Regulation of Protein Kinase CβII (PKCβII) gene expression in Chronic Lymphocytic Leukaemia (CLL) cells

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in philosophy

By

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Dedication

I dedicated this humble work to the soul of

My father who was and will still inspire me as a person who was working hard to achieve something valuable at the end

My mother Fatima, who have believed in me to arrive to this point of my studies

My brother in-law Sami Al-Omor who have encouraged me all the time until the end of his battle with the cancer

Abstract

Chronic lymphocytic leukaemia (CLL) cells are derived from mature B lymphocytes and are distinctive with respect to overexpression of the classical protein kinase C isoform protein kinase CβII (PKCβII), which is encoded by *PRKCB*. Expression of PKCβII in CLL plays a vital role in the pathogenesis of the malignant cells in this disease, and within the microenvironment cells where it provides signals for the production of factors which support the survival of CLL cells. In CLL cells *PRKCB* transcription is stimulated by vascular endothelial growth factor (VEGF) through a mechanism involving activated PKCβII. However, at the beginning of this thesis the molecular regulatory mechanism(s) governing expression of the PKCβ gene were poorly described. Thus, to characterise the factors regulating *PRKCB* transcription in CLL cells I used different approaches including mithramycin treatment, a drug which intercalates into GC-rich areas of DNA to inhibit binding of specificity protein 1 (Sp1), specific Sp1 siRNA, promoter function assays and site directed mutagenesis and chromatin immunoprecipitation (ChIP). Experiments using these techniques showed that Sp1 has a direct role in driving expression of the gene coding for PKCβII in CLL cells. My results also show that Sp1 is highly associated with the *PRKCB* promoter in CLL cells compared to that in normal B cells, and suggest that this is likely because of the presence of histone marks permissive of gene activation. Examination of other transcription factors such as Sp3, MITF, RUNX1 and E2F1 that potentially bind the *PRKCB* promoter showed that they have static or indirect effects in regulating transcription of this gene. The exception to this is STAT3 which my data suggests plays a role in suppressing PKCβ gene expression in CLL cells. Exploration of the mechanism through which VEGF induces *PRKCB* transcription revealed that this growth factor stimulates increased association of Sp1 and decreased association of STAT3 with the *PRKCB* promoter. Thus, VEGF-stimulated activation of PKCβII may play a role in this process. Taken together, Sp1 is the major driver for overexpression of PKCβII in CLL cells, and because this transcription factor is also overexpressed in these cells, the mechanisms I describe controlling *PRKCB* transcription potentially provide a foundation for further study of other genes

contributing to the phenotype of CLL cells that are regulated by this pleiotropic transcription factor.

Declaration

I, Ola Mohammad Fahad AL-Sanabra, declare that all of the data presented in this thesis is a result of my own work and effort and was generated from the experiments that I have performed during my work in this project. The only exception to this is the *PRKCB* promoter methylation studies results which were carried out by Mr. Benjamin R.B. Brown, Haematology Division, Department of molecular and Clinical Cancer Medicine, University of Liverpool.

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-Table of Contents:

-List of Figures:

-List of Tables:

