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Regulation of transcription and analysis of drug targets in lymphoma and myeloma cells

Sophia C. E Bolick

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Regulation of Transcription and Analysis of Drug Targets in Lymphoma and Myeloma Cells

by

Sophia C.E. Bolick

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Dedication

For my mother, Cornelia P.E. Bolick, who had looked forward to this day.
    Gone but never forgotten.
(October 17, 1937-April 15, 2006)
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I would like to thank my mentor, Dr. Wright, for providing me with the opportunity to continue my dissertation research in his lab. I greatly appreciate his time, effort, and mentorship in helping me complete my Ph.D. I would also like to thank my first mentor, Dr. Dalton, for his guidance and mentorship the first 2 years of graduate school.

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<tbody>
<tr>
<td>ABC</td>
<td>Activated B-cell-like</td>
</tr>
<tr>
<td>ABi</td>
<td>Ammonium bicarbonate</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ATF-6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>Blimp-1</td>
<td>B lymphocyte induced maturation protein-1</td>
</tr>
<tr>
<td>BCAP</td>
<td>B cell adaptor for phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>BCL</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>B-CLL</td>
<td>B cell chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt’s lymphoma</td>
</tr>
<tr>
<td>BLNK</td>
<td>B cell linker</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone marrow stromal cells</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSAP</td>
<td>B cell-lineage-specific activator protein</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II transactivator</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CMXRos</td>
<td>Chloromethyl-X-Rosamine</td>
</tr>
<tr>
<td>Cox</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>D&lt;sub&gt;H&lt;/sub&gt;</td>
<td>IgH diversity</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>DMS</td>
<td>Dimethyl sulfate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>F</td>
<td>Farad</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDP</td>
<td>Farnesyl diphosphate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>Follicular lymphoma</td>
</tr>
<tr>
<td>FTase</td>
<td>Farnesyl transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FTI</td>
<td>Farnesyl transferase inhibitor</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating factor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GGTase</td>
<td>Geranylgeranyl transferase</td>
</tr>
<tr>
<td>GGTI</td>
<td>Geranylgeranyl transferase inhibitor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration of 50%</td>
</tr>
<tr>
<td>Id3</td>
<td>Inhibitor of differentiation 3</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgH</td>
<td>Immunoglobulin heavy chain</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>JH</td>
<td>IgH joining</td>
</tr>
<tr>
<td>LBCL</td>
<td>Large B cell lymphoma</td>
</tr>
<tr>
<td>LM-PCR</td>
<td>Ligation mediated PCR</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane perturbation</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MZL</td>
<td>Marginal zone lymphoma</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Pax5</td>
<td>Paired box protein 5</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Pellet cell volume</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PR</td>
<td>PRDI-BF1 and RIZ1</td>
</tr>
<tr>
<td>PRDI-BF1</td>
<td>Positive regulatory domain I binding factor 1</td>
</tr>
<tr>
<td>PRDM1</td>
<td>PR domain containing 1</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rag</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl (lauryl) sulfate</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>XBP</td>
<td>X-box binding protein</td>
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</table>
Regulation of Transcription and Analysis of Drug Targets in Lymphoma and Myeloma Cells

Sophia C.E. Bolick

ABSTRACT

Hematological malignancies, such as lymphomas and myelomas, have low cure rates or remain refractory to treatment, although advances have been made in treatment regimens for these patients. Questions still remain as to what is occurring in these cells on a molecular level, specifically at the level of gene transcription. The positive regulatory domain I binding factor 1 (PRDI-BF1) has been shown to directly repress genes required for cell proliferation and maintenance of the B cell phenotype, however very little is known as to its regulation. The first study presented in this dissertation demonstrates regulation of the PRDM1 gene occurs primarily at the level of transcription in B cell receptor (BCR)-stimulated lymphoma cells and myeloma cells. It also demonstrates PU.1 binding is involved in BCR-mediated activation of lymphoma cells. Most importantly, this study presents evidence of a promoter poised and primed for activation in lymphoma cells. These studies lay the groundwork for the second study which examines modulation of PRDM1 expression in lymphoma cells by chemotherapeutic agents. Induction of PRDI-BF1 in lymphoma cells negative for PRDM1 gene expression correlates with increased apoptosis, which has important therapeutic implications for treatment of lymphomas. One common problem that arises
in treatment of cancer patients is the eventual emergence of a drug resistant population of cells. Identifying specific drug targets and whether they confer drug resistance is an important area of study, which is the focus of the third study presented in this dissertation. It demonstrates the response of myeloma cells to treatment with the farnesyltransferase inhibitor (FTI)-277 and examines whether known mechanisms of drug resistance in these cells are responsible for cross-resistance to FTI-277.
Chapter One
General Introduction

B cell to plasma cell differentiation

All hematopoietic cells are derived from pluripotent stem cells, which can self-renew and give rise to progenitor cells in the myeloid and lymphoid lineages. At each step in the development process, these progenitors become more and more differentiated (160). Early lymphoid progenitors start to express recombination activating genes 1 and 2 (Rag1 and Rag2) and can recombine at the immunoglobulin heavy chain (IgH) locus (101). They can further differentiate into either bone marrow common lymphoid progenitors (CLPs) or early T-cell lineage precursors. CLPs give rise to a multitude of cells of the lymphoid lineage, such as B cells, T cells, dendritic cells, and natural killer cells. With the onset of expression of the cell surface marker B220, CLPs are committed to the B cell differentiation process. Cells then begin to express CD19 and completion of IgH diversity (D_H)-to-joining (J_H) gene segment rearrangement by pre-B cells occurs. Rearrangement of the IgH locus at its variable (V) region continues until V_{H}-DJ_{H} alleles are generated. Rag1 and Rag2 expression is terminated and the product of IgH locus rearrangement is expressed on the cell surface. It forms the pre-B cell receptor (BCR) by assembling with the immunoglobulin (Ig) light chains and signaling molecules Igα and
Igβ, defining the immature B cell stage of development (160, 175). Immature B cells are also characterized by expression of surface IgM (213).

Immature B cells expressing surface IgM migrate to the spleen from the bone marrow for further development (213). A small minority become naïve marginal-zone B cells, homing to the splenic marginal zone (201). Most become naïve follicular B cells, circulating to follicles in the spleen, lymph nodes, and bone marrow. At this point, they either encounter antigen or die. Those encountering antigen will either further differentiate to a plasma cell or form germinal centers, at which point they become memory B cells or undergo differentiation to a plasma cell (figure 1, derived from (160, 236)).

This process is very tightly regulated at the level of transcription. Several transcription factors are involved in maintaining the B cell phenotype, while others are involved in progression to and maintenance of the plasma cell phenotype. Most of these transcriptional regulators work in a mutually exclusive manner, meaning that a factor responsible for maintaining the B cell phenotype is repressed during further development to the plasma cell state (236).
Figure 1. Stages of B cell differentiation and development. B cell to plasma cell differentiation occurs in a temporal and compartmentalized manner. Immature B cells exit the bone marrow for further differentiation in the spleen. In the spleen, they become marginal zone B cells, moving to the secondary lymphoid organs for further development. It is here that follicular B cells, germinal center B cells, and memory B cells further differentiate to plasma cells.
Inducers of plasma cell differentiation

Treatment of B cells with cytokines stimulates the differentiation process. In both murine primary B cells (223) and BCL1 lymphoma cells (263), interleukin (IL)-2 and IL-5 co-stimulation differentiate B cells to plasma cells (164, 223, 263), as demonstrated by increases in PRDI-BF1/Blimp-1 and J chain mRNA (223) and a corresponding increase of Ig-secreting cells measured (118, 223). The effects of IL-2 and IL-5 in the murine system are mimicked by IL-6 in human cells. Human CESS cells stimulated with IL-6 differentiate to plasma cells, with an increase in Ig secretion (173, 295) and PRDI-BF1/Blimp-1 levels (202) and concomitant decrease in B cell markers, like class II major histocompatibility complex (MHC) (173). In some cases, IL-6 requires an additional stimulus to act as a differentiation factor, such as CD40 ligation (25) or IL-2 stimulation (242).

Treatment of both murine and human B cells with IL-21 induces plasma cell differentiation (62, 192). In murine B cells, co-treatment with the B cell receptor (BCR) cross-linker anti-IgM not only induced B cell proliferation, but also induced expression of the plasma cell markers Syndecan-1 and surface Ig and decreased expression of MHC class II (192). This correlated with an increase in PRDI-BF1/Blimp-1 mRNA and a decrease in BSAP/Pax5 mRNA expression. IL-21 treatment either in the presence or absence of anti-IgM crosslinking of the BCR, induced Blimp-1 protein expression in murine cells (192). In human B cells, the same effect is achieved by a combination of IL-21, CD40 ligation, and cross-linking of the BCR with anti-IgM. Cell cycle analysis demonstrated the majority of plasma cells were not actively cycling, indicating they were
terminally differentiated. They were also able to secrete Ig, a hallmark of plasma cells (62, 192).

**B cell receptor cross-linking**

When immature B cells leave the bone marrow (BM), they must undergo selection to become a mature B cell or they die (154). This process is regulated by the BCR. The majority of surviving immature BM B cells become mature B cells of the follicular or mantle zone cell subtypes. Follicular B cells are resting, long-lived B cells with a half-life of approximately 4.5 months. Working with T cells, they are important in the adaptive immune response (86). Mantle zone B cells are readily able to detect foreign antigen, as they are localized to the marginal sinus region of the spleen that filters blood (149). Upon encountering antigen, they respond rapidly by proliferating rapidly and differentiating to plasma cells, independent of T cell involvement (187, 200). These plasma cells generate low affinity antibodies and are likely able to mount a first line of defense against foreign pathogens, allowing time for follicular B cells to coordinate with T cells to mount a high affinity antibody response (257).

BCR signaling plays an important role in the development of an immature BM B cell to a mature B cell. BCRs themselves lack intrinsic cell signaling ability, pairing with both Ig-α and Ig-β to form the BCR complex. Antigen binding to a BCR results in cross-linking of multiple BCRs, leading to the formation of intracellular signaling complexes responsible for different cellular outcomes like proliferation, differentiation, and survival (257). During early B cell development in the BM, the BCR only acts as a scaffold for intracellular signaling complexes, as truncation of the antigen receptor or targeting of Ig-α/β to the membrane do not hinder development past the pre-B stage of development (7,
However, as B cells transition from immature to mature B cells, termed transitional B cell maturation, the role of the BCR becomes important (257).

Immature and mature B cells exhibit different responses to BCR stimulation. This may in part be explainable by how BCR signaling works at the cell membrane. In resting B cells, the majority of BCR complexes are located outside lipid rafts. However, upon antigen stimulation, these complexes localize inside lipid rafts, activating other BCRs in an antigen-independent manner and amplifying the antigen response. Many Src family kinase members like Lyn, Fyn, Blk, and Lck are localized to lipid rafts, which puts them near BCR complexes upon antigen stimulation, allowing for the rapid phosphorylation of immune receptor tyrosine-based activation motifs (ITAM) on Ig-α and Ig-β (257). Mutating ITAMs on Ig-α and Ig-β blocks B cell development in the bone marrow (148, 258). Some of the Src family kinase members do not play an essential role in BCR activation, such as Blk and Fyn (240, 255), while loss of Lyn results in reduced numbers of transitional B cells (2, 35). Phosphorylated ITAMs recruit the cytoplasmic Syk/ZAP-70 kinase family member Syk, which then initiates the intracellular BCR response (264, 265).

ITAM activation results in recruitment of the adaptor molecules B cell linker (BLNK) and B cell adaptor for phosphoinositide 3-kinase (BCAP), which act as scaffolds for more signaling molecules. BLNK and BCAP both appear to be important in the transitional B cell stage of development, as studies with knock-out mice have shown (110, 194, 287, 290). When BLNK is absent, differentiation of immature B cells is delayed (287). BLNK and BCAP activate phospholipase Cγ2 (PLCγ2) signaling acting through separate pathways. BLNK binds PLCγ2 (103) as well as Btk (288), which then
allows for further activation of PLCγ2 (67). Syk and Btk phosphorylate BCAP, allowing it to bind phosphoinositide 3-kinase (PI3K) (10, 186), which leads to the phosphorylation of more downstream targets, leading to activation of PLCγ2 (250). Activation of PLCγ2 leads to the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3), which activates a number of downstream signaling pathways responsible for the differing outcomes of BCR signaling (257).

In addition to proximal signaling events, expression of surface molecules on B cells can modulate outcomes during transitional B cell development. The tyrosine phosphatase CD45 may dephosphorylate the inhibitory tyrosine on Src family kinases, thereby lowering the threshold for BCR-mediated B cell activation (42, 148). CD19 also positively regulates BCR signaling through PI3K and its loss or overexpression affect transitional B cell development (60, 190, 298).

BCR-mediated signaling is an important part of transitional B cell development. However, the differential response of immature and mature B cells to BCR activation suggests the signaling pathways necessary for further development are expressed in a temporal manner (257). To elucidate what is occurring in B cells in vivo, numerous in vitro studies have been done using anti-Ig cross-linking to stimulate the BCR. Anti-IgM cross-linking of the BCR of an immature B cell in the absence of co-stimulatory molecules induces growth arrest or apoptosis (89). The WEHI 231 immature B cell line undergoes growth arrest and/or apoptosis when treated with anti-IgM (12, 87), which may be due to defects in BCR signaling at multiple steps of the pathway. It has been shown transitional B cells contain half the membrane cholesterol of mature B cells (113). Studies in immature B cells have demonstrated the inability of BCRs to localize to lipid
rafts (39, 243), limiting their access to adapter molecules necessary for further BCR signaling cascades. When WEHI-231 B cells are co-stimulated by CD40 ligation, they survive, partly due to the upregulation of the anti-apoptotic Bcl-2 family member A1 (122). In murine primary B cells, survival conferred by IL-4 and anti-IgM co-treatment is mediated by upregulation of another anti-apoptotic member of the Bcl-2 family, Bcl-xL (284). Much of the work detailing BCR activation and consequent signaling cascades have been done in vitro. These studies have recently been extended to primary B cells isolated from normal donors, which have had comparable results and have been able to further elucidate the kinetics of BCR-mediated signaling following anti-IgM cross-linking (102).

**Transcription factors involved in plasma cell differentiation**

**Positive regulatory domain I binding factor 1 (PRDI-BF1)**

The positive regulatory domain I binding factor 1 (PRDI-BF1) was originally found to specifically bind the interferon beta (IFN-β) promoter and suppress IFN-β transcription following viral induction (116). PRDI-BF1/Blimp-1, a zinc-finger containing-protein (116), is induced by IL-2 and IL-5 in murine cell lines (263) and by IL-6 in human cell lines (202). IL-21 is also able to induce PRDI-BF1/Blimp-1 expression (192). Because PRDI-BF1/Blimp-1 is able to drive the differentiation of B cells to immunoglobulin-secreting plasma cells, it has been termed the master regulator of plasma cell differentiation (263).

PRDI-BF1/Blimp-1 belongs to the PR (PRDI-BF1 and RIZ1) domain family, a small family of transcription factors involved in cell differentiation and tumorigenesis. Family members include RIZ1 (PRDM2) (27), MDS1-EVI1 (PRDM3) (66), PFM1/SC-1
Similar to several of its fellow PR domain family members (66, 143, 286), the PRDM1 gene expresses two different products, the full-length PRDI-BF1α (hereafter referred to as PRDI-BF1) and PRDI-BF1β, characterized by a truncated PR domain (figure 2). PRDI-BF1β is the product of alternative transcription initiation using an internal promoter of the PRDM1 gene. PRDI-BF1 is a transcriptional repressor, while PRDI-BF1β binds DNA but lacks transcriptional repression activity (80). PRDI-BF1β mRNA expression is elevated in myeloma cell lines, suggesting the oncogenic potential of this gene (80).

**Figure 2. The PRDM1 gene expresses two different products.** Either full length PRDI-BF1α or the truncated PRDI-BF1β form are transcribed. PRDI-BF1β is the result of alternative transcription initiation using an internal promoter. PRDI-BF1β has a truncated PR domain and only one acidic domain.
PRDI-BF1/Blimp-1 is able to directly repress several downstream targets involved in cell proliferation or maintenance of the B cell phenotype, thereby allowing a B cell to differentiate to a plasma cell. Direct targets include c-myc (140), BSAP/Pax5 (137), Spi-B (229), Id3 (229), and CIITA (74, 202) (figure 3). Mechanisms for how PRDI-BF1/Blimp-1 exerts repressive activity include recruitment of histone deacetylase activity (296) or histone methyltransferase activity to target promoters (81). Studies using c-myc as a target promoter demonstrated PRDI-BF1/Blimp-1 and HDAC2 associate in vivo and are able to bind the c-myc promoter as a complex. PRDI-BF1/Blimp-1 is able to recruit HDAC activity to its target DNA because it contains two independent HDAC association domains, one in the proline-rich region and the other in a zinc-finger region (296). The histone methyltransferase G9a is recruited to the IFN-β promoter by PRDI-BF1/Blimp-1 by binding its zinc finger domain and thereby silences IFN-β gene transcription (81). Whether these mechanisms are involved in silencing of other targets, namely those specifically involved in B cell differentiation, is still unknown.
Figure 3. **PRDI-BF1 represses a number of downstream targets.** Direct targets of PRDI-BF1 are c-myc, BSAP, Spi-B, Id3, and CIITA promoter 3. Repression by PRDI-BF1 further affects the expression of additional targets, changing the global gene expression profile of B cells differentiating to plasma cells.
PRDI-BF1/Blimp-1 is known to cause either differentiation or apoptosis of B cells. The fate of a cell is determined by the stage of development a B cell finds itself in. The 5B1b and 3B3 sublines of the murine BCL1 B cell lymphoma cell line were used to demonstrate activated B cells were able to further differentiate, while immature and partially activated B cells underwent apoptosis upon IL-2 and IL-5 stimulation. The activated 3B3 subline, which was derived from the 5B1b line, underwent differentiation upon interleukin stimulation, with a concomitant increase in plasma cell marker Syndecan expression and decrease in B cell marker CD19 expression. In contrast, the partially activated 5B1b line demonstrated no change in Syndecan or CD19 expression. Transfection of Blimp-1 into these cells demonstrated Blimp-1 induced apoptosis in 5B1b cells, but not 3B3 cells. Similar results were found with forced expression of Blimp-1 in several B cell lines representing various stages of B cell development. Blimp-1 expression resulted in apoptosis of the pre-B 18-81, immature WEHI-231 and mature L10A cell lines. Therefore, induced expression of PRDI-BF1/Blimp-1 determines the ultimate fate of a cell depending on its stage of B cell development. (164)

Recent studies using Prdm1 conditional knockout mice have demonstrated PRDI-BF1/Blimp-1 is required for plasma cell development (237). Its role in this process is accomplished by affecting several downstream targets. It affects cell cycle and proliferation by targeting c-myc (140) and the anti-apoptotic gene A1 (119). Upon introduction of Blimp-1 into immature WEHI-231 lymphoma cells, these markers decreased to levels below that of control cells. These data agree with the normal short life span of plasma cells (119). A1 gene expression levels are comparable to those of mature plasma cells and myeloma cells (254), indicating there is another mechanism for
how long-lived plasma cells are able to circumvent apoptosis. PRDI-BF1/Blimp-1 is also responsible for upregulating genes involved in the plasma cell phenotype, namely those involved in Ig secretion. These include Ig heavy and light chain genes, \( J \) \( chain \) (263) and X-box binding protein (\( XBP \)) (229). Finally, PRDI-BF1/Blimp-1 is also responsible for downregulating genes associated with maintenance of the B cell phenotype, such as \( BSAP/Pax5 \) (137, 267) and \( BCL-6 \) (229).

