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Regulation of positive regulatory domain I-binding factor 1 and its role in mantle cell lymphoma

Shruti Desai
University of South Florida

Regulation of Positive Regulatory Domain I- Binding Factor 1 and Its Role in Mantle Cell Lymphoma

by

Shruti Desai

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Department of Cancer Biology
College of Arts and Sciences
University of South Florida

Major Professor: Kenneth L. Wright, Ph.D.
W. Douglas Cress, Ph.D.
Amer A. Beg, Ph.D.
Sheng Wei, M.D.

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DEDICATION

I would like to dedicate this dissertation to my family and friends for all their support throughout the years. I owe all my success in the pursuit of my doctoral degree to my parents, my sister and my husband. They have been my source of strength and inspiration. Their constant love and support helped me accomplish my goals. This work is also dedicated to my parents-in-law and my grandparents. Words cannot do justice to express my gratitude to all of you.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATF6	Activating transcription factor 6
Blimp-1	B lymphocyte induced maturation protein-1
BCAP	B cell adaptor for phosphoinositide 3-kinase
BCL2	B cell lymphoma 2
BCL6	B cell lymphoma 6
B-CLL	B cell chronic lymphocytic leukemia
BCR	B cell receptor
BLNK	B cell linker
BM	Bone marrow
BSA	Bovine serum albumin
BSAP	B cell specific activator protein
CDK4	Cyclin dependent kinase 4
ChIP	Chromatin immunoprecipitation
CIITA	Class II transactivator
CLP	Common lymphoid progenitor
DAG	Diacylglycerol
DC	Dendritic Cell

D_H	IgH diversity
DLBCL	Diffuse large B-cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GC	Germinal Center
HDAC	Histone deacetylase
HRS	Hodgkin's Reed Sternberg
HSCs	Hematopoietic stem cells
Id3	Inhibitor of differentiation 3
Ig	Immunoglobulin
INFβ	Interferon β
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine based activation motif
Mafk	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K
MCL	Mantle Cell Lymphoma
MHC	Major histocompatibility complex
MS	Mass spectrometry
NHLs	Non- Hodgkin's lymphomas
PAX5	Paired box gene 5

PBMC	Peripheral blood mononuclear cell
PMSF	Phenylmethylsulfonylfluoride
PRDI-BF1	Positive regulatory domain I binding factor 1
PRDM1	PR domain containing 1
PVDF	Polyvinylidene fluoride
qRT-PCR	quantitative real time PCR
RT-PCR	reverse transcription PCR
SDS	Sodium dodecyl (lauryl) sulfate
SHM	Somatic Hypermutation
TdT	Terminal deoxynucleotidyl transferase
T_H	T helper cell
TLE4	Transducin-like enhancer of split 4
UPR	Unfolded Protein Response
Xbp-1	X-box binding protein 1

REGULATION OF POSITIVE REGULATORY DOMAIN I- BINDING FACTOR 1 AND ITS ROLE IN MANTLE CELL LYMPHOMA

Shruti Desai

ABSTRACT

The human positive regulatory domain I binding factor 1 (PRDI-BF1/PRDM1) promotes differentiation of mature B cells into antibody secreting plasma cells. In contrast ectopic expression of PRDM1 in lymphoma cells can lead to inhibition of proliferation or apoptosis. However, little is currently known about the regulation of PRDM1. The first study presented demonstrates that in lymphoma cells stimulation through the B cell receptor rapidly induces endogenous PRDM1 at the level of transcription. This study provides evidence that the PRDM1 promoter is preloaded and poised for activation in the B cell lines. The transcription factor PU.1 is shown to be required for B cell receptor induced expression of PRDM1 in lymphoma cells and in PU.1 positive myeloma cells. Furthermore, activation is associated with loss of the co-repressor TLE4 from the PU.1 complex.

The second study establishes the requirement for PRDM1 in Mantle cell lymphoma (MCL) response to Bortezomib. MCL, an aggressive form of B cell lymphoma, has poor disease- free survival rate. The proteasome inhibitor, Bortezomib, is approved for treatment of relapsed and refractory MCL. However, the precise mechanism of action of Bortezomib is not well understood. Bortezomib rapidly induces transcription of PRDM1 along with apoptosis in MCL cell lines and primary MCL tumor samples. Knockdown of PRDM1 inhibits Bortezomib-induced apoptosis, while ectopic expression of PRDM1 alone leads to apoptosis in MCL. MKI67 and PCNA, which are required for proliferation and survival, were identified as novel direct targets of PRDM1 in MCL. Chromatin immunoprecipitation and knockdown studies reveal specific repression of MKI67 and PCNA is mediated by PRDM1 in response to Bortezomib. Furthermore promoter studies demonstrate that PRDM1 functions through a specific site in the proximal promoter region of PCNA and through a distal upstream repression domain on the MKI67 promoter. Together these findings establish PRDM1 as a key mediator of Bortezomib activity in MCL through suppression of proliferation and survival genes.

The third study presented demonstrates use of Tandem affinity purification technique followed by mass spectrometry to identify PRDM1 and Reptin52 protein interactions. The observations in this study provide preliminary evidence of novel mechanism of regulation of PRDM1 protein function.

CHAPTER ONE

GENERAL INTRODUCTION

B Cell Development

B cell lineage is generated from multipotent hematopoietic stem cells (HSC) by a series of complex processes, with the primary sites of development being the fetal liver and adult bone marrow. This forms the microenvironment that gives signals to the stem cell precursors to grow and differentiate (Billips, Lassoued et al. 1995). B cells play an important role in development of the immune system as they function as professional antigen presenting cells as well as generate antibody secreting plasma cells. B cell development can be divided into two broad phases – initial antigen independent phase and an antigen dependent phase (Cooper 1987).

Antigen-Independent Phase

The HSCs receive signals from other cells present in the microenvironment to enter the B cell specific pathway. With the onset of expression of cell surface marker B220, common lymphoid progenitors (CLPs) are committed to the B cell differentiation process. The stromal cells present in the microenvironment provide signals to pro-B cells to divide and differentiate (Kincade, Witte et al. 1987). The stromal cells interact directly with the pro-B and pre-B cells and also secrete cytokines that support the development process. The pro-B

cells begin to express CD19 and undergoes rearrangement of the heavy chain variable region D_H to $J_{H,}$ followed by V_H to D_HJ_H rearrangement (Yancopoulos and Alt 1986). If the first rearrangement is not productive then V_H-D_H-J_H rearrangement continues on the other chromosome. The initial productive heavy chain rearrangement results in expression of μheavy chain. Rearrangement of the immunoglobulin variable region of the heavy chain is followed by regulated rearrangement of the light chain locus. Before a successful light chain rearrangement takes place, the productive heavy chain is associated with a surrogate light chain and membrane bound Igα/Igβ heterodimers. This forms the pre-B cell receptor and the B cells at this stage are called pre-B cells (Melchers, Karasuyama et al. 1993). This population of pre- B cells undergoes further maturation. The surrogate light chain is down-regulated followed by generation of a productive light chain gene rearrangement. Failure to achieve a productive kappa chain arrangement leads to rearrangement of lambda light chain (Sakaguchi and Melchers 1986). A productive light chain rearrangement generates an immature B cell that expresses surface IgM (Nishimoto, Kubagawa et al. 1991). Successful expression of a light chain not only depends on productive gene rearrangement but may also be under transcriptional regulation (Alt, Reth et al. 1986). Further maturation process leads to expression of second surface immunoglobulin isotype, IgD. This generates a mature B cell population with surface markers IgM and IgD (Cooper, Kearney et al. 1980). Some cells of the B cell clone undergo switching of immunoglobulin heavy chain isotype but with identical antigen binding specificity. This process is called isotype switching.

Pre-B Cell Receptor

The pre-B cell receptor complex is present on the surface of pre-B cells. The pre-B cell receptor is composed of two immunoglobulin μ heavy chains and two surrogate light chains, which are associated with membrane bound signaling Igα/Igβ heterodimer. The surrogate light chain consists of two proteins – a V- like sequence (Vpre-B) and a C-like sequence (λ5) (Nishimoto, Kubagawa et al. 1991). These proteins associate noncovalently to form a surrogate light chain. The genes for the surrogate light chain do not undergo rearrangement for their expression. The pre-BCR can recognize a ligand on the stromal-cell membrane transmitting signal leading to allelic exclusion. Signaling from the pre-BCR may also cause proliferation of the pre-B cells (Geier and Schlissel 2006). As the pre-B cells further develop into immature B cells the surrogate light chain is down-regulated and replaced by the κ or λ light chain.

Selection of Immature B Cells

B cells generated after a successful rearrangement of both heavy and light chains are called immature B cells. Self reactive immature B cells expressing autoantibodies against self-antigens present in the bone marrow microenvironment are eliminated by negative selection. There are three known mechanisms of negative selection – deletion, anergy and receptor editing (Nossal and Pike 1980; Nemazee and Burki 1989; Nemazee and Weigert 2000). Negative selection can lead to apoptosis of the self reacting immature B cell. Self reacting immature B cells are not always deleted. Sometimes their maturation is arrested till the B cell receptor is edited. Receptor editing may take place by replacing the κ light chain with the λ light chain which is not self-reactive (Tiegs, Russell et al. 1993). This rescues the cells from

elimination and allows the cells to further mature into mature B cells and leave the bone marrow. Further maturation of the B cell requires exposure to antigens and is thus called the antigen-dependent phase.

B Cell Receptor

B cell receptor is expressed on the immature B cell. It consists of two molecules of Igα/Igβ heterodimer associated with one molecule of immunoglobulin. Signaling through the BCR is mediated by the Igα/Igβ heterodimer, this is because all the mIg except for IgG have a short cytoplasmic tail that lack signaling capacity. The signaling molecules have specific motifs in the cytoplasmic tail termed as immunoreceptor tyrosine-based activation motif (ITAM).

In resting B cells the BCR complexes are localized to the outside lipid rafts. Upon antigen stimulation these complexes localize to the inside lipid rafts activating other BCRs in an antigen independent manner and amplifying the signal (Thomas, Srivastava et al. 2006). The resting BCR is associated with Src family tyrosine kinases such as Byk, Lyn and Fyn which are activated upon receptor ligation (Burkhardt, Brunswick et al. 1991). The resting BCR is also associated with tyrosine Syk kinase. Phosphorylation of the ITAM tyrosine enhances the recruitment and activation of Syk kinase, which is a principle kinase that drives many signaling pathways (Turner, Gulbranson-Judge et al. 1997). A series of linker molecules are required for transduction of the activating signals. ITAM activation results in recruitment of adaptor molecules B cell linker (BLNK) and B cell adaptor for phosphoinositol 3-kinase (BCAP), which acts as scaffolds for other signaling molecules (Pappu, Cheng et al. 1999; Yamazaki, Takeda et al. 2002).

One pathway leads to activation of the phospholipase Cγ2 (PLCγ2) and Ras. BLNK binds to PLCγ2 as well as Btk2, which allows for further activation of PLCγ2 (Ishiai, Kurosaki et al. 1999). Syk and Btk phosphorylate BCAP, allowing it to bind to phosphoinositide 3-kinase (PI3K) leading to phosphorylation of more downstream targets and further activation of PLCγ2 (Beitz, Fruman et al. 1999; Okada, Maeda et al. 2000). This activation leads to the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate which further activates a number of downstream signaling pathways responsible for the different cellular outcomes such as proliferation, survival or differentiation (Thomas, Srivastava et al. 2006).

Signaling pathway activating NFκB is independent of the Syk kinase activation but requires activation of one or more Src family kinases. Other surface molecules on B cells can modulate B cell development. Tyrosine phosphatase CD45 may dephosphorylate the inhibitory tyrosine on the Src family kinases, thus lowering the threshold for BCR –mediated signaling (Cyster, Healy et al. 1996). CD19 can also positively regulate BCR signaling through PI3K (Otero, Anzelon et al. 2003; Otero and Rickert 2003).

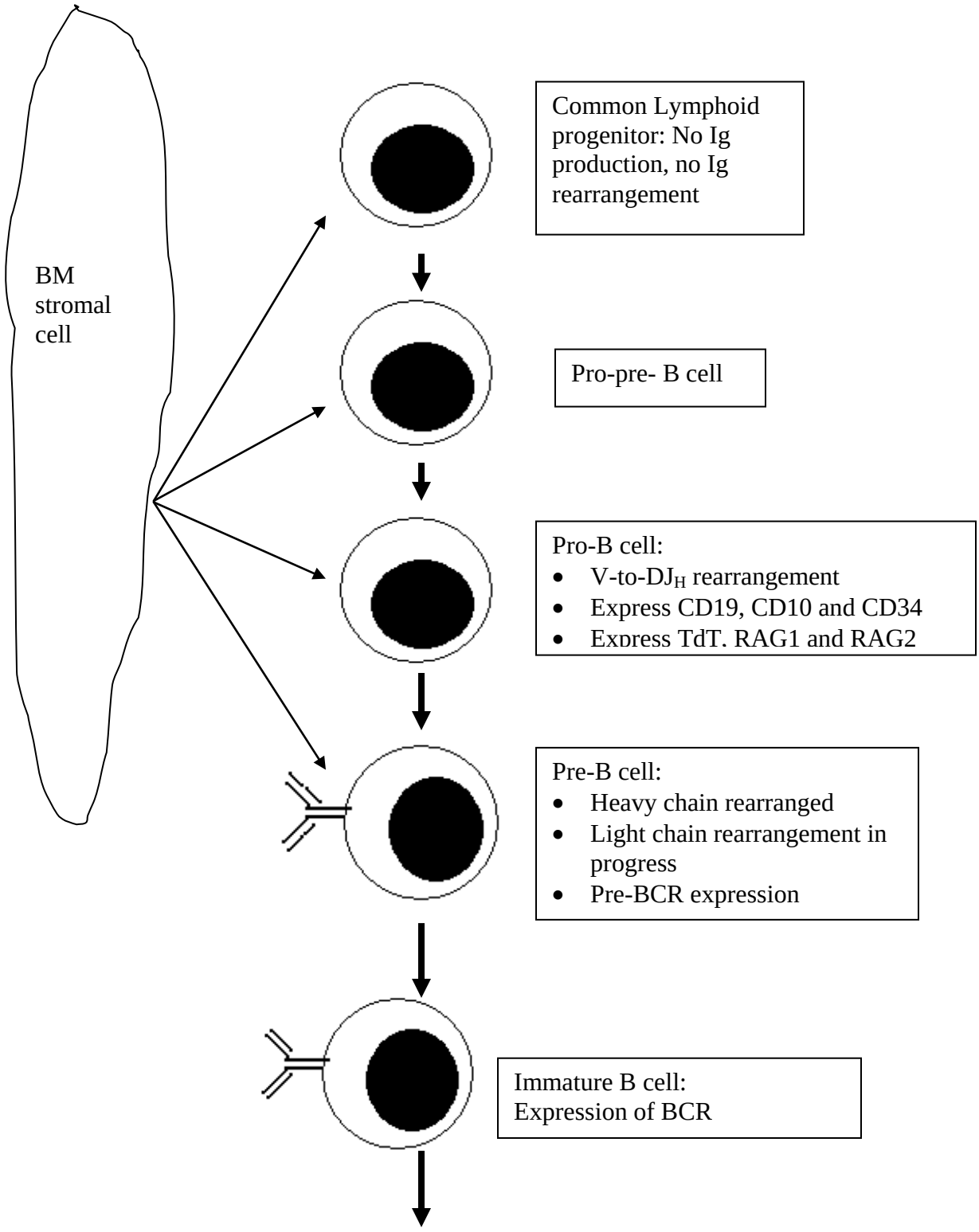


Figure 1: Antigen-Independent Phase of B Cell Development

Antigen-Dependent Phase

Absence of stimulation by antigens or non-specific mitogens leads to apoptosis of the B lymphocyte. Moreover, most of the B cells reaching the spleen die within a few days. Cross-linking of surface immunoglobulin molecules with antigens induces the resting B cells to enter cell cycle (Melchers and Andersson 1986). Depending on the antigen the B cell activation proceeds as either dependent on T_H cells (thymus dependent antigens) or independent on T_H cells (thumus independent antigens). Humoral response generated by thymus independent antigens is usually weaker with no formation of memory cells and is the predominant antibody is IgM.

The antigen molecule bound to the surface immunoglobulin receptor are internalized and partially digested. These fragments are then expressed on the surface of the B cells in association with the major histocompatibility complex (MHC) class II molecules. This is recognized by helper T cells that produce T cell factors which promote proliferation and differentiation of antigen presenting B cells (Hodgkin and Basten 1995). This leads to terminal differentiation of B cells into antibody secreting plasma cells or memory cells.

Activating Signals

Activating signal received by naïve resting B cells consists of two distinct events – signal 1 and signal 2. A thymus independent antigen is able to produce both the signals, but thymus dependent antigens do not provide both the signals. A thymus dependent antigen provides signal 1 by cross-linking the membrane bound immunoglobulin (Ig) and signal 2 is provided by a separate interaction of CD40 in the B cell membrane with the CD40L on the activated helper T cell membrane.

Sites of B Cell Activation

When the antigen enters the host system it is captured and processed by professional antigen presenting cells. This leads a sequence of events that generate a humoral immune response against the antigen. The professional antigen presenting cells, especially dendritic cells (DC) migrate from the tissues into the T cell zones of the local lymph nodes. Antigen derived peptide bind to naïve T cell receptors leading to activation and proliferation of T helper (T_H) cells. Similarly, naïve B cells enter the T cell zone of the lymph node and the B cells with antigen specific receptors get trapped in the T cell zones along with B cells that have antigen presented in association with class II MHC molecules. The trapped B cells interact with the activated T_H cells leading to formation of primary focus. Primary focus consists of the activated B cells which are clonally expanding. Most of clonally expanded activated B cells in this focus undergo apoptosis, but some differentiate into antibody secreting plasma cells, while others develop into a secondary lymphoid follicle or germinal center. The germinal center comprises B cells, activated T_H cells and follicular dendritic cells. The germinal center consists of dark zone and basal light zone. The dark zone is made up of centroblasts. The centroblasts are proliferating activated B cells with reduced expression of surface immunoglobulins. The centroblasts exit the dark zone and enter the light basal zone as centrocytes. The light zone contains a large number of follicular dendritic cells and activated T_H cells. The centrocytes bearing IgM that bind to antigen presented by the follicular dentritic cells undergo differentiation into memory B cells or plasmoblasts (Gold and DeFranco 1994; MacLennan 1994).

In the germinal center the B cells undergo modifications that lead to B cell differentiation. These modifications include somatic hypermutation, isotype switching and formation of plasma cells and memory B cells.

Somatic Hypermutations: is one of the processes that create antibody diversity. Somatic hypermutation introduces single base pair substitutions and occasionally deletions and insertions in the variable region of the heavy and light chain. The point mutations accumulate in the proliferating B cells and may negatively or positively impact the B cell receptor ability to bind to the antigen. Mutations that increase the affinity of the B cell receptor for antigens are positively selected for expansion. The positive stimulus prevents apoptosis in these cells (Neuberger and Milstein 1995).

Isotype Switching: The variable region determines the specificity of antibody. Isotype switching allows subset of B lymphocytes to associate their variable region with the constant region of any isotype such as IgA, IgE or IgG. These immunoglobulin istypes have same antigen specificity but different effector functions (Cooper, Kearney et al. 1980). Class switch requires the CD40/CD40L interactions as well as various cytokines secreted by activated T_H cells. Isotype switching involves cutting of repetitive DNA sequences at the switch (S) region upstream of the constant region of μ gene. This is spliced with a complementary S region in front of the next constant heavy chain gene region to be expressed (Li, Woo et al. 2004).

Plasma Cell Generation: Follicular dendritic cells produce IL-1 and CD23 which interacts with the IL-1 receptor and CR2 complex present on the centrocytes leading to their differentiation into plasma cells. High affinity centrocytes present in the light zone generate memory B cells.

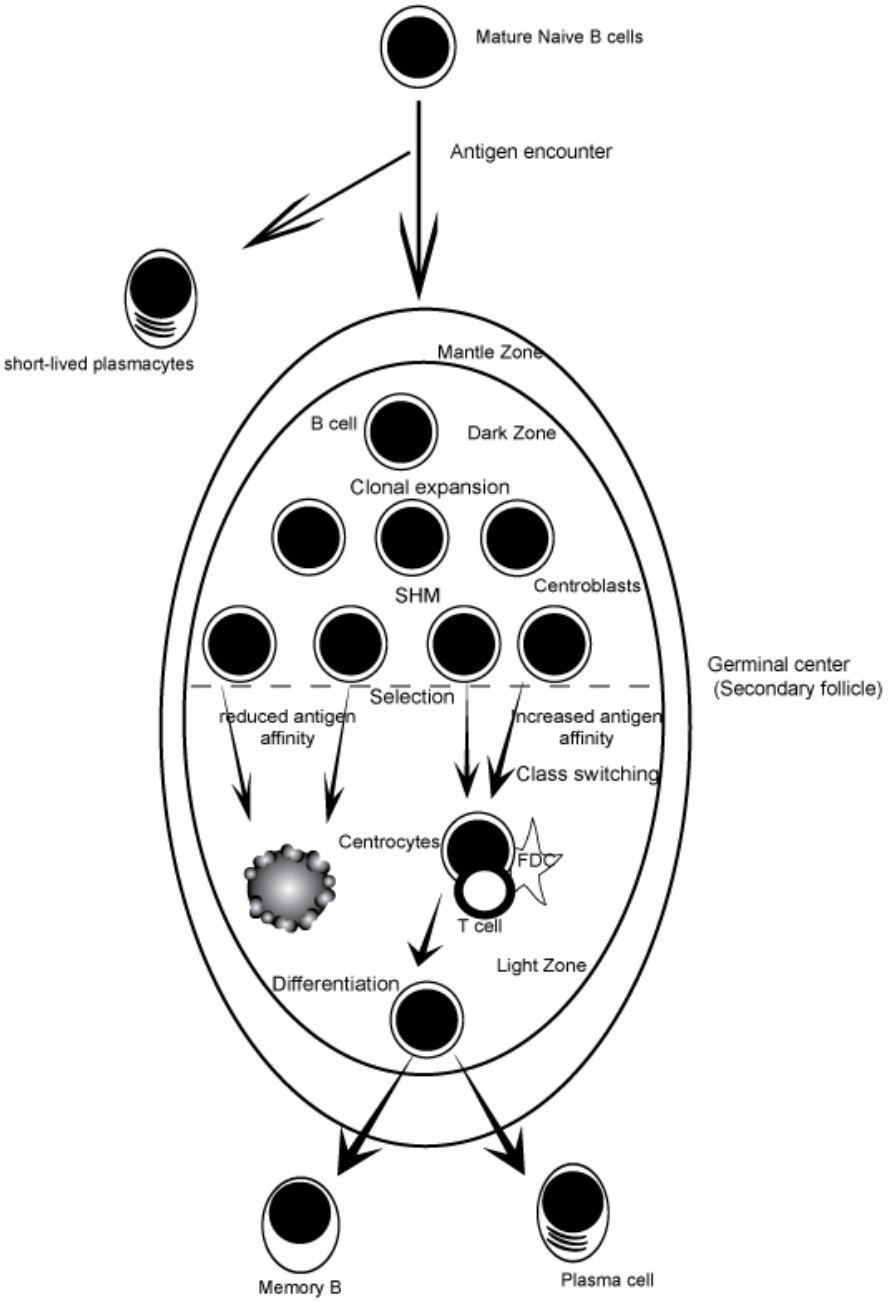


Figure 2: Antigen-Dependent Phase of B Cell Development: Mature naïve B cell encountering an antigen usually establish a secondary follicle or germinal center and undergo clonal expansion, somatic hypermutation and terminal differentiation to form plasma cells or memory B cells. Passage of the B cell through the different stages of development post antigen encounter is depicted.

Transcriptional Regulation of B Cell Development

Transcriptional regulation plays an important role in the B cell development. Different transcriptional factors are required and expressed at the different stages of B cell development.

Transcriptional Regulation in Primary B Cell Development

Early B cell development is controlled by a network of transcriptional factors that are expressed in a hierarchical manner, these factors include PU.1, E2A, EBF and PAX5. Expression of several other factors is restricted to the different stages of early B cell development. Hallmarks of lymphoid commitment in the bone marrow are up regulation of *Flt3* and *IL-7Rα* as well as up-regulation of Rag1 and Rag2.

PU.1: It is an Ets family protein that is expressed in variety of hematopoietic precursors but at different levels. A PU.1 knockout is embryonic lethal (Scott, Simon et al. 1994; McKercher, Torbett et al. 1996) PU.1 plays a role in adult bone marrow lymphopoiesis. PU.1 is absolutely required for normal differentiation of B cells and macrophages. It has been observed that high levels of PU.1 in PU.1 deficient fetal liver progenitor cells generated macrophages whereas lower levels of PU.1 leads to formation of B cells (DeKoter and Singh 2000). PU.1 is not strictly restricted to development of B cells but deficiency of PU.1 leads to a lower frequency and slower kinetics of B cell development compared to cells developed from wild type embryos (Ye, Ermakova et al. 2005). Moreover, Iwasaki et.al. have shown that PU.1 functions primarily to specify lymphoid progenitors and is not required for further B cell differentiation. PU.1 interacts with other proteins to regulate its targets genes. Several B cell specific genes have an Ets binding site and may be regulated by PU.1 (Henderson and

Calame 1998). These genes include genes encoding immunoglobulin heavy chain and light chains, RAG1, Igα, Igβ, Vpre-B, λ5, BTK, bcl-2, TdT, CD19 and J-chain (Fitzsimmons and Hagman 1996; Henderson and Calame 1998). Moreover it is thought to regulate B cell development by regulating EBF and IL7Rα genes (DeKoter, Lee et al. 2002).

Ikaros: It controls the early events important for lymphoid differentiation at the level of multipotent progenitor (Georgopoulos, Bigby et al. 1994). Ikaros can function as transcription activator or as a repressor but it predominantly functions as a repressor (Brown, Guest et al. 1997). Absence of Ikaros leads to failure of expression of Flt3, IL-7R and Rag1 (Yoshida, Ng et al. 2006) and Ikaros knockdown mice completely lack B cells from the earliest detectable stage (Georgopoulos, Bigby et al. 1994). Ikaros can regulate several genes e.g. Rag1, IL-2 receptor, Igα, VpreB, λ5 and TdT. Ikaros and PU.1 can regulate similar target genes suggesting overlapping functions in early lymphoid specification (Nutt and Kee 2007). Additional Ikaros related factor Aiolos has also been identified in B cells. Ikaros and aiolos mRNAs are expressed through out B cell development (Thompson, Cobb et al. 2007).

E2A and EBF1: E2A is required for regulation of expression of many lymphoid genes and induction of early B cell factor (EBF1) (Smith, Gisler et al. 2002). The basic helix-loop-helix proteins E12 and E47 collectively known as E2A are regulators of B cell lineage speciation as well as required for proper formation of common lymphoid progenitors (CLPs) (Bain, Robanus Maandag et al. 1997; Borghesi, Aites et al. 2005). E2A along with EBF1 are essential for early B cell development events such as expression of pre-B cell receptor component. Induction of EBF1 is a critical event in specification of B cell development. Mice that lack expression of EBF1 fail to express several B cell genes such as Igα, Igβ, and do not undergo IgH recombination in the bone marrow (Lin and Grosschedl 1995).

Knockdown of EBF1 leads to arrest of transition of common lymphoid progenitor cells to pre-pro B cell stage (Nutt and Kee 2007). Knockdown of E2A leads to a block in B cell development at the similar stage. Thus E2A along with EBF1 are essential for early B cell development events (Ye and Graf 2007).

PAX5: It is also known as B cell specific activation protein (BSAP) is a transcription factor that is required for the completion of the B cell commitment process. It is expressed throughout the B cell lineage and is down-regulated in the plasma cells (Fuxa and Busslinger 2007). It can positively or negatively regulate transcription depending on the binding protein partners (Cobaleda, Schebesta et al. 2007). PAX5 along with PU.1 can co-recruit groucho proteins for repression of B cell specific gene IgH by binding to the HS1,2 enhancer (Linderson, Eberhard et al. 2004). PAX5 can also promote B cell commitment by repressing expression of genes associated with other cell types such as cell surface receptors MCSF-R and Notch1 associated with macrophage and T cell development (Souabni, Cobaleda et al. 2002). It can also repress Flt3 which can block B cell formation (Holmes, Carotta et al. 2006). PAX5 plays a role in B cell lineage commitment by activating B cell specific genes. PAX5 can activate target genes coding for essential signaling components of pre-BCR and BCR such as Igα and λ5, co-receptor components CD19 and adapter molecule BLNK. Thus absence of PAX5 leads to blocking of B cell development in the early pro-B cell stage. These pro-B cells are not committed to B cell lineage and are capable of differentiating into a broad spectrum of hematopoietic cells (Nutt, Heavey et al. 1999). Additionally, PAX5 can activate other B cell specific genes including SpiB, Aiolos, Id3, Lef1, CIITA, IRF4 and IRF8. PAX5 has also been shown to regulate transcription of E2A and EBF1 thus indicating a feedback loop.

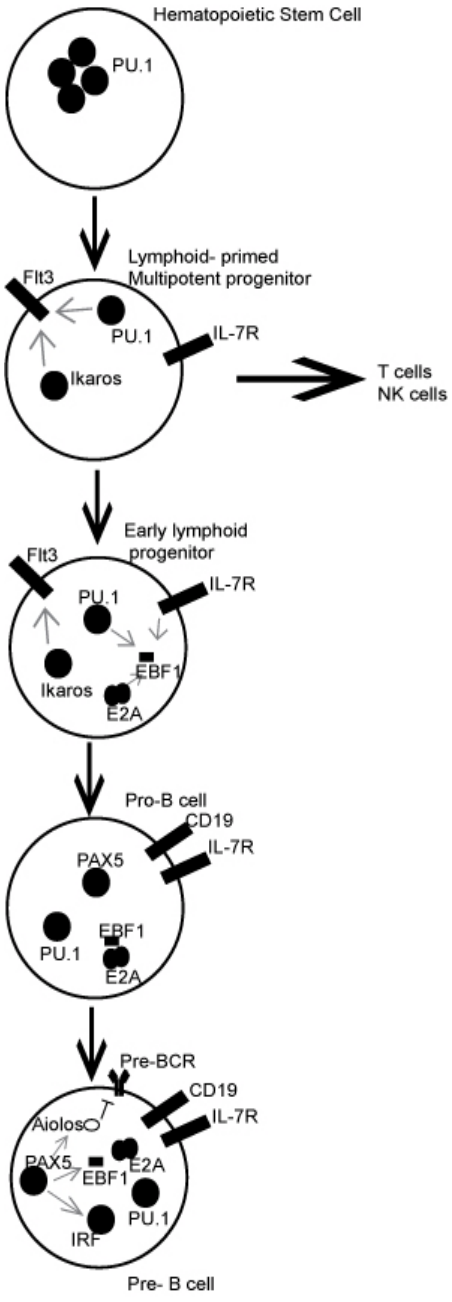


Figure 3: Transcriptional Regulation During B Cell Development: Stages of differentiation from hematopeitic stem cells to committed pre-B cell along with expression of different transcription factors, surface receptors and cell surface markers are shown. A↑ indicates positive regulation and a ⊤ indicates gene repression.

Transcriptional Regulation in Germinal Center B Cell and Plasma Cell Development

The key transcriptional factors that regulate the transition of B cells through the germinal center and eventually into antibody secreting plasma cells include B-cell lymphoma 6 (BCL6), Interferon regulatory factor (IRF), X Box Binding Protein 1 (Xbp-1) and Positive regulatory domain I- binding factor 1 (PRDM1)

BCL6: BCL6 was identified from chromosomal translocation break point in B cell lymphomas. It is highly expressed in germinal center B cells. BCL6 deficient mice lacked formation of germinal center B cells. BCL6 can repress DNA damage sensor ATR in centroblasts resulting in a germinal center B cell phenotype (Ranuncolo, Polo et al. 2007).

IRF: IRF family members that play a role in B cell development are IRF-4 and IRF-8. It is required for the initial proliferative burst of activated B cells which is followed by terminal differentiation (Mittrucker, Matsuyama et al. 1997). IRF-4 is able to bind to IFN-stimulated response elements and repress IFN-stimulated genes on its own (Yamagata, Nishida et al. 1996). Expression and activation of IRF-4 leads to up-regulation of plasma cell markers such as increase in the expression of PRDM1 and XBP-1 and down regulation of germinal center B cell markers such as a decrease in the mRNA levels of BCL6 and PAX5. IRF-4 can interact with PRDM1 and repress the expression of CD23b (Gupta, Anthony et al. 2001). IRF-8 is another member of the IRF family which has been shown to activate PRDM1 in murine myeloid progenitor cells. It is constitutively expressed in macrophages, B cells and dendritic cells (Lu, Medina et al. 2003; Schiavoni, Mattei et al. 2004). IRF-8 is highly expressed in B cells but its expression decreases in the plasma cells. In the GC B cells it has been shown to activate BCL-6 (Lee, Melchers et al. 2006). Therefore the different IRF proteins appear to play important roles in B cell development and differentiation.