-Abbreviations

ABC: protein kinase A, protein kinase B and protein kinase C

AML: acute myeloid leukaemia

Alb: Albumin

AMG: aorta-gonad-mesonephros region

AML 1: Acute myeloid leukaemia 1

α-Tub: α-tubulin

BAFF: B cell-activating factor

BCL10: B-cell CLL/Lymphoma 10

B-ALL: B-acute lymphoblastic leukaemia

BSA: Bovine Serum Albumin

β2M: β2- microglobulin

Btk: Bruton's tyrosine kinase

CARMA1: CARD-containing MAGUK protein 1

CBM complex: CARMA1/ Bcl10/ MALT1

cDNA: Complementary deoxyribonucleic acid

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

G6PDH: Glucose6-phosphatedehydrogenase

CES: Combinatorial enhancer solution

ChIP: Chromatin immunoprecipitation

CLL: Chronic Lymphocytic leukaemia

CLP: Common lymphoid progenitor cells

CRISPR-Cas9: Clustered regularly interspaced short palindromic repeats-Cas9

ΔCt: delta cycle threshold

DAG: diacylglycerol

DHFR: Dihydrofolate reductase

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

EBF: early B-cell Factor

EDTA: Ethyenediaminenetetraacetic acid

EtBr: Ethidium Bromide

EBF: early B-cell Factor

ERK: Extracellular Signal-Regulated kinases

FCS: Fetal calf serum

FLT3: fms-like tyrosine kinase 3

G-CSF: granulocyte-colony-stimulating factor growth factors

GM-CSF: granulocyte/macrophage colony-stimulating factor

HSCs: haematopoietic stem cells

HAT: Histone Acetyltransferase

HDAC: Histone Deacetylase

HATi: Acetyltransferase inhibitor

HDACi: Histone Deacetylase Inhibitor

HOX: Homeobox

h: Hour

HPRT: Hypoxanthine–guanine phosphoribosyltransferase

HSC: Haematopoietic stem cells

Ig: Immunoglobulin

IL-6: Interleukin- 6

IL-21: Interleukin- 21

IP: Immunoprecipitation

ITAM: immunomodulatory tyrosine activation motifs

LB agar: Luria-Bertani agar

MALT1: Mucosa Associated Lymphoid Tissue Lymphoma Translocation 1

MZ: marginal zone

MBL: monoclonal B lymphocytosis

µM: micro molar

min: Minutes

MIA: Mithramycin

miRNA: microRNA

miRSIC: miRNA-induced silencing complex

µg: microgram

mg: milligram

MITF: Microphthalmia-associated transcription factor

MYD88: myeloid differentiation primary response protein 88

nM: nanomolar

N.B: Normal B cells

XPO1: nuclear export protein

NF-YA: Nuclear factor-YA

PAX5: paired box protein

PI3Kδ: phosphatidylinositol 3-kinaseδ

PKCβ: Protein Kinase Cβ

PDGF-A: Platelet-derived growth factor A

PBS: Phosphate Buffer Solution

PBGD: Porphobilinogen deaminase

PH: pleckstrin homology

PHLPP: PH domain and Leucine rich repeat Protein Phosphatase

PP2A: protein phosphatase 2A

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

PLA: Phospholipase A2

PLCγ2: phospholipase Cγ2

Poly-HEMA: Poly 2-hydroxyethyl methacrylate

PTM: Post-translational modifications

Romi: Romidepsin

PPIA: Peptidyl prolyl isomerase A

Pro-B cells: Precursor-B cells

PVDF: Polyvinlydiene diflouride

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

RAG: Recombination activation gene

RPL13: Ribosomal protein L13

RasGRP3: Ras-guanine exchange factor

RNA: Ribonucleic acid

RCF: Relative centrifugal force

R.T.: Room temperature

RT-PCR: Real Time Polymerase Chain Reaction

RUNX1: Runt-related transcription factor 1

RPM: Revolutions per minute

S: Seconds

siRNA: small interfering RNA

shRNA: short hairpin RNA

SF3B1: splice factor3B1

SOC medium: Super optimal broth with catabolite repression broth medium

SOP: Standard operating procedure

SOS: son-of-sevenless

Sp1: Specificity Protein 1

Sp3: Specificity Protein 3

SFKs: Src-family kinases

TAK1: Transforming growth factor-beta-activated kinase 1

TBP: TATA-Box binding protein

T0: Initial time or Time=0

TBE: Tris-Borate- EDTA

T-BST: Tween-Tris saline buffer

TSS: Transcription start site

VEGF: Vascular Endothelial Growth Factor

UV light: Ultraviolet light

U: Unit

UK: United Kingdom

USA: United States of America

ZAP-70: zeta-chain associated protein kinase 70

❖ Chapter One: General Introduction

1.1 Overview and aim of the study

Protein kinase C βII (PKCβII) overexpression is a distinctive feature of the malignant cells in chronic lymphocytic leukaemia (CLL) where it plays an important role in modulating B cell receptor (BCR) signalling [1], a key contributor to the pathogenesis of this disease [2]. Importantly, disruption of the gene coding for PKCβII, *PRKCB*, in a mouse model of CLL suppresses development of the disease [3], suggesting that PKCβII is also key to the pathogenesis of this malignancy. Therefore, understanding the mechanisms controlling (over)expression of the PKCβ gene may provide insight into the regulation of other genes important in the pathogenesis of CLL. Previous studies have been performed to investigate the control of *PRKCB* transcription, and have identified different transcription factors involved in this process [4-8]. However, at the beginning of this thesis the principle driver of *PRKCB* transcription was unknown, and how this transcription factor was regulated was undescribed. Thus, the main aims of this thesis are focused on identification of the principle driver(s) of PKCβ gene expression, and on understanding how these driver(s) are regulated in CLL cells. Chapter 3 of this thesis identifies a key role for Sp1 in regulating *PRKCB* transcription in CLL cells. In Chapter 4 I investigate the roles of other transcription factors, and find that they largely play minor or indirect roles in regulating expression of this gene. STAT3 is an exception, and my data show that it plays a major role in suppressing transcription of *PRKCB*. Chapter 5 explores the role of epigenetics in modulating PKCβ gene expression, and I show that high levels of PKCβII expression are likely due to changes in chromatin landscape rather than gene methylation. Finally, Chapter 6 investigates the role of cell extrinsic factors, and I show that CLL cell stimulation with VEGF results in suppressed STAT3 and increased Sp1 binding to the promoter region of *PRKCB*. Taken together, the results I present in this thesis define a mechanism resulting in PKCβ gene overexpression in CLL cells, and are important to our understanding of cancer because this gene is found overexpressed in both the malignant and stromal support cells of other

