if ELL3 and ELL2 have a similar linked activity in B cells or independently function to regulate transcription of specific genes. Finally, it also remains to be determined if the presence of ELL3 alters RNA splicing similar to the proposed function of ELL2 at the immunoglobulin heavy chain locus [288, 289, 335].

ELL3 is part of the B cell transcription factor network extinguished by PRDM1 during plasma cell differentiation. Our cytokine stimulation experiments depict an inverse correlation between ELL3 and PRDM1 upon cytokine stimulation and ectopic over-expression. In addition, we demonstrated direct association of PRDM1 at the ELL3 promoter in intact cells and direct repression of the cloned ELL3 promoter. While PRDM1 is best known for its repressive functions, it has also been reported to have activating function in mouse plasmablasts [381]. This report also identified ELL2 as PRDM1 activated gene. With a similar correlation between

PRDM1 and ELL2 identified in our cytokine stimulus experiments, we propose that the ELL3-to-ELL2 switch can be attributed to both activation and repressive function of PRDM1.

GC B cell lymphoma subtype models can be stratified by ELL3 expression. With a panel of cell lines that model maturation, activation and differentiation of B cells our analysis depicts abundant levels of ELL3 in GC activated B cell lines. With all BL and three out of five DLBCL cell lines expressing abundant ELL3 protein levels and the correlation of ELL3 expression with proliferation and survival pathways, our findings conform to the idea of pathway dependencies that was recently described in B-cell malignancies [195, 382]. The absence of ELL3 in a subset of DLBCL cell lines also indicates a possible dichotomy for ELL3 expression within DLBCL. The DLBCL subtype of B-cell malignancies displays significant heterogeneity. Several molecular subtypes have been described based on differing origin, host response and genetic heterogeneity which include inactivating PRDM1 mutations [203, 204, 216, 383, 384]. The expression of ELL3 in DLBCL did not correlate with known classifications. Further exploration of this dichotomy may elucidate the usefulness of ELL3 as a marker for subtype classification or response to treatment. It is currently not known how ELL3 presents itself in B-cell lymphoma patient samples and our attempts of assessment were hampered by the lack of available B-cell lymphoma patient samples or molecular data in our repository and in public data bases. Further assessment may provide important insights into the participation of ELL3 in the transcriptional networks that are thought to be co-opted by B-cell malignancies.

These studies identified cell proliferation and survival as physiological mechanisms that require ELL3 expression in B-cell lymphoma cell lines. Knockdown of ELL3 at 120h time point demonstrated a diminishment of proliferation, due to compromised DNA replication and MCM proteins, and induction of apoptotic mechanisms. In a HEK-293 cell line model, over-expression

of family member ELL has also been implicated in cell proliferation and survival [286]. Thereafter a series of studies utilized over-expression of ELL3 to show its involvement in breast cancer cell line proliferation, cancer stem cell properties and drug resistance through stabilization of p53 and induction of ERK1/2 signaling pathway [296, 297]. Together these findings suggest that regulated expression of ELL family members is required for proper maintenance of cell proliferation and viability.

Collectively, the data within this dissertation establish ELL3 as a key regulator of B cell lymphoma proliferation and survival. In normal B cells, ELL3 expression is induced upon cytokine-mediated activation and switched for the expression of ELL2 upon cytokine-mediated differentiation through transcriptional regulation by PRDM1. Through direct and/or in-direct regulation, ELL3 modulates cell proliferation and survival mechanisms in the activated B cell (Figure 5.1). The transcriptional elongation factor ELL3, is described for the first time in human and mouse activated B cells and its expression affects the key mechanisms of proliferation and survival. These mechanisms are not only required for normal B cell activation and function but also represent those that are commonly hijacked by lymphomas. This notion positions ELL3 as viable therapeutic target in treatment of ELL3-positive B-cell lymphomas.