Xbp-1: Xbp-1 is a transcriptional activator that belongs to the CREB/ATF family and is required for plasma cell differentiation. Xbp-1 has been shown to be required for proper signaling through the BCR (Hu, Dougan et al. 2009). Moreover, loss of Xbp-1 leads to an almost complete absence of plasma cells and circulating immunoglobulins (Reimold, Iwakoshi et al. 2001). Besides its function in plasma cell differentiation, it also plays an important role in the unfolded protein response (UPR) (Iwakoshi, Lee et al. 2003). Accumulation of misfolded proteins in the ER triggers splicing of Xbp-1 (Xbp-1s), which plays in important role in the secretory process of plasma cells (Iwakoshi, Lee et al. 2003). Xpb-1 has been shown to be directly regulated by PAX5. Repression of PAX5 by PRDM1 has been shown to lead to upregulation of Xbp-1 (Lin, Angelin-Duclos et al. 2002). Moreover, Xbp-1 has been demonstrated to initiate a feedback loop on regulation of IRF4 and PRDM1 (Hu, Dougan et al. 2009).

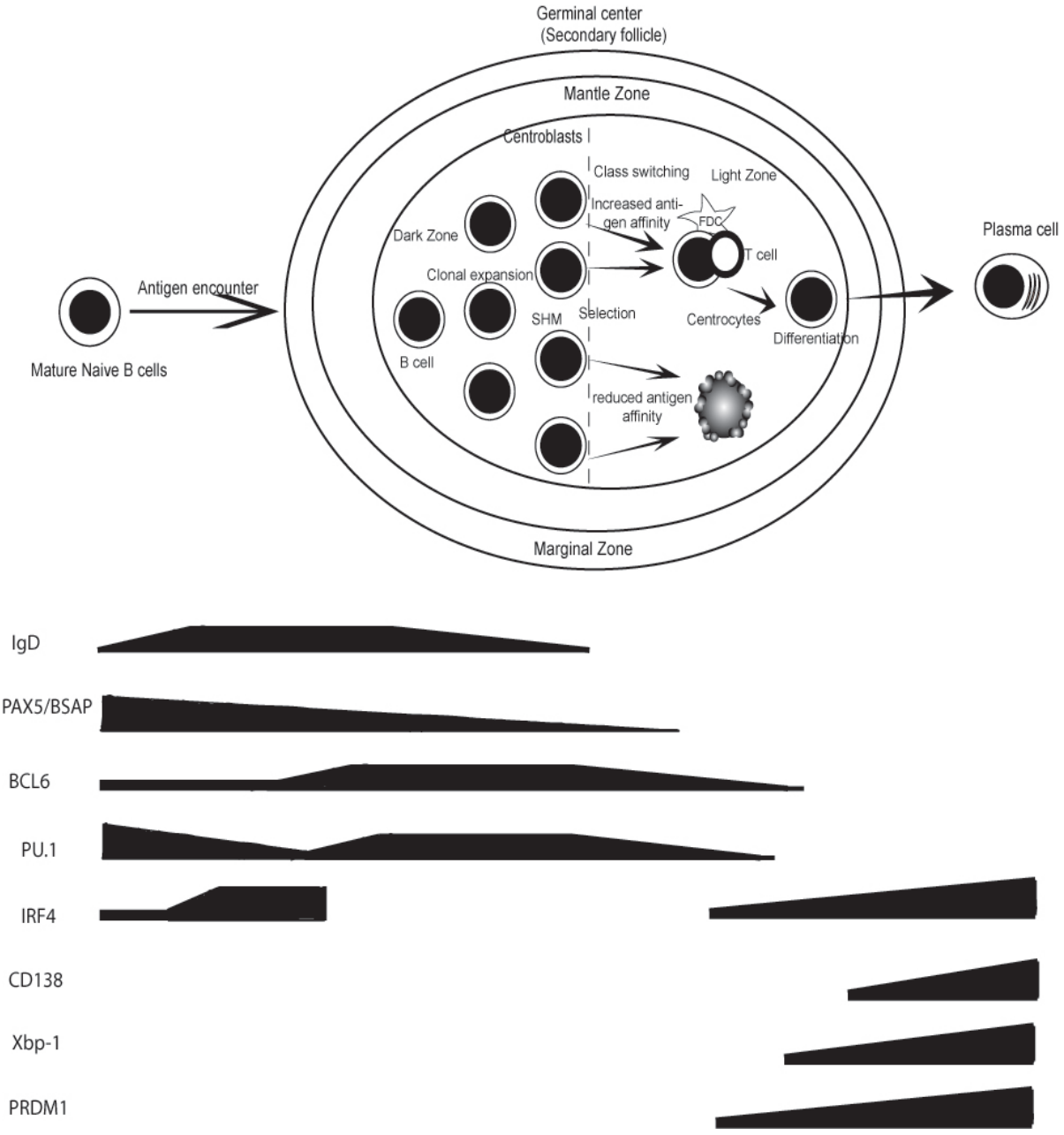


Figure 4: Transcriptional Regulation in Germinal B Cell and Plasma Cell: Changes in expression levels of some key transcription factors and cell surface markers as the B cells progress through the germinal center.

PRDM1

History of PRDM1

PRDM1 was first identified by Maniatis and colleagues about 18 years ago. It was characterized as the protein that can bind to the positive regulatory domain of human Interferon β (INFβ) and was thus named Positive regulatory domain I- binding factor 1 (PRDI-BF1/PRDM1). They have shown that PRDM1 is induced upon virus induction and is involved in post-induction repression of INFβ (Keller and Maniatis 1991). This initial discovery was made in human osteosarcoma cell lines U2OS. Later the mouse homolog B lymphocyte induced mouse protein 1 (Blimp1) was shown to be involved in B cell maturation into antibody secreting plasma cells (Huang 1994; Turner, Mack et al. 1994). The PRDM1 gene located on human chromosome 6q21 (Mock, Liu et al. 1996) encodes for a 789 amino acid protein with a molecular weight of approximately 89KDa (Martins and Calame 2008).

Structure of PRDM1

PRDM1 belongs to the PR domain gene family, which consists of 17 identified members. These proteins are characterized by the presence of a positive regulatory (PR) domain. The PR domain is a derivative of SET domain and may function as protein binding interface in the regulation of chromatin-mediated gene expression (Huang, Shao et al. 1998). The PRDM1 protein also contains five tandemly arranged zinc finger motifs of the Kruppel type (Turner, Mack et al. 1994). The zinc finger domains function as DNA binding motifs in transcriptional regulatory proteins**.** Of these 5 zinc fingers only first two zinc finger motifs are required for recognition and binding to the IFNβ promoter (Keller and Maniatis 1992).

The PRDM1 protein also contains a proline rich region. The proline rich region along with zinc fingers can mediate transcriptional repression by recruiting co-repressors such as groucho proteins and HDAC 1 and 2.

Several PR domain family members express two protein products that differ in the presence or absence of the PR domain. PRDM1 expresses two forms- full length PRDM1α and PRDM1β that lacks the N-terminal acidic region and has a disrupted PR domain (Gyory, Fejer et al. 2003). This alternative form is abundantly expressed in myeloma cell lines. The normal plasma cells have lower transcript levels of PRDM1β as compared to the multiple myeloma population (Gyory, Fejer et al. 2003; Ocana, Gonzalez-Garcia et al. 2006). The β-isoform has been suggested to induce resistance to chemotherapy in DLBCL patients (Liu, Leboeuf et al. 2007). The two isoforms can form heterodimers and potentially modulate the function of the protein.

Figure 5: Structure of PRDM1: Full length PRDM1α and truncated PRDM1β which has an impaired PR domain are shown. PRDM1β is a result of alternate transcription initiation using internal promoter region.

Expression Profile of PRDM1

PRDM1 has been shown to be required for B cell terminal differentiation into plasma cells. It is not expressed in early stages of B cell development. High level of expression of PRDM1 is observed in post-germinal center B cells which include the plasmablast and the long lived plasma cells. Memory B cells do not express PRDM1. Some GC B cells that express PRDM1 in absence of BCL6 have a partial plasma cell phenotype. Thus expression of PRDM1 is important for commitment of GC cells to a plasma cell fate.

Expression of PRDM1 has been detected in immune cells such as T cells, DCs, myeloid cells. PRDM1 has also been detected in other cell types including germ cells and sebaceous gland cells.

Regulation of PRDM1

PRDM1 gene locus is located on chromosome 6q21 covering 63.57kb with 7 exons. It has multiple initiation sites and lacks a TATA element. An alternative transcription site located 5' of exon 4 gives rise to the beta isoform lacking 101 N terminal amino acids (Gyory, Fejer et al. 2003). To prevent B cell differentiation and maintain B cell phenotype several factors repress the expression of PRDM1 mRNA. PAX5 is one such factor, which is required to express several B cell specific genes. PAX5 has been shown to bind to cis element in exon 1 of PRDM1 gene and represses the expression of PRDM1 mRNA leading to repression of plasma cell differentiation (Mora-Lopez, Reales et al. 2007). Another factor targeting the expression of PRDM1 gene is BCL6. BCL6 can repress the PRDM1 transcription by inhibiting the transcriptional activity of AP-1 protein (Vasanwala, Kusam et al. 2002). Moreover, BCL6 can bind to intron 3 of PRDM1 and recruit MTA3 at this site leading to

repression of PRDM1. MTA3 is a cell type- specific subunit of corepressor complex Mi-2/NuRD, which has the same pattern of expression as BCL6 in the germinal center (Fujita, Jaye et al. 2004). Moreover, strong activation of PI3K leads to activation of Akt leading to inhibition of BCL6, which causes derepression of PRDM1 transcription (Omori, Cato et al. 2006). The transcription repressor BTB and CNC homology 2 (Bach2) can also repress the expression of PRDM1 gene. It forms a heterodimer with Mafk and binds to Maf recognition element (MARE) of the promoter at 1.7kb upstream of the PRDM1 gene (Ochiai, Katoh et al. 2006). Moreover, recent observations have identified that binding of BCL6 at the BRE1 region and Bach2 at the MARE region within intron 5 of the PRDM1 gene, ensures repression of the PRDM1 expression (Ochiai, Muto et al. 2008).

PRDM1 expression is activated by several stimuli such as virus infection in U2OS cells (Keller and Maniatis 1991), cellular stress leading to UPR response in myeloid cells (Doody, Stephenson et al. 2006) and polysaccharides that can activate TLR4/TLR2 in B cells can also induce expression of PRDM1 (Lin, Kao et al. 2006). BCR cross linking by anti-IgM can also induce expression of PRDM1 (Desai, Bolick et al. 2009). Moreover, activated NFκB signaling has been shown to be required for induction of PRDM1 in mouse B cells (Morgan, Magnusdottir et al. 2009). Several cytokines can induce expression of PRDM1 in B cells. Davis has shown that treatment of BCL1 cells with IL-2 and IL-5 can induce Blimp1 (Turner, Mack et al. 1994). IL-6 can mimic this effect in the human cells by differentiating the B cells leading to increased Ig secretion and elevated PRDM1 levels (Natkunam, Zhang et al. 1994; Piskurich, Lin et al. 2000) IL-10 induced after activation of BCR and CD40 signals can induce expression of PRDM1. Signaling through BCR and CD40 leads to expression of IL-21 that is a strong inducer of plasmacytic differentiation and PRDM1

mRNA (Ozaki, Spolski et al. 2004; Ettinger, Sims et al. 2005). IL-2, IL-6, IL-10 and IL-21 can activate STAT3 which may activate PRDM1 transcription (Reljic, Wagner et al. 2000; Diehl, Schmidlin et al. 2008). Besides these factors IRF4 has also been shown to directly activate the expression of PRDM1 transcription (Sciammas, Shaffer et al. 2006). Moreover, recently p53 has been shown to form an autoregulatory feedback loop with PRDM1. P53 can bind to and directly activate PRDM1 transcription and PRDM1 in turn has been shown to repress p53 transcription (Yan, Jiang et al. 2007).

Biological Functions of PRDM1

PRDM1 functions as a transcription repressor which was first identified to be involved in immune responses. Immune -related functions of PRDM1 have been well studied and characterized, identifying role of PRDM1 in various immune cells. PRDM1 has been shown to play a role in differentiation of myeloid lineages. PRDM1 is induced in myeloid progenitor cells in response to macrophage- colony stimulating factor (M-CSF), leading to their differentiation into macrophages (Chang, Angelin-Duclos et al. 2000). PRDM1 has also been shown to play a role in T cell differentiation and is highly expressed in antigen-experienced T cells. Loss of PRDM1 expression in T cells leads to altered T cell function and loss of T cell homeostasis resulting in development of severe inflammatory wasting disease because of accumulation of effector and memory T cells (Kallies, Hawkins et al. 2006). PRDM1 may inhibit the T Cell Receptor (TCR) induced proliferation in stimulated naïve T cells. It may achieve this by repressing expression of IL-2 (Wang, van Panhuys et al. 2008). PRDM1 suppresses expression of IL-2 by directly binding to IL-2 promoter as well as the directly inhibiting the IL2 activator Fos, c-Fos (Martins, Cimmino et al. 2008). This

suppression of IL-2 expression is thought to inhibit T cell proliferation and promote T cell apoptosis thus regulating the immune response. Moreover, PRDM1 is highly expressed in the Th2 T helper cells than in Th1 subset and functions in Th2 differentiation by repressing critical Th1 genes, IFNγ, tbx21 and BCL6 (Cimmino, Martins et al. 2008).

The role of PRDM1 in B cells has been most extensively studied. PRDM1 is not required for the initiation of plasma cell differentiation but is essential to complete the differentiation of the pre-plasmablasts into antibody secreting plasma cells (Kallies, Hasbold et al. 2007). PRDM1 promotes B cell terminal differentiation by turning off two classes of genes- genes required for proliferation and growth, and genes involved in mature B cell functions (Shaffer, Lin et al. 2002).

- cMyc: It functions as a critical decision point of cell growth to favor proliferation and to block terminal differentiation. It is present in dividing cells but is not expressed in quiescent or terminally differentiated cells. Blimp1/PRDM1 specifically represses c-myc transcription as part of a program of terminal B cell differentiation (Lin, Wong et al. 1997). It can directly associate with HDACs and repress expression of cmyc by introducing deacetylation of H3. Overexpression of Blimp1/PRDM1 in immature mouse B cells leads to an alteration of cmyc/mad4 mRNA levels and a reduction of anti-apoptotic protein BCL2A1 (Knodel, Kuss et al. 1999). Thus leading to growth suppression.
- PAX5/BSAP: It is important for proliferation and isotype switching in germinal center B cells. PAX5 is critical for B cell identity (Nutt, Heavey et al. 1999). PAX5 is expressed throughout B cell development until terminal differentiated plasma cell stage. PAX5 is required for the rearrangement of V_H gene segments and for expression of genes that are

required for progression to the pre-B cell stage. It is an essential factor for B lineage commitment in the fetal liver. PAX5 can act as an activator or a repressor depending on its interaction with co-repressors or positive regulators (Nutt, Eberhard et al. 2001). PRDM1/Blimp1 binds to a site on the PAX5 promoter and represses the promoter in a binding-site-dependent manner (Lin, Angelin-Duclos et al. 2002). Lin et. al have shown that repression of PAX5 by PRDM1 is sufficient to regulate PAX5 targets CD19 and J chain but is not sufficient to induce Xbp-1 (Lin, Angelin-Duclos et al. 2002). Repression of PAX5 leads to differentiation of splenocytes into IgM producing cells and expression of Xbp-1.

- CIITA: The terminal differentiation of B cells into plasma cells involves repression of CIITA. CIITA is a crucial transactivator of class II MHC genes. The CIITA has four distinct functional features of which the acidic and proline, serine and threonine (PST) regions form a transcription activation domain. The activation domain can induce transcription by interacting with general transcription factors, by enhancing promoter clearance or transcription elongation and may facilitate chromatin remodeling by recruiting histone acetyltransferase CBP. The transcription of CIITA is driven by a large regulatory region that contains four distinct promoters pI, pII, pIII and pIV (Muhlethaler-Mottet, Otten et al. 1997; Reith, LeibundGut-Landmann et al. 2005). The pIII promoter is active in B cells. Factors such as E47, PU.1, IRF4 and IRF8 interact with PIII in B cells (van der Stoep, Quinten et al. 2004). PRDM1 inhibits expression of CIITA PIII and has a binding site overlapping with that of IRF4. Therefore, it could lead to differentiation by inhibition of IRF4 mediated activation of promoter. Thus the terminally differentiated B

cells do not express CIITA leading to silencing of expression of class II MHC genes (Silacci, Mottet et al. 1994).

- SpiB is an Ets family protein that is expressed in lymphoid cells with high levels expressed in B cells (Ray, Bosselut et al. 1992). SpiB and PU.1 can bind to sites identical or similar in sequence. Spi-B is required for proper signaling through the BCR (Garrett-Sinha, Su et al. 1999). Thus repression of Spi-B provides a mechanism by which PRDM1 can attenuate the central functioning of mature B cells. (Messika, Lu et al. 1998).
- ID3: The Id proteins are characterized by the lack of DNA binding domain and the retention of the HLH dimerization domain (Benezra, Davis et al. 1990). The Id proteins can heterodimerize with E proteins preventing them to bind to the DNA and activate gene expression (Jen, Weintraub et al. 1992). Id3 has been shown to be required for antibody isotype switching as well as required for BCR mediated B cell proliferation. The Id3 gene is highly expressed in proliferating cells and is down-regulated in cells undergoing differentiation and in resting cells. Id3 is expressed throughout the B cell development stages except in plasma cells (Meyer, Skogberg et al. 1995). Thus repression of Id3 by PRDM1 can allow for maintenance of plasma cell phenotype.
- BCL6: It is one of the transcription repressors required for GC B cells. PRDM1 is repressed by BCL6 and two BCL6 response elements have been mapped within the PRDM1/Blimp1 gene. There is a reciprocal ability of Blimp1/PRDM1 to repress BCL6 creating a feedback loop that enforces strict control over the B cell fate (Calame 2001).
- Genes required for class switching- Blimp1/PRDM1 down-regulates genes required for class switching such as *AID, Ku70, Ku86I* and *STAT6*. Thus PRDM1 can lead to terminal differentiation of B cells by shutting off immunoglobulin isotype switching by reducing

expression of factors required for recombination and by blocking signals that activate switch region immunoglobulin transcription (Shaffer, Lin et al. 2002). These have not been proven to be direct targets of PRDM1 but their expression levels were down-regulated in cells with overexpression of PRDM1.

Although, PRDM1 has been extensively studied in immune cells, but its role in development and differentiation of other non-immune cells and tissues has also been accepted. The mouse homolog Blimp1 has been shown to play a role in embryonic development. Blimp1 is a key regulator of primordial germ cells specification (Saitou, Payer et al. 2005). Loss of Blimp1 is embryonic lethal with mutant embryos show lack of primordial germ cells. (Vincent, Dunn et al. 2005) In addition these mutant embryos show defects in formation of the brachial arches, the placenta and loss of integrity of blood vessels. PRDM1/Blimp1 has been shown to play a role in regulating differentiation of germ cells (Ohinata, Payer et al. 2005) and the inter-follicular epidermis (Magnusdottir, Kalachikov et al. 2007). Furthermore, by targeting PRDM1/Blimp1 in the late stages of murine gestation other roles of PRDM1/Blimp1 were identified. PRDM1/Blimp1 is required for proper development of the posterior forelimb, caudal pharyngeal arches, heart and sensory vibrissae (Robertson, Charatsi et al. 2007). Additionally, PRDM1/Blimp1 has been shown to play a role in the formation of the sebaceous glands by governing the cellular input to this gland (Horsley, O'Carroll et al. 2006). In humans PRDM1 expression has been detected in breast cancer cells, with higher expression in estrogen receptor α negative breast cancers and in primary breast tumors (Wang, Belguise et al. 2009).

Besides its function in human and mouse development, PRDM1 homolog has shown to play an important role in developmental processes in a zebrafish. PRDM1 is required for

the formation of the slow-twitch muscle fibers by repressing fast-muscle specific genes (Baxendale, Davison et al. 2004; Liew, Choksi et al. 2008). Mutation in the PRDM1 gene leads to defects in specification of neural crest lineages (Roy and Ng 2004). PRDM1 has an essential role in pattern formation and organogenesis. It has been shown to play a role in the development of the photoreceptor cell layer in the eye and in the pectoral fins (Wilm and Solnica-Krezel 2005).

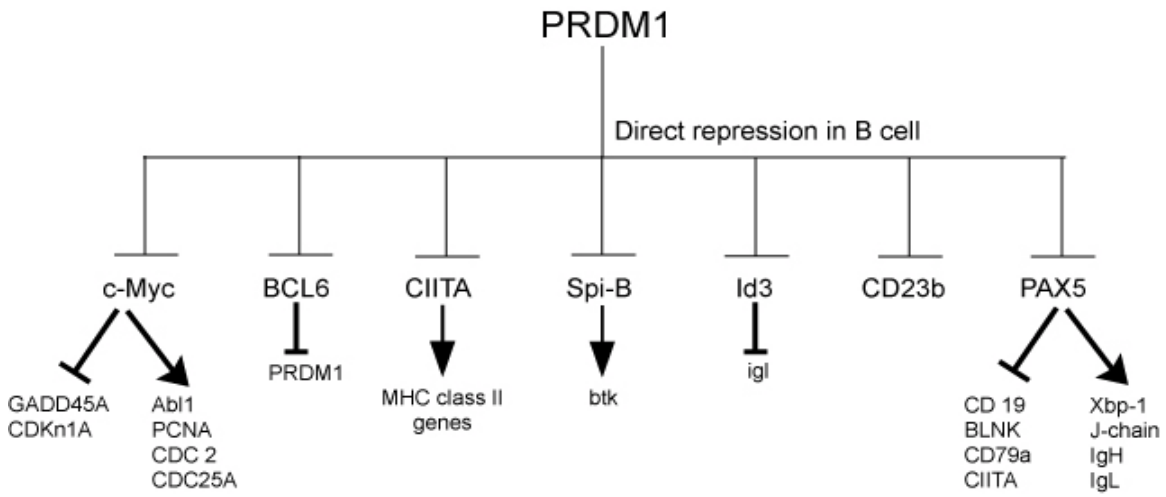


Figure 6: Down-stream Targets of PRDM1 in B Cells: Direct targets of PRDM1 in B cells are shown. Repression of these direct targets by PRDM1 then further affects the expression of additional genes that may play an important role in differentiation of B cells into plasma cells.

PRDM1 a Tumor Suppressor

PRDM1 has the ability to suppress several growth promoting pathways and is thought to play an important role as a tumor suppressor. Several other PRDM family members such as PRDM4, PRDM5 and RIZ exhibit the tumor suppressor property in different types of tumors (Yang and Huang 1999; Canote, Du et al. 2002; Deng and Huang 2004). Thus, indicating that tumor suppressor ability has been conserved throughout the family. The PRDM1 locus 6q21-q22.1 is frequently deleted in several B cell Non-Hodgkins Lymphomas (NHLs) and is associated with high grade lymphomas (Gaidano, Hauptschein et al. 1992). Several inactivating mutations in the PRDM1 gene have been identified in diffuse large B-cell lymphoma (DLBCL) indicating a tumor suppressor role for PRDM1. All the mutations identified in DLBCL lead to deletion or silencing of the paired PRDM1 allele. Mutations such as single-nucleotide changes lead to aberrant splicing and premature translation termination. Other mutations identified include frame shift mutations and chromosomal inversion that generated aberrant transcripts encoding truncated PRDM1 proteins (Pasqualucci, Compagno et al. 2006; Tam, Gomez et al. 2006). Some DLBCLs are also associated with deregulation of BCL6 expression, which in turn may repress the expression of PRDM1 leading to development of high grade lymphoma (Rao, Houldsworth et al. 1998).

Moreover expression of the truncated PRDM1β isoform has been associated with increased chemoresistance in DLBCLs (Liu, Leboeuf et al. 2007). PRDM1β is functionally impaired and has reduced capability of repressing many of the PRDM1 targets (Gyory, Fejer et al. 2003). The multiple myeloma cells which are the malignant counter parts of normal plasma cells, express relatively high levels of PRDM1β isoform (Gyory, Fejer et al. 2003;

Ocana, Gonzalez-Garcia et al. 2006). Thus higher expression of the impaired PRDM1 isoform could indicate reduced tumor suppressor activity in these cells.

In Hodgkin's lymphoma the Hodgkin Reed-Sternberg (HRS) cells have upregulated expression of miRNA that target PRDM1, thus leading to low expression of PRDM1 in these cells (Nie, Gomez et al. 2008). All these observations provide evidence that PRDM1 may act as a tumor suppressor gene in lymphoid malignancies.

Cancer

Cancer comprises a diverse group of diseases which all exhibit certain common characteristics. These characteristics are termed as Hallmarks of cancer. These hallmarks are self-sufficiency of growth signals, resistance to anti-growth signals, evasion of cell death, limitless replication potential, angiogenesis, tissue invasion and metastasis. All these events lead to uncontrolled cellular growth in the body, which eventually result in death.

It is thought that a single cell is sufficient to develop cancer. The process that leads to development of a cancerous cell is a complex multi-step process which may involve both genetic and epigenetic DNA alterations. These alterations can lead to activation of proto-oncogenes and/or inactivation of tumor suppressors.

Lymphoma is a type of cancer involving cells of the immune system- the lymphocytes from the lymphatic system. Based on the United States Cancer Statistics published by CDC for 1999-2005, lymphomas have a high incidence rate placing them in the top 10 cancers in United States. They are broadly divided into Hodgkin's lymphoma and Non- Hodgkin's lymphoma. Hodgkin's lymphoma is marked by the presence of certain type of cells called Reed- Sternberg cells. The Non-Hodgkin's lymphomas are divided into aggressive and indolent type which can be formed from B cells or T cells.

B Cell Lymphomas

The B cell lymphomas can arise from all stages of the B cell development. The malignant B cells are “frozen” at a particular stage of differentiation. Therefore, these cells possess some characteristics of normal B cells from which they are derived. Hallmarks of B cell lymphomas are the reciprocal chromosomal translocations of the immunoglobulin loci and the proto-oncogene. This translocation results in constitutive expression of the oncogene (Kuppers and Dalla-Favera 2001). Translocations result in different break points depending on the stage of the B cells. For example translocations that happen as mistakes during V(D)J recombination in early B cell development in the bone marrow. Translocations that are a by-product of somatic hypermutations, the breakpoints are found within or adjacent to rearranged V(D)J genes. DNA breaks may be introduced during class switch. Somatic hypermutation may cause lymphoma by targeting non-immunoglobulin genes (Kuppers 2005). Genes encoding for BCL6 and CD95 are targets of somatic hypermutations in normal B cells and sometimes these mutations may cause inactivation of these proteins (Pasqualucci, Migliazza et al. 1998; Muschen, Re et al. 2000).

Besides translocation, other transforming events generating B lymphomas are mutations of tumor suppressors, amplifications and virus infections such as Epstein-Barr virus in Burkitt’s lymphoma. In normal B cells BCR signaling provides survival signals, similarly BCR signaling may promote survival of lymphoma cells. Moreover, antigen binding to the BCR may provide signal for survival and proliferation to the lymphoma cells e.g. B-CLL cells can bind to autoantigens and can survive (Borche, Lim et al. 1990). Pathogenesis may also be propagated by the lymphoma microenvironment. Follicular B cell

lymphoma cells express CD40 and can interact with the helper T cells and DCs which may provide survival signals (Dave, Wright et al. 2004).

Most B cell lymphomas are derived from the germinal center or the post germinal center B cells. Some of these lymphomas and their site of origin have been depicted in figure 7.

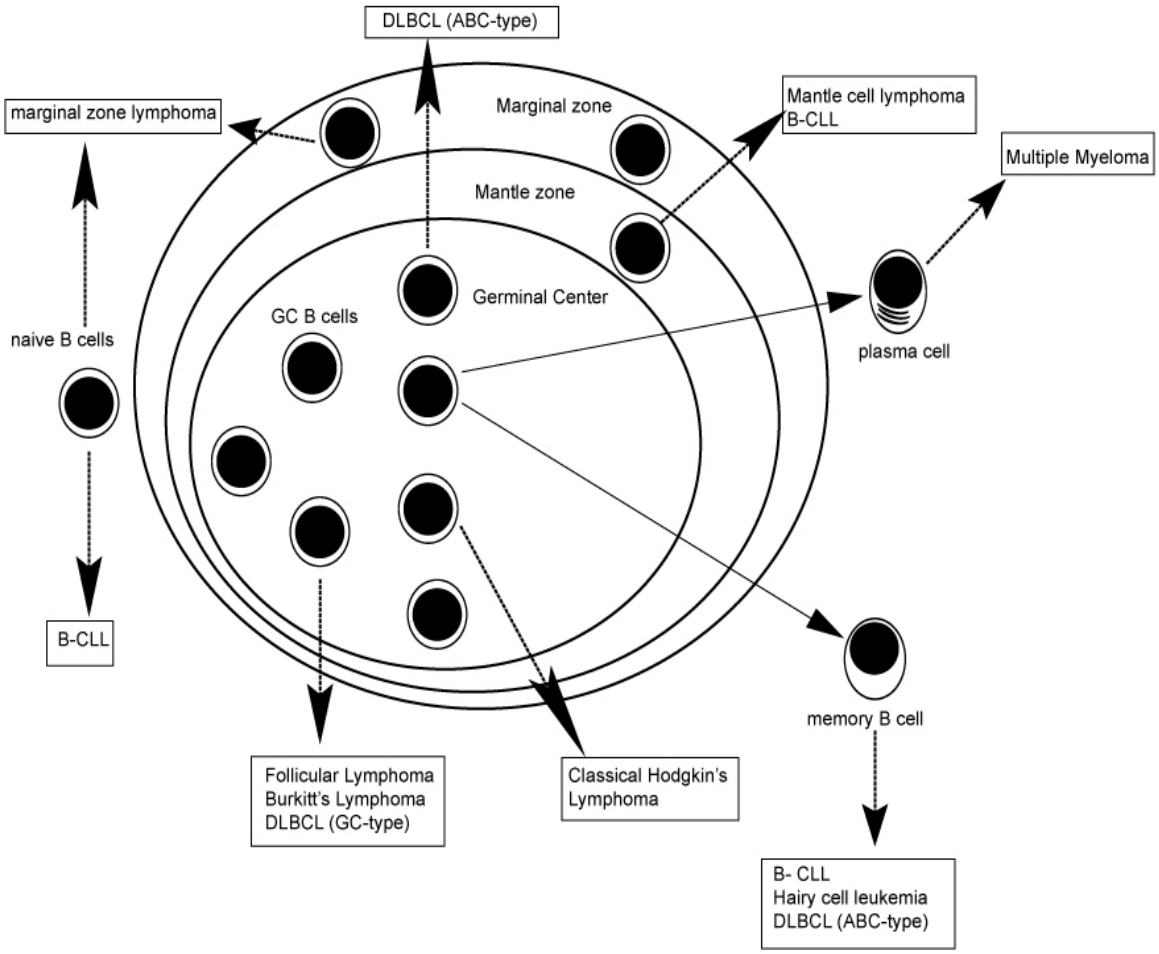


Figure 7: Generation of Lymphomas: B cell lymphomas arise from B cells at different stages of development and differentiation.

Mantle Cell Lymphoma (MCL) is an aggressive form of B cell non-Hodgkin lymphoma which makes up 5%-10% of all human non- Hodgkins lymphomas and is predominant in males with advanced age (Jares, Colomer et al. 2007). It is characterized by a rapid clinical evolution and poor prognosis to current therapies. It involves mature naïve pre-germinal center B cells expressing CD19, CD20, CD22, CD79A, IgM/IgD present in the mantle zone. These cells are usually $CD5^+$ and $CD43^+$ (Brody and Advani 2006). MCLs are generally characterized by chromosomal translocation t(11;14)(q13;q32) which involves the proto-oncogene *CCND1*, coding for cyclin D1, and Ig heavy chain gene. This translocation leads to overexpression of cyclin D1which is not expressed in normal B cells (Aguilera, Bijwaard et al. 1998). This translocation is considered as a primary lesion that could facilitate deregulation of cell-cycle at the G_1-S phase (Campo, Raffeld et al. 1999). Cyclin D1 is cell cycle regulatory protein that promotes cell proliferation by its ability to bind to and activate its associated kinases, CDK4 and CDK6. Cyclin D1-CDK4 and cyclin D1-CDK6 can cause phosphorylation of RB1 leading to inactivation of RB1. This inactivation leads to release and activation of E2F transcription factors allowing the cells to progress into the S phase (Sherr and Roberts 1999). Thus MCL has shown hyperphosphorylation of RB1 leading to deregulation of cell cycle indicating a role for overexpression of cyclin D1 (Jares, Campo et al. 1996). But findings in certain highly proliferative MCL have identified microdeletions of RB1 resulting in truncated mRNA transcripts and total lack of protein, thus indicating that cyclin D1 overexpression may play a role independent of RB1 in tumor generation (Pinyol, Bea et al. 2007). Another mechanism that may be employed by cyclin D1 overexpression to deregulate cell cycle, is by sequestering p27 from CDK4-cyclinD1 complex. This renders p27 incapable of arresting the cells in a G1 cell cycle phase (Quintanilla-Martinez, Davies-

Hill et al. 2003). Moreover, cyclin D1 can affect the cell cycle by regulating several transcription factors and transcriptional coregulators such as STAT3, CEBPβ, BMYB and nuclear receptor superfamily members (Fu, Wang et al. 2004).