neoplastic diseases. Furthermore, these results provide greater insight into the pathophysiology of CLL cells, and a strong foundation for further study of the mechanisms governing overexpression of other genes important for the phenotype of these malignant cells.

1.2 Regulatory mechanisms of gene expression

The genome is ultimately the key organizer for all cellular functions, and is largely responsible for regulating the distinctive phenotypic features of cells during their development and differentiation. Consequently, precise regulatory mechanisms, controlled by the genome, are in place at transcriptional, post-transcriptional, translational, and post-translational levels (Figure 1.1).

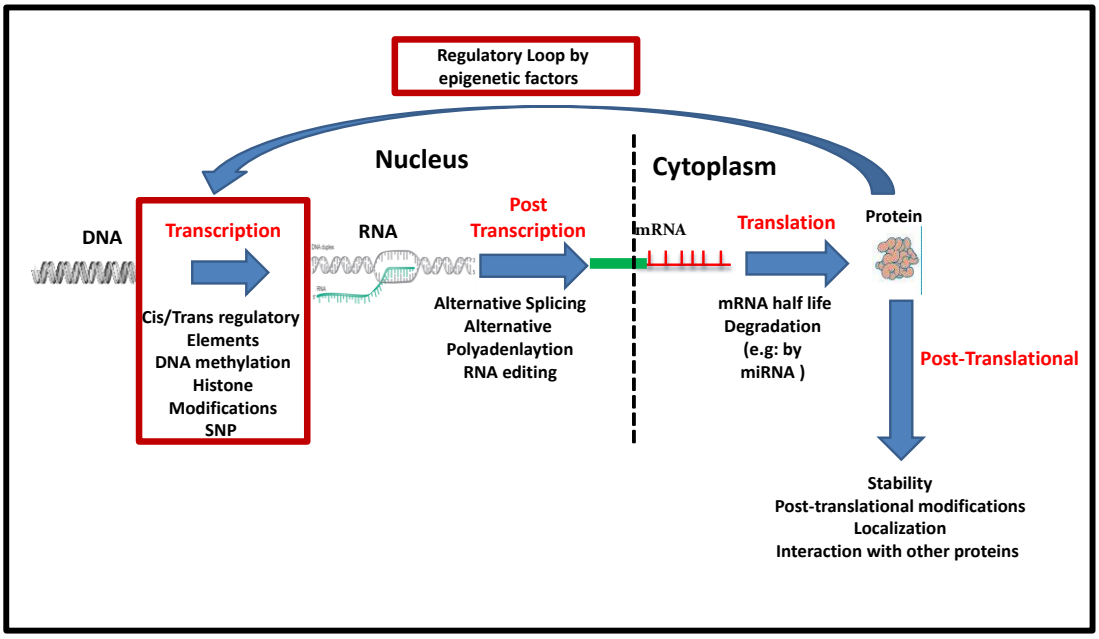


Figure 1.1: Regulatory mechanisms of gene expression. This diagram, adapted from Banks *et al.* 2000[9], illustrates the different regulatory levels of gene expression in cells (labelled in red font). The text enclosed by boxes indicates the processes that are majorly affected by epigenetic factors.

At the transcriptional level, numerous different mechanisms control both the rate of transcription as well as the time at which certain genes are transcribed. Initially, binding of transcription factors, known as trans-regulators, to specific consensus sequences within the regulatory region of DNA preceding a gene, known as the cis-

regulator, is required to recruit the machinery required for transcription to begin [10]. The specificity of this interaction is crucial to this regulatory step, allowing expression of particular genes at particular times. For example, transcription factors involved in promoting cell mitogenesis induce expression of genes involved in this process only when the cell begins to replicate itself. Furthermore, the transcriptional control regions of many eukaryotic genes are known to harbour multiple enhancer or repressor elements, which, when bound by appropriate transcription factors, are able to regulate the rate of transcription to give fine control over high expression of particular genes as the need arises [11-13]. Finally, further complication and diversity is added because some genes have alternative promoters which regulate expression either in response to different cellular stimuli, or lead to initiation from different transcriptional start sites located either in the core promoter region or in the intronic regions of the gene [14, 15].