**Role of PRDI-BF1/Blimp-1 in development**

Blimp-1 homologues play an important role in the embryogenesis of a number of organisms, including \( Drosophila \), zebrafish, sea urchin, chick, and mouse. Blimp-1 activity is required for the development of the peripheral nervous system sense organs and tracheal system in \( Drosophila \) (180) and is a key mediator in the development of slow twitch muscle in zebrafish, acting downstream of Hedgehog signaling (9). The zebrafish Blimp-1 homologue \( u-boot \) (\( ubo \)) specifies formation of the neural crest and sensory neuron progenitors in the zebrafish embryo (217). In chick embryos, the cBlimp-1 is expressed during development of limb buds, eyes, and brachial arches (82). In sea urchin development, the Blimp-1 homologue \( krox \) is alternatively transcribed and each form is expressed in a temporal and spatial specific manner (146). This is similar to what occurs during mouse embryogenesis (36). For instance, in the mouse embryo, Blimp-1 expression appears as early as day 7 in the anterior endoderm and mesoderm head regions. Expression at day 8 is restricted to the developing foregut and primordial germ cells. During organogenesis, Blimp-1 expression is found in developing bone, teeth, and hands, with expression decreasing as development is completed (36).
Direct targets of PRDI-BF1/Blimp-1

B cell-lineage-specific activator protein (BSAP)

B cell-lineage-specific activator protein (BSAP), also known as the murine homologue Pax5 (paired box protein 5), is expressed only in B cells. It both initiates commitment to the B cell lineage and maintains the B cell phenotype (165, 176) and is a direct target of PRDI-BF1/Blimp-1 (137). BSAP/Pax5 is responsible for activating the Rag-mediated $V_H$-to-$DJ_H$ recombination of IgH (297) and $Pax5^{-/-}$ B cells have severely impaired $V_H$ gene segments, thereby demonstrating a requirement for BSAP/Pax5 expression in pre-B cell development (266). Pro-B cells can differentiate into other cell types upon cytokine-induction when BSAP/Pax5 expression is absent (181). BSAP/Pax5 acts upstream of genes important to the identity of B cells, namely CD19 and B cell linker (BLNK) (98) and when BSAP/Pax5 is deleted from B cells, gene expression of these downstream targets is downregulated. Impaired BCR signaling occurs in $Pax5^{-/-}$ B cells, which is a reflection of decreased $Blnk$ expression (98).

Downregulation of BSAP/Pax5 results in the loss of the B cell phenotype and a transition to the plasma cell state. This was seen by the decreased expression of BCR signaling and elevated levels of secreted IgM in the supernatant, both characteristics of a plasma cell phenotype (176). Genes repressed by BSAP/Pax5 have recently been shown to be re-expressed in plasma cells, demonstrating the importance of BSAP/Pax5 in the plasma cell differentiation process (51) as repression of BSAP/Pax5 allows for differentiation to a plasma cell (137). BSAP/Pax5 represses X-box binding protein 1 (XBP), a transcription factor shown to be required for plasma cell differentiation (209). Inhibiting XBP is one mechanism by which BSAP is able to inhibit differentiation to a
plasma cell (210). However, loss of BSAP expression does not mean PRDI-BF1 expression is automatically induced (98). This indicates other factors are also important in maintaining the B cell phenotype and preventing the differentiation to a plasma cell.

Class II transactivator (CIITA)

Products of MHC class II genes play an integral role in immune responses. MHC class II molecules present exogenous peptides to CD4+ T cells, thereby initiating the humoral immune response. They are primarily expressed in antigen presenting cells (APCs), such as dendritic cells, B cells, and macrophages (269), but have also been shown to be expressed in cells of a non-hematopoietic origin (130). MHC class II transactivator (CIITA) is the master regulator of MHC class II transcription. CIITA is constitutively expressed in B cells and dendritic cells and is also induced by IFN-β in some systems (130).

The human CIITA gene, MHC2TA, is transcriptionally regulated by four independent promoters, which each transcribe a unique first exon (figure 4). Promoter I is active in dendritic cells, while promoter III is active in B cells. Promoter IV is the IFN-β-inducible promoter. The exact function of promoter II has yet to be elucidated (170). Although promoter III was first shown to be active and functional in B cells, further study has demonstrated it to also be used in activated T cells (97, 281), monocytes (99), and dendritic cells (124, 130).

Expression of the gene encoding CIITA, MHC2TA, is silenced during the differentiation from a B cell to a plasma cell (220, 239). As PRDI-BF1/Blimp-1 expression increases during differentiation to a plasma cell, expression of CIITA promoter III decreases (202). Additionally, in vivo footprinting of the CIITA promoter
III in plasma cells demonstrates lack of factor binding, meaning CIITA promoter III is bare (75, 239). CIITA promoter III is a direct target of PRDI-BF1 repression (74, 202). PRDI-BF1/Blimp-1 binds CIITA promoter III at a site in the proximal promoter region (74, 202). However, no interaction with this site is seen in plasma cells (75), because the promoter is bare in these cells. PRDI-BF1/Blimp-1 has also recently been shown to repress CIITA promoter IV in lymphoid lineage cells (37).

![Diagram of CIITA gene expression regulation](image)

**Figure 4. Expression of CIITA gene expression is regulated by four independent promoters.** Each promoter transcribes a unique exon 1, which occurs in a cell-type specific manner. Promoter IV is IFN-γ inducible.

### c-Myc

c-Myc plays an important role in the growth, proliferation, and apoptosis of cells (40, 179, 224) and its dysregulation plays an important role in the onset of many different types of cancers, including B cell malignancies (127, 179). c-Myc is a member of the helix-loop-helix/leucine zipper family of transcription factors (18). It is an important factor in normal B cell differentiation, with high expression in the pro-B and pre-B stages.
of development and lower expression in immature B cells which are in more of a resting stage of development. Levels of c-myc increase during antigen-induced proliferation of mature B cells (125, 163).

c-Myc is one of the five direct downstream targets of PRDI-BF1/Blimp-1. The c-myc promoter contains a PRDI-BF1/Blimp-1 binding site, which when occupied represses c-myc promoter activity. These data implicated c-myc as a direct target of PRDI-BF1/Blimp-1 (140). When BCL1 cells are induced to differentiate by IL-2 and IL-5 stimulation, PRDI-BF1/Blimp-1 mRNA levels are induced while c-myc protein expression is decreased (140). Ectopic expression of c-myc in BCL1 cells blocked differentiation of these cells, as demonstrated by the low levels of secreted IgM and unchanged levels of Syndecan-1 on the cell surface. These cells also continued proliferating (138). However, repression of c-myc transcription by a dominant negative form of c-myc did inhibit cell proliferation, but was not sufficient to cause differentiation of BCL1 cells. These data pointed to regulation of additional PRDI-BF1/Blimp-1 target genes as a requirement for plasma cell differentiation (138).

**Spi-B and Id3**

PRDI-BF1/Blimp-1 was found to directly bind Spi-B and Id3 by gel shift assays and chromatin immunoprecipitation assays, corroborating microarray data (229). The Ets family member Spi-B and inhibitor of differentiation 3 (Id3) are both involved in regulating BCR signaling (70, 193, 244). Spi-B is expressed at all stages of B cell development (226) and has also been shown to be expressed in normal plasma cells as well as those isolated from myeloma patients, but not in myeloma cell lines (172). Spi-B acts with PU.1 to regulate proper BCR signaling downstream of Syk phosphorylation
and also affects Btk transcription (171). Id3 is required for proliferation of B cells following BCR cross-linking, but is dispensable for normal B cell development, as has been shown using Id3 knockout mice (193). Both mRNA and protein levels are increased in WEHI 231 B cells stimulated with the BCR cross-linker anti-IgM, with corresponding growth arrest (88). This is due to transcriptional regulation of the Id3 promoter by anti-IgM in these cells (135). Id3 is a direct downstream target of PRDI-BF1, but the authors never studied PRDI-BF1 expression levels in these experiments.

X box binding protein 1 (XBP-1)

X box binding protein 1, or XBP, is a transcriptional activator belonging to the CREB/ATF family. It is required for differentiation to a plasma cell and is expressed at high levels in plasma cells (104, 210). Expression of PRDI-BF1 is required for the induction of XBP expression (237). This occurs by the direct repression of BSAP/Pax5 by PRDI-BF1, which leads to the upregulation of XBP (137). Splicing of XBP mRNA generates a more stable form of XBP, termed XBP$_S$, which plays an important role in the secretory process of plasma cells (105).

Plasma cells and immunoglobulin secretion

A hallmark of plasma cells is their ability to secrete immunoglobulins (236). PRDI-BF1/Blimp-1 and XBP have separate and distinct roles in this process (232). Microarray experiments were done using prdm1-deficient and xbp-deficient mice. Using lipopolysaccharide (LPS) treatment to induce plasma cell differentiation in the splenic B cells of these mice, XBP1 mRNA was shown to be induced in wild-type, but not prdm1-deficient mice, while PRDM1 mRNA expression was induced equally in wild-type and xbp-deficient mice. These data were consistent with previous findings that PRDI-
BF1/Blimp-1 acts upstream of XBP (229, 237). This work showed many genes involved in the secretory pathway were dependent on both PRDI-BF1 and XBP. Expression of the sdc1 gene, which encodes the plasma cell marker Syndecan-1, was dependent only on PRDI-BF1 expression. PRDI-BF1 was also required for Ig gene induction. Therefore, PRDI-BF1 is responsible for plasma cell gene expression, while XBP, acting downstream of PRDI-BF1, is responsible for coordinating the changes in cell structure and function as leading to the secretory phenotype of plasma cells (232).

XBP is involved in the unfolded protein response (UPR) in plasma cells. The endoplasmic reticulum (ER) is the site of protein synthesis and folding, which require an optimal mix of factors such as ATP, Ca$^{2+}$, and an environment conducive to disulphide-bond formation. Because of this, the ER environment can be disturbed by stresses affecting cellular energy levels, the redox state, and Ca$^{2+}$ concentrations. Upon ER stress, unfolded proteins accumulate in the ER, which results in the cell responding with the UPR (249). During the UPR, the ER folds and translocates proteins, protein synthesis decreases, and cell cycle arrest and apoptosis occur (225). While the endoplasmic reticulum is involved in plasma cell differentiation, decreased protein synthesis is not. Therefore, it has been suggested that there is a “physiologic” UPR in addition to the well-defined stress-induced UPR. And, it is this “physiologic” UPR that plasma cells use (72, 232).

Activating transcription factor 6 (ATF-6) (294), IL-4 (105), and Blimp-1-dependent repression of BSAP (137) are all responsible for inducing XBP mRNA. Studies using xbp-deficient mouse B cells induced to differentiate and human Raji B cells expressing the transcriptionally active, processed form of XBP demonstrated XBP
involvement in almost every aspect of the secretory process. XBP regulated genes responsible for targeting proteins to the endoplasmic reticulum (ER), translocating them across the ER membrane, folding of ER proteins, degradation of misfolded proteins, protein glycosylation, trafficking between the ER and the Golgi, trafficking in the endosomal pathway, and targeting of secretory vesicles to the plasma membrane (232). XBP is also responsible for increasing the mass of mitochondria and nucleus, size of the ER, and overall size of the cell (232).

**B-cell lymphoma 6 (BCL-6)**

B-cell lymphoma 6, or BCL-6, was discovered as a gene that is translocated in some non-Hodgkin’s lymphomas (8, 292). It is required for germinal center (GC) formation (52) and is highly expressed in these cells (33). BCL-6 represses PRDI-BF1 expression (234, 262) by inhibiting AP-1-dependent activation (271), by blocking STAT3-dependent gene transcription (211), and by direct repression (235). By repressing PRDI-BF1/Blimp-1 expression, BCL-6 allows the GC reaction to continue before plasma cell differentiation occurs (234). However, microarray analysis shows PRDI-BF1/Blimp-1 is also able to repress BCL-6 expression (229).

**Interferon regulatory factor family**

Expression of interferon regulatory factor (IRF) family members is primarily restricted to lymphoid cells (57, 159). IRF-4 is a member of the IRF family of transcriptional regulators. IRF-4 has been shown to be required for plasma cell differentiation (28) and is expressed by both plasma cells and GC B cells expressing PRDI-BF1/Blimp-1 (5, 64). It is also required for the initial proliferative burst of activated B cells (166). IRF-4 has been proposed to be responsible for preparing B cells
for the differentiation process by inducing this proliferative burst which precedes terminal differentiation (236). This is based on the observation that loss of Mitf, a factor which represses IRF-4, leads to the formation of plasma cells and a concomitant increase in IRF-4 expression (139).

IRF-4 is able to bind IFN-stimulated response elements and repress IFN-stimulated genes on its own (289). In the presence of the Ets family member PU.1, IRF-4 is able to activate the CD20 promoter and the Ig κ 3’ enhancer (57, 94, 203), while IRF-4 complexed with PU.1 can downregulate CD68 expression in lymphoid cells (183). IRF-4 can interact with BCL-6 (79) as well as PRDI-BF1/Blimp-1 (78). Its interaction with PRDI-BF1/Blimp-1 represses CD23b (78).

IRF-8 is another member of the IRF family, which has recently been shown to activate PRDI-BF1/Blimp-1 in murine myeloid progenitor cells (252). IRF-8 is constitutively expressed in macrophages, B cells, T cells, and dendritic cells (56, 152, 174, 221, 259, 276, 277). A recent study demonstrated insertion of proviral elements at the IRF-8 locus in mice with primarily B cell lymphomas or plasmacytomas (153), indicating IRF-8 might play a role in the proliferation of B cell lymphomas. IRF-8 expression was found to be high in B cells, but is decreased in plasma cells (128). In GC B cells, BCL-6 was shown to be a target of activation by IRF-8 (128). Therefore, IRF family members appear to play an important role in B cell development and differentiation.

**PU.1**

PU.1 is a transcription factor belonging to the Ets family and is an important regulator of B cell development (83). PU.1 regulates genes expressed at both early and
late stages of B cell development (49, 161, 270, 293), but is not expressed in plasma cells (172, 198). Mice lacking *PU.1* are deficient for both B cells and macrophages, demonstrating the importance of PU.1 in B cell development (162, 227). PU.1 regulates expression of the IL-7Rα, which is necessary for pro-B cell differentiation and survival signals conferred by IL-7 (49). This regulation occurs in a graded manner. If PU.1 expression is low, B cell development occurs, with concomitant increases in IL-7Rα gene expression (49, 50). When PU.1 expression is high, B cell development is repressed and macrophage differentiation is induced (50).

Ets family members can complex with other transcription factors or other family members to regulate gene expression (134). They can interact with AP-1, Sp1, BSAP, NF-κB (134), and IRF family members (61, 157) in a cell-type specific manner. In hematopoietic cells, PU.1 can interact with IRF family members to regulate gene expression (183, 188, 270). For example, IRF-8 has been shown to regulate gene expression of RANTES in cooperation with IRF-1, PU.1, and NF-κB at an NF-κB site in murine macrophages (142).

**B cell lymphomas**

Lymphoid malignancies, such as lymphomas and leukemias, can occur when the normal process of B cell differentiation is disrupted. Gene expression profiling has recently demonstrated the similarity between lymphoid malignancies and various stages of normal B cell development (46, 230, 233, 238). Diffuse large B-cell lymphomas (DLBCL) are further classified as either germinal center B-cell-like (GC) DLBCL or activated B-cell-like (ABC) DLBCL. GC DLBCLs have a gene expression profile comparable to that of normal GC B cells (1, 214, 215) and appear to be fixed at the GC
stage of B cell differentiation (230). Unlike GC DLBCLs, ABC DLBCLs have a molecular profile analogous to activated peripheral B cells (1, 214, 215). They resemble pre-plasma cells in terms of their increased expression of Ig, X-box binding protein 1(XBP), IRF-4, and PRDI-BF1/Blimp-1 (195, 215, 230, 251) while their expression of BCL-6 is lower (215, 230). Burkitt’s lymphomas also express GC B cell genes, as do follicular lymphomas (230). Burkitt’s lymphomas can be distinguished from DLBCL by high levels of c-myc target genes and a subgroup of GC B cell genes as well as low levels of MHC class I genes and NF-κB target genes (46). Distinguishing the molecular profiles of lymphomas is important, as the specific genetic alterations that occur disrupt normal B cell differentiation by affecting their progression through the cell cycle, their ability to undergo apoptosis, and their ability to differentiate normally (230).