A small percentage of MCL patients may not show cyclin D1 and immunoglobulin heavy chain translocation, thus are negative for cyclin D1. But the morphology, phenotype, global expression profile and secondary genetic lesion are undistinguishable from conventional MCL. These patients show high expression of cyclin D2 or cyclin D3, which may deregulate the cell cycle (Rosenwald, Wright et al. 2003).

In addition to cyclin D1 deregulation, MCL is one of the lymphoid malignancies associated with highest chromosomal aberrations leading to losses, gains and high copy number amplifications of certain chromosomal regions, which may play an important role in progression of the disease (Bea, Ribas et al. 1999). It has been shown that transgenic mice that have the cyclin D1 under the control of Ig gene regulatory element require cooperation from the *myc* gene to develop tumors. Highly proliferative MCL have disrupted regulatory pathways ARF-MDM2-p53 and INK4a-CDK4-RB1 (Jares, Colomer et al. 2007). The blastoid variants of MCL are associated with p53 mutations and INK4a/ARF deletion leading to high proliferation (Greiner, Moynihan et al. 1996; Perez-Galan, Roue et al. 2006). The mutations in the *TP53* gene are found in approximately 30% of MCL cases with high proliferative index (Hernandez, Fest et al. 1996). However another mechanism of inactivating p53 is by up-regulating MDM2 and high levels of MDM2 are detected in some of the MCL cases (Hernandez, Bea et al. 2005). Some MCL patients may show overexpression of *BMI1*, a gene of the polycomb group that participates in cell cycle regulation by repressing expression of INK4a/ARF locus. Though *BMI1* alterations are

uncommon in most human neoplasms, but have been shown to particularly contribute to MCL pathogenesis (Bea, Tort et al. 2001). Moreover, the INK4a-CDK4-RB1 pathway may be disrupted by presence of amplification of *CDK4* locus leading to overexpression of *CDK4* in highly proliferative MCL (Hernandez, Bea et al. 2005).

Other secondary lesions in MCL that may lead to development of the disease are deletions in the chromosomal region 11q22.3 which includes the ataxia-telangiectasia mutated (ATM) gene (Schaffner, Idler et al. 2000). The ATM mutations associated with majority of MCL patients affect the PI3K domain or lead to truncated ATM proteins thus causing inactivation of ATM protein (Camacho, Hernandez et al. 2002). Occasionally, MCL patients have germline ATM mutations which lead to the loss of wild-type allele in the tumor cells. This supports the concept that ATM mutations may be a pre-disposing event in the generation of the MCL. Moreover, constitutive activation of NFκB, which can regulate several genes involved in survival as well as apoptosis, has been found in MCL cell line and primary samples (Pham, Tamayo et al. 2003).

Treatment Regime

Chemotherapy: There is no standard treatment regime available to MCL patients. The first line of treatment usually consists of chemotherapeutic agents based on the cyclophosphamide, adriamycin, vincristine and prednisone (CHOP) combination. This regiment has shown complete response rates between 20-80%. Other chemotherapies involve dose intensification of CHOP regime or utilize the intensive regime hyper-CVAD. The hyper-CVAD regime consists of dose intensive hyper-fractionated cyclophosphamide, doxorubicin, vincristine, dexamethasone, alternated with high-dose methotrexate and

cytarabine. This treatment regime has shown a complete response of 38% and a partial response rate of 55.5% in 45 previously untreated or relapsed and refractory MCL patients (Witzig 2005). Since MCL B cells express CD20, the anti-CD20 antibody Rituximab has shown a response rate of 20%-40% as a single agent. Rituximab in combination with CHOP regime is superior to CHOP with an increased complete response rate and improved progression-free survival (Witzig 2005). In younger patients Rituximab in combination of hyper-CVAD is administered.

Stem Cell Transplant: Autologous stem cell transplant is beneficial in MCL earlier in the course of the disease. Myeloablative dose chemotherapy followed by stem cell transplant have shown a mixed response in relapsed and refractory MCL (Brody and Advani 2006). Allogenic stem cell transplant is the only curative option for MCL patients in advanced stage of the disease based on the graft-versus-lymphoma effect observed for other lymphomas. Allogenic stem cell transplant has also been given following non-myeloablative chemotherapy in case of older patients. The results have been mixed, and further study is important.

Radioimmunotherapy: Radioimmunotherapy has shown limited efficacy in MCL patients but high dose myeloablative immunotherapy coupled with monoclonal antibody such as anti-CD20 has shown improved efficacy in pilot experiments (Behr, Griesinger et al. 2002).

New Therapeutic Drugs

Though MCL patients show a complete response or partial response to most of the first line chemotherapeutic agents, but relapse is almost certain resulting in a median disease free survival of 3-4 years (Jares, Colomer et al. 2007). Several treatment strategies targeting different cellular pathways are being developed to improve the prognosis and median disease free survival rate. Some of the new therapies that are in clinical trials are

Cell cycle Inhibitors: Clinical trials are ongoing for inhibitors of CDKs such as flavopiridol which is in phase I clinical trials. The R-roscovitine is in the preclinical stage and has been shown to cause cell cycle arrest and downregulation of cyclin D1 and MCL1 (Brody and Advani 2006).

BCL2 inhibitors: BCL2 family consists of pro-apoptotic and anti-apoptotic proteins. he small molecule obatoclax can mimic BH3-only proteins by binding to multiple anti-apoptotic BCL2 members such as MCL1 and BCL-X_L releasing BAK and inducing apoptosis in MCL. Obatoclax has been used in combination with Bortezomib in phase I clinical trials (Perez-Galan, Roue et al. 2007).

TRAIL activators: TNF-related apoptosis inducing ligand is required for extrinsic apoptotic pathway. TRAIL can trigger apoptosis in MCL through the DR4 receptors. Activators of TRAIL or anti-DR4 can selectively kill lymphoid tumor cells and are a promising therapeutic option for MCL patients (Brody and Advani 2006).

mTOR inhibitors: mTOR plays a central role in multiple growth signaling pathways. Phase II clinical trail with Temsirolimus (CCI-779) as a single agent has achieved an overall response rate of 38% in relapsed MCL patients. The anti-proliferative activity of Temsirolimus is due

to down-regulation of p21 and autophagy, without significant effect on the cyclin D1 expression (Witzig, Geyer et al. 2005).

HDAC inhibitors: Treatment of MCL with HDAC inhibitors used alone or in combination with standard chemotherapy has provided promising results in MCL (Sakajiri, Kumagai et al. 2005; O'Connor, Heaney et al. 2006). HDAC inhibitors have been shown to reduce VEGF production in MCL cells along with induction of growth suppression and apoptosis. Since, overexpression of VEGF is usually associated with poor prognosis in MCL patients, regulating VEGF pathway may prove to be a key therapeutic target (Heider, Kaiser et al. 2006).

Proteasome inhibitors: In 2006 the FDA approved the proteasome inhibitor Bortezomib (PS-341, Velcade) for treatment of relapsed and refractory MCL (Kane, Dagher et al. 2007). Despite high response rates to the front-line regimens, most patients relapse, often demonstrating acquired chemoresistance and have a short duration of response to conventional chemotherapy (Goy, Bernstein et al. 2009). Bortezomib treatment has shown substantial activity in relapsed and refractory MCL patients. Bortezomib is a boronic acid dipeptide that binds reversibly to the chymotrypsin–like site in the 20S core of the 26S proteasome (Adams and Kauffman 2004).

Proteasome 26S is a central protease in the ATP and Ub- dependent pathway (Hochstrasser 1996; Voges, Zwickl et al. 1999). The 26S proteasome consists of a 20S core proteolytic subunit capped at both ends by 19S regulatory subunit. The 20S core functions as the catalytic center of the proteasome, allowing for cleaving of peptides on the carboxyl side of hydrophobic, basic, and acidic amino acids *in vitro* that have been designated as chymotrypsin-like, trypsin-like, and PGPH activities, respectively (Chouduri, Tokumoto et

al. 2008). Thus, inhibition of proteasome activity by Bortezomib can alter multiple signaling pathways and bring about cytotoxicity. These pathways are further discussed in detail in chapter four of this dissertation.

Overall treatment with Bortezomib is highly active in MCL patients with an overall response rate of 31%. It has shown efficacy even in MCL patients, who have relapsed following high- intensity therapy (Goy, Bernstein et al. 2009). Moreover, several studies have shown efficacy of Bortezomib in combination with other therapeutic agents such as BH3 mimetic GX15-070 (Perez-Galan, Roue et al. 2007) and HDAC inhibitor SAHA (Heider, von Metzler et al. 2008).

Other ongoing studies, are investigating combination therapies with Bortezomib and standard agents (based on CHOP combination or hyper-CVAD), in relapsed/refractory and previously untreated MCL, have shown some promising results. Thus, these studies indicate that novel therapies for relapsed/refractory MCL may be able to improve the disease outcome.

Gene Regulation:

Gene regulation takes place at many levels, by controlling transcription of DNA into RNA or by controlling translation of the RNA into protein. Primary step of gene regulation is thought to be at the level of transcription. Transcription regulation can take place at the initiation step requiring the recruitment of the DNA– binding transcription factors along with the transcriptional machinery. Transcriptional regulation may also occur at level of transcript elongation or alternation in splicing and stability of mRNA. Transcription initiation is regulated by the binding of regulatory proteins to the DNA. The basal regulatory proteins such as TFII-D recognize specific DNA sequences that lie near the promoter of the gene called the promoter-proximal elements (Gill 2001). Specific transcription factors form another group of regulatory proteins that can bind to promoters and regulatory sequences that are located away from the gene promoters. Transcription factors regulate gene transcription by modification of the chromatin structure around the gene regulatory elements. Transcription initiation and elongation are both associated with specific histone modifications causing chromatin remodeling leading to activation or repression of the gene (Jenuwein and Allis 2001; Sims, Belotserkovskaya et al. 2004). Methylation of histone H3 on lysine residue 9 (K9) or K27 and methylation of histone H4 on K20 or K59 are associated with gene silencing. Trimethylation of K4 residue or K36 residue on histone H3 is linked with gene activation (Schubeler, MacAlpine et al. 2004; Martin and Zhang 2005; Li, Carey et al. 2007). Acetylation of the core histones catalyzed by histone acetyltransferases (HATs) including Gcn5, TAF1 and p300/CBP is associated with transcription activation (Sterner and Berger 2000; Roth, Denu et al. 2001). Acetylation of histone H3 lysine 9 and 14 residues is present at promoters of actively transcribed genes (Liang, Lin et al. 2004; Schubeler,

MacAlpine et al. 2004). The ability of the transcription factors to introduce the chromatin changes is controlled by their interaction with other proteins which function as co-activators or co-repressors (Xu, Glass et al. 1999).

Recent genome-wide study of H3K4me3 in T cells identifies open chromatin structure being associated with active transcription or genes which are induced rapidly (Roh, Cuddapah et al. 2006). Interestingly, Guenther et.al. have shown that genome wide analysis of H3K4me3 in human embryonic cells is associated with promoters for more than half of transcriptionally inactive genes and not only limited to the genes poised for activation. They have further shown that these genes experience transcription initiation but show no evidence of elongation, suggesting transcription regulation predominantly at the post-initiation step (Guenther, Levine et al. 2007).

Thus gene regulation at the level of transcription takes place by several different methods which all lead to the controlled and regulated expression of the gene.

CHAPTER TWO

MATERIALS AND METHODS

Cell Lines, Primary Cells and Reagents

The CA-46 EBV- negative Burkitt's lymphoma cell line, RPMI-8226 multiple myeloma cell line, Mino and Jeko-1 mantle cell lines were maintained in RPMI medium. U2OS sarcoma cell line was maintained in DMEM medium. Both these mediums were (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin (Invitrogen). Goat anti-human IgM Ab (Southern Biotechnology) was used at 10µg/ml, Actinomycin D (Sigma-Aldrich) was used at 10µg/ml. Bortezomib (LC Laboratories) was resuspended in mannitol to make a 10mM stock solution.

Fresh primary MCL samples were obtained from MCL patients at H. Lee Moffitt Cancer Center. These cells were maintained in 50% stromal conditioned medium in RPMI. Stromal conditioned medium was collected after culturing HS5 stromal cells for 3 days in complete RPMI.

Flow Cytometry Assays

Annexin V staining: Cells were washed with 1X PBS followed by 1 wash in 1X Annexin V buffer. Cells were then suspended in 1X Annexin V buffer at a concentration of $1X10^5$cells/ 200µl and incubated with 5µl of Annexin V-PE (BD Biosciences) in dark at room

temperature for 15 minutes. 300µl of Annexin V buffer was added to make the total volume to 500µl. 10µl of 3mM ToPro3 was added just before analyzing the samples. Annexin V-PE was read at FL2 and ToPro3 was read at FL4 lasers.

B Cell Isolation

B cells were isolated from healthy human donors. Briefly, PBMCs were isolated by Ficoll separation and incubated with anti-CD19 microbeads (Miltenyi Biotec) followed by magnetic separation using MS columns. The purified B cells were routinely >90% B cells as confirmed by flowcytometry analysis for CD20- FITC (Miltenyi Biotec). Isolated B cells were activated by co-culturing with irradiated CD40L-expressing L cells (ref) in presence of cytokines IL-2 (20 U/ml), IL-4(50ng/ml), IL-10 (50ng/ml) and IL-12(2ng/ml) for 4 days. The activated B cells were gently detached from the CD40L cells and washed three times with complete medium. The cells were then divided into two flasks, contents of one stimulated with anti-IgM and those of the other unstimulated for 24 hours.

Quantitative PCR

RNA was isolated from cells using TriZol reagent (Invitrogen) and the manufacturer's protocol. One µg RNA was DNase-treated using RQ1 DNase (Promega), followed by first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). $1/20^{th}$ of the final cDNA reaction volume was used in each PCR reaction. Primers used are listed in table 1. Real-time PCR reactions were performed in a volume of 25 µl, which included 200 nM of each forward and reverse primer and iQ SYBR Green Supermix (Bio-Rad) or PerfeCTa SYBR Green Supermix for iQ (Quanta). Reactions were run in duplicate using an iCycler

and iQ software Version 1.0 (Bio-Rad). Average threshold cycles (Ct) for the genes of interest were normalized to the average GAPDH Ct values or β Actin Ct values for each cDNA sample and relative levels of the genes of interest were calculated by the ΔΔCt method (Livak and Schmittgen 2001): for example, $2^{-(PRDM1\text{-}GAPDH)}$ or $2^{-(PRDM1\text{-}\beta\ Actin)}$ for PRDM1 gene expression. Patient samples were normalized to GUS B.

Table 1: Expression Primers for real-time PCR

Primer	Forward primer Sequence	Reverse primer Sequence	Annealing Temperature ºC
GAPDH	5'-GAAGGTGAAGGTCGGAGT -3'	5'-GAAGATGGTGATGGGATTTC -3'	60
PAX5	5'-TGG AGG ATC CAA ACC AAA GG-3'	5'-GGC AAA CAT GGT GGG ATT TT-3'	55
MKI67	5'-GTCCAGACAGACCCTCTTCG-3'	5'-CCACCATCCAGACACACAAG-3'	55
PCNA	5'-GAGATGCTGTTGTAATTTCCTGTG-3'	5'-TCTATGGTAACAGCTTCCTCCTCT-3'	60
NOXA	5'-GCTGGAAGTCGAGTGTGCTA-3'	5'-CCTGAGCAGAAGAGTTTGGA-3'	55
Caspase8	5'-TCACAGCATTAGGGACAGGA-3'	5'-AGTCATCGTGGGGCTTG-3'	55
Caspase 9	5'-GAG AGT TTG AGG GGA AAT GC-3'	5'-AGT CGA TGT TGG AGC CAG T-3'	55
PRDM1 (PRDI-BF1) set1	5'-TACATACCAAAGGGCACACG -3'	5'-TGAAGCTCCCCTCTGGAATA -3'	60
PRDM1 (PRDI-BF1) set2	5'-GGACATGGAGGATGCGGATAT-3'	5'-GTTGCTTTTCTCTTCATTAAAGCCG -3'	55
PU.1	5'-AGC AGA TGC ACG TCC TCG ATA.-3'	5'-AGA CCT GGT GGC CAA GAC TG-3'	55

Quality control was carried out with each primer set which consisted of melt curve detecting a single product and efficiency between 90%-110%. Beta-actin, GUS B and cmyc primers were obtained from realtimeprimers.com.

Nascent RNA Isolation: Nascent RNA was isolated as previously described (Wuarin and Schibler 1994; Kuchtey, Pennini et al. 2003). Nuclei from cells was isolated in a RNase-free buffer comprised of 140 mM NaCl, 1.5mM $MgCl_2$, 0.5% NP-40, 1000 U/ml RNaseOUT (Invitrogen), 1mM DTT, and 50mM Tris, pH 8. Extracted nuclei were washed in this buffer three times to remove cytoplasmic RNA, followed by lysis in a RNase-free buffer containing 300mM NaCl, 1 M urea, 1% NP-40, 7.5mM $MgCl_2$, 0.5mM EDTA, 1mM DTT, and 20mM HEPES, pH 7.6, to isolate histone-bound chromatin. RNA isolation was done as described above. Nascent RNA levels were measured by real-time PCR.

DNA Constructs: *MKI67:* The full length MKI67 luciferase promoter construct consists of a 2067bp proximal and a 720bp distal promoter region. The distal region was PCR cloned into PCR2.1 at the EcoRI sites using the MKI67 distal primer set (Table 2). This region was then blunt end cloned into the SmaI site in the pGL3 basic. The proximal promoter region was also PCR cloned into the PCR2.1 plasmid at the EcoRI sites using the MKI67 proximal primer set (Table 2). A clone carrying the reverse MKI67 proximal construct was used. This region was then cloned into the XhoI and HindIII sites of pGL3 basic carrying MKI67 distal promoter region. This clone was the full length MKI67 luciferase promoter construct. Similarly, the MKI67 proximal only construct was cloned where the proximal region sub-cloned in the PCR2.1 was transferred into the XhoI –HindIII into pGL3 basic.

PCNA: PCNA luciferase promoter construct consists of a 720 base pair region which was PCR cloned into PCR2.1 using the PCNA primer set (Table 2). This region was cut out of PCR2.1 and cloned into pGL3 basic at the SmaI –KpnI site.

PRDM1 TAPTAG: pRAV plasmid provided by Dr Liu contains the TAPTAG comprising protein A binding domain and flag tag which are separated by TEV cleavage sites. PRDM1α was first cloned into the pRAV vector. Long 3'UTR -PRDM1α was cut from pcDNA3.1 using EcoRI. This was gel purified and ligated to pRAV which was cut with MfeI. This led to generation of pRAV containing PRDM1α. The TAPTAG PRDM1α was then moved into the adenovirus shuttle vector Adt-GFP-IRES1. This was done to be able to infect B cells. TAPTAG PRDM1α was cut from pRAV using BamHI and ligated to the shuttle vector which was cut open with BglII. This generated the TAPTAG-PRDM1α in the shuttle vector. Stop codons were used from the shuttle vector. This shuttle vector was then used to generate the adenovirus expressing TAPTAG-PRDM1α.

Table 2: PCR cloning primers

Primer	Forward primer Sequence	Reverse primer Sequence
MKI67 distal	5'-CAGGAAGTGAATGAGTTGTGTTC-3'	5'-GTTCTTTCAGACTTCCGTA-3'
MKI67 proximal	5'-CCATGAGGGTGGAGGAACTG-3'	5'-TGGCCCTACAGGCTACGTC-3'
PCNA	5'-CGGAATGAGTGCATTTTTGA-3'	5'-CTAGCTGGTTTCGGCTTCAG-3'

Transfections and Luciferase Assays in CA-46 and RPMI-8226: Cells were transfected by electroporation using the Gene Pulser II (Bio-Rad, Hercules, CA). Cells (1×10^7) were pulsed with 250 V at a capacitance of 1070 µF. Transfections for luciferase assays were

done with 10 µg of luciferase reporter construct and 50 ng of the internal control plasmid pRL-TK. Firefly luciferase activity was normalized to Renilla luciferase activity in all experiments. For siRNA knock-down experiments in CA46, cells were transfected with 3 µg control non targeting siRNA or Pu.1 siRNA (Dharmacon) for 24 hours. Live cells were separated using a Ficoll gradient and treated with 10 µg anti-IgM for 24 hours. For siRNA knock-down experiments in RPMI8226, cells were transfected with 2µg control non-targeting siRNA or Pu.1 siRNA (Dharmacon) for 48 hours.

MKI67 and PCNA Luciferase Assays in U2OS and Mino: U2OS cells were transfected using FuGENE transfection reagent (Roche). Transfections for luciferase assays were done with 200ng of luciferase reporter, 200ng of PRDM1α plasmid or control pCDNA and 10 ng of the internal control plasmid pRL-TK. Cells were placed in complete medium for 42-48 hours and harvested for luciferase activity per the Dual-Luciferase Reporter Assay System protocol (Promega). Luciferase readings were done using the $20/20^{n}$ luminometer (Turner Biosystems). Firefly luciferase activity was normalized to Renilla luciferase activity in all experiments.

Mino, MCL cell line, was transfected by electroporation using the Gene Pulser II (Bio-Rad, Hercules, CA). Cells (1×10^{7}) were pulsed with 250 V at a capacitance of 1070 µF. Transfections for luciferase assays were done with 1µg of luciferase reporter construct; 10µg of PRDM1α plasmid or control pCDNA and 100 ng of the internal control plasmid pRL-TK. Cells were placed in complete medium for 42-48 hours and harvested for luciferase activity per the Dual-Luciferase Reporter Assay System protocol (Promega). Luciferase

readings were done using the $20/20^n$ luminometer (Turner Biosystems). Firefly luciferase activity was normalized to Renilla luciferase activity in all experiments.

siRNA Knockdown in Mino: All siRNAs were purchased from Dharmacon in the Acell modified formulation. Two PRDM1 siRNAs were confirmed to be active and selective and in most experiments were used in an equal mixture of 500nM each. Delivery of the siRNAs was carried out per manufacturer's protocol. Mino, MCL cells were incubated with the siRNAs at a density of $1X10^5$ cells/ml Accell siRNA delivery medium for 24 hours followed by addition of 5nM of Bortezomib for 20 hours in presence of 2% FBS. 500µl of 15nM Bortezomib diluted in Accell siRNA delivery medium was added to the cells to make the final drug concentration of 5nM. A non-targeting siRNA (Dharmacon) was used as a control in all experiments.

Chromatin Immunoprecipitation (ChIP): ChIP experiments in CA-46: After 24 hours treatment with anti-IgM or control (no treatment) cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature and the reaction was stopped by the addition of glycine to a final concentration of 0.125M. Cells were washed twice with ice cold PBS and resuspended in ice cold TX-100/NP40 buffer (10mM Tris pH 8.1, 10mM EDTA, 0.5M EGTA, 0.25% TX-100, 0.5% NP40, 1mM PMSF, 0.5x Protease inhibitors) at a density of $4x10^6$ cells/ml. Cells were resuspended in 10ml ice cold Salt-wash buffer (10mM Tris pH 8.1, 1mM EDTA, 0.5M EGTA, 200mM NaCl,1mM PMSF, 0.5x Protease inhibitors) and incubated for 10 minutes at 4 ^oC. Cells were lysed by adding sonication buffer (10mM Tris pH8.1, 1mM EDTA, 0.5M EGTA, 1% SDS, 1mM PMSF, 1x Protease inhibitors) at a cell

density of $1x10^6$ cells/30µl. Lysate was sonicated using a water bath sonicator (Diagenode). Chromatin immunoprecipitation was carried out using $2x10^6$ cells and 5µg of specific antibody (IgG Upstate, Pu.1 Santa Cruz, TLE4 Santa Cruz). Immunoprecipitated chromatin was washed sequentially with low salt wash (20mM Tris pH8.1, 2mM EDTA, 150mM NaCl, 0.1% SDS, 1% tritonX 100), high salt wash (20mM Tris pH8.1, 2mM EDTA, 500mM NaCl, 0.1% SDS, 1% tritonX 100) and LiCl wash (10mM Tris pH8.1, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholic acid, 1mM EDTA). DNA was eluted with elution buffer (10mM Tris pH8, 1% SDS, 1mM EDTA) and crosslinks were reversed by incubating with 312 mM NaCl at 65 ^oC for 4 hours. The immunoprecipitated DNA was treated with RNase (Ambion) at 37 ^oC and proteinase K (Roche) for 1 hour at 45 ^oC. The DNA was purified with Qiagen PCR spin columns. Purified DNA was analyzed by quantitative PCR using following primers PRD_Pu.1 binding site, proximal PRDM1. Primers to the HLA-DRA gene were used as a negative promoter. All the primer sets are summarized in table 3.

ChIP experiments in MINO were carried out using similar protocol. After 20 hours treatment with 5nM Bortezomib or control (mannitol) cells were harvested. Chromatin immunoprecipitation was carried out using $6x10^6$ cells and 1µg of specific antibody per immunoprecipitation (non-specific rabbit IgG, Upstate; PRDM1, Cell Signaling; acetylated-H3K9, Diagenode; dimethyl-H3K9, Diagenode). The immunoprecipitated DNA was treated with RNase (Ambion) at 37 ^oC and proteinase K (Roche) for 1 hour at 45 ^oC. The DNA was purified with Qiagen PCR spin columns. Purified DNA was analyzed by real-time PCR using following ChIP specific primers MKI67, PCNA , CIITA P3 , PAX5 and DR alpha was used as a negative promoter. All the primer sets have been summarized in table 3.

Table 3: ChIP Primers for real-time PCR

Primer	Forward primer Sequence	Reverse primer Sequence	Annealing Temperature ºC
PRD_Pu.1 binding site	5'-ACTCACCAGCAGTTGCATGA -3'	5'-CAGTCTCACTTGCAGATGTTAAAGA -3'	60
proximal PRDM1	5'-AGGACCAGACAGCTCCACTG -3'	5'-GCTCAAATCCCCAGGTACAA -3'	60
CIITA P3	5'-TCC CAA CTG GTG ACT GGT TA-3'	5'-CAA GGA TGC CTT CGG ATG-3'	60
PAX5	5'-GAT TTG GGC GAG AAC AGG AC-3'	5'-GAA GGC ACC GTG AAA TGA TTA-3	55
MKI67	5'-TACGGAAGTCTGGAAGGAAC- 3'	5'-CTGGGTTTACAGGCGTGA-3'	55
PCNA	5' –AGGACCAGACAGCTCCACTG-3'	5'-GCTCAAATCCCCAGGTACAA-3'	55
HLA -DRA	5'-GATCTCTTGTGTCCTGGACCCTTTGCAAGAACCCT-3'	5'-CCCAATTACTCTTTGGCCAATCAGAAAAATATTTTG-3'	60

Quality control was carried out with each primer set which consisted of melt curve detecting a single product and efficiency between 90%-110%.

TAPTAG Protein Purification:

U2OS cells were infected with TAPTAG –PRDM1α expressing adenovirus or the TAPTAG-GFP adenovirus. Cells were harvested after 48 hours. Cells were lysed in TAPTAG lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 15% glycerol, 1mm PMSF, 1X protease inhibitors) at a density of 1X 10^6/100µL. 75µl IgG Sepharose 6 fast flow beads (GE healthcare) were added to Micro Bio-Spin Chromatography Column (Bio-Rad) and wash twice with TAPTAG lysis buffer (200µl). Beads were pre-blocked with 30µl GFP

lysate and 470µl TAPTAG lysis buffer. Then incubated at 4ºC for 10-20 minutes. The liquid was allowed to pass through the micro-bio spin 6 columns (Bio-rad) by gravity or centrifuged the column at 2000rpm for 20 seconds. The bottom of the column was capped and the beads were resuspended in 400µl lysis buffer and 100µl PRDM1 TAPTAG lysate. Incubated at 4ºC for 2 hours. The columns are centrifuged at 2000rpm for 20 seconds. The beads are then washed twice with 200µl IPP150 (25mM tris-Cl pH 8.0, 150mM NaCl, 0.1% NP40). This is followed by one wash in 200µl of TEV cleavage buffer (IPP150, 0.5mM EDTA, 1mM DTT). Beads were resuspended in 200µl TEV cleavage buffer and 30 units of TEV enzyme (Promega). They were incubated at 4ºC overnight and then centrifuged at 2000rpm for 20 seconds and collect the supernatant. Beads were then washed twice with 200µl of IPP150. These washes were combined with the supernatant. This combined supernatant was then applied to the prepared FLAG M2 resin. FLAG M2 resin was prepared first by rinsing a fresh column with 200µl of IPP150. To this column 60µl of M2 resin was applied. The pipette tip was rinsed with IPP150 and the rinse was applied to the column. The column was allowed to drain by gravity. The resin was then washed twice with 200µl 0.1M glycine HCl pH3.5. The resin was then washed four times with 300µl each of IPP150 (no DTT). To this prepared resin the supernatant collected after TEV cleavage was added. The resin was incubated at 4ºC for 1 hour. Resin was washed twice with 200µl IPP150 (no DTT). The tagged PRDM1 was then eluted with 60µl of 1mg/ml Flag peptide (Chemistry dept USF) incubated at 4ºC for 30 minutes.

FLAG Protein Complex Purification and Immunoprecipitation

CA46 were transfected with PRDM1α-FHH (triple tagged FLAG-HA-HIS) or control pCDNA 2.1 plasmid. Protein lysates were prepared by washing the cells with PBS and lysing in PBS containing 0.1% NP40, 10% glycerol and protease inhibitor Complete (Roche). 1ml of lysis buffer was used for $1X10^6$ cells. Lysates were incubated on ice for 20 minutes and sonicated in the water bath for 8 minutes.

Immunoprecipitation using FLAG resin: 500µl of the lysate was used for protein purification. 40µl of ANTI-FLAG M2 affinity gel (Sigma) was used. The gel was first prepared by washing 40µl (2:1) resin twice with 0.5ml TBS. 500µl of the lysate was added to the washed gel. Samples were incubated on a shaker at 4ºC for 2 hours. The resin was then centrifuged for 30 seconds at 5000-8000rpm. Supernatant was removed. The resin was washed three times with 0.5ml TBS. This was followed by elution of the bound protein with 100µl of 1mg/ml of 3X FLAG peptide (synthesized by USF chemistry dept) for 30 minutes at 4ºC.

Immunoprecipitation using PRDM1 antibody: 500µl of the lysate was used for the protein purification. 100µl of protein A/G beads (1:1) were first prepared by washing twice using 0.5ml PBS. 500µl of the lysate was added to the washed beads. Samples were incubated overnight on a shaker at 4ºC. Antibodies against PRDM1 (cell signaling) or Reptin52 (BD biosciences) were added and incubated at 4ºC for 2 hours. The samples were then centrifuged for 30 seconds at 5000-8000rpm. The beads were then washed twice with 1X PBS. 60µl of loading dye was added to the washed beads.

Immunoblot: Cells were harvested and lysed in RIPA buffer at $1X10^5$/20µl concentration. Lysates were incubated on ice for 20 minutes followed by sonication in water bath sonicator for 8 minutes. The lysates were then centrifuged at 13,000 rpm at 4ºC for 10 minutes. 20µl of the lysate were used per well. The protein samples were first run through a stacking gel for 30 minutes at 80V followed by separation with an 8% SDS/ polyacrylamide gel run at 100V for approximately 90 minutes. The separated samples were then transferred to a PVDF membrane, which had been soaked in 100% methanol followed by a rinse in distilled water. Before setting up the transfer the gel as well as the methanol soaked PVDF membrane were soaked in transfer buffer () for 10 minutes. Transfer was carried out using a ice pack at 350mA for 65 minutes with constant stirring. The membrane was then blocked using 5% non-fat milk (Carnation) for one hour at room temperature. The membranes were then incubated overnight with primary antibodies at 4ºC followed by 3 (0.05%) PBST washes. The primary antibodies used were PRDM1 (1:1000) (cell signaling), PU.1 (1:500) (Santa Cruz), Reptin52 (1:500) (BD biosciences), β Actin (1:10,000) (Sigma). The washed membrane was then incubated with secondary antibodies for 2 hours at room temperature. The secondary antibodies used were anti-Rabbit-IgG and anti-Mouse-IgG (Amersham).