Gene expression is also regulated through a number of epigenetic mechanisms. Epigenetics is an umbrella term referring to heritable reversible changes that do not affect the DNA sequence of genes but nevertheless influence cellular phenotype in normal and diseased tissues diseases [16]. These mechanisms include DNA methylation [17], histone modification leading to changes in chromatin landscape [18], alternative splicing of RNA transcripts [19], and non-coding RNA species such as miRNA [20] (Figure 1.2).

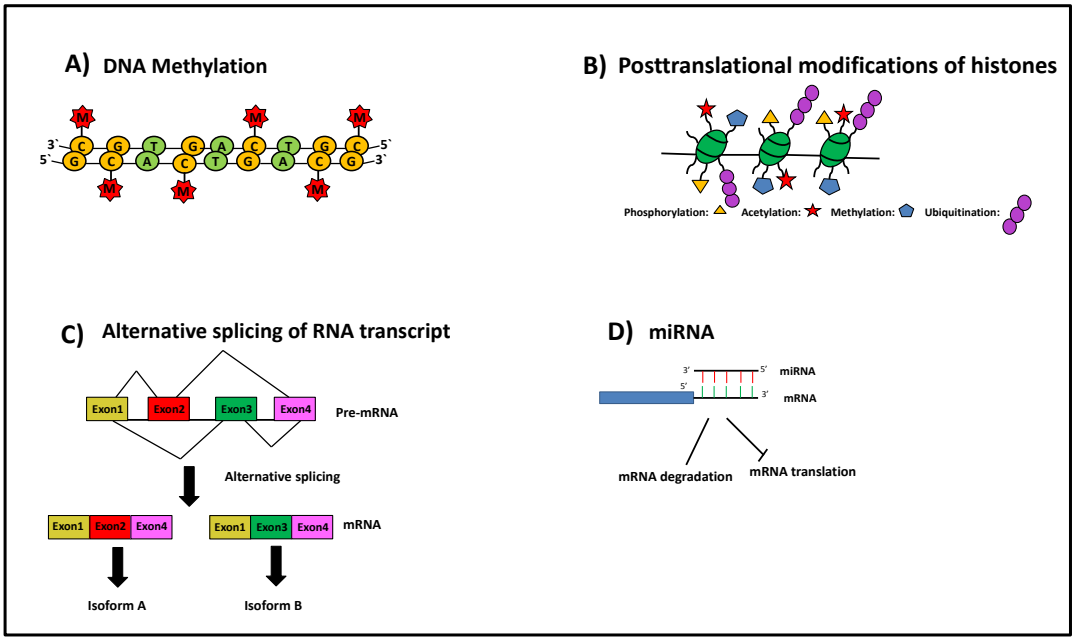


Figure 1.2: Epigenetic regulatory mechanisms for gene expression. This figure represents the epigenetic mechanisms that regulate gene expression at different levels of this process. **A)** DNA methylation. Methylation of the cytosine residues of the CpG islands within the promoter or gene body region causes silencing or activation of transcription. **B)** Posttranslational modifications of histone. Modifying the histone protein tails by acetylation (red star), methylation (blue pentacle), phosphorylation (yellow triangle), or mono ubiquitin (Purple small round circle) leads to alterations in chromatin structure. **C)** Alternative splicing of the initial RNA transcripts (pre-mRNA) results in various protein isoforms from the same gene but which may have different functions. **D)** miRNAs seed sequences recognize full complementary sequences within the target mRNA, which results in binding of both RNA species, and consequently gene silencing occurring by either blocking mRNA translation or by leading to mRNA degradation by miRNA-induced silencing complex (miRISC).