**Role of PRDI-BF1 in lymphoma and myeloma cells**

PRDI-BF1/Blimp-1 has long been demonstrated to be expressed in plasma cells, but not in B cells (172). Recent findings demonstrate expression of PRDI-BF1/Blimp-1 in a subset of lymphoma cells, namely GC B cells which are also positive for plasma cell markers (32). Diffuse large B cell lymphoma (DLBCL) can be divided into several subtypes based on recent molecular profiling data. These subtypes are representative of the origins from the different stages of B cell differentiation. Germinal center B cell-like (GCB) DLBCL is thought to derive from GC centroblasts, while activated B cell-like (ABC) DLBCL resembles the phenotype of a small group of GC cells undergoing differentiation to a plasma cell (1, 230).

However, even though a subset of ABC-DLBCLs expresses the *PRDM1* gene, it appears to be a functionally inactive form, as protein levels are not expressed in these
cells (24, 69, 195, 251). It has been proposed that lack of functional PRDI-BF1/Blimp-1 leads to inhibition of differentiation in these cells, forcing these GC B cells to remain in an active, proliferative state, eventually leading to the development of DLBCL (251).

Mutational analysis of the \textit{PRDM1} gene in both DLBCL cell lines and patients demonstrated genetic alterations, leading to a lack of protein expression in these cells. Of 35 patient samples studied, 8 had genetic alterations such as point mutations and frameshift mutations, all leading to premature translation termination and production of a non-functional form of PRDI-BF1/Blimp-1 (251). Pasqualucci et al. did a similar study of the \textit{PRDM1} gene, but looked at mutations in both ABC-DLBCL and GC-DLBCL patients. No \textit{PRDM1} gene mutations were found in GC-DLBCL patient samples, but 24% of ABC-DLBCL patient samples had genetic alterations (195). It appears \textit{PRDM1} gene inactivation by mutation in some DLBCL patients contributes to lymphomagenesis by inhibiting the normal differentiation process of GC B cells (195, 251). The loss of functional PRDI-BF1/Blimp-1 in some DLBCL cases suggests \textit{PRDM1} acts as a tumor suppressor gene in a small subset of lymphomas (195, 251).

The expression of PRDI-BF1/Blimp-1 in myeloma cells has been widely reported (21, 74, 80, 172). However, a recent report by Borson et al. shows \textit{PRDM1} gene expression is found in B cells derived from myeloma patients, but not in those from normal, healthy donors (21). Genetic alterations of the \textit{PRDM1} gene were not studied (21), but these findings indicate PRDI-BF1/Blimp-1 may be important to the pathology of lymphomas and myelomas.
Drug resistance in multiple myeloma

Multiple myeloma is a fatal plasma cell malignancy characterized by the accumulation of latent plasma cells in the bone marrow (84). These cancerous cells resist apoptosis and are characterized by a low-proliferative index (268). The disease course of patients with myeloma typically includes the emergence of drug-resistant plasma cells (76), a phenotype termed multidrug resistance (MDR). Following an initial response to treatment, many patients invariably become refractory to treatment. Therefore, it becomes important to find new drugs which can overcome this phenotype and still be effective in patients.

Several mechanisms of MDR have been well elucidated. These include decreased uptake of drugs, due to the presence of drug transporters; cellular mechanisms which diminish the capacity of drugs to kill cells, such as cell cycle alterations, DNA damage repair mechanisms, and altered drug metabolism; and, increased energy-dependent efflux of drugs that enter the plasma membrane by diffusion (248). Drug resistance in myeloma cells is also conferred by interaction of myeloma cells with extracellular matrix components of the bone marrow (45).

Activating Ras mutations have been shown to be involved with chemoresistance in melanoma cells (107) and ovarian cancer cells (96). They have also been demonstrated to be important in myeloma cells. ANBL6 myeloma cells transfected with mutated N- or K-Ras were shown to be more resistant to dexamethasone, doxorubicin, and melphalan than control cells (216). Recently, the same ANBL-6 transfectants were shown to have enhanced binding to both the extracellular matrix component fibronectin and bone marrow stromal cells (BMSC), which was found to be mediated by increased
levels of cyclooxygenase (cox)-2 in fibronectin-adhered cells. The mechanism for enhanced binding to the BMSCs remains to be elucidated (95). Regardless, increased cox-2 levels in ANBL6 cells expressing activated N- and K-Ras are correlated with increased resistance to treatment with melphalan (95). Although the exact mechanism by which activated Ras protects cancer cells from apoptosis is still unknown, activating mutations of the Ras oncogene play an important role in protecting myeloma cells from chemotherapy-induced apoptosis.

**Apoptosis and role of Bcl-x<sub>L</sub>**

Apoptosis, or programmed cell death, is a finely tuned cascade of events leading to the eventual death of cells. Apoptosis plays an important role in the daily self-renewal of organs and tissues in the body. As new cells are produced, a homeostatic balance is maintained by older cells dying in an ordered fashion (207, 208). The apoptotic process contrasts with necrosis, in that there are no inflammatory reactions or tissue scarring (207). When apoptosis is deregulated, disease states such as cancer occur and can be maintained due to defects in the apoptotic machinery.

The Bcl-2 family of proteins plays an important role in regulating cell death. Several family members are pro-apoptotic (Bax, Bok, Bid), while others have an anti-apoptotic role in cells (Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, Bfl-1/A1) (34). Bcl-2 family members regulate apoptosis by either interacting with other family members (197) or by regulating ion-channel activity (207). It is the ratio of pro- and anti-apoptotic Bcl-2 family members which determines the ultimate fate of a cell in response to an apoptotic signal (268). Anti-apoptotic family members are localized to the nuclear membrane, endoplasmic
reticulum, or mitochondrial outer membrane, while pro-apoptotic family members are generally found in the cytosol or associated with the cytoskeleton (77).

Bcl-x<sub>L</sub> expression is associated with chemoresistance in myeloma cells (261) and is expressed in normal plasma cells as well as myeloma cell lines and patient samples (205, 241). IL-6, an essential cytokine to the growth and survival of myeloma cells, upregulated Bcl-x<sub>L</sub> protein levels in both patient samples and cell lines (205). Studies using Bcl-x<sub>L</sub> transgenic mice demonstrate a nonmalignant plasma cell phenotype in both the bone marrow and lymphoid organs of these mice, whose incidence increased with the age of the animal (141). However, when the Bcl-x<sub>L</sub> mice were crossed with c-myc transgenic mice, the plasma cell phenotype became one of malignancy with death from lymphoproliferative disorder at 5.5 weeks in 50% of the double-transgenic mice (141). These data provide a link between Bcl-x<sub>L</sub> and myeloma disease pathology.

**The role of the Ras oncogene in myeloma**

The Ras proteins are members of a large superfamily of GTP-binding proteins, which are subdivided based on sequence homology. Ras family members are involved in control of cell growth. This family is comprised of 3 members, namely H-Ras, K-Ras, and N-Ras. These members can be activated by mutation at codons 12, 13, and 61 (22). When Ras is activated, it is bound to GTP and able to interact with its downstream target enzyme (55). When Ras is inactive, it is found in the GDP-bound form. In the normal situation, the ratio of bound GTP to GDP controls the activity of Ras proteins (29). This biological activity is controlled by a GDP/GTP cycle, which is regulated by GDP/GTP exchange and GTP hydrolysis. GEFs, or guanine nucleotide exchange factors, regulate the exchange of GDP for GTP, while GAPs, or GTPase activating proteins, regulate
hydrolysis of GTP (29). However, when Ras is mutated, the equilibrium of Ras-GTP to Ras-GDP is weighted in favor of the active, GTP-bound form. GAPs are prevented from hydrolyzing the GTP on Ras, locking it in the active form (55). This leads to constitutive activation of Ras proteins.

Members of the Ras family GTPases have a well-established role in oncogenesis (22). Mutations of various Ras family members have been shown to be associated with various cancers (156), including multiple myeloma. Numerous investigators have examined the frequency of Ras mutations in multiple myeloma, and have reported a correlation between activating Ras mutations and disease progression (15, 41, 144, 178, 204, 206). Activating mutations of N-Ras and K-Ras codons 12, 13, and 61 are involved in myeloma disease progression (15).

**Prenylation of the Ras oncoprotein**

Ras proteins must undergo further post-translational modification upon being synthesized to become biologically active molecules. Otherwise, Ras remains an inactive, cytosolic protein. Ras proteins undergo protein prenylation to allow targeting to the plasma membrane, where they become active players in signaling cascades (55, 85, 280). The mevalonate pathway, also commonly recognized as the cholesterol biosynthetic pathway, is responsible for the production of cholesterol and nonsterol products (167). It is also the pathway that plays an important role in protein prenylation reactions (see figure 5). Two enzymes, farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I), catalyze this prenyl posttranslational modification by transferring farnesy1 or geranylgeranyl, respectively, to the cysteine of the carboxyl terminal CAAX box ((C=cysteine, A=aliphatic amino acid (leucine,
isoleucine, or valine), X=any amino acid)) (55, 106). Following addition of the farnesyl or geranylgeranyl group to the cysteine residue, the three terminal amino acid residues (AAX) are cleaved in the endoplasmic reticulum by the endoprotease Rce1(191). Finally, the cysteine residue becomes carboxyl methylated (43, 65). H-, N-, and K-Ras are all farnesylated. When FTase is inhibited, K-Ras, but not N-Ras or H-Ras, becomes geranylgeranylated (133).
Figure 5. The mevalonate pathway plays a role in generating prenylated proteins. Farnesyl or geranylgeranyl are transferred to the carboxyl terminal cysteine residue by either the enzyme farnesyl transferase or geranylgeranyl transferase, respectively. (adapted from (167))
Figure 6. Prenylation occurs on the carboxy terminal CAAX box. Farnesyl and geranylgeranyl groups are transferred to the cysteine residue of the CAAX motif by either farnesyl or geranylgeranyl transferase, respectively. The –AAX residue is cleaved in the endoplasmic reticulum (ER) by the enzyme Rce1 and the cysteine residue is modified by addition of a carboxy methyl group (OMe). (adapted from (123))
**Downstream targets of Ras**

Activated Ras is able to mediate signaling through a variety of downstream kinase pathways that regulate cell proliferation and survival (123). These include the Raf-MEK-MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol-3 kinase)/AKT pathways. These pathways can be activated in response to a variety of growth factors (131). However, activated Ras has been shown to act independently of the exogenous myeloma growth factor IL-6 in Ras-mutated ANBL6 cells (16, 17), while the parental ANBL6 cells are IL-6-dependent and stop growing when IL-6 is removed (108). Therefore, mutated Ras confers a survival advantage to myeloma cells by activating signaling pathways responsible for cell growth and survival, independent of normal growth factors.

The Raf-MEK-MAPK pathway is one of the key signaling pathways downstream of Ras. Raf is a serine-threonine protein kinase which binds Ras to become active (23, 274). Once active, Raf is able to initiate a cascade of phosphorylation events in downstream targets, which results in the phosphorylation of MAPK. MAPK is now active and can translocate to the nucleus, activating transcription factors which regulate cell proliferation and apoptosis (20, 285). In myeloma, MIP1-α was recently shown to be a downstream target of MAPK signaling in Ras mutated myeloma cells. MIP1-α is an important survival and proliferation factor in myeloma cells as well as an important factor in myeloma-associated bone disease (158).

Myeloma growth-stimulating cytokines, such IL-6 (4, 115), activate signal transduction through the PI3K/AKT signaling pathway resulting in cell proliferation and survival (26, 117). Activated Ras interacts directly with the PI3K catalytic subunit,
which leads to the activation of the serine-threonine kinase AKT (109, 212). AKT is frequently activated in myeloma cells and correlates with disease activity (100). Inhibition of AKT resulted in decreased cell proliferation (260) and increased apoptosis (93). Treatment with both the farnesyl transferase inhibitor lonafarnib and the proteasome inhibitor Bortezomib were shown to downregulate phospho-AKT and induce apoptosis (47).

**Inhibitors of protein prenylation**

The high incidence of Ras mutations in cancers, which leads to the constitutive activation of Ras proteins, drove the development of pharmacologic inhibitors of Ras (123). Non-selective agents which targeted the mevalonate pathway, such as the HMG CoA reductase inhibitor lovastatin, were initially used to inhibit Ras prenylation. Unfortunately, patients treated with this drug developed dose-inhibiting side effects due to the nonspecific nature of this compound (256). Therefore, inhibitors to other steps of the mevalonate pathway were developed to target protein prenylation of Ras. Several classes of farnesyl transferase inhibitors (FTI) have been designed in recent years to target the biological function of Ras proteins. Screening of libraries and natural products has yielded farnesyl diphosphate (FDP) analogues as one class of FTIs. CAAX peptidomimetics are another class of FTIs which compete for farnesyl transferase binding by mimicking the carboxyl terminus CAAX motif of Ras and related proteins. Some compounds share properties of both the CAAX box and farnesyl diphosphate and are known as bisubstrate analogues because they mimic a step in the mevalonate pathway. A fourth class of FTIs are the nonpeptidomimetic inhibitors, which have been identified by random screening of compound libraries (55, 123).
FTIs have been developed by pharmaceutical companies as potential anti-cancer agents. Some of these include R115777 (tipifarnib), SCH66336 (lonafarnib), and BMS-214662 (106). In clinical trials, R115777 has been found to be effective against hematopoietic malignancies, like myeloma (3). Geranylgeranyl transferase inhibitors have also been developed because when farnesylation of N- and K-Ras are blocked, they become alternatively geranylgeranylated. However, attempting to use both together at doses required to block prenylation are not effective due to toxicity issues (147). Therefore, targeting activating mutations of Ras in myeloma cells with prenyltransferase inhibitors is an important area of study.
Chapter Two

Transcriptional regulation of PRDI-BF1/Blimp-1 in lymphoma and myeloma cells

Introduction

The positive regulatory domain I binding factor 1 (PRDI-BF1) was originally found to specifically bind the interferon beta (IFN-β) promoter and suppress IFN-β transcription following viral induction (116). Blimp-1, the murine homologue of PRDI-BF1, was originally described by Turner et al. as a transcription factor that could induce the differentiation of B cells (263). PRDI-BF1/Blimp-1 has since been found to be required for the differentiation of a B cell to a plasma cell (237). During the differentiation of mature B cells to plasma cells, PRDI-BF1 represses multiple genes involved in maintaining the B cell phenotype and in maintaining cellular proliferation, such as CIITA (74, 202), c-myc (140), and BSAP (137). Microarray studies have outlined the PRDI-BF1 repression profile and led to the identification of two additional direct targets, Spi-B and Id3 (229). Additionally, PRDM1 has recently been linked to cellular stress and the unfolded protein response (54).

Anti-IgM cross-linking of the B cell receptor has been reported in multiple studies to induce apoptosis in lymphoma (12, 30, 87, 112, 299). This response has been correlated with decreased levels of c-myc (140). Inducing PRDI-BF1/Blimp-1 expression in lymphoma cells with histone deacetylase inhibitors also decreased
expression of the downstream targets c-myc and BSAP (129). More specifically, introduction of Blimp-1 into lymphoma cells can induce apoptosis, suggesting PRDI-BF1 may be an important mediator of the anti-IgM and apoptotic response (119, 164). However, no direct link between expression of PRDI-BF1/Blimp-1 and anti-IgM mediated B cell receptor activation has been described.

Recently, PRDI-BF1/Blimp-1 expression has been detected in some diffuse large B cell lymphomas (DLBCL) (69, 195, 251). However, inactivating mutations were described indicating a potential tumor suppressor role for this gene (195, 251). Similarly, proliferating myeloma cells and myeloma cell lines abundantly express the truncated PRDI-BF1 isoform, PRDI-BF1β, which has impaired function (80). In addition, Borson et al. (21) suggest that PRDM1 expression is present in B cells isolated from myeloma patients while normal donors lack expression as expected. The mutation status of PRDM1 in these myeloma derived B cells is unknown. Together these findings indicate PRDI-BF1/Blimp-1 may be important to the pathology of various hematopoietic malignancies, including lymphoma.

Very little is known about how PRDI-BF1 expression is regulated. 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 mRNA levels and promoter activity while mRNA stability is unchanged. Using promoter deletion constructs and in vivo genomic footprinting, we demonstrate two regions of activation in the PRDM1 promoter and multiple in vivo protein-DNA interactions at the proximal promoter and at more distal activation regions in both lymphoma and myeloma cells. Three of the detected
interactions occur in a cell-type specific manner and PU.1 binding is revealed to be 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 PRDI-BF1 expression in lymphoma cells may be a viable target to inhibit lymphoma progression.