Immunoflourescence Staining: $1X10^5$ Mino cells treated with either 5nM Bortezomib for 20 hours or mannitol were spun down onto Shandon Cytoslides .(Thermo Scientific) The cells were fixed by treating with 4% formaldehyde for 15 minutes at room temperature. Fixation for PCNA staining was carried out by fixing with 100% methanol for 5 minutes at -20°C. This was followed by two washes in PBS. The cells were then permeabilized with 0.1% Triton X-100 for 7 minutes at room temperature. The cells were incubated with primary

antibodies Ki67 (1:200) (Abcam) or PCNA (1:100) (Cell Signaling) for 1hour at room temperature in a humid chamber. This is followed by 1 hour of incubation at room temperature with secondary antibodies conjugated to fluorochrome. Nucleus was stained with DAPI (Sigma). Anti- Rabbit conjugated to Alexa594 (Invitrogen) was used for Ki67 and Anti- Mouse conjugated to FITC (Sigma) was used for PCNA. The 5-6 images per slide were collected using the Zeiss fluorescence upright microscope and further analyzed using Definiens image analysis software with the rule set designed by the Moffitt microscopy core.

CHAPTER THREE

PU.1 REGULATES PRDM1 TRANSCRIPTION IN LYMPHOMA CELLS

Introduction

The positive regulatory domain I binding factor 1 (PRDI-BF1/ PRDM1), encoded by the PRDM1 gene, was originally found to specifically bind the interferon beta (IFN-β) promoter and suppress IFN-β transcription following viral induction (Keller and Maniatis 1991). Blimp-1, the murine homologue of PRDM1, was originally described by Turner *et al.* as a transcription factor that could induce the differentiation of B cells (Turner, Mack et al. 1994). PRDM1/Blimp-1 has since been found to be required for the differentiation of a B cell to a plasma cell (Shapiro-Shelef, Lin et al. 2003). During the differentiation of mature B cells to plasma cells, PRDM1 represses multiple genes involved in maintaining the B cell phenotype and in maintaining cellular proliferation, such as CIITA (Piskurich, Lin et al. 2000; Ghosh, Gyory et al. 2001), c-myc (Lin, Wong et al. 1997), and BSAP (Lin, Angelin-Duclos et al. 2002). Microarray studies have outlined the PRDM1 repression profile and led to the identification of two additional direct targets, Spi-B and Id3 (Shaffer, Lin et al. 2002). Additionally, expression of the PRDM1 gene has recently been linked to cellular stress and the unfolded protein response in B cells (Doody, Stephenson et al. 2006).

Anti-IgM cross-linking of the B cell receptor has been reported in multiple studies to induce apoptosis in lymphoma cells (Benhamou, Cazenave et al. 1990; Hasbold and Klaus

1990; Kaptein, Lin et al. 1996; Zupo, Isnardi et al. 1996; Carey and Scott 2001). This response has been correlated with decreased levels of c-myc (Lin, Wong et al. 1997). Inducing PRDM1/Blimp-1 expression in lymphoma cells with histone deacetylase inhibitors also decreased expression of the downstream targets c-myc and BSAP (Lee, Bottaro et al. 2003). More specifically, introduction of PRDM1/Blimp-1 into lymphoma cells can induce apoptosis, suggesting PRDM1 may be an important mediator of the anti-IgM-mediated apoptotic response (Messika, Lu et al. 1998; Knodel, Kuss et al. 1999). However, no direct link between expression of PRDM1/Blimp-1 and anti-IgM mediated B cell receptor activation has been described.

Recently, PRDM1 expression has been detected in a subset of diffuse large B cell lymphomas (DLBCL) (Garcia 2006; Pasqualucci, Compagno et al. 2006; Tam, Gomez et al. 2006). However, inactivating mutations in the PRDM1 coding sequence were described, indicating a potential tumor suppressor role for this gene (Pasqualucci, Compagno et al. 2006; Tam, Gomez et al. 2006). Similarly, proliferating myeloma cells and myeloma cell lines abundantly express the truncated PRDM1 isoform, PRDM1β, which has impaired function (Gyory, Fejer et al. 2003). Additionally, Borson *et al*. demonstrated PRDM1 expression in B cells isolated from myeloma patients while normal donors lack expression (Borson, Lacy et al. 2002). The mutation status of PRDM1 in these myeloma-derived B cells is as yet unknown. Together, these findings indicate PRDM1/Blimp-1 may be important to the pathology of various hematopoietic malignancies, including lymphoma.

Very little is known as to the regulation of PRDM1 expression. Our data now demonstrate PRDM1 is regulated primarily at the level of transcription in both myeloma cells and in lymphoma cells stimulated by cross-linking of the B cell receptor. B cell receptor

stimulation leads to rapid increases in newly transcribed PRDM1 RNA levels, while mRNA stability is unchanged. Using promoter deletion constructs, we demonstrate several regions of activation in the PRDM1 promoter in both lymphoma and myeloma cells. *In vivo* genomic footprinting demonstrates multiple protein-DNA interactions in both lymphoma and myeloma cells. Further analysis of these interactions reveals PU.1 binding is functionally important for promoter activity in stimulated lymphoma cells. These findings demonstrate the PRDM1 promoter is poised for rapid activation in lymphoma cells, which suggests inducing PRDM1 expression in lymphoma cells may be a viable target to inhibit lymphoma progression.

Results

Induction of PRDM1 *Expression and Activity in Lymphoma Cells.*

Burkitt's lymphoma cell lines respond to signaling through the B cell receptor via anti-IgM treatment by undergoing either growth arrest or apoptosis. Similarly, although most Burkitt's lymphoma cell lines lack detectable levels of PRDM1, ectopic expression of PRDM1 leads to growth arrest or apoptosis (Messika, Lu et al. 1998; Knodel, Kuss et al. 1999). In order to dissect the regulation of PRDM1 in B cell lymphoma, we have initially investigated the effects of B cell receptor cross-linking of the EBV-negative lymphoma line, CA46. Twenty-four hour exposure to anti-IgM results in a significant increase in Annexin-V staining, indicative of the early stages of apoptosis (Figure 8A) but does not significantly increase the presence of late apoptotic or dead cells (control 3% vs anti-IgM 5%) as detected by ToPro3 staining. This finding is similar to that reported by Kaptein *et al* in which B cell receptor cross-linking of CA46 cells induced growth arrest and limited apoptosis along with a decrease in c-myc expression (Kaptein, Lin et al. 1996). This treatment also significantly increases PRDM1 mRNA levels approximately 8-fold above untreated controls ($p<0.05$) (Figure 8B). The induction of PRDM1 is also detectable at the protein expression level as revealed by immunoblot analysis (Figure 8C). We have previously reported that a PRDM1-beta isoform can also be expressed from a distinct promoter within intron 3 (Gyory, Fejer et al. 2003). Neither mRNA nor protein for the PRDM1-beta isoform was detected in the anti-IgM treated cells. Whether or not the PRDM1 protein is functional after induction was determined by examining its ability to silence target gene promoters. The steady state levels of BSAP and cmyc mRNA were examined by quantitative RT-PCR. Expression of both genes decreased after B cell receptor cross linking consistent with suppression by PRDM1

(Figure 8D). The observed 2-fold level of suppression is consistent with previous reports using over-expression of murine PRDM1 (Blimp-1) (Lin, Wong et al. 1997; Lin, Angelin-Duclos et al. 2002). Given the rapid turn-over rate of c-myc mRNA (Dani, Blanchard et al. 1984) this may suggest that PRDM1 can attenuate expression of some target genes but does not necessarily silence them.

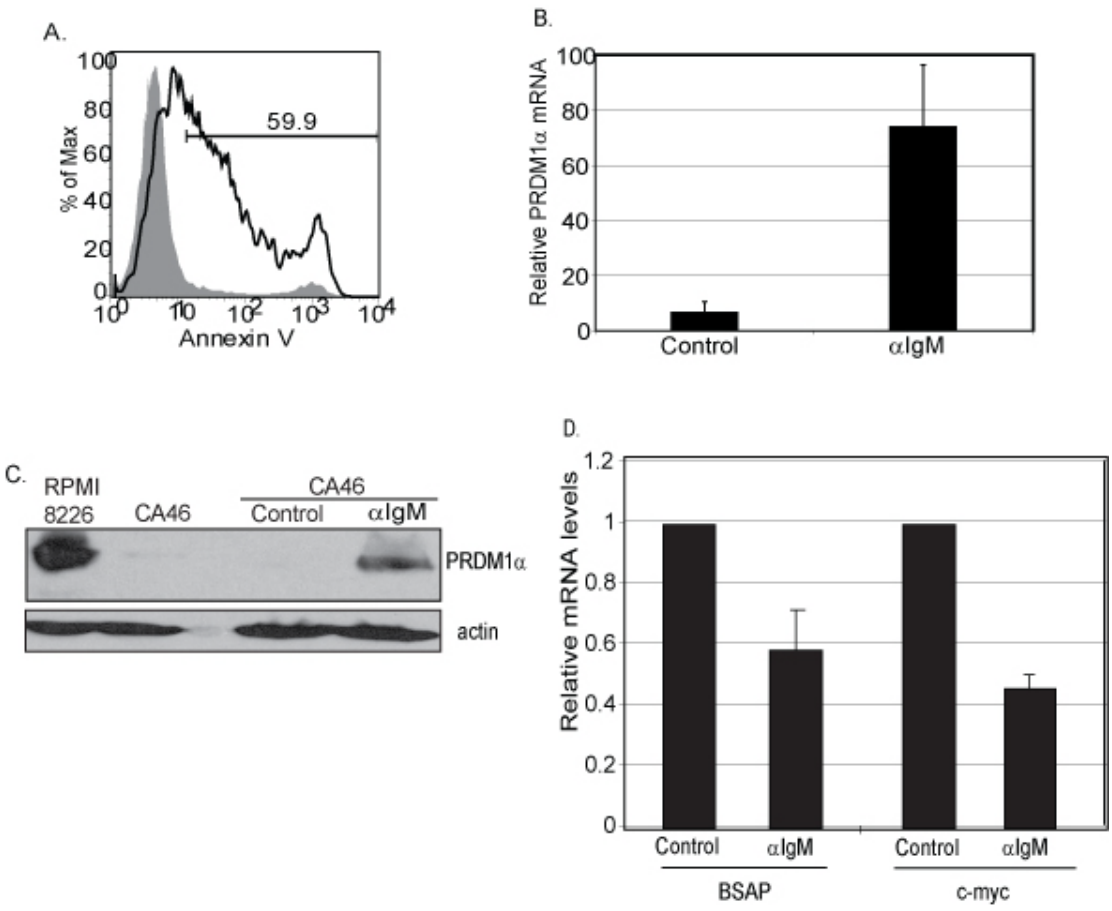


Figure 8. Anti-IgM Treatment Induces PRDM1 (PRDI-BF1) and Apoptosis in CA46 Lymphoma Cells. A) Treatment with anti-IgM (10 μg/ml) for 24 hours induces apoptosis approximately 60% above the control in CA46 lymphoma cells as assessed by Annexin V staining followed by FACS analysis. The data is representative of 3 independent experiments. (Gray histogram, untreated control cells; black outlined histogram, treated cells); **B)** Treatment of CA46 lymphoma cells for 24 hours with 10 μg/ml anti-IgM induces PRDM1 mRNA levels as assessed by quantitative RT-PCR. The data shown is mean of 3 experiments normalized to GAPDH with SEM shown ($p<0.05$). **C)** Immunoblot analysis of PRDM1 protein expression. CA46 lymphoma cells express PRDM1 protein only after treatment with anti-IgM for 24 hours. The RPMI8226 myeloma cell line constitutively expresses PRDM1 and serves as a positive control. **D)** Quantitative RT-PCR analysis of PRDM1 target genes, BSAP and c-myc. Anti-IgM exposure represses mRNA steady state levels. Data shown is a mean of 3 independent experiments with SEM shown ($p<0.05$ for c-myc).

PRDM1 Regulation Occurs at the Transcriptional Level

The mechanism by which PRDM1 expression is induced in lymphoma cells is unknown and could occur at multiple levels. We first examined basal levels of nascent RNA production in both lymphoma and myeloma cells. Nascent RNAs, defined as those RNAs still in the process of being transcribed, are an accurate measure of endogenous transcriptional activity (Wuarin and Schibler 1994). The nascent RNA were purified from nuclei after extensive washing to remove the released transcripts and quantified by real-time RT-PCR with specific primers directed to the 5' end of the RNA transcript. Levels of nascent RNA production in CA46 lymphoma cells are significantly lower than that measured in myeloma cells (Figure 9A). This finding is consistent with the high level of PRDM1 protein expression in myeloma cells (Figure 8C). Stimulation of the lymphoma cells with anti-IgM increased production of PRDM1 nascent RNA three fold after 1 and 4 hours (Figure 9B), indicating a rapid transcriptional activation. Changes in mRNA stability could also contribute to the increase in PRDM1 levels. mRNA stability changes were directly measured by inducing PRDM1 mRNA for one hour and then blocking subsequent transcription initiation with Actinomycin D. The mRNA half-life was indistinguishable before and after anti-IgM treatment of the lymphoma cells (Figure 9C). The mRNA half-life was very short (<1 hour) in the lymphoma cells and less than two-fold longer in the myeloma cells. This is consistent with the recent genome wide analysis of mRNA decay rates in mouse embryonic stem cells in which PRDM1 was one of the rare transcripts with a less than 1 hour half-life (Sharova, Sharov et al. 2009). The PRDM1 mRNA is present is three predominant molecular weights which vary only in the length of the 3'UTR (Tunyaplin, Shapiro et al. 2000; Gyory, Fejer et al. 2003). Using real-time PCR probes spanning the 3'UTR (Figure 10A) we determined the

largest mRNA species had a slightly faster decay rate but that this was not affected by anti-IgM treatment (Figure 10B). Similarly, a proximal 3'UTR probe which detects each of the mRNA species showed a similar decay rate and no change upon anti-IgM exposure. While this does not exclude changes in a minor mRNA species, this indicates that anti-IgM does not have a major role in altering PRDM1 mRNA stability. Together these data indicate regulation of PRDM1 occurs primarily at the level of transcription while the mRNA has a relatively short half-life in both stimulated lymphoma cells and in myeloma cells.

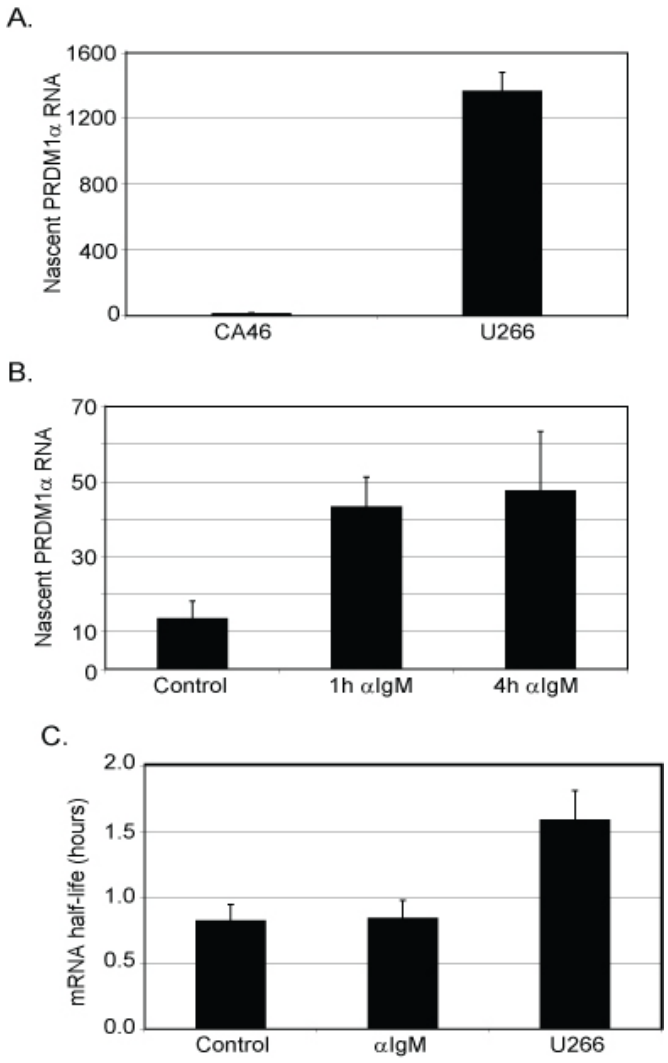


Figure 9. PRDM1 Regulation Occurs at the Level of Transcription. A) Relative levels of active transcription as measured by nascent RNA levels were determined by quantitative RT-PCR. Levels in U266 myeloma cells are significantly higher than that of unstimulated CA46 lymphoma cells. Data represents three independent experiments with SEM shown ($p<0.005$). **B)** Anti-IgM induces nascent PRDM1 RNA synthesis as early as one hour. CA46 cells were stimulated with 10μg/ml anti-IgM for 1 or 4 hours before harvest of nascent RNA and analysis by quantitative RT-PCR. Data represents three independent experiments with SEM shown. **C)** Stability of PRDM1 mRNA is unchanged upon treatment with anti-IgM. CA46 cells were pre-treated for 1 hour with anti-IgM, followed by a inhibition of transcription with Actinomycin D. PRDM1 mRNA levels were determined by quantitative RT-PCR of cells harvested at 15 minute intrvals over 2 hours. Data shown is the mean of at least 3 experiments with SEM shown.

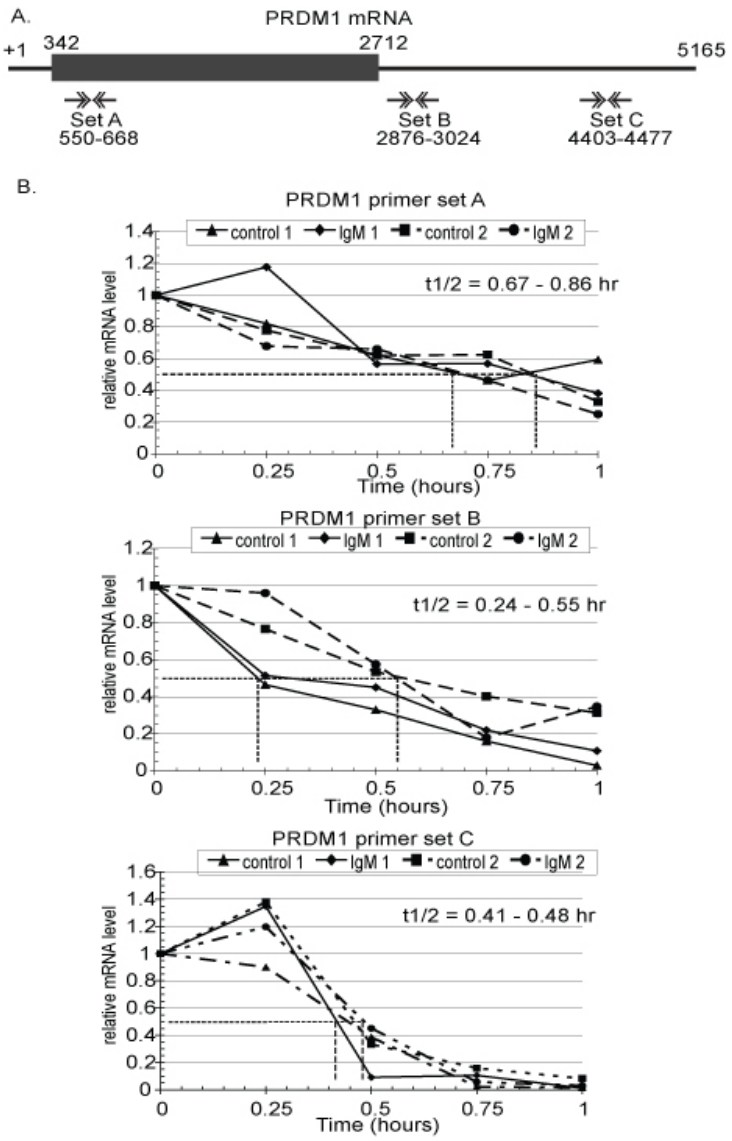


Figure 10. PRDM1 mRNA Decay Rates: Analysis for different PRDM1 mRNA isoforms show that the stability of PRDM1 mRNA is unchanged upon treatment with anti-IgM. **A)** Diagram of PRDM1 mRNA. The coding region is indicated by the solid rectangle and the 5' and 3' UTR is indicated by the thin line. Position of the three quantitiative RT-PCR primer sets is indicated and the numbers refer to the position downstream of the mRNA cap site. **B)** mRNA decay plots. CA46 cells were pre-treated for 1 hour with anti-IgM or mock treated (control) followed by a inhibition of transcription with Actinomycin D. PRDM1 mRNA was measured at 15 minute intervals over a one hour period. PRDM1 mRNA levels were determined by quantitative RT-PCR using the primer sets indicated in each graph and illustrated in panel A. In all panels, the data was normalized to the level of PRDM1 mRNA detected at time 0 and the data are shown for 2 independent experiments. The mRNA half-life was not affected by anti-IgM treatment although primer sets within the 3'UTR estimated a slightly faster overall decay rate.

Characterization of PRDM1 Promoter Activity

Because PRDM1 expression is primarily regulated at the level of transcription, we cloned the human promoter to assess the regions necessary for activity. Using a series of promoter deletion constructs spanning 2618 base pairs upstream of the transcription start site, potential regulatory regions were identified in lymphoma and myeloma cells (for schematic see Figure 11). PRDM1 promoter constructs containing 521, 863, or 1528 bp display robust and similar promoter activity in the myeloma cell line, RPMI-8226 (Figure 12A). Addition of promoter sequences up to 1921 bp results in a significantly higher level of activity. Similar results were obtained in a second myeloma cell line, U266. PRDM1 promoter activity was also analyzed in the lymphoma cell line CA46 (Figure 12B). The pattern of promoter activity in the unstimulated lymphoma cells is similar to that observed in the myeloma cell lines. The 521 bp promoter was sufficient for activity and the activity increased significantly with the addition of the region between -1528 and -1921 bp but larger constructs show a partial but consistent inhibition of activity. The overall level of promoter activity in the lymphoma cell line was lower than that in the myeloma cell lines consistent with the levels of nascent RNAs detected at the endogenous promoter in figure 9A. However comparing activity in two different cell lines requires the assumption that a co-transfected minimal Tymidine Kinase promoter has similar activity in both cell lines. This common assumption has not been proven for these cell lines. The effect of anti-IgM treatment was next examined in the CA46 lymphoma cell line. Constructs containing 2618 bp of the promoter were analyzed 24 hours after stimulation. Stimulation resulted in a small but not statistically significant increase in promoter luciferase activity (Figure 12C). This finding may indicate that a region required for induction lies outside of the 2618 bp promoter. One likely candidate is the intronic

regions previously shown to bind the repressor BCL6 (Tunyaplin, Shaffer et al. 2004; Parekh, Polo et al. 2007). It is also possible that the transiently transfected promoter constructs do not fully recapitulate the chromatin structure of the endogenous gene. This may prevent further activation of these promoter constructs by anti-IgM. However, these results reveal that the lymphoma cells have the necessary components to transcribe the PRDM1 gene.

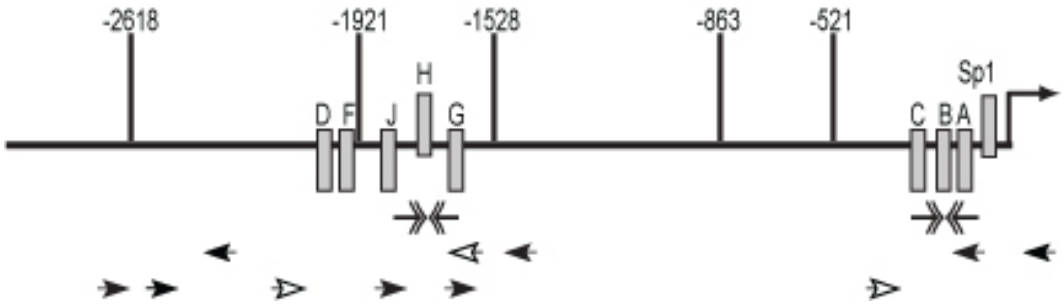


Figure 11. Schematic of the PRDM1 promoter: Schematic of the first 3000 base pairs of the PRDM1 promoter. The transcription start site is indicated by the bent arrow on the right side. The relative position of the end of the promoter luciferase deletion constructs is indicated above the promoter. The grey boxes mark the position of the in vivo protein DNA interaction sites. Open arrowheads represent the relative position of the in vivo footprinting primers shown in figure 13. Closed arrowheads represent additional in vivo footprinting primers. Double headed arrows indicate position of the primers used for chromatin immunoprecipitation analysis.

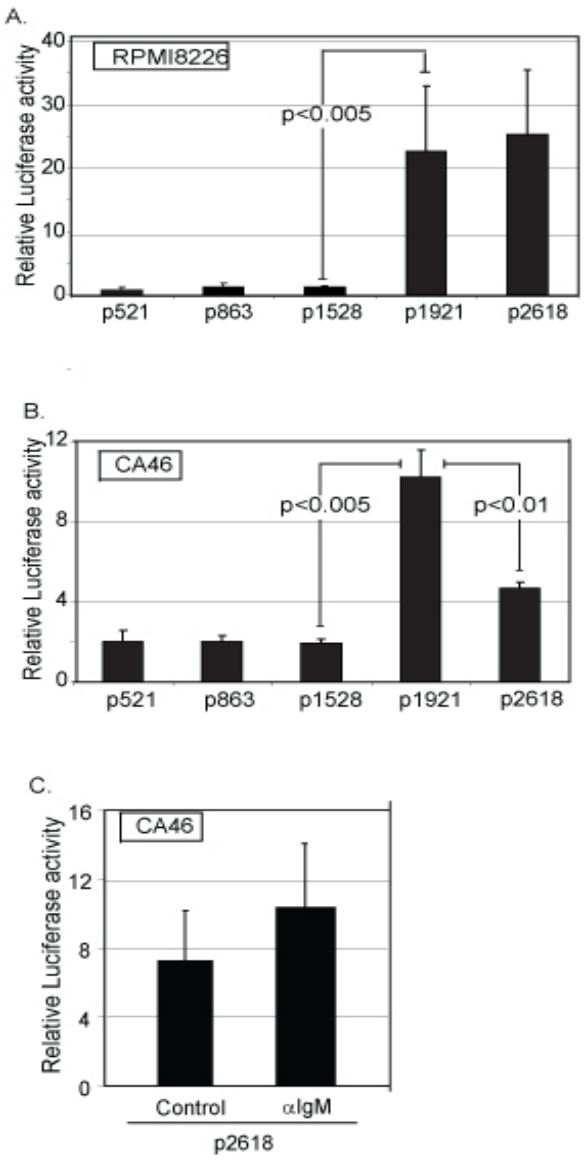


Figure 12. Characterization of PRDM1 promoter activity. A) RPMI8226 myeloma cells and **B)** CA46 lymphoma cells were transiently transfected with the indicated PRDM1 promoter deletion constructs fused to a luciferase reporter gene. **C)** CA46 lymphoma cells were transiently transfected with p2618 construct and stimulated with anti-IgM for 24 hours. Luciferase activity was measured 42 hours after transfection. Data presented is normalized to expression of a co-transfected minimal TK promoter-renilla luciferase construct. Construct names along the x-axis represent the number of PRDM1 promoter base pairs upstream of the transcription start site included in the construct. The region between -1528 to -1921 relative to the transcription start site was required for maximal transcription activity in both cell types. Data shown are the mean of 3 independent experiments with SEM shown.

In vivo Protein/DNA Interactions Occur Across the PRDM1 Promoter.

In order to define the important cis-acting DNA elements within the PRDM1 promoter, high resolution mapping of the protein/DNA contact sites was done by in vivo genomic footprinting. Unstimulated CA46 lymphoma cells and RPMI-8226 myeloma cells were treated briefly with dimethylsulfate to induce limited methylation of guanine residues. Close protein/DNA interactions have been demonstrated to inhibit or enhance the methylation activity which can be visualized after chemical cleavage and resolution by sequencing gel electrophoresis. These footprints of altered methylation represent the contact points of transcription factors bound to the promoter. Examination of the first 237 base pair region proximal to the transcription start site demonstrated factor binding in both the lymphoma and myeloma cells (Figure 13A). Four clusters of interaction are detected and labeled with brackets on the left side of the sequence. These contacts are indistinguishable between the two cell types. Closest to the transcription start site, nine strongly protected guanine residues map across a sequence with homology to an Sp1 consensus binding element. Sp1 binding to this element was confirmed by in vitro electrophoretic gel mobility shift assay (EMSA) and specific antibody reactivity (Figure 14). This extends the recent findings by Mora-Lopez *et al* and establishes Sp1 as a regulator of PRDM1 transcription in vivo (Mora-Lopez, Pedreno-Horrillo et al. 2008). Three additional occupied sites have been designated P.A, P.B, and P.C. These contact sites do not have obvious homology with known elements.

The distal promoter region from -1497 to -2641 base pairs was next examined by genomic footprinting using eight overlapping primer sets. Five additional clusters of contact were detected in the distal promoter (Figure 13 B,C and data not shown). The region which

conferred transcriptional activation (-1528 to -1921) contained three contacts designated P.J, P.H, and P.G. Site-P.J, associated with contacts at -1805 and -1802 and site-P.G associated with contacts at -1648, -1645, -1643, and -1641 overlap with AP1 consensus sequences. Both of these sites were previously predicted due to sequence homology by Vasanwala et al but neither direct assessment of AP1 binding nor functional activity in B cells or unstimulated myeloma cell lines were done (Vasanwala, Kusam et al. 2002). These sites show conservation across species (Figure 15). Our data demonstrates that both sites are occupied in vivo. Site-P.H associated with strong contacts at -1733, -1732, -1731, -1728 has sequence homology to consensus binding sites for Ets family members known to regulate multiple genes in the B cell lineage. These sites also show conservation across species (Figure 15). The region associated with a partial repression of transcription in the lymphoma cell line (-1921 to -2618) contained only two consistent clusters of in vivo contacts, designated sites P.D and P.F. Site P.F was strongly protected in the CA46 lymphoma cell line and weakly protected in the myeloma cell line. Mutation of the P.F site did not affect promoter activity of the 2618 PRDM1-luciferase construct in either lymphoma or myeloma cell lines. Site-P.D associated with contacts at -2038 and -2032 only in the lymphoma cell line.