DNA methylation is a process whereby methyl groups are added to carbon #5 within cytosine residues by methyltransferases. DNA methylation can act to silence or facilitate gene expression depending on the location of methylation. For instance, methylation of CpG islands within a promoter region results in gene silencing because transcription factor binding may be prevented, or because chromatin silencing complexes are recruited [21]. Alternatively, DNA methylation within the body of a gene can result in transcriptional activation because transcription factor binding to repressor elements is blocked [22, 23]. DNA methylation is a process that occurs naturally during cell development and helps to

maintain cell commitment to its overall phenotypic fate [24]. However, this process often becomes deregulated in cancer cells allowing phenotypic change away from that of its normal counterpart, and, in many types of cancer, DNA methylation is used as a biomarker to identify patients with progressive disease [25-27]. That DNA methylation changes in cancerous cells compared to their normal counterpart is because expression of the genes coding for and/or activity of the methyltransferases and demethylases that control this process becomes deregulated in these cells [28-30]. Importantly, DNA methylation cooperates with other epigenetic mechanisms, such as those involved in histone modification, to regulate chromatin structure [31, 32].

Histone proteins play an important role in regulating gene expression because they control the basic structure of DNA, condensing this linear molecule into chromatin so that it is able to fit inside the nucleus of a cell. Chromatin is comprised of basic structural units known as nucleosomes, each consisting of approximately 147bp of DNA wrapped around a core octamer histone complex of 2 each of H4, H3, H2A and H2B histone proteins. Nucleosome units are 10nm in diameter, and are arranged on the DNA fiber in a structure known as “beads on a string” where each nucleosome is separated from the other with a 10-80bp DNA linker. Nucleosomes are further arranged around histone core H1 linkers that allow the condensation of helical chromatin into 30nm diameter structures. Further folding of these structures allows the formation of larger aggregates that are 300nm in diameter and result in overall chromosome structure [33]. The degree to which chromatin structure is condensed regulates gene expression. Thus, within the nucleus of a cell transcriptionally active regions of the genome (euchromatin) segregates from transcriptionally silent (heterochromatin) regions [34].

DNA wraps around the core octamer histone complex through interactions between the chargeable tails of histone proteins and phospho-groups in the DNA backbone. The chargeable tails of histone proteins can be post-translationally modified by acetylation, phosphorylation, sumoylation, ubiquitination, and methylation. These changes alter the charge associated with the histone tail, which, in turn, changes the interaction of the core octamer histone complex with DNA

forcing either winding or unwinding of the beads on a string structure. Ultimately, overall chromatin structure is changed to either allow or disallow accessibility of transcription factors to gene regulatory regions. Histone post-translational modification is carried out by chromatin-remodelling enzymes, which are divided into three types depending on their function. Writers are enzymes that function to add chemical groups or small proteins such as mono ubiquitin and SUMO (all known as histone marks) to the amino acid residues in histone tails. Writers include enzymes such as methyl- /acetyltransfearases, enzymes involved in ubiquitination/sumoylation and kinases. Erasers are enzymes that function to remove histone marks, and include proteins such as demethylases, deacetylases, deubiquitinases and phosphatases. Readers are the third type of chromatin-remodelling enzyme, and are not really enzymes at all. Readers function to recruit further proteins whose role is to energize the process of change in chromatin structure [18, 35, 36]. All post-translational modifications of histones are considered as simple modifications because they usually involve the addition of a single chemical group or small proteins to such as mono ubiquitin and SUMO to an amino acid residue within the histone tail. The exception to this is methylation, where lysine and arginine residues within histone tails can be multiply methylated [37]. Chromatin-remodelling enzymes act as either activators or repressors of transcription, depending on the modifications they make to histone proteins, and on both the location and extent of these modifications relative to the gene regions [37]. Furthermore, the gene activator/repressor function of chromatin-remodelling enzymes operates within a cell-specific context [38]. For instance, within particular cells methylation of H3K9/H3K27 within core octamer histone complexes associated with DNA of gene promoter regions are markers for heterochromatin, whilst in other cells methylation of H3K4 in the same regions is a marker of euchromatin [37].

For the purpose of this thesis I will focus on the role of histone acetylation in influencing chromatin structure. Acetylation is an active reversible type of lysine residue modification, and affects histone and non-histone proteins [39]. This chemical modification is catalysed by histone acetyltransferases (HATs) which

transfer acetyl groups from acetyl-CoA to lysine residues of target proteins. In contrast, histone deacetylases (HDACs) reverse this modification and catalyse the removal of acetyl groups from the lysines of target proteins (Figure 1.3). The acetylation of histones is not exclusive for any particular lysine residue within the chargeable tail, and there is no consistent pattern in terms of position or extent. The role of acetylation is to neutralize the positive charge associated with the histone tails which consequently weakens the binding between histones and DNA to ultimately result in relaxing the chromatin structure so that it is open and permissive of transcription [40]. Histone acetylation often occurs with other modifications, either within the same histone tail, or between different histone tails within the core octamer histone complex [41]. For example, acetylation of H3K9/14 is linked to methylation of H3K4, both of which are marks of euchromatin [42].