Results

Expression of PRDM1 mRNA levels in lymphoma and myeloma cells

Most B cells lack detectable levels of PRDM1 until they commit to differentiate (5, 24, 32, 69). As shown in figure 7, Burkitt’s lymphoma cell lines lack detectable levels of PRDM1 while myeloma cells have abundant PRDM1 expression. Analysis of myeloma patient bone marrow samples show that compared to normal bone marrow, myeloma bone marrow expresses very high levels of PRDM1 mRNA. We next examined the effects of B cell receptor cross-linking on PRDM1 mRNA levels in CA46 Burkitt’s lymphoma cells. Treatment with anti-IgM to cross-link the B cell receptor significantly increased PRDM1 mRNA levels approximately 8-fold above untreated controls (p<0.05) (figure 8A). This induction strongly correlated with increased apoptosis, as measured by Annexin V staining (figure 8B).
Figure 7. PRDI-BF1 mRNA levels are elevated in myeloma cells versus lymphoma cells. A, Basal levels of PRDI-BF1 mRNA were determined in both lymphoma and myeloma cell lines by real-time RT-PCR. Lymphoma cells have nearly undetectable mRNA, while myeloma cell lines have significant but variable levels. PRDI-BF1 mRNA levels were normalized to GAPDH mRNA levels. The data is representative of at least three experiments. B, PRDI-BF1 mRNA levels are elevated in bone marrow samples derived from myeloma patients when compared to normal healthy donor samples. NBM, normal bone marrow (Stratagene); N1 and N2, donors with other disease but without any evidence of bone marrow involvement (H. Lee Moffitt Cancer Center Tissue Bank); M1-M5, myeloma patient bone marrow containing 53-76% plasma cells.
Figure 8. Anti-IgM treatment induces PRDI-BF1 mRNA levels and apoptosis in CA46 lymphoma cells. A, Treatment of CA46 lymphoma cells for 24 hours with 10 µg/ml anti-IgM induces PRDI-BF1 mRNA levels significantly. The mean of 3 experiments is shown (p<0.05, Student’s t-test). B, Treatment with anti-IgM 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; black outlined histogram, treated cells)
PRDI-BF1 regulation occurs at the transcriptional level

As was shown in figure 7A, lymphoma cells do not have detectable levels of *PRDM1* mRNA, while myeloma cells express very high levels. Therefore, to determine the level at which PRDM1 regulation occurs, we 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 (283). 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 (p<0.005) than that of U266 myeloma cells (figure 9A). Because anti-IgM stimulation significantly increases *PRDM1* mRNA levels (figure 8A), nascent RNA production in CA46 lymphoma cells stimulated with anti-IgM was examined. Treatment of these cells for 1 hour with anti-IgM significantly increased production of PRDI-BF1 nascent RNA (p<0.05) (figure 9B).

To further establish the transcriptional activation of PRDM1, we cloned 2618 basepairs of the promoter (80) and examined the effect of B cell receptor stimulation on transcriptional activity (figure 9C). A statistically significant increase in luciferase activity was observed 24 hours after stimulation. This indicates that, at least in part, the transcription activation is mediated by elements within the 2618 bp promoter. Examination of PRDM1 mRNA stability did not reveal any changes upon anti-IgM treatment (figure 9D). Together these data indicate regulation of PRDI-BF1 occurs primarily at the level of transcription.
Figure 9. PRDI-BF1 regulation occurs at the level of transcription. A, Relative levels of nascent RNA were determined by real-time RT-PCR for both CA46 lymphoma and U266 myeloma cells. Levels in U266 myeloma cells are significantly higher than that of CA46 lymphoma cells (p<0.005, Student’s t-test). B, In CA46 lymphoma cells treated with anti-IgM for 1 hour, relative levels of PRDI-BF1 nascent RNA increase significantly (p<0.05, Student’s t-test). C, Anti-IgM treatment (24 hours) of transiently transfected CA46 cells with PRD 2618 shows a significant increase in luciferase activity (p<0.05, Student’s t-test). D, 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 time course with Actinomycin D to determine mRNA half-lives. In all panels, data shown is the mean of at least 3 experiments.
Identification of regulatory regions in the PRDI-BF1 promoter

Because PRDI-BF1 is primarily regulated at the level of transcription, further examination of the PRDI-BF1 promoter is necessary to determine specific areas of regulation. Using promoter deletion constructs, potential regulatory regions in the promoter were identified in both CA46 lymphoma and U266 myeloma cells. As shown in figure 10A, strong promoter activity is detected in U266 myeloma cells with the proximal 521 bp promoter region as compared to the pGL3-basic control. Two distal regions conferring additional activation were also detected. The first region of activation is found -1528 to -1921 upstream of the transcription start site, while the second region is found at -2271 to -2618. These two regions both confer an increase of approximately 2-fold in activity and suggest important regulatory factors bind in these areas.

Transfection of the same PRDM1 constructs into the CA46 lymphoma cell line revealed a similar pattern of activation through the proximal 521 promoter and the distal -1528 to -1921 activator (figure 10B). However, the region between -2271 and -2618 was not active in lymphoma cells. While it is not possible to definitively compare absolute activity levels in transient transfections between different cell lines, the 2618 promoter construct shows consistently higher expression in the U266 myeloma cells, which correlates with higher expression of the endogenous gene expression in these cells.
Figure 10. Characterization of PRDM1 promoter activity in CA46 lymphoma cells and U266 myeloma cells. CA46 lymphoma cells and U266 myeloma cells were transiently transfected with PRDI-BF1 promoter deletion constructs fused to the luciferase reporter gene to map regions of activation. A, U266 cells show elevated levels of promoter activity in the proximal promoter. Regions of activation are located -1528 to -1921 and -2271 to -2618 relative to the transcription start site. B, CA46 lymphoma cells show increased activity at the proximal promoter. There is also one region of activation located -1528 to -1921 relative to the transcription start site. Data shown are the mean of 3 experiments for both figures.
Identification of putative factor binding sites in the PRDI-BF1 promoter

To further elucidate what transcription factors are responsible for the activation of the promoter in these cells, we employed in vivo genomic footprinting to identify where factors are bound to the promoter in these putative regulatory regions. Examination of the region proximal to the transcription start site demonstrated factor binding in both the lymphoma and myeloma cells (figure 11). These sites are similarly occupied in both cell types. A cluster of contacts closest to the transcription start site are centered over a sequence with homology to a Sp1 element. Sp1 binding was confirmed by electrophoretic gel mobility shift assay and specific antibody reactivity (figure 12). The P.A and P.B contact points do not have obvious homology with known elements.

To examine the more distal promoter regions by in vivo footprinting, approximately 600 bp were scanned between -1600 to -2200 and 400 bp between -2200 and -2600. We utilized ten in vivo footprinting primer sets to display both the upper and lower strands across these regions. The majority of contacts were in the region -1700 to -2100. Three cell line-specific interactions, which are denoted as P.C, P.F, and P.H (figure 13), were found in this region. The other indicated protein-DNA interactions (i.e., P.D, P.E, P.F, P.I, and P.J) are found in both lymphoma and myeloma cells. Site P.C is located at -2067 and is a myeloma-specific factor. Sites P.F and P.H are located at -1974 and -1742, respectively, and are lymphoma specific factors. Site P.J is located at -1807, and has been previously characterized as the AP-1 element binding site by Vasanwala et al. (271). Because the -2271 to -2618 region is a myeloma-specific region of activation, we thoroughly examined this region of the promoter, but found no unique contacts in this region (figure 14).
Figure 11. *In vivo* genomic footprinting of the PRDI-BF1 proximal promoter reveals numerous protein-DNA interactions in both lymphoma and myeloma cells. A, 8226 myeloma and CA46 lymphoma cells were analyzed for interactions at the first 160 basepairs of the promoter region using the PRDLOW2 primer set. The control lanes show the *in vitro* methylated guanine residues, while the DMS lanes show the *in vivo* methylated residues. Protections (closed arrowheads) and enhancements (open arrowheads) are shown on the right side of each footprint panel and indicated in the sequence by black or gray, respectively, triangles. Potential elements are labeled site P.A and P.B. B, 8226 myeloma and Raji lymphoma cells were analyzed for interactions at the first 275 basepairs of the promoter region. The Sp1 element was confirmed in vitro (see figure 12). 8226 and Raji footprints used PRD UP primer 1 combined with PRDPI primers 2 and 3.
Figure 12. Sp1 binds the PRDI-BF1 proximal promoter. EMSA assays were done using an oligonucleotide containing the Sp1 consensus sequence and Raji lymphoma cell nuclear extract. Competitor oligonucleotides were added to the binding reaction at 25- to 50-fold molar excess. 0.2 µg of either Sp1 or ATF-1 antibody were used where indicated. The Sp1 complex is supershifted by addition of Sp1 antibody (lane 3), while addition of non-specific ATF-1 antibody (lane 4) shows no change from the probe only (lane 1).
Figure 13. *In vivo* genomic footprinting of the PRDI-BF1 promoter region -1528 to -1921 bp distal to the transcription start site reveals numerous protein-DNA interactions in both lymphoma and myeloma cells. A, U266 myeloma and CA46 lymphoma cells were analyzed for in vivo interactions in the region between -1897 to -2072 bp using PRD C primer set. The control lanes show the *in vitro* methylated guanine residues, while the DMS lanes show the *in vivo* methylated residues. Protections (*closed arrowheads*) and enhancements (*open arrowheads*) are shown on the right side of each footprint panel and indicated in the sequence by black or gray, respectively, triangles. B, U266 myeloma and CA46 lymphoma cells were analyzed for in vivo interactions in the region between -1718 to -1895 bp using PRD H primer set. Site P.H is a novel protection found only in the CA46 cells. C, H929 myeloma and CA46 lymphoma cells were analyzed for in vivo interactions in the region between -1811 to -1966 bp using PRD D set. There are no unique interactions in this specific region.
Figure 14. *In vivo* genomic footprinting of the PRDI-BF1 promoter -2271 to -2491 bp distal to the transcription start site reveals no protein-DNA interactions in both lymphoma and myeloma cells. A, U266 myeloma and Raji lymphoma cells were analyzed for in vivo interactions in the region between -2273 to -2431 bp. B, U266 and 8226 myeloma and Raji lymphoma cells were analyzed for in vivo interactions in the region between -2381 and -2544 bp. The control lanes show the *in vitro* methylated guanine residues, while the DMS lanes show the *in vivo* methylated residues. There are no apparent protections in this region of the promoter in either myeloma or lymphoma cells. This region was analyzed using the PRD 0.4K FWD and PRD 0.4K REV primer sets, respectively.
Identification of a functional factor binding site on the PRDI-BF1 promoter

Sites P.C, P.F, and P.H, which were occupied in a cell type-specific manner, were mutated within the PRD 1921 and 2618 promoter constructs. Transfection of the mutated constructs into either lymphoma or myeloma cells demonstrates sites P.C and P.F are not required for PRDM1 promoter activity (figure 15). However, mutating the lymphoma-specific factor P.H in both constructs decreases promoter activity by approximately 50% when transfected into CA46 lymphoma cells. There is a slight effect on promoter activity in U266 myeloma cells, but the decrease in activity of the mutated constructs when compared to the wild-type constructs is much less dramatic (figure 16). These data correlate with the occupation of site P.H in a lymphoma-specific manner in the footprints.

Gel shift assays were done to confirm factor-binding in a specific manner at site P.H. Examination of both CA46 lymphoma and U266 myeloma cells demonstrate specific binding of factor P.H in the lymphoma cells. Site P.H is homologous to an Ets factor binding site. PU.1 is a B cell specific Ets family member (172), so we examined its ability to bind P.H in lymphoma and myeloma cells. Addition of antibody against PU.1 competes out binding to P.H in CA46 lymphoma cells (figure 17A), but has no effect on P.H binding in U266 myeloma cells (figure 17B), which correlates with the lack of PU.1 expression in myeloma cells (172). PU.1 is known to bind DNA in a complex with IRF family members on other promoters (58, 61), so we examined numerous IRF family members to determine if they also bound the PRDM1 promoter in CA46 lymphoma cells at site P.H. However, using IRF-4 and IRF-8 antibodies did not shift or cause a disappearance of the P.H binding site, indicating that these factors do not bind site P.H in CA46 lymphoma cells (figure 18).
Figure 15. Site-directed mutagenesis demonstrates sites P.C and P.F are not necessary for activity of the PRDI-BF1 promoter in either CA46 lymphoma cells or U266 myeloma cells. A, Mutating the P.C and P.F sites in the PRD 2618 promoter deletion construct had no effect on promoter activity in CA46 lymphoma cells. B, In the U266 myeloma cells, there is also no change in mutant promoter activity when compared to wild type promoter activity. Promoter activity was normalized to Renilla activity and pGL3 basic activity set to 1. The mean of 3 independent experiments is shown with standard deviations.
Figure 16. Site-directed mutagenesis demonstrates site P.H is necessary for activity of the PRDI-BF1 promoter in CA46 lymphoma cells, but not in U266 myeloma cells. A, Mutating the P.H site in both the PRD 1921 and PRD 2618 promoter deletion constructs decreases promoter activity in CA46 lymphoma cells by approximately 50%. B, In the U266 myeloma cells, there is minimal change in mutant promoter activity when compared to wild type promoter activity. Promoter activity was normalized to Renilla activity and pGL3 basic activity set to 1. The mean of 6 independent experiments is shown with standard deviations.
Figure 17. Site P.H is a PU.1 factor binding site in CA46 lymphoma cells, but not U266 myeloma cells. Gel shift assays were done using an oligonucleotide containing the P.H consensus sequence and CA46 or U266 nuclear extract. A, Competitor oligonucleotides were added to the binding reaction at 150- to 300-fold molar excess. 1 µg IRF-4 antibody and 0.5-4 µg PU.1 antibody was used. The P.H complex is shifted by addition of PU.1 antibody, while addition of IRF-4 antibody does not affect the complexes. B, Competitor oligonucleotides were added to the binding reaction at 20- to 40-fold molar excess. 0.2 ug IRF-4 antibody, 0.5 ug PU.1 antibody (BD Pharamingen), 0.2 ug PU.1 antibody (Santa Cruz), or 0.2 ug Sp1 antibody were used. There is no factor binding to site P.H in U266 myeloma cells.
Figure 18. Site P.H is not a IRF-4 or IRF-8 factor binding site in CA46 lymphoma cells. Gel shift assays were done using an oligonucleotide containing the P.H consensus sequence and CA46 nuclear extract. Competitor oligonucleotides (specific cold P.H, mutant P.H, and non-specific Sp1) were added to the binding reaction at 25- to 50-fold molar excess. 0.2 µg IRF-4 antibody and 0.5 µg IRF-8 antibody was used. Addition of IRF-4 or IRF-8 antibody does not affect the P.H binding.
PU.1 is involved in anti-IgM-mediated transcriptional regulation of PRDI-BF1

To determine if PU.1 is involved in the activation of the PRDM1 promoter by B cell receptor cross-linking, CA46 lymphoma cells were transfected with PU.1 siRNA and treated with anti-IgM. Treatment of cells for 24 hours with anti-IgM demonstrated a decrease in anti-IgM-mediated induction of PRDI-BF1 mRNA levels (figure 19A). Additionally, treatment of cells for 1 hour with anti-IgM demonstrated a marked decrease in anti-IgM-mediated PRDI-BF1 nascent RNA induction (figure 19B). PU.1 siRNA specifically targeted PU.1, but not PRDI-BF1, as demonstrated in figure 19C. These data indicate that PU.1 is one of the factors involved in anti-IgM induced transcription of PRDM1.

The PI3K and MAPK signaling pathways are involved in anti-IgM-mediated induction of PRDI-BF1 in lymphoma cells

To investigate the role of signaling pathways involved in anti-IgM-mediated induction of PRDI-BF1 in CA46 lymphoma cells, preliminary experiments were done utilizing the MAPK inhibitor U0126 and the PI3K inhibitor Wortmannin. Pretreatment of cells with these compounds inhibited anti-IgM-mediated induction of PRDI-BF1 mRNA levels (figure 20), indicating these pathways are involved in the induction of PRDM1. However, further investigation will need to be done to elucidate the role of these pathways in regulating PRDI-BF1.
Figure 19. PU.1 is involved in anti-IgM-mediated transcriptional regulation of PRDI-BF1. A, CA46 lymphoma cells transfected with either MARK (neg) or PU.1 siRNA were treated with anti-IgM for 24 hours and PRDI-BF1 mRNA levels were detected. B, Cells were also treated for 1 hour with anti-IgM and PRDI-BF1 nascent RNA levels were measured. C, PU.1 mRNA levels are decreased using PU.1 siRNA 48 hours after transfection. Additionally, PU.1 mRNA levels are unaffected by anti-IgM treatment. The data are representative of 2 experiments done in duplicate.
Figure 20. The PI3K and MAPK signaling pathways are involved in the anti-IgM-mediated induction of PRDI-BF1. CA46 lymphoma cells were pretreated with the PI3K inhibitor Wortmannin or the MAPK inhibitor U0126 for 2 hours, followed by treatment with 10 μg/ml anti-IgM for 4 hours. Control cells had a 5-fold induction in PRDI-BF1 mRNA levels, while cells undergoing pretreatment with either inhibitor lacked an induction of PRDI-BF1.
Discussion

The transcription factor PRDI-BF1/Blimp-1 is required for the differentiation of a mature B cell to a plasma cell (237). It does this by directly repressing downstream targets, which in turn has a widespread effect on further downstream targets (236). These downstream effector cascades have been well studied, however very little is known as to how PRDI-BF1/Blimp-1 expression is regulated. Bcl-6 is known to repress PRDI-BF1 by acting through AP-1 elements (271) and IRF-8 is known to activate PRDI-BF1 in murine myeloid progenitor cells (252).

This study demonstrates a direct link between B cell receptor cross-linking by anti-IgM and transcriptional activation of PRDI-BF1/Blimp-1. Treatment of CA46 lymphoma cells with anti-IgM significantly induced PRDM1 mRNA levels, caused apoptosis, and increased activity of the PRDM1 promoter. Because there was no change in mRNA stability with anti-IgM treatment, while actively transcribing nascent RNA levels were rapidly induced, this indicated regulation occurs primarily at the level of transcription. Nascent RNA levels are very low in CA46 lymphoma cells, but in myeloma cells they are significantly higher. While nascent RNA levels can be rapidly induced upon BCR cross-linking to levels significantly higher than that of control cells, luciferase activity is only nominally induced by BCR cross-linking. This could be due to the IgM response element on the PRDM1 promoter being located more than 2618 bp distal to the transcription start site, which is the PRDM1 promoter construct tested. Rapid induction of nascent RNA levels by B cell receptor cross-linking and the evidence of a promoter with many protein-DNA interactions detected by in vivo genomic
footprinting indicate the PRDM1 promoter is occupied and primed for transcription in lymphoma cells, even though PRDI-BF1 gene expression is not detectable.