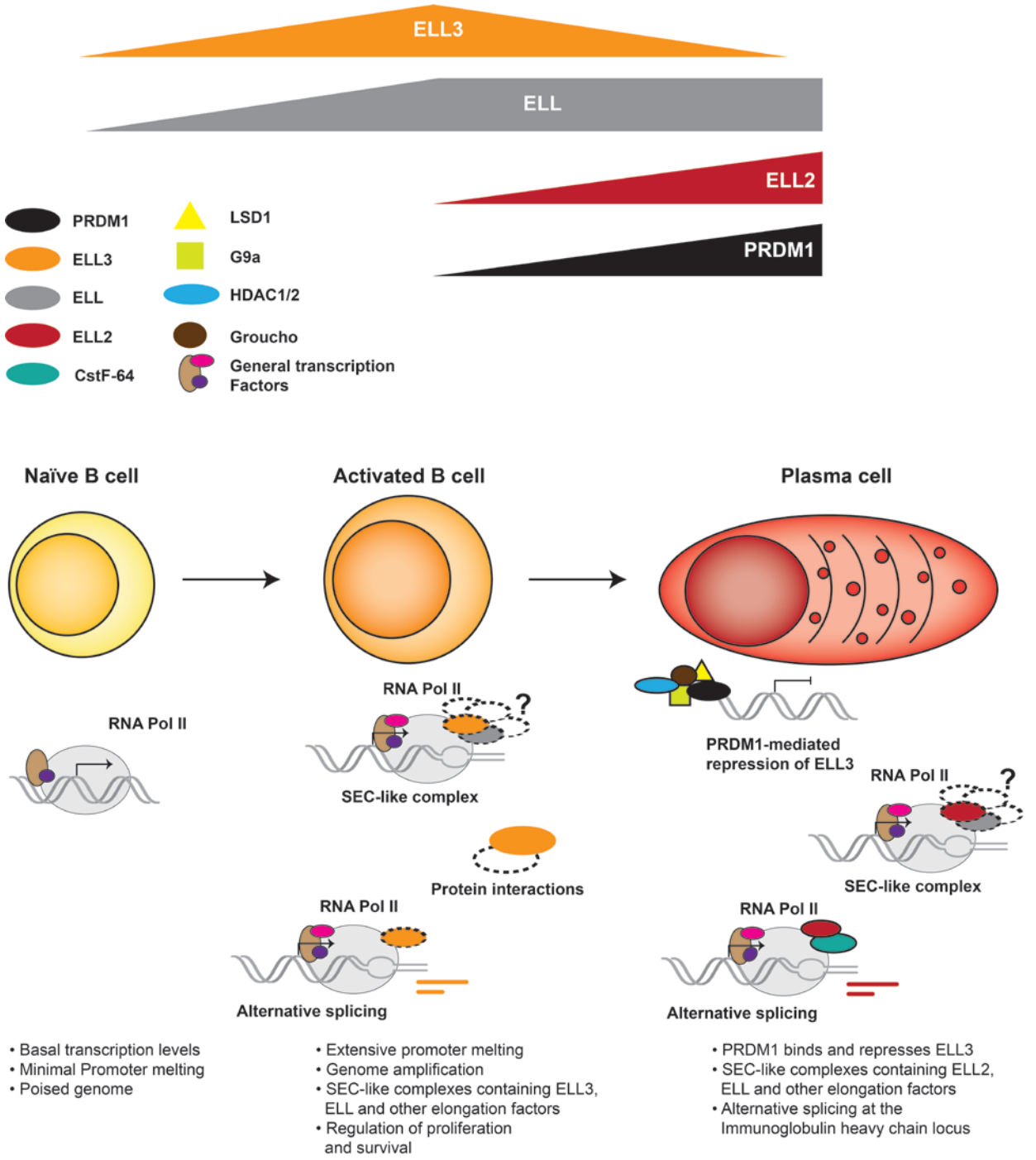

Figure 5.1 Model of ELL Family Expression and Function in B cells. Schematic depiction of B cells at various stages of differentiation with the expression pattern of ELL, ELL2, ELL3 and PRDM1 as well as a depiction of their regulation and their proposed transcriptional and physchiological functions. Rectangles indicate previously identified proteins; ovals indicate findings presented in this dissertation; dashed boarders represent hypothesized associations; solid boarders indicate associations presented in this dissertation.

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APPENDICES

Appendix I Rights and Permissions for Molecular Immunology Journal

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Appendix II Rights and Permissions for Data in Brief Journal

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ABOUT THE AUTHOR

Lou-Ella M.M. Alexander was born and raised on the island of Curaçao, a dutch colony island located just off the coast of South America. After completing athenaeum at Peter Stuyvesant College in 2004, she moved to the Netherlands to attend Saxion Universities of Applied Sciences at the Enchede location. Lou-Ella majored in Biology and Medical Laboratory Research and obtained her Bachelor in Science in 2007. During this time, she performed a rotation in the laboratory of Frits Peters, Ph.D. at Vrije Universiteit Medical Center in Amsterdam, Netherlands. There she participated in the research that led to two publications in *Journal of Medicinal Chemistry* and *Nucleosides Nucleotides Nucleic Acids* journal.

Following graduation, she and entered Radboud University Nijmegen to pursue a Master of Science in Medical Biology. During this degree she performed two rotations. The first was at the Nijmegen Center for Molecular and Life Sciences under the guidance of Ger Pruijn, Ph.D, which led to a publication in *Arthritis Research & Therapy*. The second rotation brought her to Moffitt Cancer Center in Tampa, FL to work with Kenneth L. Wright, Ph.D for her thesis project. There she also got accepted into the Cancer Biology Ph.D. Program, which she joined in 2010. Her work in the laboratory of Dr. Wright, for the past 7 ½ years, has led to poster presentations at both national and local meetings. This work culminated in two first author publications in *Molecular Immunology* and *Data in Brief* journals. Upon graduation, she will continue working in the area of Immunology at Emory University in the laboratory of Jeremy Boss, Ph.D.