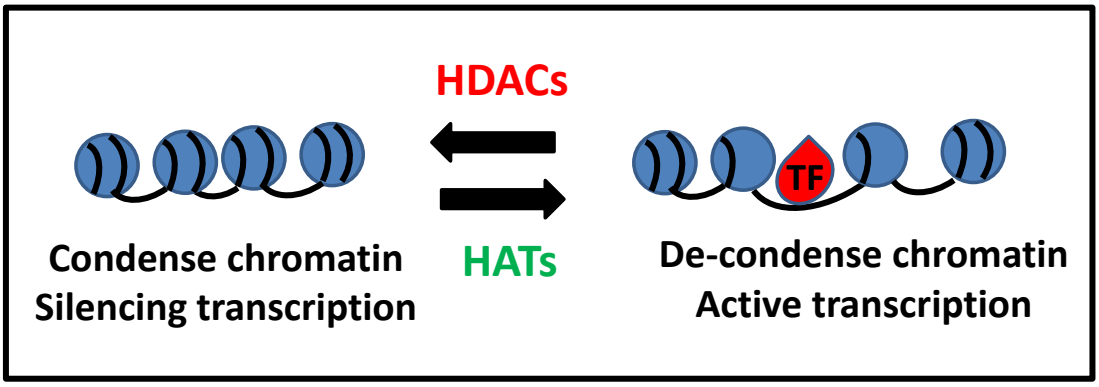


Figure 1.3: Histone acetylation/deacetylation which alters chromatin structure and regulates transcription is catalysed by HATs and HDACs enzymes.

The next epigenetic phenomenon affects the transcribed product of gene expression. Genes are transcribed directly from DNA into pre-mRNA which includes gene intron and exon sequences. This pre-mRNA is then subjected to splicing whereby intron sequences are removed from exon sequences in order to produce the final mRNA from which the amino acid sequences of proteins are translated. However, in certain genes the way in which exons are spliced together can lead to the expression of different isoforms of the same protein from a single gene [19, 43].

For example, the gene coding for PKCβ, *PRKCB*, can be differentially spliced to yield two isoforms; PKCβI and PKCβII [44]. These protein isoforms can have differential functions within the cell; PKCβI is described to phosphorylate Bruton's tyrosine kinase in monocytic cell lines [45], whereas PKCβII is reported to phosphorylate $p66^{shc}$ [46].

The process of translation of the final mRNA can be regulated by a class of non-coding RNAs known as miRNA. miRNAs are short 21-25bp sequences which have a complementary sequence to the target mRNA species. Binding of miRNA to their cognate mRNA species leads to either blocked translation, or facilitated destruction through an ancient process which activates miRNA-induced silencing complex (miRSIC) complexes [20, 47]. Certain miRNA species are able to feedback to chromatin remodelling enzymes or to transcription factors themselves, thereby indirectly affecting transcription of genes in a specific cellular context [48].

The final step of regulation occurs at the post-translational level. This mainly leads to modifications in the structure of the end protein product, which can lead to a change in protein stability, its functional activity, and its ability to interact with other proteins [49].

1.3 Chronic lymphocytic leukaemia (CLL)

Chronic lymphocytic leukaemia (CLL) is a type of mature B neoplasm, where the malignant cells in this disease accumulate in the bone marrow as well as in the peripheral blood and lymphatic tissues [50]. This disease is the most common form of leukaemia affecting adults in America and Europe, and in the UK nearly 34% of all haematological cancers are CLL [51, 52]. The incidence of CLL is highest in people over the age of 65 years old [53], and favours males over females at a ratio of 2:1 [54]. The aetiology of CLL remains unclear; however studies have identified risk factors contributing to the development of this disease. Genetic familial studies have suggested the risk of developing CLL is higher in persons who are the first degree relatives of CLL patients [55], or who have a family history of leukaemia and lymphoma [56, 57]. In addition, environmental work factors such as benzene or

pesticide use respectively in industry workers [58] and farmers have also been identified as potential risk factors [59]. Finally, the incidence of CLL is higher in people who have experienced multiple episodes of respiratory infection [60].