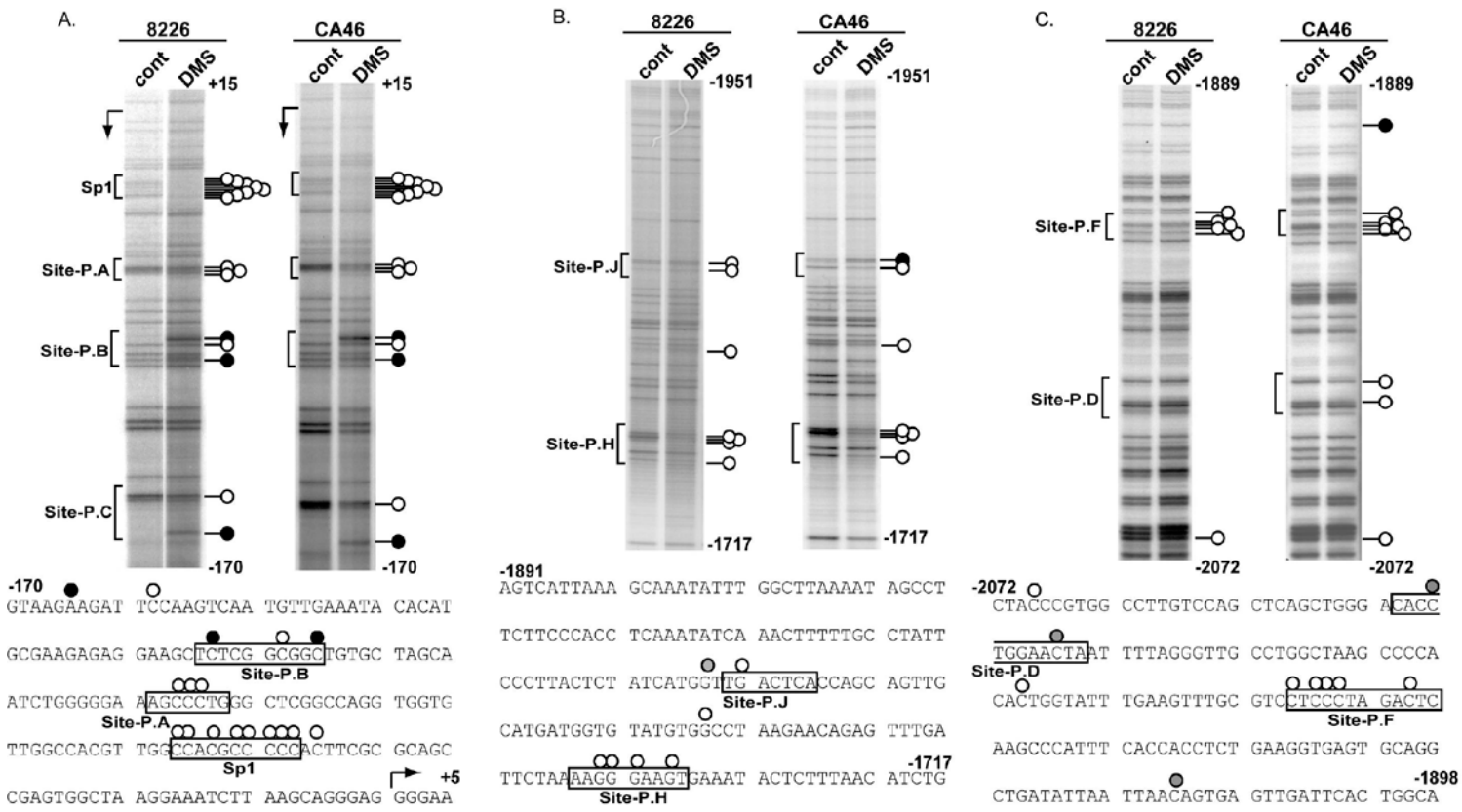

A.
8226
CA46
cont
DMS
+15
Sp1
Site-P.A
Site-P.B
Site-P.C
-170
GTAAGAAGAT TCCAAGTCAA TGTTGAAATA CACAT
GCGAAGAGAG GAAGCTCTCG GCGGCTGTGC TAGCA
Site-P.B
ATCTGGGGGA AAGCCCTGGG CTCGGCCAGG TGGTG
Site-P.A
TTGGCCACGT TGGCCACGCC CCCACTTCGC GCAGC
Sp1
+5
CGAGTGGCTA AGGAAATCTT AAGCAGGGAG GGGAA
B.
-1951
-1717
Site-P.J
Site-P.H
-1891
AGTCATTAAA GCAAATATTT GGCTTAAAAT AGCCT
TCTTCCCACC TCAAATATCA AACTTTTTGC CTATT
CCCTTACTCT ATCATGGTTG ACTCACCAGC AGTTG
Site-P.J
CATGATGGTG TATGTGGCCT AAGAACAGAG TTTGA
-1717
TTCTAAAAGG GAAGTGAAAT ACTCTTTAAC ATCTG
Site-P.H
C.
-1889
-2072
Site-P.F
Site-P.D
-2072
CTACCCGTGG CCTTGTCCAG CTCAGCTGGG ACACC
TGGAACTAAT TTTAGGGTTG CCTGGCTAAG CCCCA
Site-P.D
CACTGGTATT TGAAGTTTGC GTCCTCCCTA GACTC
Site-P.F
AAGCCCATTT CACCACCTCT GAAGGTGAGT GCAGG
-1898
CTGATATTAA TTAACAGTGA GTTGATTCAC TGGCA

Figure 13. *In vivo* Genomic Footprinting of the PRDM1 Promoter Reveals Multiple Protein-DNA Interactions. The RPMI8226 myeloma and CA46 lymphoma cell lines were analyzed with eight different primer sets to reveal interactions across the proximal 2618 base pairs of the PRDM1 promoter. Three regions which revealed contacts are shown: **A)** +15 to -170 bp, **B)** -1717 to -1951 bp, and **C)** -1889 to -2072 bp. In each pane the control lanes show the guanine residue sequence from deproteinized *in vitro* methylated DNA. The DMS lanes show the *in vivo* methylated residues. Protections (*open circles*) and enhancements (*filled circles*) are shown on the *right* side of each footprint panel and indicated in the sequence below. Clusters of contacts have been assigned arbitrary names as indicated along the left side and are boxed in the sequence. The bent arrow in panel A indicates the position of the transcription start site. A schematic of the footprint primers is shown figure 11.

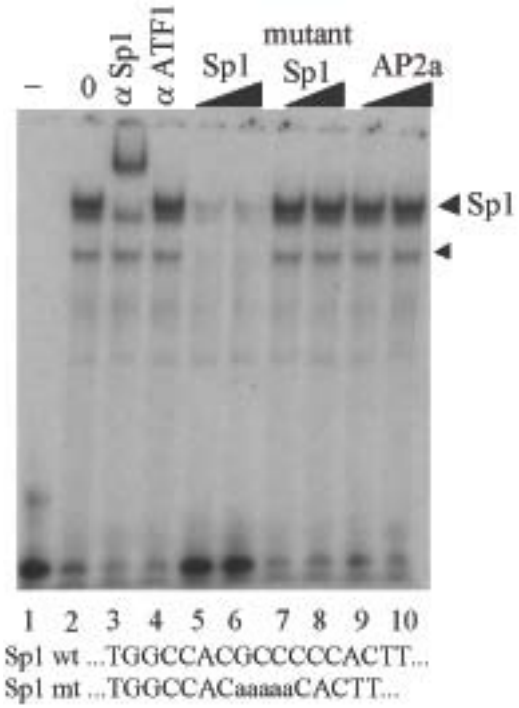


Figure 14. Sp1 Interacts at the PRDM1 Proximal Promoter. Electrophoretic mobility shift assay using an oligonucleotide spanning the Sp1 consensus sequence identified at position -52 to -43 in the PRDM1 promoter. Lanes 1 and 2 contain 0 and 2 uL of nuclear extract respectively. The binding reactions in lanes 3 and 4 were incubated with the specific antibody indicated at the top of each lane. Unlabeled competitor oligonucleotides as indicated at the top of lanes 5-10 were added to the binding reaction at 150 or 300-fold molar excess. The sequence of the Sp1 oligonucleotide and the mutant probe are shown at the bottom of the figure. The Sp1 containing complex is indicated by the labeled arrowhead. The smaller arrowhead represents a related specific GC-box binding protein antigenically unrelated to Sp1.

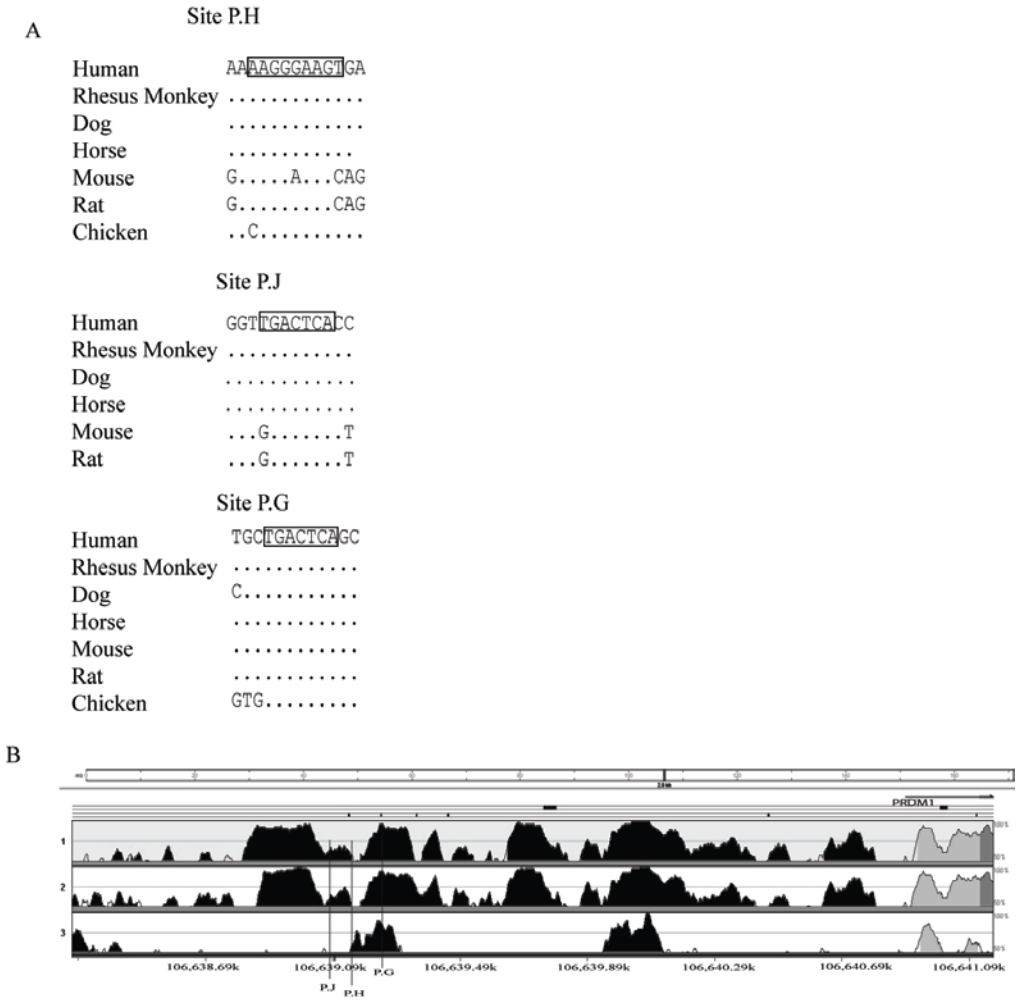


Figure 15. Binding Site and Promoter Conservation. A) Human sequences corresponding to sites P.H, P.J, and P.G are shown. Dots indicate site of conservation between the human sequence and the species indicated on the left. **B)** Promoter alignment of the PRDM1 promoter between human and mouse, rat and chicken using GenomeVISTA. 2618 base pairs of the promoter upstream of the transcription start site are shown along with exon 1 as indicated at the upper right. Black histograms represent conservation within the promoter and grey histograms represent conservation in exon 1. Alignment parameters were set at a 50 base pair window with 60% conservation. Location of sites P.J, P.H, and P.G are indicated below the histogram.

PU.1 Binds to the P.H Site and Regulates PRDM1 Promoter Activity

In order to identify the transacting factor bound at site P.H both in vivo and in vitro assays were used. First EMSA binding assays were performed with a 30 base pair probe spanning the P.H site in conjunction with nuclear extracts from CA46 lymphoma cells (Figure 16A). A fast migrating protein/DNA complex was detected which was specifically competed by unlabeled P.H probe but not a mutated P.H probe. The P.H site has homology to consensus Ets-family binding sequences. Antibody to PU.1 induced the formation of a super-shifted protein/DNA complex indicating that PU.1 is contained within the complex bound to P.H in vitro (Figure 16A). Similar results were obtained with nuclear extracts from the myeloma cell line RPMI8226.

PU.1 association at the P.H site was also measured at the endogenous PRDM1 promoter. Chromatin immunoprecipitation (ChIP) was performed with antibodies specific to PU.1 and association with the P.H site was assessed by quantitative PCR (Figure 16B). In lymphoma cells PU.1 factor binding at the PRDM1 promoter was approximately 9 fold greater than the negative control promoter HLA-DRA. Similarly, PU.1 binding was also observed in the myeloma cell line. We next examined normal primary B cells for PU.1 binding in vivo. ChIP analysis clearly identified PU.1 binding at the region of the P.H site while essentially no binding was observed at the negative control locus (Figure 16C). PU.1 binding was unchanged by anti-IgM treatment. Consistent with this finding the level of PU.1 protein expression in the primary B cells did not change with anti-IgM treatment (Figure 16D).

Functional assessment of site P.H. was performed initially by mutating the site in the context of either the p1921 and p2618 PRDM1 promoter-luciferase constructs. Transfection

of these mutated constructs into the lymphoma cell line revealed an approximately 50% reduction in transcriptional activity indicating that this site is required for maximal activity of the PRDM1 promoter (Figure 17A). PU.1 function in the activation of the endogenous PRDM1 gene was also examined by inhibiting PU.1 protein expression using siRNA. CA46 lymphoma cells were transiently transfected with the PU.1 specific siRNA and incubated with or without anti-IgM stimulation. Anti-IgM mediated induction of endogenous PRDM1 mRNA is significantly abrogated by PU.1 knockdown (Figure 17B). Furthermore induction of PRDM1 protein was also inhibited by the reduction in PU.1 expression (Figure 17C). The low level of apoptosis as assessed by PARP cleavage was not altered by PU.1 knockdown (Figure 17C). This indicates that PU.1 is involved in anti-IgM induced transcription of PRDM1 and that additional factors also contribute to the activity.

Similarly, to demonstrate the involvement of PU.1 in the expression of PRDM1 in myeloma cells, RPMI8226 were co-transfected with PRDM1 full-length luciferase promoter construct and siRNA specific for PU.1. PU.1 knockdown in these cells decreases the PRDM1 promoter activity by approximately 60% (Figure 18A). Mutation of the P.H site results in a greater than 80% loss of PRDM1 promoter activity. Knock-down of PU.1 does not further alter PRDM1 transcription in the context of a mutated P.H site. This confirms that the P.H site is functional in myeloma cells and that PU.1 exerts its effects through the P.H site. Loss of PU.1 also decreases endogenous PRDM1 mRNA levels in the RPMI8226 cell line (Figure 18B) further confirming a role for PU.1 in regulating PRDM1 transcription.

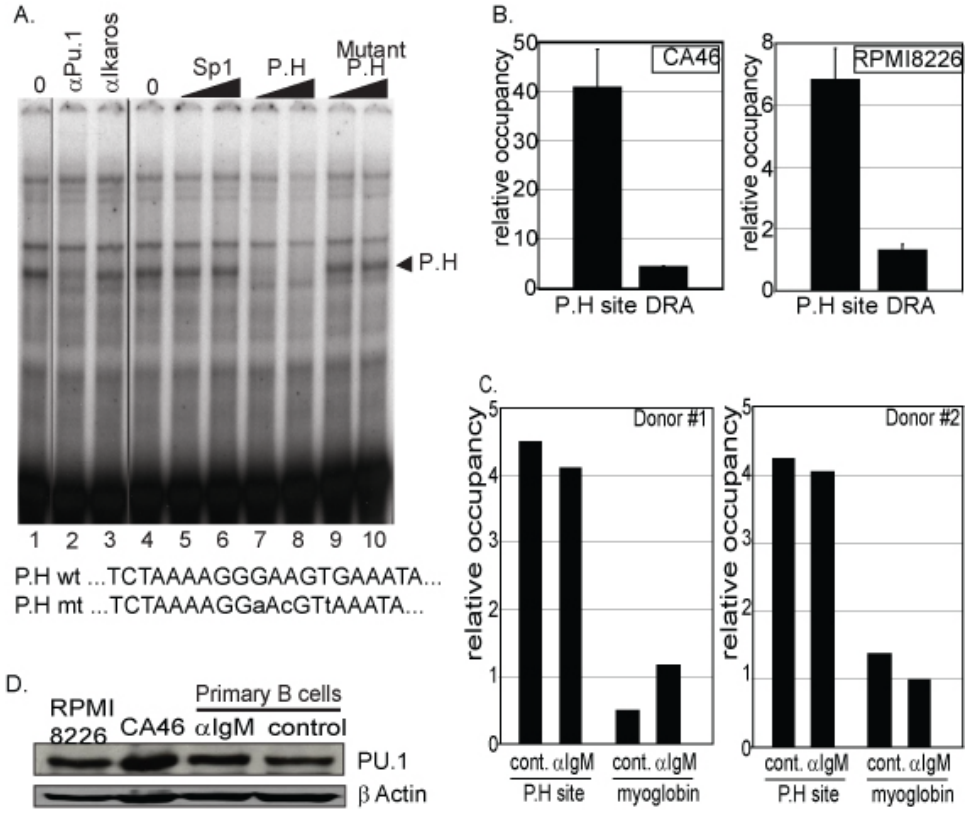


Figure 16. Site P.H is a PU.1 Factor Binding Site. A) In vitro binding assays were done using an oligonucleotide containing the P.H site sequence identified at position -1745 to -1737 of the PRDM1 promoter and CA46 nuclear extracts. Lanes 1 and 4 contain 2 uL of nuclear extract. Unlabeled competitor oligonucleotides as indicated at the top of lanes 5-10 were added to the binding reaction at 150 or 300-fold molar excess. A single complex indicated by an arrowhead is specifically competed by the wild type but not a mutant or unrelated oligonucleotide. In lanes 2 and 3 antibodies as indicated at the top were added to the binding reaction. The formation of the specific P.H complex is inhibited by addition of PU.1 antibody. The wild type and mutant P.H site sequences are shown at the bottom. **B)** Chromatin immunoprecipitation assay was performed in CA46 lymphoma cells (left panel) and RPMI8226 myeloma cells (right panel) using PU.1 antibody and quantitative PCR primers spanning the P.H site. PU.1 binding at the P.H. site was significantly higher than on the negative control promoter HLA-DRA. Data shown are mean of 3 independent experiments with SEM shown ($p<0.01$). **C**) Chromatin immunoprecipitation assay in activated primary human B cells. The experiment is as described in panel B except a myoglobin B locus is shown as the negative control. Lanes labeled αIgM were treated for 24 hours with anti-IgM while lanes labeled control were mock treated for 24 hours. PU.1 binding is specifically detected at the P.H site and is not altered by anti-IgM treatment. Similar data were obtained in two independent donor samples. **D)** Immunoblot analysis of PU.1 expression.

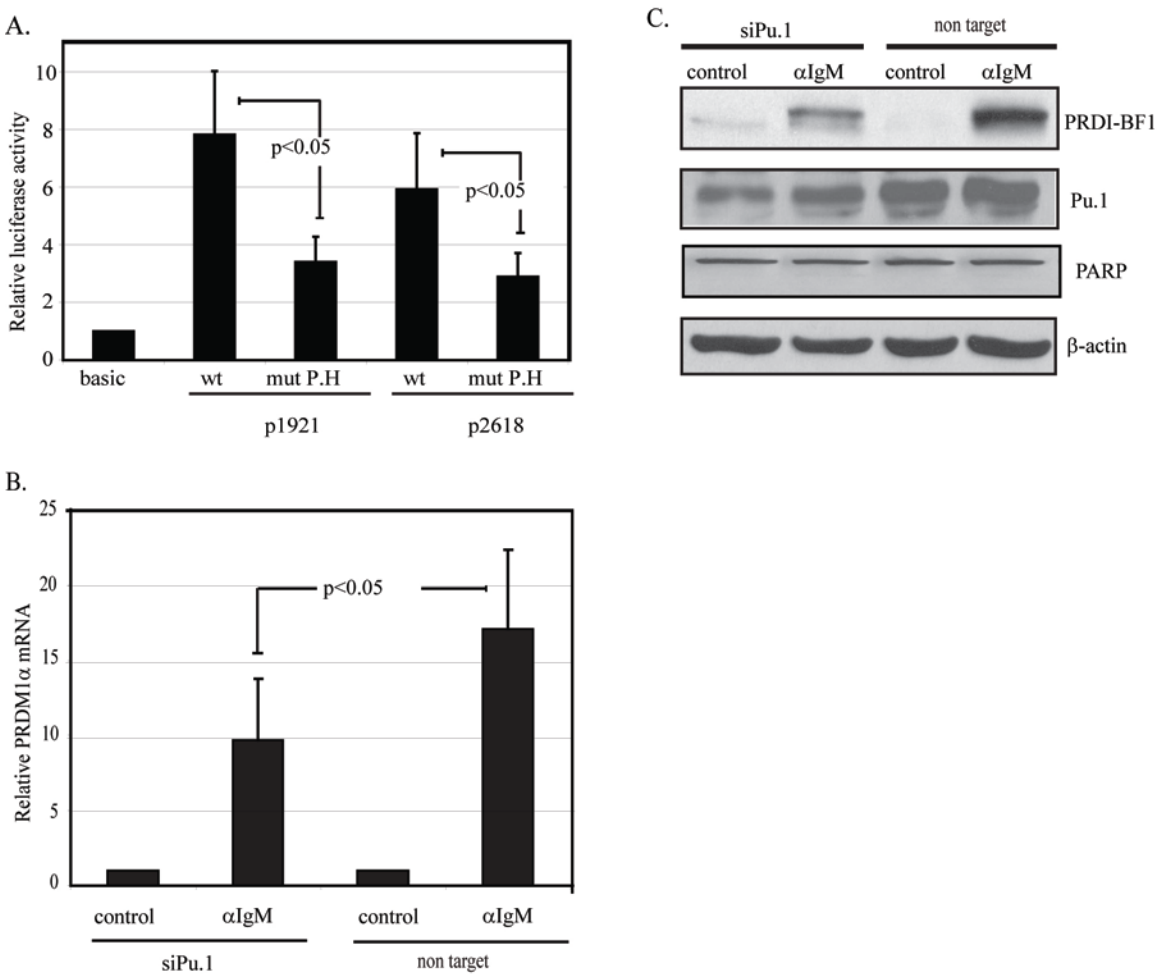


Figure 17. Site P.H and Transcription Factor PU.1 are Involved in Anti-IgM-Mediated Transcriptional Activation of PRDM1. A) The p1921 and p2618 PRDM1 promoter-luciferase constructs containing a wild type or mutated sequence at the P.H site were transfected into CA46 cells. Mutation of the P.H site in either construct decreased promoter activity. The mutation is the same as shown in figure 6A. The lane marker "basic" represents the activity from the promoterless vector, pGL3-Basic. Promoter activity was normalized as in figure 3 and represents six independent experiments with SEM shown. **B)** Endogenous PRDM1 expression is inhibited by loss of PU.1. CA46 lymphoma cells transfected with either non targeting control siRNA or an siRNA specific to PU.1 for twenty four hours and then either stimulated with anti-IgM (αIgM) or untreated (control) for an additional twenty four hours. PRDM1 mRNA was assessed by quantitative RT-PCR. Data was normalized to GAPDH and shown as fold induction relative to untreated (control) sample. Data is the mean of 3 independent experiments with SEM shown. **C)** Immunoblot analysis of PU.1 and PRDM1 expression. PU.1 siRNA decreased PU.1 protein levels and diminished induction of PRDM1 in response to anti-IgM but did not change the PARP cleavage. Experimental conditions are as in panel B.

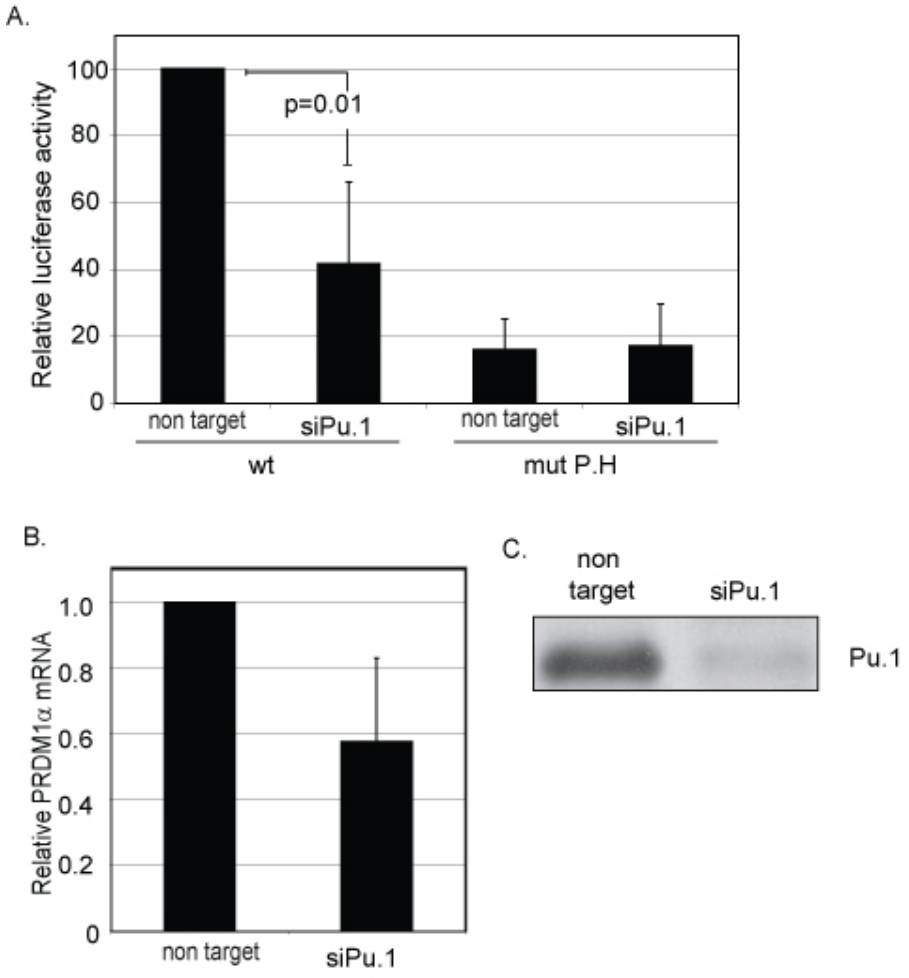


Figure 18. Site P.H and transcription factor PU.1 are involved in transcriptional regulation of PRDM1 in myeloma cells. A) RPMI8226 myeloma cells were transfected with either wild type or mutant Site-P.H p2618 PRDM1 promoter luciferase constructs. In addition the cells received siRNA against PU.1 or the non targeting control as indicated below each graph. siRNA against PU.1 diminished wild type promoter activity. Mutation of the P.H site also significantly lowered transcription but addition of siRNA to PU.1 did not further diminish activity in the context of a mutated P.H site. Data is normalized as in figure 12 and represents four independent experiment with SEM shown. **B**) Endogenous PRDM1 mRNA levels are decreased by siRNA knockdown of PU.1 in RPMI8226 cells. mRNA was assayed 48 hours after transfection of either a non-targeting siRNA or a PU.1 specific siRNA. Levels were measured by quantitative RT-PCR and shown as the average of 3 independent experiments with SEM shown. **C**) Immunoblot of PU.1 expression after siRNA knockdown in RPMI8226 cells.

Loss of TLE4 at the P.H Site in Response to Transcriptional Stimulation

PU.1 has been described to function both as an activator of transcription and as a repressor (Yamamoto, Kihara-Negishi et al. 1999; Linderson, Eberhard et al. 2004). These divergent activities have been linked to differential PU.1 mediated recruitment of the co-repressor TLE4 and the co-activator CBP. The observed PU.1 dependent activation in response to anti-IgM might be due to changes in PU.1 binding to the PRDM1 promoter or to changes in the co-activator or co-repressors recruited by PU.1. In order to address this question we used chromatin immunoprecipitation to profile binding of the factors in response to anti-IgM. PU.1 binding at the P.H site is robust and unchanged by anti-IgM treatment (Figure 19A). Minimal binding was detected near the transcription start site of PRDM1 confirming the localized recruitment of PU.1 to the P.H site. In contrast chromatin immunoprecipitation of the co-repressor TLE4 revealed a significant loss of binding in response to the activation stimulus (Figure 19B). This is consistent with a loss of repressive activity and the observed increase in PRDM1 transcription. Similar analyses with antibodies to the co-activator CBP were variable with a general increase in binding observed upon stimulation. However, the results for CBP did not reach statistical significance (Figure 19C).

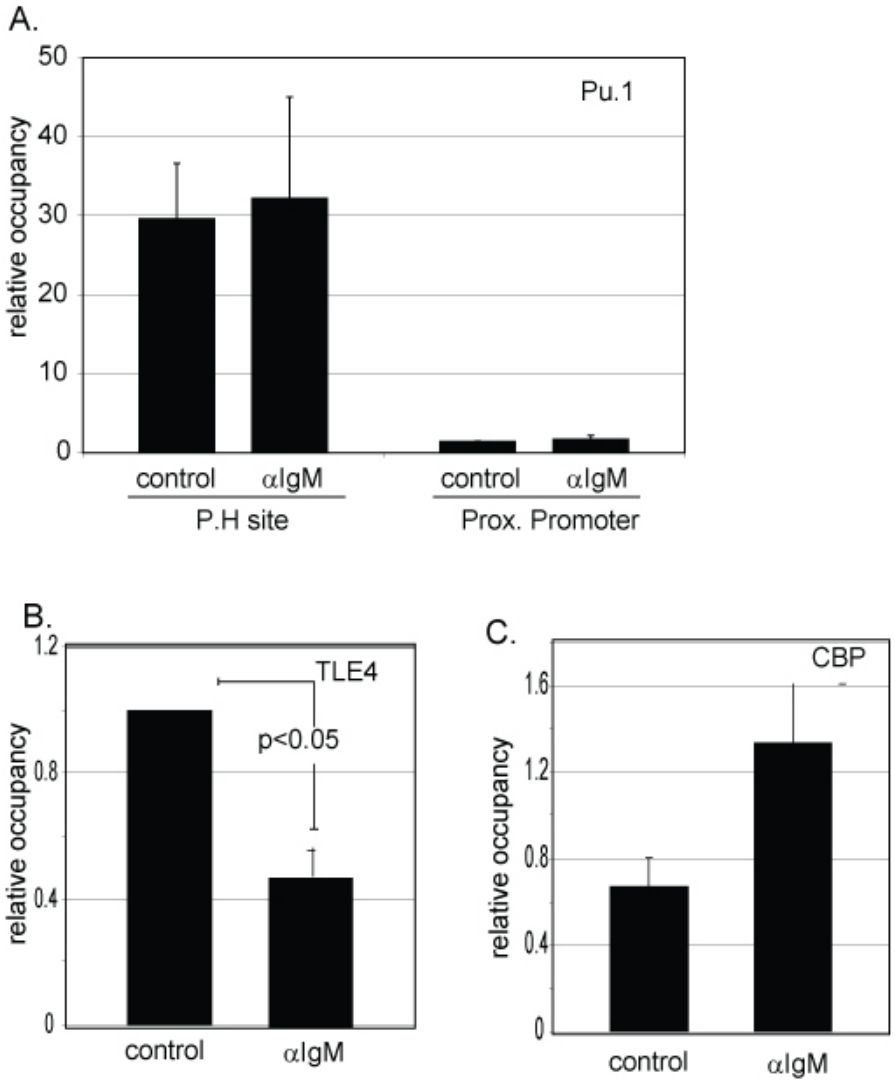


Figure 19. Chromatin immunoprecipitation of PU.1 and TLE4 at the distal PRDM1 promoter. A) ChIP assay using a PU.1 antibody to detect binding at the P.H site before and after anti-IgM stimulation. CA46 cells were left unstimulated (control) or stimulated with anti-IgM (αIgM) for 24 hours prior to harvest. Binding was assessed by quantitative PCR using primers surrounding the P.H site or located at the proximal PRDM1 promoter. PU.1 binding at the P.H site on PRDM1 distal promoter is unaffected by anti-IgM treatment. Data represents the mean of six (P.H site) or four (prox. promoter) independent experiments with SEM. **B**) ChIP assay using TLE4 antibody to detect binding at P.H site. The same samples assessed in panel A were reassessed for TLE4 binding. TLE4 binding at the P.H site significantly decreases upon treatment with anti-IgM. **C)** ChIP assay using CBP antibody to detect binding at P.H site. Change in CBP binding was not significant upon treatment with anti-IgM. Data shown in each panel are mean of 4 independent experiments with SEM shown.

Discussion

The transcription factor PRDM1/Blimp-1 is required for the differentiation of a mature B cell to a plasma cell (Shapiro-Shelef, Lin et al. 2003). It does this by directly repressing downstream targets, which in turn has a widespread effect on further downstream targets (Shapiro-Shelef and Calame 2005). These downstream effector cascades have been well studied, however very little is known as to how PRDM1/Blimp-1 expression is regulated.