This is comparable to the situation in T cells, which also do not express PRDM1. However, it has recently been shown that activating CD4+ T cells in vitro will elevate PRDM1 levels equivalent to that of splenic B cells treated with LPS (111). Our findings in lymphoma cells are also important in light of recent evidence which demonstrates the presence of PRDI-BF1 gene expression in DLBCL (32, 195, 251). Mutations of PRDM1 were found in 23% of DLBCL patient samples examined (251). Another group further characterized their DLBCL patient population and determined only the activated B cell-like DLBCL samples were positive (24%) for mutated PRDM1 expression (195). Alterations in PRDM1 included frameshift mutations, point mutations, chromosomal inversions and, most importantly, all led to PRDM1 gene inactivation (195, 251). Mutations in DLBCL suggest a potential role for PRDI-BF1 as a tumor suppressor, consistent with its differentiation and apoptosis promoting effects.

Our work is the first to directly link the expression of PRDI-BF1/Blimp-1 to anti-IgM mediated B cell receptor activation. It has long been known that anti-IgM induces apoptosis in lymphoma cells (12, 30, 87, 112, 299) and has been correlated with decreased levels of c-myc expression (112), a direct target of PRDI-BF1 (140). The BCL1 murine B cell model was used to demonstrate phenotypic differences resulting from the introduction of Blimp-1 into partially activated mature 5B1b and activated 3B3 cells, a cell line derived from the 5B1b line. Blimp-1 is able to induce apoptosis in the mature 5B1b cell line (164), as well as numerous other B cell lines in the early stages of development (119, 164). However, in the activated 3B3 cells, ectopic expression of
Blimp-1 induces differentiation (164). Our study directly correlating apoptosis with increased PRDM1 mRNA expression is important because it may be possible to induce apoptosis in PRDM1-negative lymphoma cells by inducing PRDI-BF1 expression levels. Furthermore, PRDI-BF1 has been shown to be an important target in immunotherapy of myeloma by induction of PRDI-BF1-specific CTLs (150).

Regions of PRDM1 promoter activation are localized to two distal regions in U266 myeloma cells and one distal region in CA46 lymphoma cells. In vivo genomic footprinting of the regions of activation in both lymphoma and myeloma cells yielded multiple protein-DNA interactions. One of these was site P.J, which is the AP-1 factor binding site shown to be important for Bcl-6-mediated repression of the PRDI-BF1 promoter by Vasanwala et al (271). Our in vivo footprinting data show site P.J is occupied in both CA46 lymphoma and U266 myeloma cells. Another study in the murine system suggested c-Fos acting through distinct elements is a key transcription factor for Blimp-1 expression in activated B cells, regardless of Bcl-6/AP-1 element interactions (185). Our findings of factor binding at site P.J in both lymphoma and myeloma cells support a BCL-6 independent role for c-fos/AP1 at site P.J, as myeloma cells do not express Bcl-6 (272). The distal region of activation specific for only myeloma cells demonstrated no protein-DNA interactions, indicating factors may be bound to this region but are undetectable using DMS in vivo footprinting which recognizes methylated guanine residues.

Several cell-type specific interactions, namely P.C, P.F, and P.H, were observed by in vivo footprinting. Basal and anti-IgM stimulated PRDM1 promoter activity was significantly inhibited by mutating the P.H site in lymphoma cells. This indicates P.H,
which shares homology with Ets family member binding sites, is an important functional site for *PRDM1* promoter regulation. PU.1 is an Ets family member found only in B cells (172) and is bound to P.H, as demonstrated by electrophoretic mobility gel shift assay. PU.1 antibody supershifted the P.H complex. Ets and IRF family members have been reported to complex with one another and bind promoters (58, 61). In addition, the transcription factor IRF-8/ICSBP has been shown to directly induce *Blimp-1* expression in myeloid cells (252). However, we did not detect specific binding of IRFs at the P.H site in CA46 lymphoma cells. This suggests that either other undetected IRF elements exist or the activity of IRF-8 on *PRDM1* is specific for myeloid cells. This study further demonstrated a role for PU.1 in anti-IgM-mediated induction of *PRDM1* in lymphoma cells, as introduction of PU.1-specific siRNA in lymphoma cells diminished the anti-IgM-mediated induction of PRDI-BF1 mRNA expression. However, *PRDM1* expression was only reduced approximately 50%, indicating PU.1 is not the sole factor responsible for anti-IgM-mediated induction of gene expression. Preliminary data using inhibitors to the MAPK and PI3K signaling pathways indicate these pathways are involved in inducing PRDI-BF1 in CA46 lymphoma cells. However, further experiments are required to elucidate additional factors responsible for inducing PRDI-BF1 in lymphoma cells using anti-IgM.

In conclusion, we have demonstrated *PRDM1* regulation occurs primarily at the transcriptional level in lymphoma and myeloma cells. We have also demonstrated the involvement of PU.1 in basal and anti-IgM induced activation of the PRDM1 promoter in lymphoma cells. Importantly, we report that the promoter is poised and ready for activation in lymphoma cells, suggesting that inducing PRDI-BF1 expression in
lymphoma cells lacking PRDM1 gene mutations is a viable therapeutic approach to inducing apoptosis in these cells.
Materials and Methods

Cell lines and drugs

The RPMI-8226, U266, and NCI-H929 multiple myeloma and CA46, Raji, and BJAB Burkitt’s lymphoma cell lines were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone), and 1% penicillin/streptomycin (P/S) (Invitrogen). The NCI-H929 cell line was also supplemented with 0.05 M \( \beta \)-mercaptoethanol (Sigma, St. Louis, MO). Bone marrow cells derived from myeloma patients were obtained from the H. Lee Moffitt Cancer Center Tissue Bank under Institutional Review Board approval. Normal adult bone marrow samples were obtained from the H. Lee Moffitt Cancer Center Tissue Bank and Stratagene. Goat anti-human IgM antibody (Southern Biotechnology) was used at 10 \( \mu \)g/ml. Actinomycin D (Sigma) was used at 10 \( \mu \)g/ml to determine mRNA stability. Trypan blue exclusion was used to determine total live cell counts.

Apoptosis assay

Cells were treated with anti-IgM continuously for 24 hours, followed by Annexin V-PE and 7-AAD staining per manufacturer’s protocol (BD Pharmingen). Flow cytometry acquisition was done on a FACSCalibur and analyzed with CellQuest software (Becton Dickinson, Carpinteria, CA).
**Real-time 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\textsuperscript{th} of the final cDNA reaction volume was used in each PCR reaction. Primers sequences are as follows: PRDI-BF1 FWD, 5’- TACATACCAAAGGGCACACG -3’, PRDI-BF1 REV, 5’- TGAAGCTCCCCTCTGGAATA -3’, PU.1 FWD, 5’- AGCAGATGCACGTCTCGATA -3’, PU.1 REV, 5’- AGACCTGGTGGCCAAGACTG -3’, GAPDH FWD, 5’- GAAGGTGAAGGTCGGAGT -3’, and GAPDH REV, 5’- GAAGATGGTGATGGGATTTC -3’. Primer sets were run using an annealing temperature of 60°C. 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). 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 for each cDNA sample and relative levels of the genes of interest were calculated by the ∆∆Ct method (145): for example, $2^{- (∆ΔCt)_{PRDI-BF1-GAPDH}}$ for PRDI-BF1 gene expression.

**Isolation of nascent RNA**

Nascent RNA was isolated as previously described (121, 283). Briefly, nuclei from lymphoma and myeloma cells was isolated in a RNase-free buffer comprised of 140 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 0.5% NP-40, 1000 U/ml RNase OUT (Invitrogen), 1 mM
DTT, and 50 mM 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 300 mM NaCl, 1 M urea, 1% NP-40, 7.5 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT, and 20 mM 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**

PRD 2618, originally referred to as PRDM1α, was cloned as previously described (80). Using PRD 2618 as a cloning template, PRD 521 was sub-cloned using a NheI/SmaI digest to remove the 521 bp fragment, blunt-ended, and cloned into pGL3 basic. PRD 1648 was cloned by digesting PRD 2618 with BglII to yield a 1648 bp fragment, which was then ligated into pGL3 basic. PRD 1921 was cloned by digesting PRD 2618 with Kpn/PacI to remove 703 bp. PRD 2391 was derived from PRD 2618 by digestion with HindIII to remove 2391 bp and cloned into pGL3 basic. PRD 983 and PRD 2041 were PCR-cloned.

Site-directed mutagenesis was done by PCR cloning the mutated sequence into the PRD 1921 or PRD 2618 constructs. Briefly, mutations were introduced into either construct using a common PRD 2618 forward or reverse primer, paired with either a reverse or forward primer, respectively, containing the site mutation. Primer sets used are as follows, with the mutated bases indicated in lower case letters: PRD 2618 FWD, 5’- TTCCTATTATGGAGCAAGCTTCC -3’, PRD 2618 REV, 5’-CATTTCTCCTTCGACCTGCA -3’, P.C mut FWD, 5’-CCTAAAGTTCTAttttaagCCTTGTCCAGCTC -3’, P.C mut REV, 5’-
GAGCTGGACAGGctttaaaTAGAACTTTAAGG -3’, P.F/G mut FWD, 5’-
GTGGCGTCTCTaaagctagcCAAGCCCATTTCA -3’, P.F/G mut REV, 5’-
GTGAAATGGGCTTGAgtacctttAGGACGCAAAAC -3’, H3 mut FWD, 5’ -
ATTCTAAAGGAcGTTAAAATCTCTTTAAC - 3’, and H3 mut REV, 5’ -
GTAAAGAGTATTTAcGAcTGcTcTTTAGAAT - 3’. PCR products were gel-purified and paired with their respective mutation partner and amplified using PRD 2618 common primers. PRD 1921 mut P.H was derived from the PRD 2618 mut P.H construct by digesting with KpnI/PacI, removing a 703 bp fragment, and re-ligating the vector. All mutations were confirmed by sequencing.

**Luciferase assays**

Cells were transfected by electroporation using the Gene Pulser II (Bio-Rad, Hercules, CA). Cells (1 x 10^7) were pulsed with 250 V at a capacitance of 1070 µF. Transfections were done with 10 µg of luciferase reporter construct and 50 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.

**Dimethyl sulfate in vivo footprinting**

*In vivo* methylation of cells with dimethylsulfate (DMS) and subsequent isolation of DNA was done as previously described (75, 199). Cells were *in vivo*-treated with 1
µl/ml DMS and methylation of guanine residues was ensured by occasional swirling for 5 minutes at 37°C. The reaction was stopped by adding ice-cold PBS plus 60 mM Tris, pH 7.5, and washing twice. Cells were lysed in lysis buffer (100 mM EDTA, 60 mM Tris, pH 7.5, 0.1% SDS, 500 µg/ml Proteinase K (Roche)) for 4 hours at room temperature. Samples were stored at 4°C or genomic DNA was extracted immediately.

To extract genomic DNA, samples were brought to room temperature to bring the SDS back into solution. DNA was extracted using phenol (Roche)/chloroform:isoamyl alcohol (24:1) and washed twice with chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding 1/10 volume 3 M NaAc (pH 7) and 2.5 volumes 95% ethanol, followed by spooling with a hooked Pasteur pipet to remove the DNA. DNA was resuspended in 3-5 ml Tris:EDTA (10 mM:0.1 mM). Genomic DNA was digested with either 100 units HindIII or PstI (NEB) and extracted as above.

Control samples were treated with 1 µl DMS for 20 seconds. The reaction was stopped by adding DMS stop buffer (1.5 M NaAc, pH 7, 1 M β-mercaptoethanol, 100 µg/ml tRNA (Ambion)). DNA was precipitated by adding -20°C 95% ethanol and snap-freezing in a dry-ice/ethanol bath for 10 minutes, washed twice with cold 75% ethanol, and dried by Speed-Vac. DNA was cleaved at guanine residues by adding 200 µl piperdine (diluted 1:10 in ice-cold H2O) and incubating at 90°C for 30 minutes. DNA was precipitated and lyophilized to dryness in a Speed-Vac. DNA was washed twice with H2O, lyophilized to dryness, and resuspended in 50 µl H2O.
Ligation-mediated PCR (LM-PCR)

Primers for the ligation-mediated PCR, LM-PCR, are shown in Table 1 with Tms used in each reaction. Full-length primers were purified using denaturing polyacrylamide gel electrophoresis. Briefly, primers were incubated with an equal volume formamide at 55°C for 5 minutes. Products were separated using a pre-run 16% polyacrylamide gel (7 M urea, 20:1 acrylamide/bisacrylamide, 1x TBE) run at 1500 V for 1 hour. UV shadowing was used to distinguish between full-length primers and prematurely terminated products. Primers were extracted from the gel using an EluTrap (Schleicher and Schuell) and passed over a Sep-Pack column (Waters) using a 10 ml syringe and slow flow rate. The column was equilibrated by adding 10 ml 100% acetonitrile and 20 ml water. The extracted primer was added in 1.5 ml volume water and eluted using 10 ml fresh 25 mM ammonium bicarbonate (ABi), 10 ml ABi/5% acetonitrile, 20 ml water/5% acetonitrile, and 4 ml water/30% acetonitrile. Primers were dried in a Speed-Vac and resuspended in water.

LM-PCR was performed as previously reported (169, 282). Approximately 7.5 µg DMS-treated genomic DNA was annealed to 0.3 pmol primer 1 by first denaturing for 4 minutes at 95°C and then annealing for 30 minutes at the Tm reported in Table 2 in a 15 µl solution containing 1x Mg-free Sequenase buffer (40 mM Tris, pH 7.7 and 50 mM NaCl). Primer extension was performed by adding 7.5 µl Mg/DTT/dNTP solution ((20 mM MgCl₂, 20 mM DTT, 0.2 mM each dNTP (USB)) and 1.5 µl diluted Sequenase 2.0 (USB) (diluted 1:4 in ice-cold TE) and incubating for 10 minutes at 47°C, 5 minutes at 60°C, and holding at 22°C. The Sequenase extension reaction was stopped by adding 6 µl 310 mM Tris, pH 7.7 and incubating at 67°C for 10 minutes. Ligation of the
hybridized linker (hybridized long linker and short linker primers) to the above reaction product was done by adding 20 µl dilution solution (17.5 mM MgCl₂, 42.3 mM DTT, 125 µg/ml BSA) and 25 µl ligation mix (10 mM MgCl₂, 20 mM DTT, 50 µg/ml BSA, 50 mM Tris, pH 7.7, 3 mM rATP (Promega), 100 pmol hybridized linker per reaction, 400 U T4 DNA ligase (NEB)) and ligating overnight at 16°C. The ligation products were precipitated with 1/10 volume 3 M NaAc, pH 7, 10 µg tRNA (Ambion), and 95% ethanol, air-dried, resuspended in 70 µl water, and 30 µl PCR mix (40 mM NaCl, 10 mM Tris, pH 8.9, 5 mM MgCl₂, 0.01% (w/v) gelatin, 20 nmol dNTPs, 10 pmol primer 2, 10 pmol long linker primer, 5 U Ampli-Taq (Applied Biosystems)) was added. The PCR reaction was heat denatured at 97°C for 3.5 minutes, primer 2 annealed at the appropriate Tm (see Table 1), and extended at 76°C for 3 minutes. PCR products were amplified with 16 cycles of heat denaturation for 1 minute at 97°C, annealing of primer 2 at the appropriate Tm, and extension at 76°C for 3 minutes, 5 seconds, with an additional extension of 5 seconds each cycle. One final cycle of PCR was performed by heat denaturation for 1 minute at 97°C, primer 2 annealing for 2 minutes at appropriate Tm, and final extension for 10 minutes at 76°C. PCR products were purified by phenol/chloroform extraction and ethanol precipitation and resuspended in 15 µl loading dye (80% formamide, 0.5x TBE, bromophenol blue, xylene cyanol). PCR products (10 µl) were size-fractionated using a pre-run 6% polyacrylamide wedge sequencing gel made with 7.75 M urea, 20:1 acrylamide/bisacrylamide (Bio-Rad), and 1x TBE. The gel was dried and products were visualized by autoradiography.

Primer 3 was radioactively labeled in a kinase reaction mix containing 20 pmol primer, kinase buffer (NEB), 150 µCi γ-³²P-ATP (Amersham), and 10 U polynucleotide

69
kinase (NEB). Primer labeling occurred by incubating for 45 minutes at 37°C, followed by heat inactivation of the enzyme for 10 minutes at 68°C. Unincorporated ATP was removed by using the Qiagen nucleotide removal kit and eluting labeled primer with 200 µl H₂O (pH 7.7). The PCR product was radioactively labeled by annealing 20 pmol primer 3 in a reaction mix containing 40 mM NaCl, 10 mM Tris, pH 8.9, 5 mM MgCl₂, 0.01% (w/v) gelatin, 20 pmol dNTPs, and 5 U Ampli-Taq and annealing by cycling twice through 3.5 minutes of heat denaturation at 97°C, annealing at appropriate primer 3 Tm for 2 minutes, and extending at 76°C for 10 minutes.

Nuclear extract preparation and electrophoretic mobility shift assays

Nuclear extracts were prepared according to Dignam et al. (53). To summarize, a minimum of 3 x 10⁸ cells were pelleted and the pellet cell volume (PCV) was estimated. Cells were resuspended in 5x PCV buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF) and swelled in this hypotonic buffer for 10 minutes on ice. Cells were resuspended in 2x original PCV buffer A and dounced with B pestle of the Dounce homogenizer until 95% of the cells were lysed. The nuclei were isolated by a quick centrifugation at 30000 x g in a Beckman JA25.50 rotor. The nuclear pellet was resuspended in 1 ml buffer C (modified) (20 mM Hepes, pH 7.9, 20% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 8.0, 0.42 M KCl, 0.5 mM DTT, and 0.5 mM PMSF)/1 x 10⁹ cells. Nuclei were extracted by agitating on a nutator for 20 minutes at 4°C, centrifuging for 30 minutes at 25000 x g in a 4°C Beckman JA25.50 rotor, and resuspending in dialysis buffer D (20 mM Hepes, pH 7.9, 20% v/v glycerol, 0.2 mM EDTA, pH 8.0, 0.5 mM DTT, and 0.5 mM PMSF).
The Sp1 oligonucleotide sequence is 5’-GATCTGGCCACGCCCCCACTTCGCGC-3’. The Sp1 mutant oligonucleotide sequence is 5’-GATCTGGCCACaaaaaaACTTCGCGC-3’. The P.H oligonucleotide sequence is 5’-GATCCAGAGTTTGATTCTAAAAGGGAAGTGAAATACTCTTTAAC-3’. The mutant P.H oligonucleotide contains the same mutation as in construct PRD 2618 mut H3. The AP2α oligonucleotide sequence is 5’-GATCAAGCCCTGGGCTCGGCCAG-3’. Gel shift probes were end-labeled using T4 polynucleotide kinase (NEB). Five picomoles of radioactive probe were end-labeled with γ-32P-ATP and purified using mini Quick Spin DNA columns (Roche). 50 picomoles of cold competitor probe was synthesized using the same conditions as above.