Phenotypically the malignant cells of CLL are distinguished from those of other types of leukaemia by their expression of the cell surface markers CD19, CD5, CD23, coupled with weak expression of CD20 and the surface immunoglobulins (Ig) IgM and IgD. Early studies using gene expression profiling have shown that CLL is distinct from other types of B cell malignancies, and that the phenotype of CLL cells is more closely related to memory than to naïve B cells [61, 62] . More recently, a study by Seifert *et al.* has shown the gene expression profile of CLL cells resembles that of normal $CD5^+$ B cells in humans [63]. This latter description provides a solution to a long standing problem regarding the origin of CLL cells [64], and also provides some insight into the relationship between CLL and a clinical condition known as monoclonal B lymphocytosis (MBL). Like CLL, the cells associated with MBL develop from $CD5^+$ B cells and have similar genetic aberrations such as 13q14 deletion and trisomy 12, but do not have 17p or 11q deletions. The frequency of these cells increases with age, and are higher in elderly men than women, and in persons who have a familial history of CLL. In many ways CD5/CD23 positive MBL cells are similar to the malignant cells associated with the clinically indolent subtype of CLL. This similarity is important because it suggests that MBL cells may be a good model to study the transformational events that lead to CLL [65]. Here it is important to note that only a small proportion of patients with MBL develop CLL [66, 67].

B lymphocytes are part of the adaptive immune system in the body where they mediate humoral immunity against various foreigner invaders. The development of B cells begins during embryogenesis from a multipotent lineage of progenitor cells which divide to yield undifferentiated cells with self-renewal capacity known as haematopoietic stem cells (HSCs) [68]. Initially, the precursor cells of HSCs derive from the mesoderm of the embryo, and during embryogenesis differentiate to HSCs and migrate to the aorta-gonad-mesonephros region (AMG). The blood forming cells then migrate to the placenta, and finally to the fetal liver, thymus, omentum,

and bone marrow during the late stages of fetal development. After birth, haematopoiesis, including B cell development, takes place in the bone marrow [69].

The first stage of B cell development starts when HSC differentiate into common lymphoid progenitor cells (CLP) and requires the expression of the transcription factors Ikaros, fms-like tyrosine kinase 3 (FLT3) and low levels of PU.1. CLP cells then have the ability to further differentiate into natural killer cells, dendritic cells, B, or T lymphocytes depending on the expression of certain transcription factors and stimulatory factors (e.g. cytokines) received from surrounding stromal cells. CLP cells develop into precursor B cells (pro-B) when they activate the expression of genes (TdT and RAG) involved in immunoglobulin production. TdT and RAG are under the control of the transcription factors E2A, early B-cell Factor (EBF), and paired box protein (PAX5), which are important in carrying the development of CLP to pro-B cell and then the latter Pre-B cell stages. The pro-B cell stage is divided into two sub-stages, early pro-B stage is where the first Ig loci of the heavy chain, known as D_H (diversity) and J_H (joining) are joined, followed by the late pro-B cell stage where D_H-J_H segments are joined to V_H (variable) gene segments. Late pro-B cells express accessory proteins for BCR proteins Igα and Igβ on their surface supported with chaperon protein, Calnexin. The pre-B cell stage proceeds causing VDJ segments rearrangement and results in the expression of heavy chains joined with surrogate light chains composed of VpreB and Vλ5 comprises the pre-BCR receptor. At the end of the pre-B cell stage joining of the V_L and J_L segments of the λ and κ light chain loci takes place which allows differentiation to immature B cells. During the immature B cell stage the rearrangement of VL segments occurs and results in the formation of a mature BCR receptor that able to act as immunological checkpoint (Negative selection) [70, 71]. The Ig gene rearrangements that take place to produce mature BCR on B cells result in a diverse repertoire of B cells with the ability to recognize different antigens. Clones which show strong self-reactivity are negatively selected by apoptosis, and those that show weak self-reactivity become anergic whereby the BCR no longer responds to antigen stimulation. The B cell clones which do not show any form of self-reactivity are able to migrate from the bone marrow environment as naïve B cells [71].

The next stage of B cell development continues mainly in secondary lymphoid organs such as the spleen and lymph nodes. Here B cells are exposed to foreign antigen. Those B cells that experience strong antigen stimulation subsequently pass through a gene rearrangement phase whereby Ig heavy chain sequences can be switched from IgM/IgD to IgG, IgE, or IgA, and through a stage involving somatic hypermutation of the antigen binding regions [71]. This latter process allows increased antigen binding affinity and antibody avidity and is known as affinity maturation. The end point of these processes is the development of antibody-producing plasma cells, or retention of immunological memory by differentiation of mature B cells into memory B cells. Two other types of B cells that can develop are marginal zone (MZ) and peritoneal (B-1) B cells. The former develop in the spleen while the latter develop in the peritoneum, and result from B cells which experience weak antigen stimulation [72]. Here it is important to note that B-1 cells also develop during embryogenesis and are present at birth. Both MZ and B-1 cells only differentiate into plasma cells, and are largely responsible for innate antibody production [70, 73, 74] (Figure 1.4).