This study demonstrates a direct link between B cell receptor cross-linking by anti-IgM and transcriptional activation of PRDM1/Blimp-1. Treatment of CA46 lymphoma cells with anti-IgM significantly up-regulated PRDM1 mRNA and protein levels and induced apoptosis. This is consistent with observations in other B cell lymphoma cell lines (Benhamou, Cazenave et al. 1990; Hasbold and Klaus 1990; Kaptein, Lin et al. 1996; Zupo, Isnardi et al. 1996; Carey and Scott 2001). One previous study using the EBV-negative CA46 cell line reported that this line was unique in responding to anti-IgM with only growth arrest raising the possibility that EBV-negative B cell lymphomas were heterogeneous in the apoptosis response (Kaptein, Lin et al. 1996). However, this report used very late markers of apoptosis (DNA fragmentation) while we measured early markers suggesting that only the kinetics of apoptosis induction may vary. The anti-IgM induced growth arrest and apoptosis has been linked to down-regulation of c-myc (Kaptein, Lin et al. 1996). This is consistent with the induction of PRDM1/Blimp1 and its known role in directly repressing c-myc transcription (Lin, Wong et al. 1997). Suppression of PRDM1 target genes was approximately 2-fold which is consistent with previous reports using over-expression of murine PRDM1 (Blimp-1) (Lin, Wong et al. 1997; Lin, Angelin-Duclos et al. 2002). The

lower levels of PRDM1 induced by anti-IgM treatment could also be responsible for the attenuation of the suppressive activity of PRDM1/Blimp1. Alternatively, post-translational modifications of PRDM1 after anti-IgM treatment may alter the functional activity of PRDM1. Therefore, future investigations in the post-translational modifications of PRDM1/Blimp1 may further explain the functional activity of PRDM1. The increase in PRDM1 expression occurs primarily at the level of transcription as we did not detect any change in mRNA stability but actively transcribing nascent RNA levels were induced within one hour. Unexpectedly in vivo genomic footprinting revealed that the PRDM1 promoter was extensively occupied by transcription factors even in the absence of stimulation or promoter activity. Together these findings indicate that the PRDM1 promoter is in an open and poised state in the lymphoma cells. This provides support that therapeutic approaches to trigger endogenous PRDM1 expression are feasible and could be a viable approach to induce apoptosis in lymphoma cells. Furthermore, PRDM1 has been shown to be an important target in immunotherapy of myeloma by induction of PRDM1-specific cytotoxic T cells, an approach which could also be exploited to kill lymphomas after PRDM1 induction (Lotz, Mutallib et al. 2005).

The transcription factors and cis-acting elements controlling PRDM1 promoter activity have only begun to be investigated. A region of the murine PRDM1 promoter spanning -918 to +207 base pairs was previously shown to have minimal promoter activity but did not confer any cell type specific activity (Tunyaplin, Shapiro et al. 2000). This is consistent with our finding that the sequences between -1528 to -1921 base pairs of the human promoter are required for activation of the promoter in lymphoma and myeloma cells. In vivo genomic footprinting of the proximal promoter region revealed four occupied

elements within the first 170 base pairs. These include a bound Sp1 site proximal to the transcription initiation point and is consistent with the absence of a canonical TATA box element and the recent findings by Mora-Lopez et al (Mora-Lopez, Pedreno-Horrillo et al. 2008). In vivo genomic footprinting of the upstream activation domain revealed three occupied elements in both lymphoma and myeloma cells. Sites P.J and P.G both have homology to an AP1 consensus binding site sequence. These sites were previously predicted by sequence homology (Vasanwala, Kusam et al. 2002). Investigation of the murine PRDM1 promoter has also provided evidence that c-fos can regulate the gene (Ohkubo, Arima et al. 2005). The authors identified an AP1 binding site at a region homologous to the site we designated P.G and demonstrated that c-fos can bind to this site. c-fos was required for maximal activity of the murine PRDM1 promoter. Together these findings strongly support that the PRDM1 gene expression is directly regulated by AP1 through sites P.J and P.G.

The third in vivo occupied element within the PRDM1 promoter required for transcriptional activation is site P.H. In vivo chromatin immunoprecipitation assays and in vitro DNA binding assays established that Ets family member PU.1 specifically binds to site P.H. Basal and anti-IgM stimulated PRDM1 promoter activity was significantly inhibited by mutating the P.H site in lymphoma cells. Additionally, knock-down of PU.1 expression by siRNA decreased PRDM1 transcription after B cell receptor cross-linking by anti-IgM. This indicates that PU.1 and the Ets site is a critical and required component of the PRDM1 promoter which must be present for the promoter to fully respond to anti-IgM stimulation. However, this site is not sufficient for the anti-IgM response. Moreover these data do not exclude the possibility that other Ets family members may function in regulating PRDM1 expression such as Elf-1 which has a DNA recognition sequence similar to that of PU.1. In

the myeloma cell line RPMI8226 the P.H site and PU.1 expression were also required for maximal promoter activity. PU.1 is known to have an important role in regulating early B cell development and is continued to be expressed throughout B cell maturation (Scott, Simon et al. 1994). A recent report has shown that PU.1 is also expressed in primary human plasma cells but that expression in myeloma cells and cell lines is variable (Tatetsu, Ueno et al. 2007). Our results suggest that PU.1 may contribute to the initial activation of PRDM1 expression in B cells. Furthermore, PRDM1 expression in myeloma cells is significantly enhanced by PU.1. PU.1 is bi-functional and can act to either increase or repress transcription of its target promoters (Yamamoto, Kihara-Negishi et al. 1999; Suzuki, Yamada et al. 2003). This opposing activity is mediated by differential recruitment of co-regulators by PU.1. The co-activator CBP, a histone acetyltransferase, binds to PU.1 and promotes activation of the promoter (Yamamoto, Kihara-Negishi et al. 1999). In contrast PU.1 can also recruit TLE4, a corepressor which in turn recruits the histone deacetylases HDAC1 and HDAC2 (Linderson, Eberhard et al. 2004). We observed TLE4 recruitment to the PRDM1 promoter at the region of PU.1 binding. This recruitment was significantly diminished upon activation by anti-IgM while CBP binding was largely unaffected. This indicates that the components of the PU.1 complex bound at the PRDM1 promoter are modulated by B cell receptor cross linking to promote gene activation. Conversely knockdown of PU.1 in unstimulated B cells did not induce PRDM1 expression suggesting that the PU.1/TLE4 complex is not acting as a dominant repressor.

BCL6 can repress PRDM1 expression (Shaffer, Yu et al. 2000). Vasanwala et al showed that AP1 and BCL6 could interact and suggested that the two AP1-like sites, which we have designated as P.J and P.G, may mediate BCL6 repression of PRDM1 (Vasanwala,

Kusam et al. 2002). Bach2 has also been suggested to repress murine PRDM1 though association with Mafk bound at the site homologous to site P.G. (Ochiai, Katoh et al. 2006). Our in vivo data support the hypothesis that the sites are bound by transcription factors but the region conferred activation not repression in the BCL6-positive CA46 cell line. This suggests that BCL6 does not repress through these elements although cell line differences between the reports may have an effect. Recent direct evidence of BCL6 binding to intron 5 of human PRDM1 or introns 3 and 5 of murine PRDM1 are consistent with the absence of a dominant repression domain detected by our promoter studies (Tunyaplin, Shaffer et al. 2004; Parekh, Polo et al. 2007). Our studies revealed that the distal region of the promoter (-1921 to -2686 base pairs) partially decreased overall promoter activity in the B cell line. This region contained only 2 clear in vivo occupied elements and the site P.D was observed only in the B cell line but not in the myeloma line. The factor binding at site P.D remains to be elucidated but the sequence does have partial homology to the transcription repressor ZEB1 (Genetta, Ruezinsky et al. 1994).

In conclusion, we have demonstrated PRDM1 regulation occurs primarily at the transcriptional level in lymphoma and myeloma cells. We show for the first time that PRDM1 is transcriptionally regulated by PU.1 and the co-repressor TLE4. Furthermore we have shown that two AP1 sites and an Sp1 site within the PRDM1 promoter are occupied in vivo. Importantly, we report that the promoter is poised for activation in lymphoma cells, suggesting that inducing PRDM1 expression in lymphoma cells lacking PRDM1 gene mutations is a viable therapeutic approach to inducing apoptosis in these cells.

CHAPTER FOUR

PRDM1 IS REQUIRED FOR MANTLE CELL LYMPHOMA RESPONSE TO BORTEZOMIB

Introduction

Mantle Cell lymphoma (MCL) is an aggressive form of B cell non-Hodgkin lymphoma which makes up 5% - 10% of all human non-Hodgkin's lymphomas (Jares, Colomer et al. 2007). It involves pre-germinal center B cells present in the mantle zone. MCL is generally characterized by the chromosomal translocation t(11;14)(q13;q32) leading to over-expression of cyclin D1 (Brody and Advani 2006). In addition to cyclin D1 deregulation, MCL is one of the lymphoid malignancies associated with high chromosomal aberrations likely to play an important role in progression of the disease. TP53 mutations (Kane, Bross et al. 2003; Kane, Dagher et al. 2007) and INK4a/ARF deletion are some of the secondary genetic lesions associated with MCL that lead to high proliferation. The majority of MCL patients show a complete or partial clinical response to first line chemotherapeutic agents mainly based on the CHOP combination or hyperCVAD (Brody and Advani 2006), but relapse is almost certain resulting in a median disease free survival of 3-4 years (Jares, Colomer et al. 2007).

In 2006 the FDA approved the proteasome inhibitor Bortezomib (PS-341, Velcade) for treatment of relapsed and refractory MCL (Kane, Dagher et al. 2007). Bortezomib has also been approved for treatment of refractory multiple myeloma (Kane, Bross et al. 2003).

Bortezomib is a boronic acid dipeptide that binds reversibly to the chymotrypsin–like site in the 20S core of the 26S proteasome (Adams and Kauffman 2004). Inhibition of the cellular proteasome activity by Bortezomib can alter multiple signaling pathways and bring about cytotoxicity. Bortezomib has been shown to inactivate the NFκB pathway in MCL as well as in multiple myeloma (Pham, Tamayo et al. 2003). However, recent findings have shown that Bortezomib is active in MCL with proteasome-insensitive activation of NFκB (Rizzatti, Mora-Jensen et al. 2008; Yang, Young et al. 2008). This indicates Bortezomib must also target other pathways. Bortezomib has been shown to induce apoptosis through the generation of reactive oxygen species (ROS) and activation of the NOXA pathway in MCL (Perez-Galan, Roue et al. 2006). NOXA is a pro-apoptotic Bcl2 protein that can bind to anti-apoptotic Mcl-1 protein, thus releasing Bak from the Mcl-1 complex and promoting apoptosis of the cell. Besides involvement of these pathways, studies in multiple myeloma and some solid tumors such as head and neck cancers have revealed that Bortezomib can induce apoptosis by inducing ER stress due to the accumulation of misfolded proteins (Obeng, Carlson et al. 2006)(Fribley, Zeng et al. 2004). Improperly folded proteins can build up in the ER leading to activation of the stress signaling pathway known as the unfolded protein response (UPR). UPR is a three-pronged pathway comprising IRE1, pancreatic ER kinase (PERK) and activating transcription factor 6 (ATF6) (Todd, Lee et al. 2008). If ER stress is prolonged or severe UPR activation leads to cell cycle arrest and induction of apoptosis (Brewer, Hendershot et al. 1999; Yamaguchi and Wang 2004).

PR Domain Zinc Finger Protein 1 (also known as PRDM1, Blimp-1, and PRDI-BF1) is a transcriptional repressor, required for terminal differentiation of B cells into antibody secreting plasma cells. During differentiation of mature B cells to plasma cells, PRDM1

represses several key target genes required for maintaining the B cell phenotype and in maintaining cellular proliferation such as CIITA, PAX5, Spi-B, Id3 and c-myc (Lin, Wong et al. 1997; Piskurich, Lin et al. 2000; Ghosh, Gyory et al. 2001; Lin, Angelin-Duclos et al. 2002; Shaffer, Lin et al. 2002). PRDM1 functions as a repressor by recruiting to the DNA multiple co-repressor proteins including the histone H3 methyltransferase, G9a (Gyory, Wu et al. 2004), the histone deacetylase HDAC2 (Yu, Angelin-Duclos et al. 2000), and the arginine methyltransferase PRMT5 (Ancelin, Lange et al. 2006; Eckert, Biermann et al. 2008). In addition PRDM1 may displace IRF transcriptional activators through DNA binding site competition at some promoters (Kuo and Calame 2004). PRDM1 exists in two isoforms, the full length PRDM1α and a truncated form, PRDM1β. The truncated PRDM1β which is abundantly expressed in proliferating myeloma cells and myeloma cell lines is functionally impaired (Gyory, Fejer et al. 2003). Recently, PRDM1 expression has been detected in a subset of diffuse large B cell lymphomas (DLBCL) (Garcia 2006; Pasqualucci, Compagno et al. 2006; Tam, Gomez et al. 2006). However, inactivating mutations were observed in each case, indicating a tumor suppressor role for PRDM1 (Pasqualucci, Compagno et al. 2006; Tam, Gomez et al. 2006). Additionally, ectopic expression of PRDM1 in lymphoma cells can induce apoptosis (Messika, Lu et al. 1998). Moreover, induction of PRDM1 transcription in lymphoma cells by anti-IgM treatment induces apoptosis in these cells (Hasbold and Klaus 1990; Kaptein, Lin et al. 1996; Desai, Bolick et al. 2009). PRDM1 also has been linked to cellular stress and the unfolded protein response (Doody, Stephenson et al. 2006). Together this suggests that PRDM1 is capable of inducing apoptosis in B cells when expressed outside of the plasma cell transition stage.

PRDM1 has not previously been investigated in the context of Bortezomib treatment of lymphoma. This report demonstrates that in MCL PRDM1 is required for the apoptotic effect of Bortezomib. Bortezomib induced PRDM1 functions at least in part through direct repression of MKI67 and PCNA and inhibits NOXA activity. These findings reveal that PRDM1 is an essential component of the apoptotic response in MCL and a potential important marker for the effectiveness of Bortezomib therapy.

Results

Bortezomib Induces Expression of PRDM1 Along with Induction of Apoptosis in Mantle Cell Lymphoma

Bortezomib is an FDA-approved drug for treatment of refractory and relapsed MCL. In order to better understand the mechanism of action of Bortezomib in MCL we treated the MCL cell lines with a low dose of 5nM Bortezomib for 20 hours. Incubation with 5nM of Bortezomib induced apoptosis as indicated by PARP cleavage (Figure 20A) as well as a two fold increase in mRNA levels of the pro-apoptotic protein NOXA (Figure 20B). This apoptotic effect is consistent with previous observations of other MCL cell lines treated with Bortezomib (Perez-Galan, Roue et al. 2006). We also observed that the induction of apoptosis is accompanied by a large increase in expression of PRDM1α protein (Figure 20A). Similarly, analysis of PRDM1 mRNA levels revealed a significant elevation of PRDM1α mRNA while PRDM1β mRNA was not affected (Figure 20C). To determine if the PRDM1 present is functionally active we analyzed the mRNA levels of PRDM1 target genes CIITA and PAX5 (Figure 20D). Expression of both CIITA and PAX5 were repressed in Bortezomib treated MCL cells consistent with PRDM1-mediated repression. We next sought to establish if a similar response is present in primary MCL cells freshly isolated from lymph nodes of MCL patients. First, optimal conditions to culture the primary cells in in-vitro were established. Maintaining primary MCL cells in complete RMPI induced spontaneous apoptosis. Primary cells maintained in 50% RPMI and 50 % stromal conditioned medium prevented this spontaneous apoptosis (Figure 21A). The stromal conditioned medium from HS5 bone marrow stromal cells mimicked the natural microenvironment providing the necessary cytokines to the MCL primary cells to survive in vitro. Next, primary MCL cells were

incubated with increasing doses of Bortezomib revealed a consistent induction of PRDM1 protein expression (Figure 21B). The optimal dose varied between 5 and 10nM in the patient samples indicating some heterogeneity in Bortezomib sensitivity. PRDM1 expression was paralleled by PARP cleavage indicating the cells were beginning to undergo apoptosis. PRDM1 mRNA levels also increased with Bortezomib treatment (Figure 21C). Consistent with the results in the Mino and Jeko-1 MCL cell lines only PRDM1α and not PRDM1β, was detected in all the primary MCL cells.

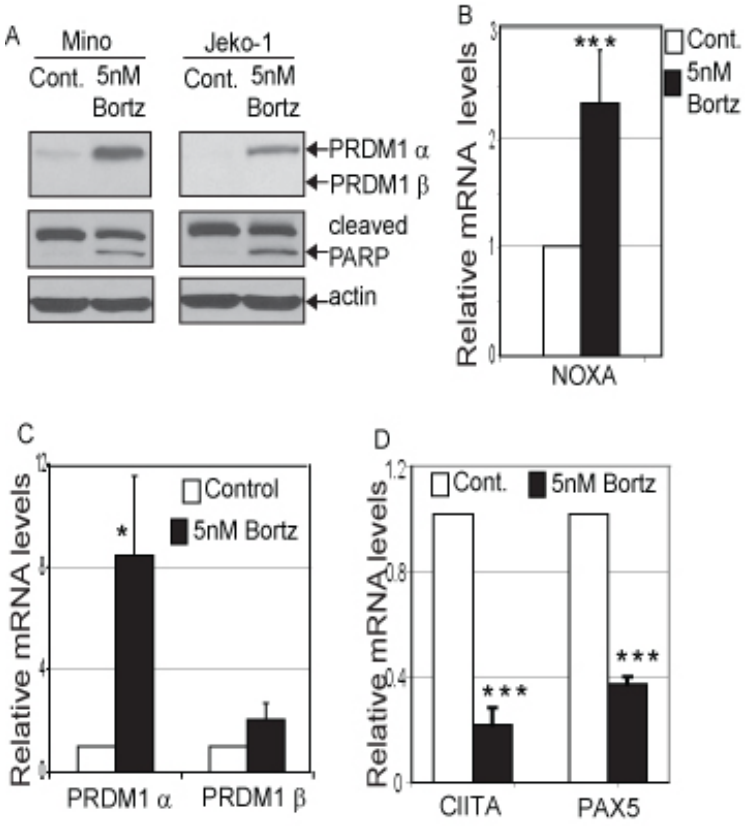


Figure 20. Bortezomib treatment induces PRDM1 and apoptosis in MCL cell line. **A)** Immunoblot analysis for expression of PRDM1 and PARP cleavage in MCL cell lines Mino and Jeko-1 after treatment with 5nM Bortezomib for 20 hours. Beta-actin is the loading control. Treatment with 5nM Bortezomib for 20 hours induces **B)** mRNA expression of NOXA as well as **C)** PRDM1α mRNA levels but not PRDM1β. **D)** PRDM1 target genes CIITA and PAX5 mRNA levels are repressed by Bortezomib treatment. All mRNA levels were assessed by quantitative RT-PCR (RT-qPCR). The data is presented relative to the control cells after normalization to GAPDH and represents the mean of 3 independent experiments with the SEM (*** indicates $p<0.002$ * indicates $p<0.05$). Relative levels of PRDM1α and β mRNA in the control cells were 0.5 and 0.014 respectively.

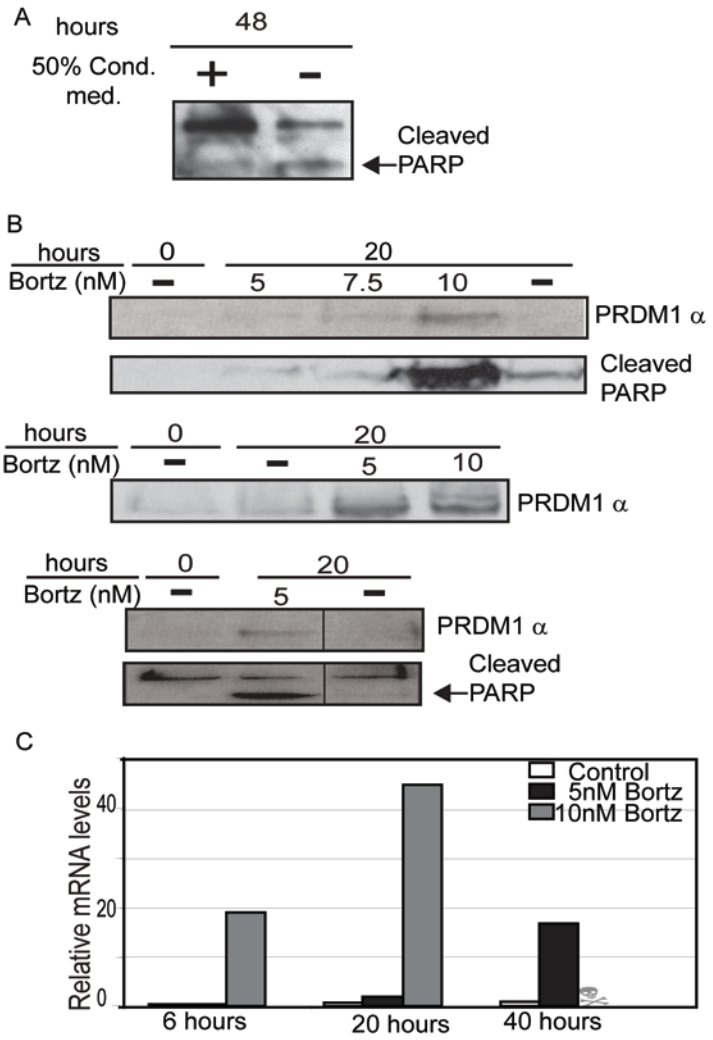


Figure 21. **Bortezomib treatment induces PRDM1 and apoptosis in primary MCL samples. A)** Immunoblot analysis of PARP cleavage detecting spontaneous apoptosis in MCL patient samples maintained in presence or absence of 50% conditioned medium after 48 hours. **B)** Immunoblot analysis of PRDM1α expression and PARP cleavage in three representative MCL patient samples treated with Bortezomib. The Bortezomib dose and duration of treatment is indicated above each lane. The dash indicates cells treated with an equal concentration of mannitol only. Only a single molecular weight size was detected for PRDM1 and it migrated at the position of PRDM1α. Each lane contains lysate from $5X10^5$ cells and loading was confirmed by beta-actin immunoblot (data not shown). **C)** RT-qPCR analysis of PRDM1α mRNA levels in MCL primary cells treated with 5nM and 10nM Bortezomib over a 40 hour time course. The data is normalized to the housekeeping gene, GUS-B. Data is representative of 5 MCL patient samples.

PRDM1 is Required for the Apoptotic Effect of Bortezomib

Induction of PRDM1 by Bortezomib could be a required event for apoptosis to occur or alternatively could be a downstream result of apoptosis. In order to directly test these two alternatives, PRDM1 expression was blocked during Bortezomib exposure and the impact on apoptosis was examined. Mino MCL cells were incubated with two PRDM1 specific siRNA for 24 hours followed by a 20 hour treatment with 5nM of Bortezomib. Apoptosis was analyzed by Annexin V staining and PARP cleavage. As shown in Figure 22A, the PRDM1 specific siRNA was able to reduce expression of PRDM1α to near basal levels while the non-targeting control siRNA did not block expression. This reduction in PRDM1α was accompanied by reduction in PARP cleavage (Figure 22A). Knockdown of PRDM1 also significantly prevented the increase in Annexin V staining associated with Bortezomib induced apoptosis (Figure 22B). To further establish the role of PRDM1 in Bortezomib induced apoptosis we analyzed pro-apoptotic genes involved in the Bortezomib response. Bortezomib has been shown to up-regulate expression of NOXA in MCL and activate Caspase-8 and Caspase-9 in Multiple Myeloma (Gomez-Bougie, Wuilleme-Toumi et al. 2007). Knockdown of PRDM1 in presence of Bortezomib led to approximately 60% reduction in NOXA expression (Figure 22C) and approximately 30% reduction in expression of Caspase-8 (Figure 22D) and Caspase-9 (Figure 22E). Thus the absence of PRDM1 significantly impairs the apoptotic outcome of Bortezomib treatment in MCL.

Bortezomib treatment in Multiple Myeloma induces a stress response because of accumulation of unfolded or misfolded proteins (Obeng, Carlson et al. 2006). To determine if a similar stress response occurs in MCL cells and if it is dependent on PRDM1 we analyzed the stress response protein XBP1. XBP1 mRNA undergoes unique cytoplasmic splicing in

response to ER stress to switch from encoding a negative regulator of UPR to a potent transcriptional activator of UPR (Tirosh, Iwakoshi et al. 2006; Yoshida, Oku et al. 2006). Analysis of both splicing isoforms of XBP1 revealed that Bortezomib does induce XBP1 splicing but that splicing does not diminish upon PRDM1 knockdown (Figure 23). This indicates that Bortezomib-mediated ER stress induction alone is not sufficient to induce apoptosis. Together these data reveal that PRDM1 expression is required for MCL cells to respond to Bortezomib.

We next wanted to determine if PRDM1 expression alone is sufficient to promote apoptosis in MCL cells or if additional events induced by Bortezomib are required. Since we observed that Bortezomib treatment induced only expression of PRDM1α and not the truncated PRDM1β, we over-expressed only the full length PRDM1α form. Mino MCL cells were transduced with a recombinant adenovirus expressing PRDM1α in the absence of Bortezomib treatment. Apoptosis was measured by Annexin V staining as well as PARP cleavage after 48 hours of infection. There was an approximate 50% increase in Annexin V staining in cells over-expressing PRDM1α when compared to control cells transduced with an adenovirus expressing only green fluorescent protein (Figure 24A). To confirm a specific apoptosis effect we examined PARP cleavage which is down stream of caspase activation. PARP cleavage is observed only in cells over-expressing PRDM1α (Figure 24B). This indicates that ectopic expression of PRDM1α in absence of Bortezomib leads to MCL apoptosis. Together these findings establish a central role for PRDM1 in the effect of Bortezomib and demonstrate that PRDM1 is both sufficient and required for the response.

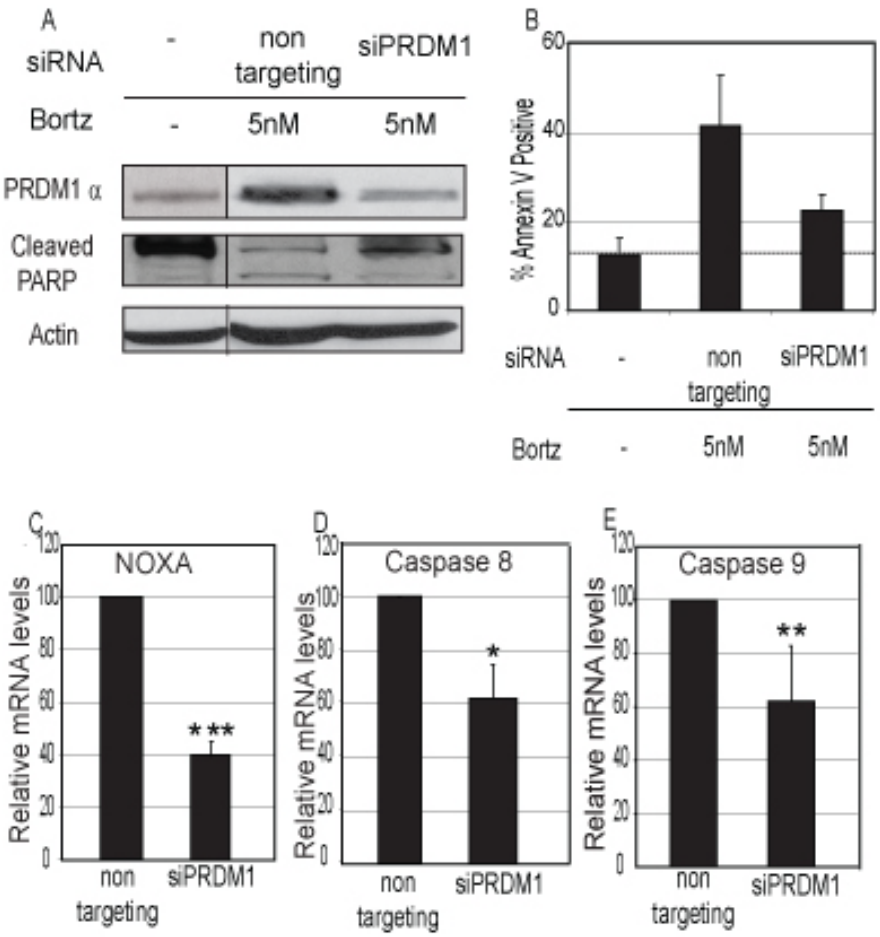


Figure 22. Knockdown of PRDM1 inhibits apoptotic effect of Bortezomib. A) Immunoblot analysis of knockdown of PRDM1α expression in presence of Bortezomib and detection of apoptosis by PARP cleavage in MCL Mino cells. Non-targeting siRNA indicates siRNA specifically designed not to inhibit any known genes. siPRDM1 indicates the PRDM1 specific siRNA. **B)** Flow cytometric analysis of Annexin V staining. Bar graph represents percent Annexin V positive Mino MCL cells treated with Bortezomib in absence or presence of PRDM1 specific siRNA. Data shown are mean of 3 independent experiments with SEM. The dashed line marks the background level of Annexin V staining detected in untreated Mino cells. Knockdown of PRDM1 also leads to significant reduction in mRNA expression of pro-apoptotic genes **C)** NOXA , **D)** CASP8 (caspase 8) and **E)** CASP9 (caspase 9). mRNA levels were assessed by RT-qPCR. The data is normalized to Beta-actin and represents the mean of 3 independent experiments with the SEM (*** indicates $p<0.002$, ** indicates $p<0.03$, * indicates $p<0.05$).

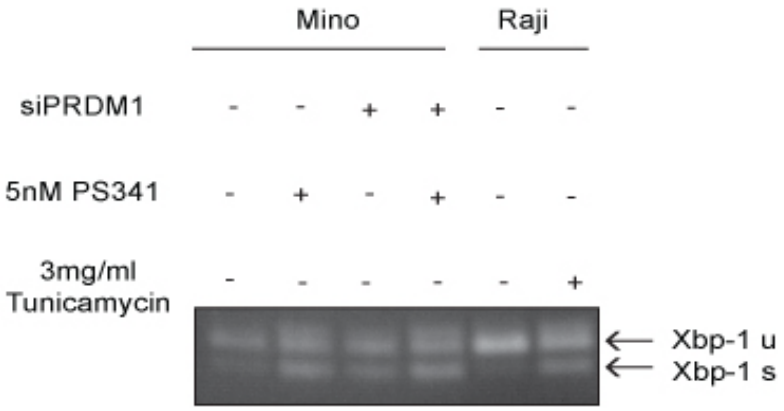


Figure 23: PRDM1 knockdown does not affect stress response. PCR analysis of XBP1 in Mino MCL cells treated with Bortezomib in presence or absence of siRNA mediated knockdown of PRDM1. The XBP-1 PCR primers used span the region of stress-induced cytoplasmic mRNA splicing and detects both the unspliced and spliced forms. Raji B cells treated with tunicamycin for 8 hours is used as a positive control to detect cellular stress response. Presence of smaller spliced isoform (Xbp-1s) confirms cellular stress.

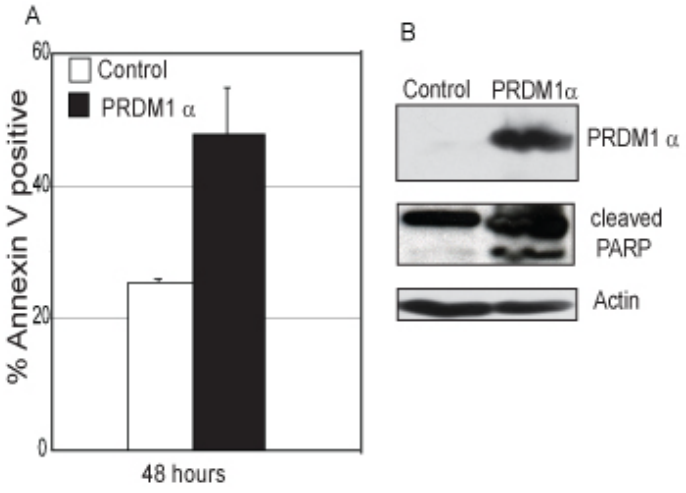


Figure 24. Ectopic expression of PRDM1α leads to apoptosis of MCL cells in the absence of Bortezomib. A) Flow cytometric analysis of Annexin V positive Mino MCL cells transduced with adenovirus expressing PRDM1α or GFP (control) for 48 hours. The data is mean of 3 independent experiments with SEM shown. **B)** Immunoblot analysis of PRDM1α expression and PARP cleavage indicating apoptosis in the adenoviral transduced Mino cells. Beta-actin is shown as the loading control.

Identification of Direct PRDM1 Targets in MCL

A limited number of direct PRDM1 targets have been identified during B cell differentiation into plasma cells including PAX5, CIITA, Myc, ID3 and Spi-B. In particular, down-regulation of Myc by PRDM1 upon anti-IgM treatment has been shown to induce apoptosis in Burkitt's lymphoma cells (Kaptein, Lin et al. 1996; Desai, Bolick et al. 2009). To determine if Bortezomib induced apoptosis in MCL also involves Myc down-regulation, we assessed Myc mRNA changes. Treatment with Bortezomib did not affect the mRNA levels of Myc (Figure 25) indicating that other PRDM1 targets must be involved in the Bortezomib response.