For each EMSA reaction, 4 µl of nuclear extract were combined with 0.5 mM DTT, 1 µg poly (dl:dC), and either antibody (0.2-4 µg) or 20-300-fold molar excess cold competitor oligonucleotides. Antibody incubations occurred on ice for 1-2 hours, followed by labeling with 100000 counts of probe for 0.5 hours. Gels were run cold for 1.5 hours and DNA binding was observed by autoradiography. PU.1 antibodies were obtained from BD Pharmingen and Santa Cruz, while IRF-1, IRF-2, IRF-4, ATF-1, and Sp1 antibodies were from Santa Cruz.

**Transfections with siRNA**

CA46 lymphoma cells were transfected with 3 µg MARK or PU.1 siRNA (Dharmacon) by electroporating 1 x 10^7 cells in 0.3 ml serum-free RPMI medium. Live cells were isolated using a Ficoll gradient and washed in complete medium. Cells were
treated for either 1 hour or 24 hours with 10 μg/ml anti-IgM. Following 1 hour incubation, cells were harvested for nascent RNA as previously described. Following 24 hour incubation, cells were harvested for mRNA, as previously described.
Table 1. Primers used for LM-PCR. Primers used to detect promoter region of interest, sequence of primers, and Tm for each LM-PCR reaction are listed.

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<th>Tm</th>
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<tr>
<td>Long linker</td>
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<td>PRDI primer 3</td>
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<td>PRDLOW primer 2</td>
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<tr>
<td>PRDLOW primer 3</td>
<td>5’- TTGCGAAGGAGCAAGGACAGCGCCACCG -3’</td>
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<tr>
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Chapter Three

Modulation of PRDM1 gene expression in lymphoma cells

Introduction

Lymphomas encompass a group of cancers localized to the lymphoid system. The most frequent type of lymphomas are the B cell lymphomas (BCL) (219), a group to which both mantle cell lymphoma and Burkitt’s lymphoma belong. Mantle cell lymphoma (MCL) is an aggressive form of non-Hodgkin’s lymphoma (NHL), accounting for approximately 5-8% of NHL cases. Even though there are a variety of treatment regimens available to patients, it remains an incurable disease (279). MCL is a distinct biologic subtype of NHL, demonstrated by high levels of cyclin D1 expression, which is caused by the t(11;14) (q13;q32) chromosomal translocation. However, experiments using transgenic mice have demonstrated that this alone is not sufficient to cause MCL (151). Another form of NHL is Burkitt’s lymphoma (BL), which has a very aggressive pathology and presents clinically as three different subtypes. The endemic form of BL occurs at a 50-fold higher rate in equatorial Africa than in the United States. The immunodeficiency subtype is found in patients with human immunodeficiency virus (HIV), while sporadic BL accounts for 1-2% of all adult lymphomas in the United States and Western Europe (19). BL is characterized by overexpression of c-myc (136), often
caused by a t(8;14) translocation (73, 90, 177) or genetic alterations in the c-myc gene (92).

While Burkitt’s lymphoma adolescent patients have cure rates of 70%, older patients typically have a poorer response rate and many are unable to finish their treatment regimens due to cytotoxic side effects (63). In the case of mantle cell lymphoma (MCL), many patients become refractory to treatment and their prognosis is poor (132). Much excitement has been generated by newer chemotherapeutic drugs, such as the proteasome inhibitor Bortezomib, which has been fast-tracked for approval in MCL by the FDA based on phase 2 trial data (182). In pre-clinical studies, Bortezomib was shown to induce apoptosis in both mantle cell lines and patient samples (196). Histone deactylase (HDAC) inhibitors are also effective drugs in lymphoma. The new HDAC inhibitor LBH589 has potent antitumor activity in myeloma cells (155), while sodium butyrate and SAHA have been shown to induce apoptosis in mantle cell lymphoma cells (91). HDAC inhibitors were shown to induce *Blimp-1* gene expression in L10A murine lymphoma cells and induce differentiation, as shown by increased expression of the plasma cell surface marker Syndecan-1 (129). However, targets of Bortezomib and HDAC inhibitors have not been fully elucidated to date.

Modulating PRDM1 expression in lymphoma cells may have therapeutic potential, as the promoter of lymphoma cells is poised for activation, as demonstrated by experiments using the B cell cross-linker anti-IgM (see Chapter Two). The *PRDM1* gene has recently been shown to be expressed in one form of lymphoma, namely DLBCL. However, inactivating mutations of *PRDM1* were described in DLBCL, implicating this gene as a potential tumor suppressor in lymphoma (69, 195, 251). In this study, the
activity of the proteasome inhibitor Bortezomib and the HDAC inhibitors Trichostatin A and LBH589 were assessed in CA46 Burkitt’s lymphoma and Mino mantle cell lymphoma cell lines. These chemotherapeutic agents induce PRDM1 gene expression in both cell lines and patient samples, indicating these could drugs could have therapeutic potential in these aggressive forms of leukemia.

Results

Treatment with HDAC inhibitors or Bortezomib induce PRDM1 levels in lymphoma cell lines and mantle cell patient samples

Most B cells lack detectable levels of PRDM1 until they commit to differentiate (5, 24, 32, 69). As shown in figure 7, lymphoma cell lines lack detectable levels of PRDM1. These data correlate with low levels of nascent RNA purified from nuclei of these cells. Levels of nascent RNA are an indicator of endogenous transcriptional activity in a cell (283). When compared to U266 myeloma cells which express abundant levels of PRDI-BF1 nascent RNA, both CA46 and Mino cells have significantly lower levels of nascent RNA production (figure 21).
Figure 21. Levels of nascent RNA in myeloma cells are elevated when compared to lymphoma cells. Relative levels of nascent RNA were determined by real-time RT-PCR for both CA46 and Mino lymphoma and U266 myeloma cells. Levels in U266 myeloma cells are significantly higher than that of CA46 and Mino lymphoma cells (p<0.005, Student’s t-test).
HDAC inhibitors have been shown to induce Blimp-1 mRNA expression in murine lymphoma cells (129) as well as inducing apoptosis (91, 218). Trichostatin A treatment of CA46 cells induced PRDM1 expression in CA46 BL cells (figure 22). Because it has been recently established PRDM1 expression is regulated primarily at the level of transcription (see Chapter Two), cells were treated with Trichostatin A and analyzed for protein-DNA interactions at the proximal promoter by in vivo DMS footprinting. However, analysis of one region of the promoter revealed no protein-DNA interactions (figure 23). The clinically relevant HDAC inhibitor, LBH589, is able to induce PRDM1 expression in the Mino mantle cell lymphoma line, with a concomitant induction of apoptosis (figure 24). However, when a patient sample was treated with the HDAC inhibitor SAHA, PRDM1 expression decreased at both 6 and 20 hours of treatment (figure 25).

![Figure 22. Treatment with the HDAC inhibitor Trichostatin A induces PRDI-BF1 mRNA levels in CA46 lymphoma cells.](image)

**Figure 22.** Treatment with the HDAC inhibitor Trichostatin A induces PRDI-BF1 mRNA levels in CA46 lymphoma cells. Treatment of CA46 lymphoma cells for 24 hours with 100 ng/ml Trichostatin A significantly induces PRDI-BF1 mRNA levels. The mean of 7 experiments is shown (p<0.0005, Student’s t-test).
Figure 23. *In vivo* genomic footprinting of the PRDI-BF1 proximal promoter reveals no protein-DNA interactions in lymphoma cells treated with Trichostatin A. CA46 lymphoma cells were analyzed for interactions at the first 200 basepairs of the promoter region. The control lanes show the *in vitro* methylated guanine residues, while the DMS lanes (Control and TSA) show the *in vivo* methylated residues. No interactions are induced by Trichostatin A treatment at this specific region of the proximal promoter.
Figure 24. Activity of the HDAC inhibitor LBH589 in Mino mantle cell lymphoma cells. LBH589 induces PRDM1 gene expression and induces apoptosis in Mino mantle cell lymphoma cells following 24 hours of treatment.

Figure 25. Treatment with the HDAC inhibitor SAHA decreases PRDI-BF1 mRNA levels in freshly isolated cells from a mantle cell lymphoma patient. Treatment of patient cells for 6-20 hours with increasing doses of SAHA decreased PRDI-BF1 mRNA levels.
Figure 26. Treatment with the proteasome inhibitor Bortezomib induces PRDI-BF1 mRNA levels in CA46 lymphoma cells. Treatment of CA46 lymphoma cells for 24 hours with increasing doses of Bortezomib induces PRDI-BF1 mRNA levels. The mean of 3 experiments is shown with standard deviations.

Figure 27. Treatment with the proteasome inhibitor Bortezomib induces PRDI-BF1 mRNA levels and induces apoptosis in Mino mantle cell lymphoma cells. Treatment of Mino mantle cell lymphoma cells for 24 hours with increasing doses of Bortezomib induces PRDI-BF1 mRNA levels and apoptosis, as measured by Annexin V staining. The data shown are representative of 3 independent experiments.
Figure 28. Treatment with the proteasome inhibitor Bortezomib induces PRDI-BF1 mRNA levels in freshly isolated cells from a mantle cell lymphoma patient. Treatment of patient cells for 6-20 hours with increasing doses of Bortezomib induced PRDI-BF1 mRNA levels. A, This patient sample was comprised of approximately 90% mantle cell lymphoma cells. B, Total mantle cell lymphoma cells unknown for this patient sample.
The proteasome inhibitor Bortezomib induced PRDM1 mRNA levels in a dose-dependent manner in both the Mino and CA46 lymphoma cell lines. Induction of PRDI-BF1 mRNA levels correlates with an increase in apoptosis of these cells (figures 26 and 27). Fresh patient samples from lymphoma patients were treated for 6-20 hours with this drug and some induction was seen at lower doses. A high dose of 50 nM in one patient demonstrated a decrease in PRDM1 expression, compared to the 25 nM dose (figure 28). This could be due to apoptosis and cell death caused by higher drug doses. However, this was not examined.

**Human B cells differentiated to plasmablasts have increased PRDM1 mRNA levels**

Using lymphoma and myeloma cell lines with differing levels of PRDM1 gene expression is one method to study the regulation of PRDM1 gene expression. Another way is to utilize normal B cells capable of differentiation. However, even though this procedure has been previously described (253), it was necessary to work out the proper conditions in our lab and determine if PRDM1 levels increased upon differentiation. Using peripheral blood from normal human donors, we were able to differentiate the purified B cells to plasmablasts expressing increased levels of PRDM1α and PRDM1β and reduced levels of PRDI-BF1 downstream targets CIITA promoter 3 and BSAP (figure 29). On day 6 of culture, the cell yield was decreased compared to what had been previously described (253). The availability of low numbers of plasmablasts at day 6 has limited the ability to do experiments beyond mRNA analysis at this stage. This problem is currently being addressed in the lab.
Figure 29. Purified B cells differentiated to plasmablasts have increased levels of PRDI-BF1 mRNA expression. Activated B cells (day 4 of culture) have increased levels of CIITA promoter III and BSAP mRNA expression, while PRDI-BF1α and PRDI-BF1β mRNA levels are not expressed. However, upon differentiation to a plasmablast (day 6 of culture), PRDI-BF1α and PRDI-BF1β mRNA expression are increased, while the PRDI-BF1 downstream targets BSAP and CIITA promoter III are decreased.
Discussion

The transcription factor PRDI-BF1/Blimp-1 is required for the differentiation of a B cell to a plasma cell (237) and accomplishes this by directly repressing a number of downstream targets (236). Work from our lab has demonstrated a rapid induction of PRDI-BF1/Blimp-1 mRNA in lymphoma cells as measured by nascent RNA levels in anti-IgM-stimulated cells as well as factor binding to the promoter as seen by in vivo DMS footprinting. These data indicate the promoter is poised and ready for activation in these cells (see Chapter Two). Very recent evidence demonstrates expression of PRDI-BF1/Blimp-1 mRNA in a DLBCL cells (32, 195, 251). However, PRDM1 gene mutations were found in approximately one quarter of all DLBCL patients studied and led to transcription of a nonfunctional protein (251). The work presented in this chapter demonstrates a correlation between PRDM1 induction and apoptosis in both BL and MCL cell lines and patient samples.

Overexpression of c-myc is a classical hallmark of BL. c-Myc is a direct downstream target of PRDI-BF1/Blimp-1 (140), so induction of PRDM1 in this type of lymphoma may be a way to target dysregulated cell growth and survival of these cells. When CA46 BL cells were treated with the HDAC inhibitor Trichostatin A, PRDM1 expression levels increased significantly. Exactly what the mechanism for this remains to be elucidated. It could be due to decreased BCL-6 expression, which is known to repress PRDI-BF1 (262) and is known to be regulated by histone acetylation (13). Another possible explanation is downregulation of c-myc by induction of PRDI-BF1.

Mantle cell lymphoma is an incurable disease characterized by high levels of cyclin D1 (275). Therefore, discovery of chemotherapeutic agents which may have
therapeutic potential in treating this disease is of upmost importance. The proteasome inhibitor has been effective alone or in combination with other drugs in the treatment of myeloma (120). Its use has been extended to treatment of lymphoma patients (132). Our work demonstrates the ability of Bortezomib to induce apoptosis in these cells, which correlates with the induction of PRDM1 gene expression. Bortezomib was shown to induce the UPR in myeloma cells (184), which involves XBP. XBP is downstream of PRDI-BF1, so inducing PRDM1 could affect XBP expression in these cells. Our studies have been broadened to study the therapeutic potential of both Bortezomib and SAHA in mantle cell patient samples. By increasing PRDM1 expression in these cells, it may be possible to modulate downstream target gene expression responsible for the aggressive phenotypes of these lymphomas. However, our studies do not currently address the role of XBP in MCL upon PRDM1 induction. Bortezomib does induce PRDM1 expression in patient samples tested. However, the HDAC inhibitor SAHA decreased PRDM1 levels in the one patient examined (figure 25). This does not correlate with data acquired in Mino cells using LBH589 (figure 24). This could be due to this particular patient being unresponsive to drug treatment in general. The current and past treatment regimens of the patients tested are unknown to us, as is their disease status. Studies with both Bortezomib and SAHA, or other HDAC inhibitors, will need to be extended to a greater number of patients.

Cyclin D1 is not one of the known direct targets of PRDI-BF1 repression. In normal plasma cells, which express high levels of PRDI-BF1, microarray data demonstrates absence of the cyclin D1 gene CCND1 expression (48). Analysis of the cyclin D1 promoter indicates there are at least three putative PRDI-BF1 binding sites.
One possible mechanism for apoptosis seen in drug-treated MCL cells is PRDI-BF1-mediated repression of cyclin D1 promoter activity. Cyclin D1 is important in cell cycle progression and, as such, repression of its activity could cause growth arrest and apoptosis. Analyzing the role PRDI-BF1 plays in cyclin D1 repression would provide further insight into how MCL cells may die upon drug treatment.
Materials and Methods

Cell lines and drugs

The CA46 Burkitt’s lymphoma cell line, Mino mantle cell lymphoma line, and U266 myeloma cell lines were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone), and 1% penicillin/streptomycin (P/S) (Invitrogen). The HDAC inhibitor Trichostatin A (Sigma) was dissolved in 100% ethanol and used at a concentration of 100 ng/ml. The Novartis HDAC inhibitor LBH589 was obtained from Dr. K. Bhalla (Moffitt Cancer Center), while the proteasome inhibitor Bortezomib was obtained from Dr. W.S. Dalton (Moffitt Cancer Center).

Apoptosis assay

Cells were treated with either Trichostatin A, SAHA, LBH589, or Bortezomib as denoted, followed by Annexin V-PE and 7-AAD staining per manufacturer’s protocol (BD Pharmingen). Flow cytometry acquisition was done using a FACSCalibur and data was analyzed with CellQuest software (Becton Dickinson, Carpinteria, CA).

Real-time 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). 1/20th of the final cDNA reaction volume was used in each PCR reaction. Primers sequences are as follows: PRDI-BF1 FWD, 5’- TACATACCAAAGGGCACACG -3’,
PRDI-BF1 REV, 5’- TGAAGCTCCCCTCTGGAATA -3’, CIITA promoter 3 FWD, 5’-CAATGCGTTGCCTGGCCTC -3’, CIITA promoter 3 REV, 5’-GCTGTTAAGAAGCTCCAGGTAG 3’, BSAP FWD, 5’-TGGAGGATCCAAAACCAAAGG -3’, BSAP REV, 5’-GGCAAAACATGGGGGATT TT -3’, GAPDH FWD, 5’-GAAGATGGTGATGGGATTTC -3’. PRDI-BF1, CIITA promoter 3, and GAPDH primer sets were run using an annealing temperature of 60°C, while the BSAP primer set was run using an annealing temperature of 55°C. 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). 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 for each cDNA sample and relative levels of the genes of interest were calculated by the ∆∆Ct method (145): for example, $2^{(\Delta \Delta Ct)}$ for PRDI-BF1 gene expression.

**B cell isolation and differentiation to plasmablasts**

Peripheral blood B cells were obtained from healthy donors and differentiated to plasmablasts, as previously described (253). Briefly, B cells were purified from peripheral blood lymphocytes by positive selection with anti-CD19 MACS Microbeads (Miltenyi) or negative selection with the human B cell isolation kit II (Miltenyi) using the AutoMACS magnetic bead sorter (Miltenyi). Purification and cell viability was verified by staining cells with CD19-PE (BD Pharmingen) and 2 nM ToPro3 (Invitrogen) and
analyzing for viable CD19+ B cells using the FACSCalibur. Purified B cells were plated on irradiated CD40L cells (71) and treated with IL-2 (20 U/ml), IL-4 (50 ng/ml), IL-10 (50 ng/ml), and IL-12 (2 ng/ml) (Peprotech) in RPMI supplemented with 10% FBS and 1% P/S for 4 days. The activated B cells were gently detached from the CD40L cells and washed three times with complete medium and grown an additional 2 days in complete medium supplemented with IL-2 (20 U/ml), IL-6 (5 ng/ml), IL-10 (50 ng/ml), and IL-12 (2 ng/ml) (Peprotech). On day 6 of culture, cells were stained with CD20-FITC, CD38-APC, and 7-AAD, and plasmablasts were sorted out by selecting CD20-/CD38+ viable cells on the FACSARia cell sorter. RNA was collected and analyzed by real-time PCR.
Chapter Four

The farnesyl transferase inhibitor, FTI-277, inhibits growth and induces apoptosis in drug-resistant myeloma cells

Introduction

Multiple myeloma is a fatal plasma cell malignancy characterized by the accumulation of latent plasma cells in the bone marrow (84). Numerous investigators have examined the frequency of ras mutations in multiple myeloma, and have reported a correlation between activating ras mutations and disease progression (144, 178, 204). Furthermore, Rowley et al. demonstrated that ANBL6 myeloma cells transfected with activated N- or K-Ras were more resistant to apoptosis induced by dexamethasone, doxorubicin, and melphalan, suggesting a role for Ras mutations in resistance to chemotherapeutic drugs (216). Following initial response to therapy, patients with multiple myeloma invariably relapse and are refractory to additional treatment regimens. Thus, myeloma has long served as a model system for investigation of the drug resistant phenotype. These model systems have led to the characterization of several mechanisms of resistance, including overexpression of the anti-apoptotic protein, Bcl-xL (261); induced expression of the ATP dependent efflux pump P-glycoprotein (44); and altered expression of topoisomerases (68).

The Ras oncoprotein is a small G-protein signal transducer that requires a prenyl lipid modification and membrane association for signal transduction activity (85, 280). Two enzymes, farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I),
catalyze this prenyl posttranslational modification by transferring farnesyl or geranylgeranyl to the cysteine of the carboxyl terminal CAAX (C=cysteine, A=aliphatic, X=any amino acid). H-, N-, and K-Ras are all farnesylated. When FTase is inhibited, K-Ras, but not N-Ras or H-Ras, becomes geranylgeranylated (133). Because prenylation is required for the oncogenic activity of Ras, we, and others designed FTase and GGTase I inhibitors as potential anticancer drugs (228). To date, the majority of studies using prenylation inhibitors have focused on solid tumors, including lung, bladder, breast, and colon carcinomas (14, 133, 246, 247). In this study, we examined the activity of prenylation inhibitors in myeloma cell lines with different Ras mutation status, using the CAAX peptidomimetics FTI-277 (273) and GGTI-2166 (245). These inhibitors are structural analogues based on the tertiary structure of the CAAX tetrapeptide (228). Cytotoxicity and growth inhibition were examined for correlation with inhibition of Ras protein processing. In addition, myeloma cell lines with well-characterized mechanisms of drug resistance were analyzed for response to farnesyltransferase inhibitors (FTI). No cross-resistance to FTI was identified in myeloma cells selected for resistance to doxorubicin or melphalan, nor did forced constitutive overexpression of Bcl-xL confer resistance to FTI. These studies provide support for further pre-clinical and clinical trials with prenylation inhibitors in drug resistant myeloma.

Results

Myeloma cells with a N-Ras mutation are highly sensitive to FTI-277

We used MTT and clonogenic assays to determine the sensitivity of myeloma cells to growth inhibition by FTI-277. The MTT assay measures the metabolic activity of
cells, while the soft agar clonogenic assay assesses the ability of cells to divide and form colonies in an anchorage-independent fashion. H929 cells, which express activated N-Ras, were found to be significantly more sensitive to growth inhibition by FTI-277 than either U266 cells, which express wild-type Ras, or 8226 cells, which express activated K-Ras (figure 30A). The 8226 cell line was three times more resistant to the growth inhibitory activity of FTI-277 than H929 cells (IC$_{50}$ 41.6 µM vs. IC$_{50}$ 12.7 µM), while U266 cells demonstrated an intermediate sensitivity (IC$_{50}$ 25.4 µM). Soft agar clonogenic assays demonstrated a similar pattern of sensitivity, with H929 cells more sensitive to treatment with FTI-277 than 8226 cells with activated K-Ras over all doses (p<0.05, Student’s t-test) (figure 30B). Neither mutant N- nor K-ras containing cells were significantly different in sensitivity to FTI-277 compared to the wild-type ras U266 cells. Because prenylation inhibitors are generally considered to be cytostatic agents, we also examined the ability of FTI-277 to induce apoptosis in myeloma cells. Myeloma cells were treated with FTI-277 for 48 h, followed by staining with Annexin V-FITC and flow cytometry analysis. In correlation with the growth inhibition assays, a dose dependent response was demonstrated, with greatest cell death in the N-Ras mutant H929 cells, followed by the wild-type U266 cells. Similar to the growth inhibition and clonogenic analysis, the K-ras mutant 8226 cell line was found to be the least sensitive to FTI cytotoxicity (figure 30C). These data are also compatible with previous reports, in which tumor cells of epithelial origin with activated K-Ras were shown to be less responsive to FTI treatment compared to cells with activated N-Ras or wild-type Ras (6, 59).
A

IC50 (uM)

H929  U266  8226

B

Percent survival

FTI-277

5 uM  10 uM  15 uM

Comparison of 2 cell lines  95% LC1  95% UC1

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<td>8226 vs. H929</td>
<td>0.63*</td>
<td>3.72*</td>
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<tr>
<td>8226 vs. U266</td>
<td>-0.54</td>
<td>2.69</td>
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<td>H929 vs. U266</td>
<td>-2.93</td>
<td>0.73</td>
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C

Percent apoptosis above control

Concentration FTI-277

5 uM  15 uM  30 uM
Myeloma cells with activated N-Ras mutation are more sensitive to cytotoxicity by FTI-277 compared to cells with wild-type Ras or K-Ras mutation. A, Myeloma cells were treated with FTI-277 continuously for 96 hours and assayed using the MTT cytotoxicity assay. IC50s were calculated by linear regression of percent survival versus log drug concentration and 95% confidence intervals were determined by statistical analysis. Data shown are a minimum of three independent experiments, and summarized with the Ras mutation status, corresponding regression estimate of the IC50, and 95% confidence intervals. B, H929, U266, or 8226 cells were treated with vehicle (DTT/DMSO), 5 µM, 10 µM, or 15 µM FTI-277 and grown in 0.3% agar layered over 0.6% agar. Colonies were stained with MTT dye and quantitated by counting. H929 cells with activated N-Ras were significantly more sensitive to FTI-277 treatment over all doses than 8226 cells with activated K-Ras (p<0.05). The data are represented as mean percent survival above control and are the mean of a minimum of five independent experiments done in triplicate. The table summarizes 95% confidence intervals for a comparison of two cell lines. (* indicates statistical significance by regression analysis.) C, Myeloma cells were treated for 48 hours with 5-30 µM FTI-277, then assayed for apoptosis using Annexin V-FITC staining as determined by flow cytometry. FTI-277 caused apoptosis in myeloma cells in a dose-dependent manner, with N-Ras mutated H929 cells being most sensitive to FTI-277-induced apoptosis. The data are represented as percent apoptosis above control and are the mean of a minimum of three independent experiments.
Inhibition of Ras processing and relationship to FTI-induced apoptosis

To determine if there was a correlation between inhibition of Ras processing and either growth inhibition or apoptosis, we examined the ability of FTI-277 to block Ras prenylation in the myeloma cell lines. Lipid modification of the p21 Ras protein alters the protein mobility on SDS-PAGE, resulting in a shift to a lower apparent molecular weight on Western blots and allowing detection of intracellular drug activity. Although FTI-277 demonstrates greater toxicity in the H929 cell line, inhibition of N-Ras processing is more effective in the U266 cells compared to H929 cells even when treated with doses as low as 1.25 µM FTI-277 (figure 31). Additionally, N-Ras processing in U266 cells is inhibited at much lower doses (range: 1.25-10 µM FTI-277) than that required for induction of apoptosis (15-30 µM FTI-277), suggesting that the mechanism by which FTI-277 induces apoptosis in U266 most likely does not involve inhibition of the prenylation and function of N-Ras.

Processing of K-Ras in the 8226 cells was completely unaffected by any dose of FTI-277 up to 30 µM following 48 hour treatment. Because K-Ras becomes geranylgeranylated when FTase is inhibited (133, 278), we also examined K-Ras prenylation following exposure to the geranylgeranyltransferase inhibitor, GGTI-2166, either alone or in combination with FTI-277. No effect was seen with either FTI-277 or GGTI-2166 alone (figure 31C). When 8226 cells were treated simultaneously with FTI-277 and GGTI-2166 for 48 hours, inhibition of K-Ras processing occurs in a dose-dependent manner. Maximal response for inhibiting K-Ras processing occurred at 5 µM GGTI-2166 in combination with 10 µM FTI-277 (figure 31D).
Figure 31. FTI-277 inhibits N-Ras processing in myeloma cells with wild-type Ras (U266) and activated N-Ras (H929), while both FTI-277 and GGTI-2166 inhibit K-Ras processing. Myeloma cells were treated with FTI-277 for 48 hours. Inhibition of Ras processing was demonstrated by a band shift to a higher apparent molecular weight by Western blot analysis (U, unprocessed Ras, P, processed Ras). A, N-Ras processing of H929 cells following treatment with 0 (vehicle control), 1.25, 2.5, 5, or 10 µM FTI-277 for 48 hours. B, N-Ras processing of U266 cells following treatment with 0 (vehicle control), 1.25, 2.5, 5, or 10 µM FTI-277 for 48 hours. C, K-Ras processing of 8226 cells following treatment with 0 (vehicle control), 5, 10, 15, or 30 µM FTI-277, and 5 or 10 µM GGTI-2166 for 48 hours. D, K-Ras processing of 8226 cells following treatment with 0 (vehicle control), 5 µM GGTI-2166 plus 5-30 µM FTI-277 or 10 µM GGTI-2166 plus 5-30 µM FTI-277 for 48 hours. Equal loading was determined by immunoblotting with β-actin (data not shown).
Co-treatment of the 8226 cell line with FTI-277 and GGTI-2166 also demonstrated an enhanced apoptotic response above the levels for each drug alone. 8226 cells treated with 30 µM FTI-277 alone induced approximately 12% drug-specific apoptosis, while GGTI-2166 treatment alone was completely ineffective in these cells. However, the combination of 10 µM GGTI-2166 with FTI-277 increased the cell death two-fold compared to FTI-277 alone in both MTT growth inhibition and Annexin V apoptosis assays (figure 32). Statistical analysis using linear regression with integrated and reduced models demonstrated that growth inhibition and apoptosis following co-treatment with FTI-277 and GGTI-2166 occurs in an additive manner. These data are compatible with previous studies demonstrating that inhibition of farnesyl transferase is insufficient to affect K-ras processing, and concomitant inhibition of geranylgeranyl transferase is required. Furthermore, inhibition of N-Ras processing in H929 and U266 cells occurs at doses well below those necessary to cause growth inhibition and apoptosis, while inhibition of K-Ras processing and significant apoptosis in 8226 cells requires co-treatment with FTI-277 and GGTI-2166.
A

Percent apoptosis above control

5 μM 15 μM 30 μM FTI-277
5 μM 10 μM GGTTI-2166
30 μM FTI-277 + 10 μM GGTTI-2166

B

Percent of control

0 GGTI-2166
5 μM GGTI-2166
10 μM GGTI-2166

FTI-277 (M)
Figure 32. Both FTI-277 and GGTI-2166 inhibit K-Ras processing. A, Myeloma cells were treated for 48 hours with 5-30 µM FTI-277 alone, 5-10 µM GGTI-2166 alone, or 10 µM GGTI-2166 combined with 30 µM FTI-277, then assayed for apoptosis using Annexin V-FITC staining by flow cytometry. FTI-277 caused apoptosis of 8226 myeloma cells in a dose-dependent manner, while GGTI-2166 had no effect on the cells. A combination of FTI-277 and GGTI-2166 had an additive effect on inducing apoptosis in 8226 cells. The data are represented as percent apoptosis above control and are the mean of a minimum of three independent experiments. B, Myeloma cells were treated with FTI-277 alone, FTI-277 plus 5 µM GGTI-2166, or FTI-277 plus 10 µM GGTI-2166 for 96 hours, then assayed using the MTT cytotoxicity assay. Increasing the dose of GGTI-2166 enhanced the cytotoxic effect above that of FTI-277 treatment alone. The data are represented as percent survival above control cells. The data are the mean of three independent experiments done in quadruplicate.

Bcl-x\textsubscript{L} and classical mechanisms of drug resistance are not determinants of resistance to FTI-277. The anti-apoptotic protein Bcl-x\textsubscript{L} is a known determinant of drug resistance in multiple myeloma and has previously been shown to protect myeloma cells from cytotoxic drugs including doxorubicin, melphalan, mitoxantrone, and VP-16 (189). We explored the ability of FTI-277 to induce apoptosis in myeloma cells overexpressing Bcl-x\textsubscript{L}. Using U266 cells engineered to constitutively overexpress Bcl-x\textsubscript{L} (U266/Bcl-xl), we compared the cytotoxic activity of FTI-277 in U266 cells expressing low basal levels of Bcl-x\textsubscript{L} (U266 and U266/Neo) to those overexpressing Bcl-x\textsubscript{L} (figure 33A-B). Using both MTT cytotoxicity and CMXRos/Annexin V apoptosis assays, no significant difference was found in the response to FTI-277 between the cells expressing low levels of Bcl-x\textsubscript{L} and those with high constitutive expression. Bcl-x\textsubscript{L} has been shown to prevent cell death by preventing the release of cytochrome c and mitochondrial perturbation (222). Equal sensitivity of cells expressing varying levels of Bcl-x\textsubscript{L} indicate that apoptosis induced by the FTIs in myeloma cells occurs through a mitochondrial-independent mechanism. Furthermore, the observation that Bcl-x\textsubscript{L} does not confer
resistance to prenylation inhibitors supports the use of these agents in patients with drug resistant disease.
Figure 33. Bcl-xL is not a determinant of resistance to FTI-277. A, U266, U266/Neo, and U266/Bclxl cells were treated with FTI-277 continuously for 96 h and assayed for cytotoxicity using the MTT assay. IC\textsubscript{50}s were calculated and the 95% confidence intervals estimated by linear regression of percent survival vs log drug concentration. The data shown are of three independent experiments done in quadruplicate. The corresponding table summarizes the IC\textsubscript{50} estimate and 95% confidence intervals from linear regression for the U266 cell lines. B, Bcl-X\textsubscript{L} protein expression. The myeloma cell lines H929, U266, U266/Neo and 8226 express minimal levels of endogenous Bcl-X\textsubscript{L} protein relative to the overexpressing transfectant, U266/Bcl-xl. Equal loading was determined by immunoblotting for β-actin. C, U266, U266/Neo, and U266/Bcl-xl cells were treated with FTI-277 continuously for 48 hours and assayed for apoptosis using CMXRos (Chloromethyl-X-Rosamine)/Annexin-V staining. Loss of MMP and Annexin-V staining of cell surface phosphatidylserine are independent markers of apoptosis, therefore viable cells appear in the upper left quadrant of the flow cytometry dot plot, while the other three quadrants represent cells in various stages of apoptosis. There is no significant difference in apoptosis in the U266 and U266/Bcl-xl cell lines, indicating that Bcl-xL protein overexpression in U266 cells is not a determinant of resistance to FTI-277 in U266 myeloma cells. The data shown are representative of three individual experiments.
Patients with multiple myeloma frequently relapse with multi-drug resistant disease, and myeloma cells selected in vitro for resistance to chemotherapeutic drugs provide a model for examining mechanisms of acquired drug resistance. We utilized cells selected for resistance to doxorubicin, in the presence (68) or absence (44) of the calcium channel blocker, verapamil, and cells selected for resistance to melphalan (11) for cross resistance to FTI-277. No significant differences were found in the response of the parental cells, 8226/S, as compared to the drug resistant variants, 8226/Dox40, 8226/Dox1V, or 8226/LR5 (figure 34). The 8226/Dox40 cell line has been shown to overexpress the ATP-dependent efflux pump, P-glycoprotein, and is approximately 180-fold resistant to doxorubicin. Additionally, this cell line demonstrates cross-resistance to mitoxantrone, etoposide, and vincristine (44). However, no cross-resistance to FTI-277 was demonstrated, indicating that the prenylation inhibitor is not a substrate for P-glycoprotein efflux. Additionally, 8226 drug resistant cells with other mechanisms of drug resistance, such as altered topoisomerase II activity and elevated levels of intracellular glutathione, are also not significantly more resistant to treatment with FTI-277 compared to parental cells. These data support the potential utility of FTI inhibitors in relapsed myeloma patients, either as single agents, or in combination with standard chemotherapeutic drugs.
Figure 34. Drug-resistant myeloma cells are not cross-resistant to FTI-277. 8226/S, 8226/Dox40, 8226/Dox1V, and 8226/LR5 were treated with FTI-277 continuously for 96 hours and assayed using the MTT cytotoxicity assay. IC50s were calculated by linear regression of percent survival versus log drug concentration and 95% confidence intervals were determined by statistical analysis. The IC50 estimates from linear regression were calculated from three independent experiments done in quadruplicate. The table summarizes the IC50s and 95% confidence intervals for the myeloma cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (uM)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>8226</td>
<td>36</td>
<td>30-42</td>
</tr>
<tr>
<td>8226/Dox40</td>
<td>40.5</td>
<td>30.8-50.3</td>
</tr>
<tr>
<td>8226/Dox1V</td>
<td>23.9</td>
<td>17.9-29.8</td>
</tr>
<tr>
<td>8226/LR5</td>
<td>38</td>
<td>30.8-45.3</td>
</tr>
</tbody>
</table>
Discussion

Ras mutations are among the most common oncogenic mutations identified in many malignant diseases, including multiple myeloma. Prenylation inhibitors are a new class of agents developed to inhibit Ras protein processing and prevent constitutive signal transduction by the activated protein. Clinical trials with FTIs have demonstrated a therapeutic response in leukemia patients, even though Ras mutation status was not a determinant for clinical response (6, 114). Similarly, preclinical studies using solid tumor xenografts have not supported a correlation of response with Ras mutation status (6, 59). Thus, the biologic basis for FTI activity in tumor cells is yet to be fully defined.