In order to identify novel PRDM1 direct targets in B cells we have used chromatin immunoprecipitation combined with hybridization to human promoter tiling arrays (ChIP-on-chip). This approach identified multiple targets involved in cell cycle regulation and proliferation, including MKI67 and PCNA. MKI67 codes for the antigen Ki67 which is a proliferative marker and is used as a predictor of survival in MCL. Increased levels of Ki67 in MCL have been associated with de-regulation of various cell cycle regulatory components such as over-expression of cyclin D1, HEC and BUB1B which are important for mitotic machinery and down-regulation of Protein Phosphate 2C, which can regulate growth by promoting expression of p53 (Ek, Bjorck et al. 2004). Furthermore, studies have shown that knockdown of Ki67 leads to cell death in human renal carcinoma cells (Zheng, Ma et al. 2006). PCNA codes for proliferating cell nuclear antigen which is found in the nucleus. PCNA is a multifunctional protein that plays a role in both DNA replication and DNA repair (Paunesku, Mittal et al. 2001). It is a subunit of DNA polymerase delta and can interact with p21 to pause replication while allowing DNA repair to occur. In addition loss of PCNA can

lead to a p21 mediated growth arrest in lung epithelial cells exposed to hyperoxia (Garcia 2006). Moreover, cells that lack PCNA expression undergo apoptosis. Studies have shown that in the WST knockout mouse model PCNA is absent from the thymus and spleen leading to reduced size of the tissues and expression of apoptotic markers in these tissues (Libertin, Weaver et al. 1994; Woloschak, Chang-Liu et al. 1996). Together these observations suggested that PCNA and Ki-67 may be functionally important targets of PRDM1.

ChIP-on-chip data analysis and sequence analysis of the MKI67 and PCNA promoter regions suggested potential binding sites for PRDM1 at a distal enhancer region of MKI67 (-4290 to -3594 bp) and the proximal promoter of PCNA (-818 to -235 bp, relative to the transcription start site). Binding of PRDM1 at these sites was determined by chromatin immunoprecipitation (ChIP) and quantitative PCR (Figure 26A). Mino MCL cells treated with 5nM Bortezomib show a significant binding of PRDM1 at both MKI67 and PCNA. The known PRDM1 targets, PAX5 and CIITA also demonstrated similar levels PRDM1 binding as expected. This binding is specific as no signal was detected at the HLA-DRA promoter. PRDM1 is known to repress its targets in part by recruiting the histone deacetylase, HDAC2, and the histone methyltransferase, G9a (Yu, Angelin-Duclos et al. 2000; Gyory, Wu et al. 2004). This results in a loss of acetylation of the histones and specific di-methylation of histone H3 at the lysine 9 position both of which are associated with gene silencing. As shown in Figure 26B Bortezomib treatment leads to a decrease in histone H3 acetylation on the MKI67 and PCNA promoters. A similar decrease in acetylation is observed for PAX5 and CIITA while the control promoter HLA-DRA is not changed. The change in acetylation was accompanied by increases in di-methylation of histone H3 lysine 9 residues consistent with PRDM1-mediated silencing (Figure 26C).

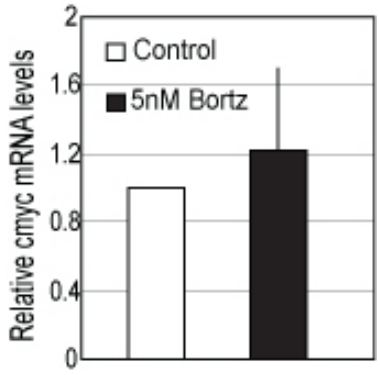


Figure 25: MYC mRNA levels are unaffected by Bortezomib treatment. RT-qPCR analysis for expression of MYC in Mino MCL cells treated with 5nM Bortezomib for 20 hours. Data represent mean of 3 independent experiments normalized to GAPDH. Error bars represent SEM.

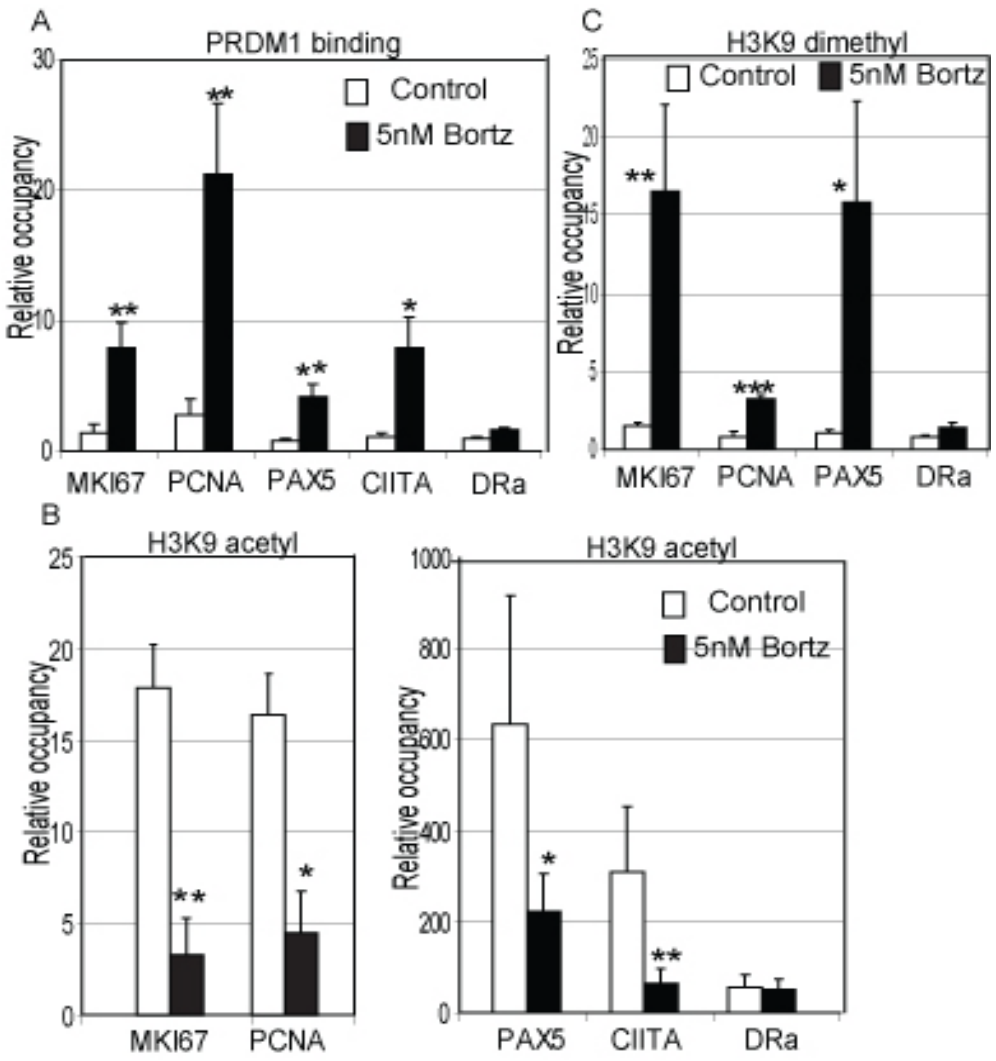


Figure 26. Chromatin immunoprecipitation (ChIP) of PRDM1 and associated epigenetic marks at the MKI67 and PCNA promoter regions. A) ChIP using the PRDM1 antibody in Mino MCL cells treated with 5nM Bortezomib for 20 hours. PRDM1 binding at MKI67 and PCNA promoters was significantly higher in Bortezomib treated cells compared to control cells (untreated). PAX5 and CIITA are positive controls for PRDM1 binding and HLA-DRA (DRα) is a negative control. Binding was quantified by qPCR and is presented as relative occupancy (antibody specific signal over signal obtained with non-specific IgG antibodies). The data is the mean of 3 independent experiements with the SEM shown (*** indicates $p<0.002$, ** indicates $p<0.03$, * indicates $p<0.05$). **B)** ChIP analysis of acetylation on histone H3 lysine 9. The conditions are as described for panel A except an antibody specific to H3 acetylated lysine was used and shows that acetylation is significantly decreased concordant with PRDM1 binding. **C)** ChIP analysis of histone H3 lysine 9 dimethylation levels. The conditions are as described for panel A except an antibody specific to dimethylated H3 lysine 9 was used and shows that dimethylation is significantly increased concordant with PRDM1 binding.

PRDM1 Regulates Promoter Activity of PCNA and MKI67

Since PRDM1 regulates its targets at the level of transcription, we cloned the human MKI67 and PCNA promoters to assess if PRDM1 can repress the promoter activity. A PCNA promoter spanning 576 base pairs upstream of the transcription start site and 152 base pairs downstream was cloned into a luciferase reporter plasmid. A potential PRDM1 binding site was identified by sequence homology at position -296 and was selectively mutated to create a PCNA mutant promoter construct. Luciferase assays were performed in two different cell types U2OS osteosarcoma cell line and Mino MCL cell line. Luciferase activity of the wild type PCNA promoter in both U2OS and Mino was reduced by 60% and 70% respectively, in presence of PRDM1α (Figure 27A and Figure 27B). In contrast, PRDM1α failed to repress the mutant PCNA promoter construct in both the cell types. This indicates that PRDM1 functions specifically though this DNA element to suppress PCNA. Moreover, the basal luciferase activity of the mutant PCNA promoter was significantly reduced only in the Mino cell lines. This indicates that certain activators may bind to this region in a cell type specific manner and regulate the PCNA promoter. IRF proteins and PRDM1 have been shown to bind overlapping DNA sequences. As seen in Figure 28 Mino cells express high levels of IRF4 compared to the U2OS cells. Thus the cell line specific inhibition in luciferase activity may be due to the ability of activator such as IRF4 to bind to and regulate the PCNA promoter in Mino cells.

A similar study was carried out on the human MKI67 promoter. The PRDM1 binding site is located about 3.5kb upstream of the transcription start site. A basal promoter construct was created spanning 2709 base pairs upstream of the transcription start site and 74 base

pairs downstream. In addition a 720 base pair fragment containing the distal PRDM1 binding site was cloned upstream of the proximal promoter. Analysis reveals that the proximal promoter construct was active but not altered by the presence of PRDM1α (Figure 29A). In contrast when the distal region containing the PRDM1 binding site is present the MKI67 promoter activity is repressed approximately 40%. A similar, effect is observed in Mino MCL cells (Figure 29B). This indicates that PRDM1 functions though specific DNA elements present in both the PCNA and MKI67 promoters.

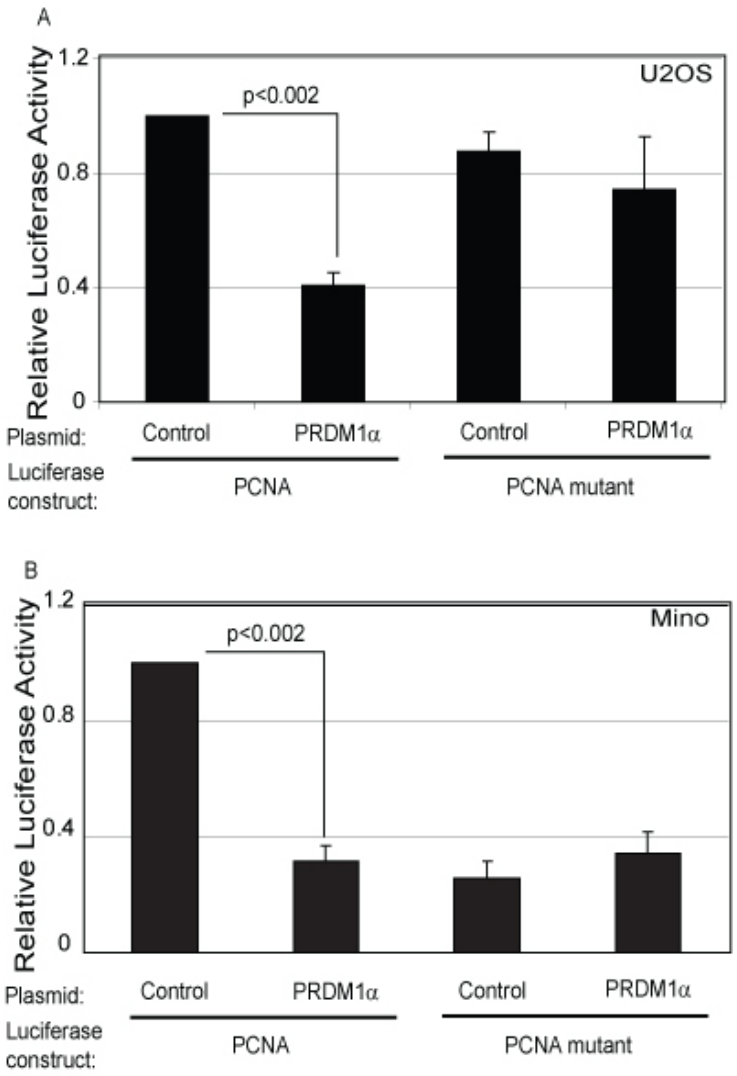


Figure 27. PRDM1 represses PCNA luciferase promoter activity. A) Cells were transfected with luciferase constructs containing either the PCNA wild type promoter (576-PCNA-Luc) or the promoter with a point mutation in the PRDM1 binding site (576-PCNA-mutPRD-Luc). Cells were co-transfected with a PRDM1α expression plasmid or an empty plasmid (-) as indicated in a 1:1 ratio (reporter to expression plasmid). Results are normalized to a co-transfected Renilla control vector and shown as the mean of three independent experiments with the SEM. **B)** Mino Cells were transfected with either the PCNA wild type promoter (576-PCNA-Luc) or the promoter with a point mutation in the PRDM1 binding site (576-PCNA-mutPRD-Luc). The experiment was done and analyzed as described in panel A.

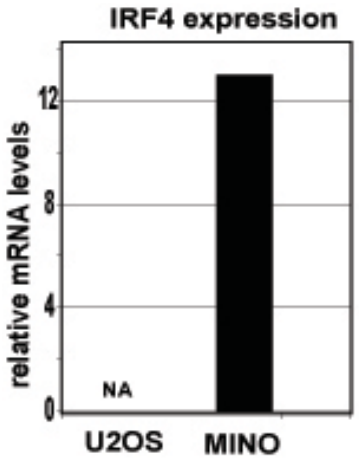


Figure 28: Differential expression of IRF4 in U2OS and Mino: IRF4 is exclusively expressed in Mino, MCL cells but is absent in the osteosarcoma cell line U2OS. The bar graph represents average relative IRF4 mRNA levels in 2 independent RNA harvests.

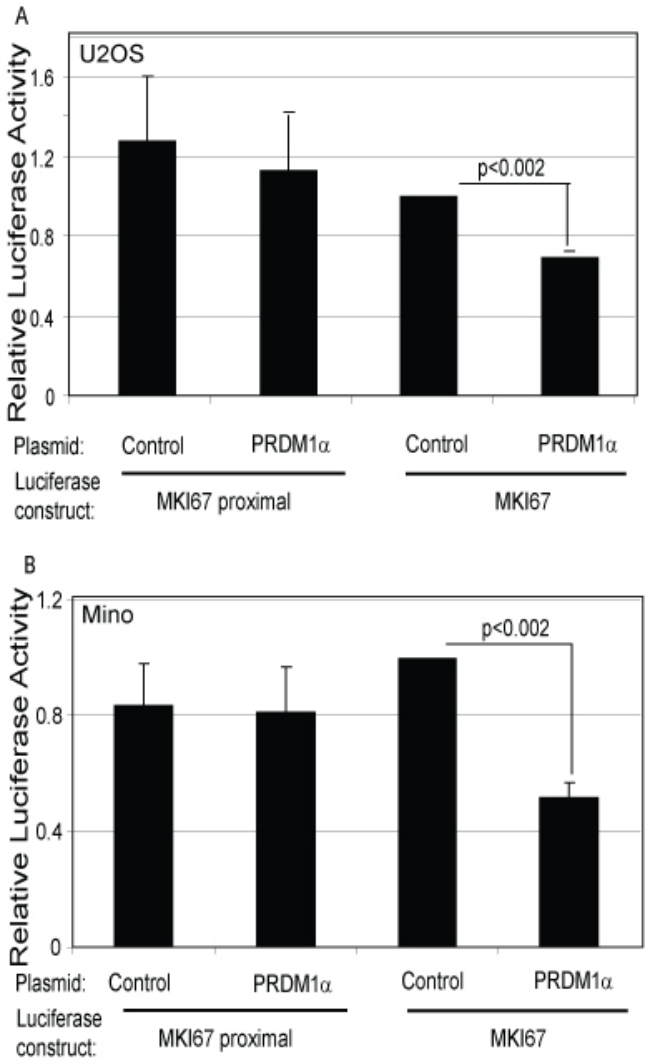


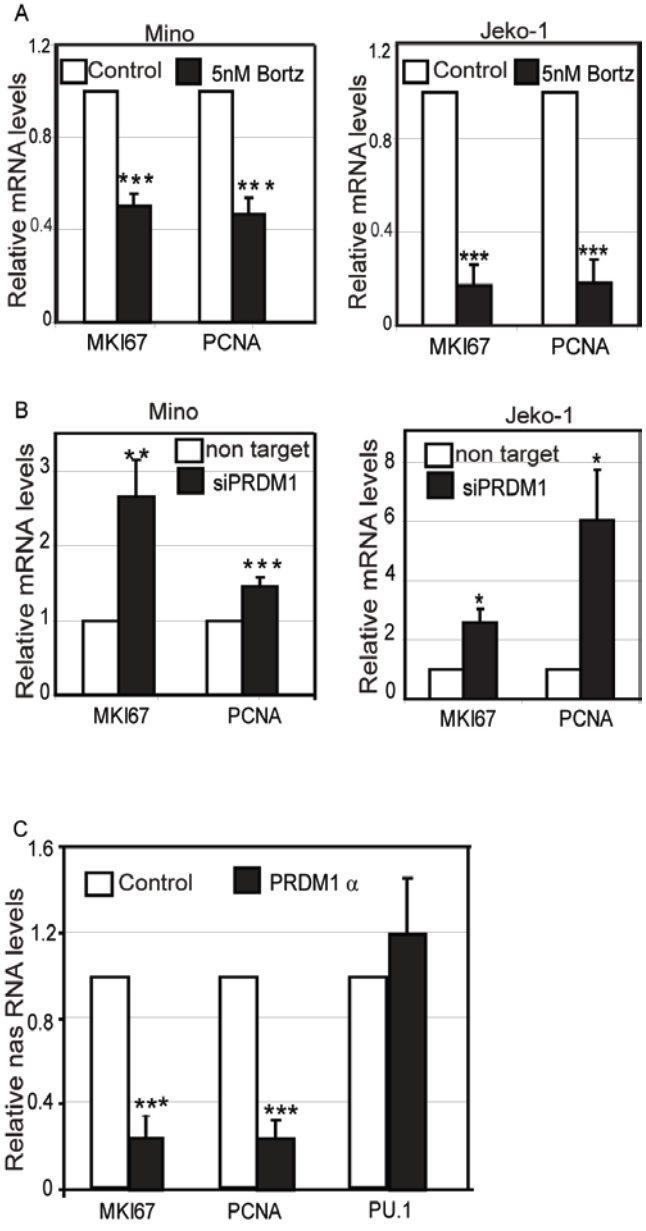
Figure 29. PRDM1 represses MKI67 luciferase promoter activity. A) U2OS cells were transfected with either MKI67 proximal luciferase construct (2709-MKI67-Luc) which does not contain the PRDM1 binding site or the MKI67 promoter also containing the distal PRDM1 binding domain (720/2709-MKI67-Luc).Cells were co-transfected with a PRDM1α expression plasmid or an empty plasmid (-) as indicated in a 1:1 ratio (reporter to expression plasmid). Results are normalized to a co-transfected Renilla control vector and shown as the mean of three independent experiments with the SEM. **B)** Mino MCL cells were transfected with either MKI67 proximal luciferase construct (2709-MKI67-Luc) which does not contain the PRDM1 binding site or the MKI67 promoter also containing the distal PRDM1 binding domain (720/2709-MKI67-Luc). Cells were co-transfected with a PRDM1α expression plasmid or an empty plasmid (-) as indicated in a 10:1 ratio (reporter to expression plasmid). Results are normalized to a co-transfected Renilla control vector and shown as the mean of three independent experiments with the SEM.

PRDM1 Directly Represses Endogenous Target Genes at Level of Transcription

The ChIP assay revealed that PRDM1 can directly bind to the novel target genes MKI67 and PCNA in MCL cells. Moreover, luciferase promoter activity for these promoters is repressed in presence of PRDM1. Thus, to validate the endogenous repressive activity of the PRDM1 induced by Bortezomib treatment, the RNA levels for MKI67 and PCNA were analyzed. Treatment of Mino MCL cells with 5nM of Bortezomib for 20 hours induces PRDM1 (Figure 20A) along with approximately 50% repression of both MKI67 and PCNA at the mRNA level (Figure 30A). Knockdown of PRDM1 expression leads to a de-repression of MKI67 and PCNA mRNA (Figure 30B). This provides strong evidence that PRDM1 expression is required for the repression of MKI67 and PCNA in response to Bortezomib.

To further confirm that PRDM1 represses MKI67 and PCNA at the transcriptional level, we analyzed the nascent RNA levels for these genes after PRDM1α over-expression. Nascent RNAs are those RNAs that are still in the process of being transcribed and are an accurate measure of endogenous transcriptional activity (Wuarin and Schibler 1994). The nascent RNA was purified from nuclei after extensive washing to remove the released transcripts and were quantified by quantitative RT-PCR. The nascent RNA levels for MKI67 in MCL cell line expressing PRDM1α showed a 70-80% repression (Figure 30C) when compared to control cells transduced with a control GFP expressing adenovirus. A similar extent of repression was observed for PCNA at the nascent RNA level (Figure 30C). Over-expression of PRDM1 did not repress the nascent RNA levels of PU.1, which is not a PRDM1 target. These data confirm that PRDM1 can specifically repress endogenous MKI67 and PCNA at the level of transcription in MCL.

To further assess that repression of MKI67 and PCNA by PRDM1 is functionally relevant, we analyzed the protein levels of these genes. Immunofluorescence staining reveals that Bortezomib treatment dramatically reduces both PCNA and Ki-67 levels. However, siRNA knockdown of PRDM1 expression prevents the loss of endogenous PCNA and Ki-67 protein levels in response to Bortezomib (Figure 31).


A
Mino
Control
5nM Bortz
Relative mRNA levels
1.2
0.8
0.4
0
***
***
MKI67
PCNA
Jeko-1
Control
5nM Bortz
Relative mRNA levels
1.2
0.8
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MKI67
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B
Mino
non target
siPRDM1
Relative mRNA levels
3
2
1
0
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***
MKI67
PCNA
Jeko-1
non target
siPRDM1
Relative mRNA levels
8
6
4
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MKI67
PCNA
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Control
PRDM1 α
Relative nas RNA levels
1.6
1.2
0.8
0.4
0
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***
MKI67
PCNA
PU.1

Figure 30. PRDM1α regulates endogenous MKI67 and PCNA at the level of transcription. A) MKI67 and PCNA mRNA levels are repressed by Bortezomib treatment. RT-qPCR analysis of mRNA isolated from Mino and Jeko-1 cells treated with Bortezomib or vehicle only (control) for 20 hours. **B)** Knockdown of PRDM1 rescues expression of MKI67 and PCNA. RT-qPCR analysis of mRNA isolated from Mino and Jeko-1 cells treated with Bortezomib in presence or absence of siRNA knockdown of PRDM1. Non-target indicates the control siRNA designed to not target any known genes. The data in A and B is presented relative to the control cells after normalization to GAPDH and represents the mean of 3 independent experiments with the SEM(*** indicates $p<0.002$, ** indicates $p<0.03$). **C)** Nascent RNA levels of MKI67 and PCNA are suppressed in Mino MCL cells ectopically expressing PRDM1α. PRDM1α indicates cells transduced with an adenovirus expressing PRDM1α while the control cells were transduced with adenovirus only expressing GFP. The data presented is relative to control cells after normalization to GAPDH and represents mean of 3 independent experiments with the SEM.(*** indicates $p<0.002$)

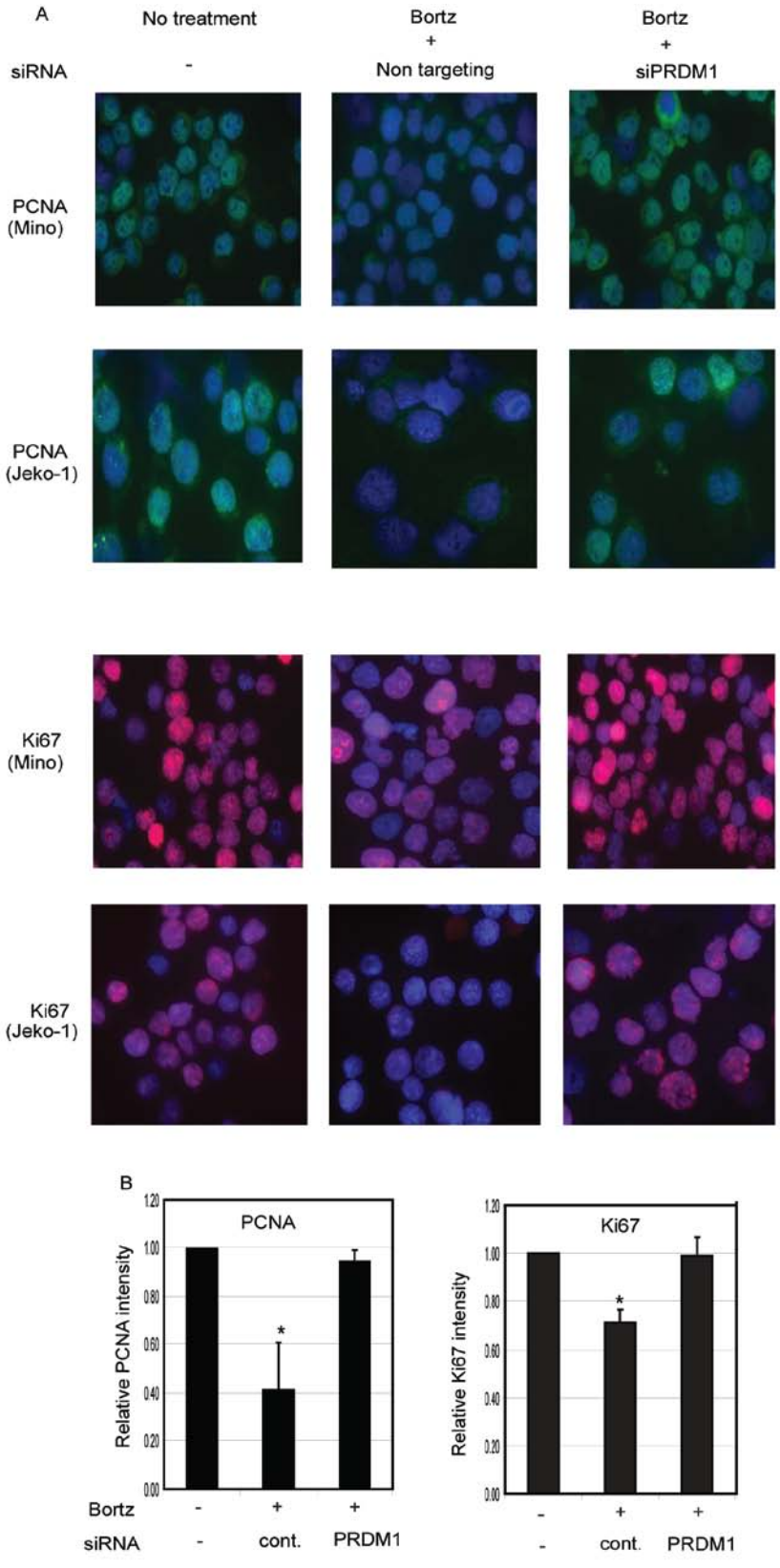
A
No treatment
Bortz
+
Non targeting
Bortz
+
siPRDM1
siRNA
-
PCNA
(Mino)
PCNA
(Jeko-1)
Ki67
(Mino)
Ki67
(Jeko-1)
B
PCNA
Relative PCNA intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
Bortz
siRNA
-
+
+
-
cont.
PRDM1
Ki67
Relative Ki67 intensity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
-
+
+
-
cont.
PRDM1

Figure 31. PRDM1 is required for the Bortezomib mediated suppression of PCNA and Ki-67. A) Immunofluorescence staining of Mino and Jeko-1 MCL cells treated with Bortezomib in the presence or absence of siRNA mediated knockdown of PRDM1. PCNA protein detected by FITC (green), Ki-67 protein detected by Alexa594 (red), and DAPI nuclear staining (blue) is shown from a representative panel. Images shown at 63X magnification of original. **B)** Quantitative analysis of the immunofluorescence. Data was collected from 2 independent experiments in Mino with duplicate slides and at least 5 individual images per slide were analyzed for each condition by automated Difiniens software. (* indicate $p<0.05$)

Discussion

Mantle cell lymphoma continues to have a poor prognosis and a low disease free survival rate. The proteasome inhibitor Bortezomib was approved for treatment of relapsed and refractory MCL in 2006 (Kane, Dagher et al. 2007) and shows promise with an overall response rate of 32% (Goy, Bernstein et al. 2009). However, the specific mechanisms by which Bortezomib is cytotoxic to MCL remains unclear and presents a significant barrier to understanding how to improve or tailor Bortezomib therapy (Pham, Tamayo et al. 2003; Fribley, Zeng et al. 2004; Obeng, Carlson et al. 2006; Perez-Galan, Roue et al. 2006; Rizzatti, Mora-Jensen et al. 2008). The findings presented in this report now demonstrate that Bortezomib induction of apoptosis in MCL is accompanied by and requires induction of the transcriptional repressor protein, PRDM1. Induction of PRDM1 occurs at the level of transcriptional activation. Proteasome-mediated regulation of transcription has been reported in several systems and shown to impact activation, elongation as well as chromatin structure (Muratani and Tansey 2003; Lee, Ezhkova et al. 2005; Sulahian, Sikder et al. 2006; Kinyamu and Archer 2007). In addition, PRDM1 has a PEST domain homology region which could target it for proteasomal degradation. While our studies have not excluded an additive effect of protein stabilization, clearly activation of PRDM1 transcription is required for MCL cells to respond to Bortezomib.

NOXA is a key pro-apoptotic sensor protein that leads to an increased activity of mitochondrial apoptotic pathway by activating BAK. Several recent studies have clearly linked NOXA to the Bortezomib response in both sensitive and intrinsically resistant MCL cells (Perez-Galan, Roue et al. 2006; Rizzatti, Mora-Jensen et al. 2008). Bortezomib selectively induced expression of NOXA but not other BH3-only proteins. Importantly,

siRNA knockdown of NOXA resulted in an approximately 70% reduction of apoptosis indicating that NOXA is a key step in the Bortezomib response. Our findings indicate that induction of NOXA is dependent on induction of PRDM1. siRNA knockdown of PRDM1 not only inhibited apoptosis approximately 70% but also significantly blocked NOXA expression. This is consistent with a central role for NOXA but now also places PRDM1 activation upstream of NOXA induction in the response to Bortezomib. The mechanism of NOXA induction is not understood however it is unlikely that PRDM1, a repressor protein, directly activates the NOXA promoter. There is no evidence for PRDM1 binding to the NOXA promoter, rather PRDM1 is more likely to suppress additional gene(s) whose absence permits NOXA activations although this remains to be resolved.

Recent studies in DLBCL have identified inactivating mutations in the PRDM1 gene, indicating a tumor suppressor role for PRDM1 (Pasqualucci, Compagno et al. 2006; Tam, Gomez et al. 2006). The PRDM1 protein has two isoforms, PRDM1α and the truncated PRDM1β which are transcribed from alternative promoters. PRDM1β has been shown to be highly expressed in myeloma cells and is associated with impairment of PRDM1 repressive activity (Gyory, Fejer et al. 2003; Ocana, Gonzalez-Garcia et al. 2006). Additionally, expression of PRDM1β in DLBCL has been associated in one study with chemoresistance and poor disease outcome (Liu, Leboeuf et al. 2007) indicating an impaired tumor suppressor activity of the β isoform. Related observations have been made in human myeloid leukemia cell lines in which cellular stress led to expression of PRDM1α but not PRDM1β (Doody, Stephenson et al. 2006). Similarly, our findings demonstrate that Bortezomib exposure leads to selective expression of the PRDM1α isoform in MCL and support the idea that the

PRDM1α isoform is the functionally active form and that the PRDM1β isoform arises to potentially squelch the activity.

To date mutations of PRDM1 have not been reported in MCL. MCL arises from pre-germinal center B cells, a stage preceding normal PRDM1 expression which first occurs in late germinal center B cells. Thus the abundant PRDM1 expression in MCL induced by Bortezomib is out of its normal physiological context which may facilitate the apoptotic outcome. This is consistent with our observation that ectopic expression of PRDM1α alone in MCL promotes apoptosis. Interestingly, a recent report has suggested that long term exposure of MCL cell lines to low doses of Bortezomib to induce drug resistance is accompanied by a plasmacytic like gene expression pattern, including PRDM1 expression (Perez Galan 2009). This supports our findings that Bortezomib induces PRDM1 but suggests that suboptimal exposure to Bortezomib can induce a partial differentiation program. It will be interesting to determine if these resistant lines acquire expression of the PRDM1β isoform, similar to myeloma cells or if they acquire PRDM1 mutations similar to DLBCL in order to abrogate normal PRDM1 activity.

A very limited number of genes have been identified to be directly regulated by PRDM1. The majority of these genes are transcription factors related to B cell development and differentiation. Our discovery that PRDM1 directly represses two genes required for proliferation establishes a novel role for PRDM1 in regulating cell growth and viability. Furthermore, down-regulation or knockdown of either PCNA or MKI67 in tumor cells can induce apoptosis (Zheng, Ma et al. 2006; Gehen, Vitiello et al. 2007). Thus, PCNA and MKI67 may be highly potent targets of Bortezomib-induced PRDM1 by inhibiting proliferation as well as inducing apoptosis in MCL. Little information is available concerning

the transcriptional regulation of MKI67 and this is the first report that PRDM1 directly suppresses MKI67. However, a recent finding in sebaceous glands has shown that cells expressing PRDM1 are devoid of Ki67expression (Horsley, O'Carroll et al. 2006). Similarly, microarray studies in B cells have shown an inverse correlation between PRDM1 expression and PCNA (Shaffer, Lin et al. 2002). Moreover, the significance of our data may not be limited to the response of MCL to chemotherapeutic agents but may also have significance in T cell homeostasis. PRDM1 has been shown to play a role in maintaining T cell homeostasis by increasing apoptosis of effector and memory T cells (Kallies, Hawkins et al. 2006). In these studies there were no substantial changes observed in the key survival regulatory proteins such as myc, Bcl-2, Bcl-x_L and CTLA4. It will be important to determine if PRDM1 can directly suppress MKI67 and PCNA in these T cells and induce apoptosis. It may also be possible that MCL tumor cells may have a unique response to PRDM1 when exposed to Bortezomib compared to normal cells. Further defining the global network of PRDM1 regulated genes in multiple cell types will be important to shed light on this question.

In conclusion, this is the first study identifying an important role for PRDM1 in Bortezomib induced apoptosis of MCL. We propose a mechanism of action in which PRDM1 induced by Bortezomib leads to direct repression of the proliferation markers MKI67 and PCNA inducing apoptosis in these cells. Finally, our data supports that approaches to directly target induction of PRDM1 may be an attractive means to enhance current therapies of MCL patients.

CHAPTER FIVE

IDENTIFICATION OF PRDM1 PROTEIN COMPLEX

Introduction

PRDM1 is a member of the PR domain family, which is characterized by the presence of zinc finger domain and positive regulatory (PR) domain. PRDM1 represses its target genes, in part by introducing histone modifications, affecting the chromatin structure and leading to silencing of the gene. PRDM1 can induce histone modifications such as methylation of histone 3 lysine 9 (H3-K9) residues, de-methylation of arginine residues of H3 and H4 and histone deacetylation which are all associated with gene silencing. The PR domain, which is a derivative of the SET domain, does not have an intrinsic methyltransferase activity. It mediates gene suppression by recruiting SET domain- containing protein G9a through its zinc finger region (Gyory, Wu et al. 2004). G9a can regulate methylation of euchromatic H3-lysine 9 (H3-K9) residue and repress transcription of the genes (Tachibana, Sugimoto et al. 2002). PRDM1 can recruit groucho family co-repressor proteins through its proline rich domain (Ren, Chee et al. 1999). Groucho proteins function in part by interacting with histone deacetylases (HDACs) (Chen, Fernandez et al. 1999). Moreover, PRDM1 has been shown to interact with HDAC2 through the groucho interaction domain and the zinc finger region (Yu, Angelin-Duclos et al. 2000). Recently, interactions between PRDM1 and PRMT5 and Lysine- specific demethylase 1 (LSD1) have been identified (Ancelin, Lange et al. 2006; Su,

Ying et al. 2009). PRMT5 is an arginine methyltransferase that mediates di-methylation of arginine 3 on histone H2A and H4 tails (Ancelin, Lange et al. 2006). LSD1 is a histone demethylase that can function as a co-repressor by specifically demethylating mono- or dimethyl groups on H3K4 (Shi, Lan et al. 2004; Shi, Matson et al. 2005). However, none of these interacting proteins are recruited by the SET domain. These findings indicate that PRDM1 can act as a scaffold to recruit multiple co-repressor proteins directly to the promoters leading to silencing of the genes.

To further study and identify other novel PRDM1 interacting proteins, we performed a TAPTAG complex purification analysis. This led to identification of Reptin52 as a novel PRDM1 interacting protein. Reptin52 (also known as TIP49b, TIP48, RUVBL2, Rvb2, TAP54β and TIH2p) has 43% identity with Potin52 (also known as TIP49a, RUVBL1) (Parfait, Giovangrandi et al. 2000; Cho, Bhoumik et al. 2001). Both Reptin52 and Pontin52 possess intrinsic single-stranded DNA stimulated ATPase activity and ATP dependent helicase activities of opposite polarities (Kanemaki, Kurokawa et al. 1999; Makino, Kanemaki et al. 1999). Reptin52 and Pontin52 are ubiquitously expressed in all tissues examined and abundantly expressed in testis and thymus (Kanemaki, Kurokawa et al. 1999; Parfait, Giovangrandi et al. 2000). Reptin52 and Pontin52 have been found in complex with c-Myc regulating its transcription activity (Wood, McMahon et al. 2000). Reptin52 has been identified as part of the TIP60 HAT complex (Ikura, Ogryzko et al. 2000), in INO80 chromatin remodeling complex (Shen, Mizuguchi et al. 2000) and in the β-catenin-TCF complex, and has been shown to modulate the function of the complexes (Bauer, Huber et al. 1998; Bauer, Chauvet et al. 2000). Recently, Reptin52 has been shown to inhibit

transcriptional activity of ATF2 (Cho, Bhoumik et al. 2001). These findings indicate that Reptin52 may function as a repressor by modifying chromatin structure.

This report identifies specific interactions between PRDM1 and Reptin52 indicating that PRDM1 recruits the helicase Reptin52 introducing chromatin modifications at the target promoter regions resulting in repression of expression.

Results

Generation of TAPTAG PRDM1 Expression Vector

To identify novel PRDM1 binding partners we used Tandem Affinity Purification (TAP) method. This is an efficient tool for protein complex purification under non-denaturing conditions (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001). The original TAP tag developed for yeast system consists of two affinity tags, protein A and calmodulin-binding peptide (CBP) separated by TEV protease cleavage sites. To generate the PRDM1α TAP tag protein we used a modified TAPTAG vector called pRAV, which was provided by Dr. Liu (Knuesel, Wan et al. 2003). The pRAV is a bicistronic retroviral expression vector which has the modified TAP tag incorporated in it. The modified TAP tag consisted of two tandem TEV cleavage sites and the CBP tag was substituted with FLAG tag. PRDM1α was cloned into this vector as described in the materials and methods section. The tagged PRDM1α was then transferred from retroviral expression vector to an adenoviral shuttle vector (CMV-IRES1-GFP) to generate PRDM1α- TAP tagged expressing adenovirus (Figure 32).

Cells were transduced with adenovirus expressing PRDM1α or control adenovirus expressing GFP. Lysates prepared from these transduced cells were subjected to a standard tandem affinity purification procedure. As shown in Figure 33 multiple bands were visible, that were selected for tandem mass spectroscopy (MS) protein identification. A partial list of the proteins identified by tandem MS along with the number of peptides identified for each protein is given in table 4.

Figure 32: PRDM1α TAP tag adenoviral construct. Schematic representation of PRDM1α-TAP tag integrated with adenoviral DNA. L-ITR is the left inverted terminal repeat, CMV promoter is the cytomegalovirus promoter region, PRDM1α-TAP is the TAPTAG PRDM1α gene separated from the GFP region by the IRES region. PolyA site is derived from the SV40 virus which is followed by the adenovirus DNA and R-ITR right inverted terminal repeat.

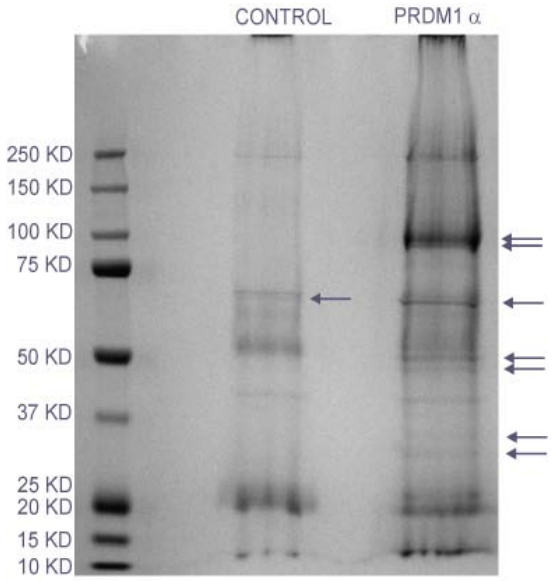

Figure 33: Coomassie Staining of TAPTAG PRDM1α complex. Samples loaded are as labeled. Control lane contains sample purified from cells overexpressing control GFP and PRDM1α lane contains sample purified from cells overexpressing PRDM1α TAPTAG protein. Arrows indicate the bands isolated to be further analyzed by tandem MS to identify the proteins.

Table 4: Partial list of proteins identified by TAPTAG

Gene Name	Alternative Name	No. of Peptides detected
Reptin52	TIP49b, RUVBL2	9
MCM4	Minichromosome maintenance complex component 4	16
YBOX1	Y box binding protein 1	11
SAF-A	Scaffold attachment factor A	4
RBBP4	Retinoblastoma-binding protein 4	4
HDAC2	Histone deacetylase 2	2

Conformation of Interactions Detected by Mass Spectroscopy

Reptin52 an ATPase dependent helicase, which has been shown to promote suppression of transcription activity of ATF2 and suppress *KAI-1* gene, was identified as a protein interacting with PRDM1α. To further confirm this interaction, Burkitt's lymphoma cells CA-46 were transiently transfected with PRDM1α tagged with FLAG at the amino terminus and an HA-HIS tag at the carboxy terminus. Immunoprecipitation of PRDM1α with anti-FLAG specifically isolated endogenous Reptin52 (Figure 34). Immunoprecipitation

analysis also identified that PRDM1α can interact with known partners SAF-A (unpublished) and HDAC2 (Yu, Angelin-Duclos et al. 2000), thus confirming the MS data (Figure 34).

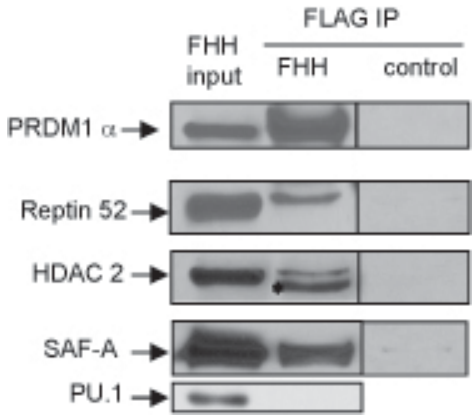

Figure 34: PRDM1 associates with Reptin52: CA-46 cells transiently transfected with FLAG-HA-HIS (FHH) tagged PRDM1α. Control cells were transfected with empty vector. Lysates were immunoprecipitated using anti-Flag and immunoblotted with antibodies specific to PRDM1 (first panel), Reptin52 (second panel), HDAC2(third panel) SAF-A (fourth panel) and PU.1 (fifth panel). HDAC2 and SAF-A are positive controls. PU.1 is a negative control.

Identifying the Protein Interacting Domain

Next, to identify the domain of PRDM1 required for Reptin52 interaction different PRDM1 constructs were used. The full-length PRDM1α, or the deletion mutants which contained deletion of one of the following domains Zinc finger (Δ Znf), C terminal acidic domain (Δ CAc), PEST domain (Δ PEST) and Proline rich domain (Δ PRO) were used. PRDM1α deletion construct containing deletion of 1-331 amino acids as well as the truncated PRDM1β, which has a deletion of the amino terminal domain and an impaired PR domain were also expressed in the CA-46 cells. Anti-PRDM1 was used to isolate protein complex by immunoprecipitation. Using a monoclonal antibody against Reptin52, the immunoprecipitated complexes were tested for presence of Reptin52 protein by immunoblot analysis. Reptin52 was detected in association with all the PRDM1α deletion constructs except for the construct containing the deletion of amino acids 1-331 and PRDM1β (Figure 35). This indicates that the interaction domain may be the PR domain or the amino terminus region.

The data demonstrates that endogenous Reptin52 complexes with over-expressed PRDM1α. To further understand the biological relevance of this interaction, we wanted to assess if endogenous Reptin52 can complex with endogenous PRDM1. To answer this question NCI-H929, multiple myeloma cells, were used. Multiple myeloma cells express high levels of PRDM1α as well as the truncated isoform, PRDM1β (Gyory, Fejer et al. 2003). Immunoprecipitating for endogenous Reptin52 identified that Reptin52 specifically interacted with PRDM1α but not PRDM1β (Figure 36). This observation indicates that the two PRDM1 isoforms alpha and beta, have the ability to form complex with different proteins. PRDM1β has a truncated PR domain and has been shown to have impaired

function. Therefore, the inability of PRDM1β to complex with Reptin52 indicates that the protein interaction domain may be the PR domain or the amino terminus domain. This observation provides preliminary evidence for the impaired function of PRDM1β.

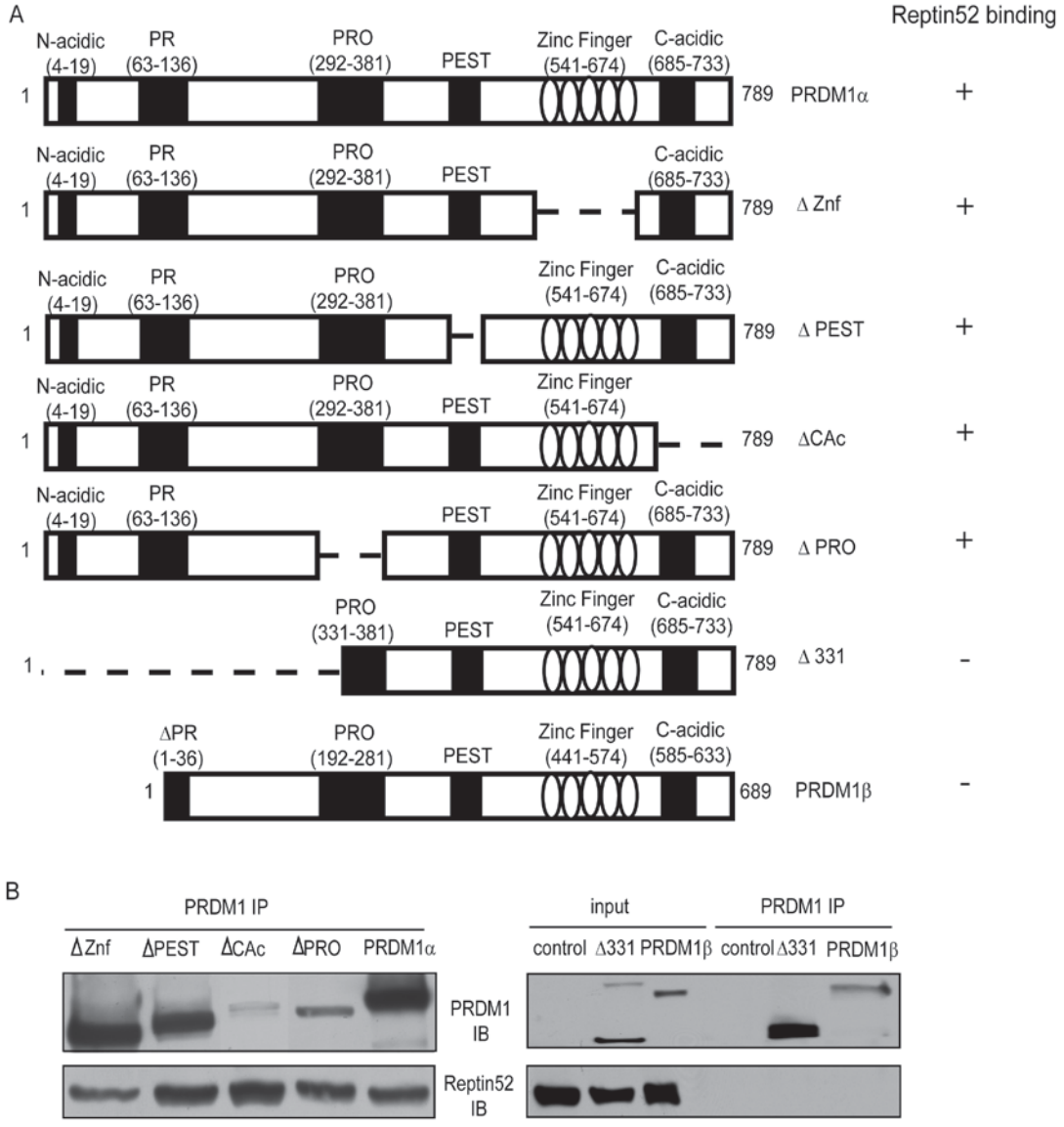

Figure 35: Reptin52 association with PRDM1 domains: A) CA-46 cells transiently transfected with full length PRDM1α, deletion constructs ΔZnf, ΔPEST, ΔCAc, ΔPRO, Δ331 and truncated PRDM1β. **B)** Lysates were immunoprecipitated using anti-PRDM1 and immunoblotted with antibodies specific to PRDM1 and Reptin52. Control lane contains lysate from cells expressing empty pcDNA.

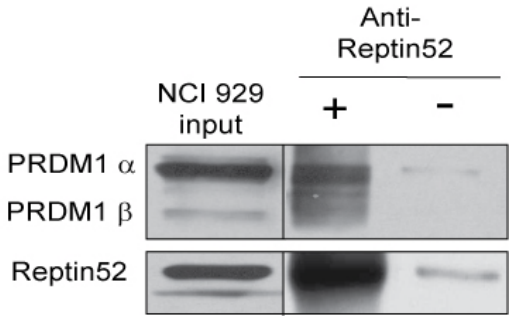

Figure 36: Reptin52 associates with endogenous PRDM1α but not PRDM1β. Protein extracts from NCI-H929 were subjected to immunoprecipitation with anti-Reptin52 antibody followed by immunoblot with anti-PRDM1 (top panel) and anti-Reptin52 (lower panel). NCI929 input represents 4% of protein lysate used for immunoprecipitation.

Discussion

PRDM1 is an important mediator of B cell differentiation into antibody secreting plasma cells. It also functions as a regulator of T cell differentiation into memory and effector cells as well as is required in germ cells (Kallies, Hawkins et al. 2006; Ohinata, Payer et al. 2005; Saitou, Payer et al. 2005). PRDM1 achieves this by recruiting co-repressors such as G9a, Groucho, HDAC2, PRMT5 and LSD1 (Ren, Chee et al. 1999; Gyory, Wu et al. 2004). However, recruitment of these co-repressors may be tissue specific such as PRMT5 which is recruited specifically in the germ cells but is not associated with PRDM1 in the myeloma cell lines (Su, Ying et al. 2009). The recruitment of the co-repressors may also be dependent on the target promoter, such as repression of Myc by PRDM1 requires HDACs but it is not essential for the repression of CIITA. The present study demonstrates the association of Reptin52 with PRDM1, which may give further insight in the mechanism of action of PRDM1. We have used tandem affinity purification technique followed by tandem MS analysis to isolate and identify PRDM1 binding partners.

Reptin52 is ubiquitously expressed and has been found in association with Pontin52 in several chromatin remodeling complexes such as the INO80 chromatin- remodeling complex (Kanemaki, Kurokawa et al. 1999). Recent findings have identified that Reptin52 is also a part of the DNA damage repair multisubunit TIP60 HAT complex (Ikura, Ogryzko et al. 2000). It is an ATPase helicase that can function to reduce the open structure of DNA leading to repression of the gene expression. Moreover, Reptin52 has been found to antagonize the transcriptional effect of T-cell factor/ lymphoid enhancer factor -1-β catenin complex. It has also been identified to be associated with β-catenin complex and is required for promoting repression of metastasis suppressor gene *KAI-1* (Bauer, Chauvet et al. 2000;

Kim, Kim et al. 2005). Besides its ability to complex with chromatin remodeling complexes and other transcription regulation complexes, Reptin52 has also been shown to directly inhibit transcriptional activity of ATF2 (Cho, Bhoumik et al. 2001). Reptin52 has been shown to function as a repressor by binding to or interact with co-repressors such as HDAC1 and TLE proteins. Similarly, our observations indicate that, association of Reptin52 with PRDM1 may be essential for the transcription repressor activity of PRDM1.

PRDM1 protein has two isoforms, the full length alpha form and the truncated beta form. The beta isoform is functionally impaired and has a truncated PR domain, which is thought to be an essential protein interacting domain. Our initial findings show that PRDM1-Reptin52 interaction may require the region between the amino acids 1- 100 which includes the amino acid terminus domain and part of the PR domain. The inability of Reptin52 to complex with PRDM1β may provide clues to the difference in the functionality of the two isoforms. This indicates that the PRDM1 isoforms may interact with different proteins, which may result in a difference in the functionality of the two isoforms.

Further experiments to understand the functional importance of PRDM1-Reptin52 interaction are required. Preliminary chromatin immunoprecipitation experiments were unable to reveal coimmunoprecipitation of PRDM1 with Reptin52 at the PRDM1 target promoters. Alternatively, luciferase assays measuring the ability of PRDM1 to repress its target promoters in presence or absence of endogenous Reptin52, may reveal the functional importance of PRDM1-Reptin52 association. Moreover, recent findings have identified that sumoylation of Reptin52 governs its ability to bind to its protein partners (Kim, Choi et al. 2006). Similarly, it would be important to identify if such post translational modification are required for interactions between Reptin52 and PRDM1.

Initial observations from the report suggest that interaction of PRDM1 with Reptin52 may influence the transcriptional ability of PRDM1. Thus further studies to confirm these findings are essential.

CHAPTER SIX

DISCUSSION AND SCIENTIFIC SIGNIFICANCE

PRDM1 is a transcription repressor that plays a key role in the terminal differentiation of B cells into antibody secreting plasma cells. Recently, PRDM1 has been shown to be involved in maintaining the T cell homeostasis and is required for differentiation of T cells into effector and memory cells (Kallies, Hawkins et al. 2006). Besides its role in immune cells, PRDM1 has been shown to be expressed in several other cell types. PRDM1 has been shown to be required for differentiation of primordial germ cells (Ohinata, Payer et al. 2005; Saitou, Payer et al. 2005). Moreover, PRDM1 has been shown to regulate the formation of sebaceous glands and its presence in breast cancer cells affects the migration of these cells (Horsley, O'Carroll et al. 2006; Wang, Belguise et al. 2009). These findings are indicative that PRDM1 plays a critical role in several different cell types and thus understanding its regulation is necessary.

The work presented in this dissertation provides an insight into the regulation of PRDM1 at the level of transcription in lymphomas and specifically identifies its role in mantle cell lymphoma. The work also provides initial evidence of regulation of PRDM1 protein by its ability to recruit ATPase helicase as a co-repressor that may affect the chromatin structure leading to gene suppression.

PRDM1 is regulated in the B cells by transcription factors AP-1 and Bach2 as well as B cell specific transcription factors such as PAX5 and BCL6 (Vasanwala, Kusam et al. 2002; Ochiai, Katoh et al. 2006; Mora-Lopez, Reales et al. 2007). Stimuli leading to differentiation of these cells causes release of repression and induction of PRDM1. Our data demonstrates that activation of B lymphoma cells, which are malignant counter-parts of GC B cells, by anti-IgM induces expression of PRDM1. Induction of PRDM1 within four hours of anti-IgM stimulation elutes to the fact that the promoter is poised and ready for activation. This is confirmed by the EMSA data which shows that several factors are bound to the PRDM1 promoter in the lymphoma cells. Utilizing ChIP assay we have demonstrated that PU.1 is one of the factors binding to the PRDM1 promoter.

This is the first study to show that, anti-IgM mediated B cell receptor activation in lymphoma cells, induces expression of PRDM1. The data presented demonstrates that PU.1 is required for the expression of PRDM1 in anti-IgM stimulated B lymphoma cells. PU.1 is a transcription factor that can either activate or repress transcription of its target genes. This opposing activity is mediated by differential recruitment of co-regulators by PU.1 (Yamamoto, Kihara-Negishi et al. 1999; Suzuki, Yamada et al. 2003). We have shown that PU.1 can recruit TLE4, a co-repressor, to the PRDM1 promoter which may lead to repression of PRDM1. Our observations indicate that PU.1/TLE4 complex is not acting as a dominant repressor but recruitment of TLE4 is significantly diminished upon activation by anti-IgM. This observation is further supported by the finding that TLE4 transcript levels are reduced in plasma cells which have high levels of PRDM1 (Underhill, George et al. 2003).

PU.1 is an Ets family transcription factor that is required for the normal differentiation of B cells and is expressed throughout B cell maturation (Scott, Simon et al.

1994). A recent report has shown its expression in primary human plasma cells but expression in myeloma cells and cell lines is variable (Tatetsu, Ueno et al. 2007). RPMI-8226, myeloma cell line used in the study presented expresses PU.1. Thus in terms of expression of PU.1 RPMI-8226 may be similar to primary human plasma cells. We observed that knockdown of PU.1 in these cells leads to a reduction in the PRDM1 mRNA indicating PU.1 is required to maintain expression of PRDM1 in certain myeloma cells.

Thus our results suggest that PU.1 may contribute to the initial activation of PRDM1 in lymphoma cells and is required for continued expression of PRDM1 in myeloma cells. Moreover, this is the first study to directly link apoptosis induced by anti-IgM treatment in lymphoma cells to induction of PRDM1. This ability of PRDM1 to induce apoptosis in lymphoma cells was the bases for the second study described in this dissertation.

The second study identifies the specific involvement of PRDM1 in MCL in response to Bortezomib. MCL cells are pre-germinal center B cells that have little to no expression of PRDM1. Treatment of MCL cells with Bortezomib induces expression of PRDM1 along with apoptosis. Our observations are in line with the notion that PRDM1 has the ability to induce apoptosis in lymphoma cells and plays a tumor suppressor role in several B cell lymphomas (Messika, Lu et al. 1998; John and Garrett-Sinha 2009). Initial observations in Bortezomib treated MCL cells have shown that the PU.1 levels are unaffected by the treatment. It would be interesting to identify if the TLE4 expression is reduced after Bortezomib treatment. This would provide a possible mechanism of regulation of PRDM1 expression in these cells upon Bortezomib treatment.

Bortezomib induced apoptosis in MCL is associated with up regulation of pro-apoptotic gene NOXA (Gomez-Bougie, Wuilleme-Toumi et al. 2007). We observe that

knockdown of PRDM1 in MCL leads to reduction in pro-apoptotic gene NOXA along with reduction in apoptosis in the cells. This observation further illustrates the requirement of PRDM1 to achieve a full apoptotic response to Bortezomib. Though, NOXA is not a direct target of PRDM1, its levels are affected by presence or absence of PRDM1. It will be crucial to identify PRDM1 targets that could directly affect the expression of NOXA.

Xbp-1 splicing indicates that Bortezomib treatment induces ER stress in MCL which may cause the cells to undergo apoptosis. The data presented in this study shows that ER stress alone is not sufficient to bring about apoptosis in these cells. Interestingly PRDM1 is able to induce apoptosis in these cells in absence of any ER stress. Furthermore, our data confirms that induction of PRDM1 is required to bring about the full apoptotic effect of Bortezomib. These findings indicate that PRDM1 represses certain downstream targets that may be required for the survival of the cells. ChIP-on-chip assay identified two novel PRDM1 targets, MKI67 and PCNA, involved in cell survival and viability. Analysis of histone modifications at the promoters of these genes, along with analysis of their RNA levels further confirms repression by PRDM1. This is the first study that identifies that MKI67 and PCNA are directly regulated by PRDM1. This discovery may explain the cell cycle arrest caused by Bortezomib treatment. Bortezomib has been shown to lead to a G2/M cell cycle arrest (Lioni, Noma et al. 2008) and this may be because of lack of expression of PCNA and Ki67.

PRDM1 has also been shown to be expressed in hematopoietic as well as non hematopoietic cell lineages. In most of these cells, expression of PRDM1 leads to loss of proliferation as observed in plasma cells or induction of apoptosis as observed in effector T cells and sebaceous gland cells (Horsley, O'Carroll et al. 2006; Kallies, Hawkins et al. 2006).

It would be important to determine if PRDM1 can directly repress expression of MKI67 and PCNA in these different cell types. This would give important clues in understanding the mechanism of action of PRDM1 in development and cell differentiation.

This is the first study identifying an important role for PRDM1 in Bortezomib-induced apoptosis of MCL. This finding may provide clues as to the ineffectiveness of other therapeutic agents. Preliminary experiments in our laboratory have shown that treatment of MCL with DNA damaging agents was unable to induce PRDM1. PRDM1 induction is observed specifically upon treatment with FDA approved Bortezomib and certain HDAC inhibitors (SAHA and LBH589), which are in clinical trials. Thus this indicates that PRDM1 expression is necessary for a higher and robust response rate in MCL patients. Moreover, the ability of the treatment to induce PRDM1 in these cells may prove to be a useful tool to predict response outcome. Finally, data presented in the above study supports that approaches to directly target induction of PRDM1 may be attractive means to enhance current therapies of MCL patients.

PRDM1 is a transcription repressor which acts as a scaffold, and is known to recruit several co-repressors to the promoters of its target genes. The third study presented in this dissertation uses Tandem Affinity Purification technique followed by tandem mass spectrometry to identify novel PRDM1 interacting proteins. This study identifies and confirms interactions between PRDM1 and Reptin52.

PRDM1 has been known to repress its targets, in part by introducing histone modifications. It can achieve this by recruiting co-repressors such as G9a, Groucho proteins, HDAC2, LSD1 and PRMT5 (Ren, Chee et al. 1999; Yu, Angelin-Duclos et al. 2000; Gyory, Wu et al. 2004; Ancelin, Lange et al. 2006; Su, Ying et al. 2009). All these proteins recruited

by PRDM1 are known to directly introduce histone modifications that eventually lead to a closed chromatin structure causing repression of the target genes. The above study is the first to identify that PRDM1 has the ability to also recruit ATPase dependent helicase – Reptin52 to modify chromatin structure inducing repression. TIP49b/Reptin52 is a ubiquitously expressed protein with both ATPase and helicase activities. TIP49b/Reptin52 has been identified as a repressor of ATPase helicase Pointin52 because of its ability to unwind DNA opposite to that of Pontin52 (Bauer, Chauvet et al. 2000). It has been shown to interact with and elicit inhibition of c-Myc, ATF2 and β-catenin transcription (Wood, McMahon et al. 2000; Cho, Bhoumik et al. 2001). Moreover, Reptin52 has also been shown to repress NFκB target KAI1 as well as β-catenin targets by complexing with co-repressors TLE1 and HDAC1 and HDAC2 (Kim, Kim et al. 2005). Thus the identified PRDM1-Reptin52 interaction may play an important role in the functionality of PRDM1 as a repressor. The findings presented in this dissertation provide initial evidence to further study the functional significance of PRDM1 and Reptin52 interaction. Moreover, future studies to identify the ability of PRDM1-Reptin52 complex to be recruited to the PRDM1 targets after Bortezomib treatment in MCL, may provide further understanding of role of PRDM1 in MCL.

This dissertation provides an insight in the regulation of PRDM1 at the level of transcription in myelomas and lymphomas and specifically addresses its role in mantle cell lymphoma. The dissertation also presents work which provides clues that PRDM1 protein function may be regulated by the proteins it interacts with.

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ABOUT THE AUTHOR

Shruti Desai attended M.E.S. Garware College, University of Pune, India from 2000-2003 where she received her Bachelors of Science in Microbiology with specialization in Industrial Microbiology. During this time she gained valuable laboratory experience working under the guidance of Dr Milind Watve and Dr. B.D. Bhole working on several projects understanding the role of microbes in long term preservations and water treatment systems. After graduation she went on to pursue her Masters of Science in Health Sciences at University of Pune. She completed her thesis in the field of drug resistance in infectious diseases. In 2005 she joined the Cancer Biology Ph.D program at H. Lee Moffitt Cancer Center. Here she joined the laboratory of Dr. K.L. Wright. Shruti participated in outside meetings and research retreats, where she was awarded Best Poster Presentation in the Immunology category. She completed her dissertation work in the field of B cell lymphomas and the focus of her research projects was regulation of PRDM1/Blimp1 in these lymphomas. Shruti will continue her research in the field of cancer as a post doctoral fellow at Mitchell Cancer Institute, Mobile.