In this study we examined the cytotoxic activity and inhibition of Ras processing in three myeloma cell lines with differing Ras mutation status. The U266 cell line, which expresses wild type Ras, is the most sensitive to inhibition of Ras processing; however, these cells are not sensitive to FTI-mediated cytotoxicity. The H929 cell line, which expresses mutant N-ras, demonstrates an intermediate sensitivity to FTI-277-induced inhibition of N-Ras processing, but is the most sensitive to growth inhibition and apoptosis. In all assays, the 8226 cell line containing a K-ras mutation is the most resistant to the cytotoxic effects of FTI. Similar to previous studies in epithelial carcinomas, K-Ras processing could only be inhibited with a combination of FTI and GGTI in 8226 cells. In a study by Lerner et. al, pancreatic, bladder, and pulmonary tumor cells also demonstrated resistance to inhibition of K-Ras processing when treated with FTI-277 or GGTI-298 alone (133). Furthermore, inhibition of K-Ras processing in these cell lines did not correlate with growth inhibition as measured by soft agar clonogenic assays. In contrast, in this study the most sensitive cell line is the one with N-
Ras mutation suggesting that N-Ras mutation status may be a determinant of response. Consistent with this is the fact that the IC50 for growth inhibition is similar to that of inhibition of N-Ras processing in H929 but not in U266. In U266, inhibition of N-Ras processing (IC50=2 µM) does not result in inhibition of growth (see Figure 31).

Expression of the anti-apoptotic protein Bcl-xL has been shown to contribute to resistance to a broad range of chemotherapeutic agents in myeloma cells (189, 261). However, the data presented here demonstrates that FTI-277 is effective in myeloma cells in vitro independent of Bcl-xL expression. The U266 myeloma cell line expresses a basal level of Bcl-xL, that confers resistance to both Fas and chemotherapeutic agents (31, 189). In contrast, H929 myeloma cells express low levels of Bcl-xL and are more sensitive to the cytotoxicity of the FTIs. This observation led us to examine FTI activity in U266 myeloma cells engineered to express very high levels of Bcl-xL (31). In these myeloma cell lines, Bcl-xL expression had no effect on the cytotoxicity of the FTI. These data are in contrast to a previous study demonstrating resistance to the farnesyltransferase inhibitor L-739,749 by Bcl-xL overexpression in Rat1 cells (126). This discrepancy is most likely due to the different cell types and their relative dependence on Bcl-xL for survival.

Classical mechanisms of acquired drug resistance include increased expression of drug efflux proteins such as P-glycoprotein, reduced expression of intracellular drug targets, such as topoisomerase II (68), and enhanced drug detoxification by glutathione S-transferase (11). The novel observation that these classical mediators of drug resistance do not confer cross-resistance to FTI-277 provides strong rationale for clinical trials of FTI in myeloma patients that are refractory to standard chemotherapeutic agents.
Therapeutic agents with molecular targets provide a unique opportunity to specifically inhibit malignant growth with minimal systemic toxicity. Many of these agents are being extensively investigated as potential combination therapies, and in fact, previous studies using a xenograft model have demonstrated that FTI in combination with cisplatin, taxol, or gemcitabine is more effective than either agent alone (245). Our data demonstrate that farnesyl transferase inhibitors inhibit growth and induce apoptosis in both drug sensitive and drug resistant myeloma cells in vitro. These results support ongoing clinical studies in myeloma patients, and may contribute to the development of a novel treatment for this incurable disease. Recently, phase II clinical trial data has demonstrated stabilization of disease in over 60% of myeloma patients treated with the FTI Zarnestra (3).

Materials and Methods

Cell lines and drugs

The RPMI 8226, U266, and H929 multiple myeloma cell lines were maintained in RPMI medium (CellGro, MediaTech, Herndon, VA) supplemented with 5% or 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA), 1% penicillin/streptomycin (P/S), and 100 mM L-glutamine (Gemini Bio-Products, Calabasas, CA). The H929 cell line was also supplemented with 0.05 M 2-mercaptoethanol (Sigma, St. Louis, MO). U266 cells transfected with Bcl-xL were cloned and selected for high (U266/Bcl-xL) or low (U266/Neo) Bcl-xL protein expression and have been previously described (189). The drug resistant cell lines, 8226/Dox40 (44), 8226/Dox1V (68), and 8226/LR5 (11) have all
been previously described. The farnesyltransferase inhibitor (FTI)-277 (273) and geranylgeranyltransferase inhibitor (GGTI)-2166 (245) were initially dissolved in 10 mM dithiothreitol in dimethyl sulfoxide (DTT/DMSO) and diluted to the appropriate concentrations with RPMI complete medium.

**MTT cytotoxicity assay**

Cells were seeded at 8000-14000 cells/well in 96-well plates (Becton Dickinson, Lincoln Park, NJ). To establish a dose-response to FTI-277, cells were incubated 96 hours in two-fold serial dilutions ranging from $3.75 \times 10^{-7}$ M to $1 \times 10^{-5}$ M. Following continuous drug exposure, 50 µl 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye was added. The insoluble formazan complex was solubilized with DMSO and absorbance measured at 540 nm. IC$_{50}$s and 95% confidence intervals were calculated by regression analysis of the linear portion of the dose-response curve.

**Soft agar clonogenic assay**

Cells were seeded at densities established for optimal cloning efficiency (range: 24000-30000 cells per well) and grown in 0.3% agar (Difco, Detroit, MI) layered over 0.6% agar. FTI-277 or vehicle control (10 mM DTT/DMSO) was included in the 0.3% agar layer of cells. Approximately 2-3 weeks after plating, colonies were stained with 1 mg/ml MTT and counted.
**Ras processing assay**

Cells were treated with FTI-277, GGTI-2166, or combinations thereof for 48 hours. Following drug exposure, cells were washed in PBS and lysed in a buffer containing 30 mM HEPES, pH 7.5, 10 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM MgCl\(_2\), 1 mM EGTA, 25 mM NaF, 25 µg/ml leupeptin, 2 mM phenylmethylsulfonylfluoride (PMSF), 2 mM sodium orthovanadate, 10 µg/ml soybean trypsin inhibitor, and 10 µg/ml aprotinin. The lysates were cleared and total protein was determined by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (50 µg) were separated on 12.5% SDS-PAGE and transferred to PVDF membrane (Bio-Rad). Membranes were probed with polyclonal antisera specific for N- or K-Ras (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-x\(_L\) (Pharmingen, San Diego, CA), or monoclonal antiserum specific for β-actin (Sigma, St. Louis, MO), followed by detection with chemiluminescence (Roche, Indianapolis, IN).

**Apoptosis assays**

Cells were treated with FTI-277 and/or GGTI-2166 for 48 hours continuously, followed by Annexin V-FITC staining according to the manufacturer's protocol (BioVision, Mountain View, CA). In experiments that included CMXRos staining for mitochondrial perturbation, cells were incubated for ½ hour prior to collection with 200 nM CMXRos (Molecular Probes, Eugene OR). Flow cytometry acquisition was done on a FACSCalibur and analyzed with CellQuest software (Becton Dickinson, Carpinteria, CA).
**Statistical analysis**

IC$_{50}$ values were calculated and 95% confidence intervals were estimated using a simple linear regression analysis following logistic transformation of the response variable where applicable. Differences across cell lines or doses were tested using either the Student’s t-test or Wilcoxon Rank Sum test. Differences in the mean of the transformed responses were determined by 95% confidence intervals. In the combination FTI-277/GGTI-2166 study, a linear regression analysis was used to test for synergism and additivity across dose levels.
Chapter Five

General Discussion

The work presented in this dissertation provides insight into the regulation of the human PRDM1 gene and explores the possibility of modulating PRDM1 gene expression as a target for inducing apoptosis of lymphoma cells. Very little was known as to the regulation of PRDI-BF1 upon initiation of this project. The finding that regulation of this gene occurs primarily at the transcriptional level is very novel. It is also at the foundation of preliminary data presented here demonstrating induction of PRDM1 by chemotherapeutic agents both in cell lines and patient samples. This induction correlates with a dose-dependent increase of apoptosis, suggesting the possibility PRDI-BF1 may play an important role in more than just differentiation.

PRDI-BF1 was traditionally thought to play a role in the differentiation from a B cell to a plasma cell. However, since inception of this project, PRDI-BF1 has been shown to be expressed by a number of organisms at different stages of development as well as in cells where it was originally thought to not be expressed, such as T cells (111) and B cells of myeloma patients (21). These findings are intriguing and lead to many more questions as to the role PRDM1 might play here.

The finding that the PRDI-BF1 promoter is loaded with factors in lymphoma cells is a very interesting one, as one would anticipate a bare promoter in a cell which does not
express this gene. However, when taken with the data demonstrating the rapid induction of the transcriptional activation, as is seen in figure 9 with 1 hour anti-IgM treatment, it indicates the PRDI-BF1 promoter is poised and primed for activation. PU.1 is shown to be a factor involved in anti-IgM-induced activation of the promoter, but is definitely not the sole factor involved in this rapid induction of transcriptional activity, as is seen by data presented in figures 19 and 20. Further elucidation of the roles played by the PI3K and MAPK signaling pathways may reveal additional factors involved in regulating PRDI-BF1. Utilizing the chromatin immunoprecipitation assay (ChIP) in these cells could also be a useful tool for analyzing potential candidate factors as to their role in regulating the PRDM1 promoter as well as identifying chromatin modifications involved in promoter regulation. However, this assay is very difficult to do in B cells and is currently being optimized in our lab.

Luciferase activity was shown to be high in myeloma cells, but is overall much lower in lymphoma cells. These data agree with the expression pattern of PRDM1 in lymphoma and myeloma cells. However, factors required for activation are present in these cells, as demonstrated by the rapid induction of nascent RNA production in lymphoma cells (figure 9). There is one distal region of activation common to both lymphoma and myeloma cells and an additional region specific to only myeloma cells. These regions were further analyzed by DMS in vivo footprinting to identify putative factors responsible for promoter activity in both cell types. Each of these approximately 400 bp regions were extensively analyzed for unique contacts, but only three factor binding sites were eventually identified.
The proximal promoter was loaded with factors in both lymphoma and myeloma cells (figure 11), however there were no distinct differences observed between the cell types. There was a completely protected site in both cell types which was later verified to be a Sp1 binding site (figure 12). However, because there were no cell-type specific DNA-protein interactions in this region of the promoter, we further analyzed the distal promoter regions of activation.

Analysis of the distal promoter regions revealed several factors unique to either lymphoma or myeloma cells. However, upon further examination, only the PU.1 binding site was demonstrated to be functionally relevant to promoter activity, as shown by site-directed mutagenesis (figures 15 and 16). Using a footprinting technique specific for residues other than guanine may be helpful in identifying additional DNA-protein interactions which may be functionally relevant for promoter activity. Myeloma cells had one unique distal region of activation, as measured by luciferase assay. However, no contacts were detected in this region by DMS \textit{in vivo} footprinting. Using one footprinting method may have limited our ability to detect protein-DNA interactions.

PU.1 was found to bind the PRDI-BF1 promoter in a cell-type specific manner. It is a B cell specific factor and was found to only play a role in promoter activity of lymphoma cells, but not myeloma cells. It is not expressed in myeloma cells, but promoter activity was found to also be increased two-fold in the same distal region of the promoter in these cells (figure 10). This indicates another as yet undetermined factor is important for increased promoter activity in myeloma cells. This undetermined factor also binds a site other than site P.H, as gel shift analysis did not demonstrate occupancy of this site in myeloma cells (figure 17). Since PRDM1 gene expression is elevated in
myeloma cells, this factor may also be one which is ubiquitously expressed in plasma-like cells, but not B cells.

Because PU.1 has been known to bind promoters in complex with IRF family members (58, 61), we examined whether the IRF family members IRF-4 or IRF-8 were binding to site P.H in complex with PU.1 (figure 17 and 18). IRF-8 has been shown to induce *Blimp-1* expression in myeloid cells (252), while IRF-4 has been shown to be required for plasma cell differentiation (28). Competition with antibodies to either IRF-4 or IRF-8 did not affect P.H binding to DNA, indicating these factors do not appear to bind site P.H in complex with PU.1.

Work presented in Chapter Two is the first of its kind to directly link anti-IgM mediated B cell receptor activation to expression of PRDI-BF1/Blimp-1. It also demonstrates regulation of PRDI-BF1/Blimp-1 occurs primarily at the level of transcription. The promoter is poised and ready for activation, which is a significant finding in light of the preliminary data presented in Chapter Three, demonstrating the induction of PRDM1 gene expression levels PRDM1-negative lymphoma cells in parallel with induction of apoptosis. However, there is no direct link between induction of PRDM1-BF1/Blimp-1 and induction of apoptosis. This could be due to an indirect effect. It has previously been shown introduction of PRDI-BF1 is able to induce apoptosis in cells (164), so one could hypothesize that there is a direct link between induction of PRDI-BF1/Blimp-1 and induction of apoptosis. Introduction of PRDI-BF1 into lymphoma cells and measuring apoptosis is the direct approach that would answer this question. However, B cells are very difficult to transfect using methods other than electroporation. Electroporation causes a large amount of background cell death, which
makes a study of apoptosis caused by introduction of PRDI-BF1 difficult. These studies are currently the focus of our lab, as this is a critical question that remains to be answered.

Work presented in Chapter Four focuses on myeloma cells and the ability of the farnesyl transferase inhibitor, FTI-277, to induce apoptosis. The goal of this study was to determine whether Ras mutation status correlated with induction of apoptosis in several myeloma cells with differing Ras mutation status. Protein prenylation has been shown to be required for oncogenic activity of activated Ras, therefore using an inhibitor which blocks the transfer of the prenyl group to Ras itself would diminish its oncogenic activity. Our data demonstrate greatest inhibition of Ras processing in U266 myeloma cells with wild-type Ras, while they are not sensitive to FTI-mediated cytotoxicity, as measured by MTT assay and soft agar clonogenic assay. However, H929 myeloma cells with mutated N-Ras were most sensitive to treatment with FTI, but demonstrated intermediate sensitivity to FTI-277-induced inhibition of Ras processing. Inhibition of mutant K-Ras processing in 8226 myeloma cells was only accomplished by a combination of FTI-277 and GGTI-2198, which correlates with findings in epithelial cell lines (133). These findings indicate N-Ras mutation status might be a determinant of response to FTI treatment in myeloma patients, as the cell line harboring the N-Ras mutation was most sensitive to FTI-277 treatment.

Drug resistance is a common problem in myeloma patients, therefore it is important to study the effectiveness of new drugs in both drug-sensitive and drug-resistant cell lines. The anti-apoptotic protein Bcl-xL has been shown to confer resistance to chemotherapeutic agents in myeloma cells (189, 261), which led us to study cytotoxic
effects of FTI-277 in a cell line engineered to overexpress Bcl-xL (31). Our studies indicate Bcl-xL expression had no effect on FTI cytotoxicity (figure 32). We extended our studies to include several 8226 cell lines selected for various mechanisms of drug resistance, including increased P-glycoprotein expression (44), reduced expression of topoisomerase II (68), and enhanced drug detoxification by glutathione-S-transferase (11). These had no effect on cytotoxicity of FTI-277, indicating they are not cross-resistant to FTI-277 treatment. This is an important finding, as many myeloma patients initially respond to drug treatment, but then become refractory to treatment upon emergence of a drug-resistant population. This work provides the rationale to study FTIs in clinical trials with myeloma patients who no longer respond to standard treatment regimens.

This dissertation presents work on the transcriptional regulation of the PRDI-BF1 gene and extends these findings to studies on the modulation of the PRDM1 gene in PRDM1-negative lymphoma cells. Work is also presented on the correlation between Ras mutation status and sensitivity to FTI-277 treatment. These studies are extended to cell lines with known mechanisms of drug resistance in myeloma patients. Cell lines with the drug resistant phenotype are not cross-resistant to FTI-277 treatment, demonstrating a potential role for use of this drug in clinical trials.
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Sophia ("Sophie") Cornelia Elisabeth Bolick was born May 25, 1972 in Delft, the Netherlands. She graduated from Vrije Universiteit Amsterdam (the Netherlands) with a M.Sc. in Medical Biology in 1999. As an undergraduate, Sophie did research in the lab of Dr. R.J. Scheper in the Department of Pathology at the Academisch Ziekenhuis Vrije Universiteit. Sophie joined the graduate program in the Department of Biochemistry and Molecular Biology (currently the Department of Molecular Medicine) in Fall 1999 and started her dissertation work in the lab of Dr. W.S. Dalton. Upon his departure from USF in 2001, she began a new project in the lab of Dr. K.L. Wright. Sophie has published in several peer-reviewed journals, including *Leukemia*. While a graduate student, Sophie also served on the USF Graduate Council and was the Association of Medical Sciences Graduate Students (AMSGS) President.