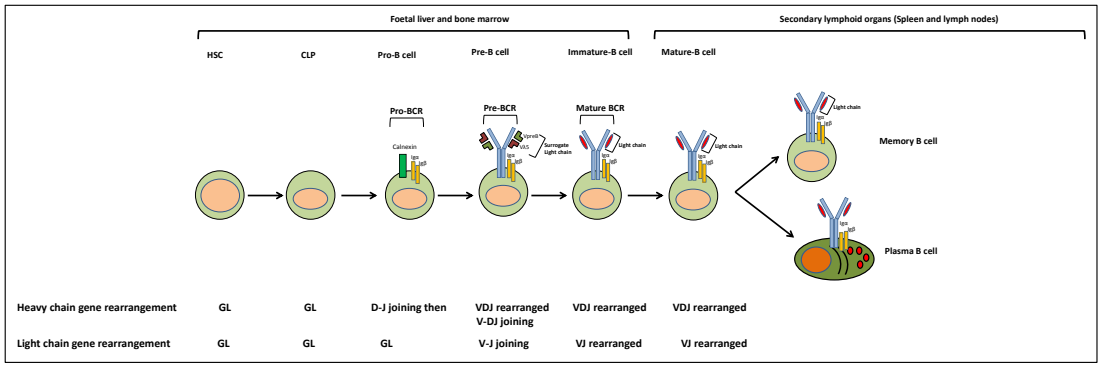


Figure 1.4: Stages of normal B cell development. (adapted from pieper *et al*., [74] and cambier *et al*., 2007, [75]).

The multiple rearrangements of Ig genes that take place during B cell development make these cells susceptible to genetic abnormalities such as chromosomal translocations resulting in expression of protein chimaeras which may lead to malignant cell transformation [76]. Additionally, misdirected somatic mutation can lead to critical deletions as well as epigenetic alterations [77]. In the first instance, a

number of chromosomal translocations have been identified and linked to specific types of leukaemia (when these translocations occur in the bone marrow) or lymphoma (when these translocations occur in the spleen or lymph node) [78]. Particular point mutations are also known to lead to the development of lymphoma. For example, in diffuse large B cell lymphoma a mutation within CD79a can lead to constitutive BCR signalling and malignant transformation [79]. However, with respect to CLL cells it seems that neither of these genetic abnormalities is responsible for neoplastic transformation. The frequency of chromosomal translocation in patients with CLL is very rare [80]. Certain subsets of CLL patients have malignant cells with chromosomal deletions occurring at 17p, 11q, or 13q as well as other genetic aberrations (Table 1.1), and mutations within genes coding for p53, NOTCH1, splice factor3B1 (SF3B1), myeloid differentiation primary response protein 88 (MYD88) and nuclear export protein (XPO1) [81-83]. However, these chromosomal deletions/gene mutations are only linked to disease prognosis/progression and are not causative of the disease. For example, deletions at 17p and 11q are typically associated with poor disease prognosis in CLL (Table 1.1) [81, 84, 85]. Therefore, the key genetic events that lead to CLL development remain undefined.

Table 1.1: Percentage (incidence), overall survival time and genes linked to genetic aberrations in chronic lymphocytic leukaemia patients [81, 84].

Genetic aberration	Percentage (%)	Overall survival time (years)	Linked genes
13q deletion only	55	11	miRNA 15a/16
11q deletion	18	7	ATM
17p deletion	7	2.5	TP53
12q Trisomy	16	10	unknown
Normal karyotype	18	10	None

Clinically CLL patients can be divided into two subsets. Patients who show no symptoms or disease progression are described as having indolent disease and have a median survival time of 20 years. In contrast, the second subset of patients show rapid progression as well as suppressed bone marrow and immune function, and have a median survival time of 7 years despite receiving intensive therapy [86, 87]. The first clinical systems devised to differentiate between these subsets of CLL disease were stablished by Rai [88] and Binet [89] (Table 1.2). These systems staged the extent of disease and related this to patient survival, but have limited application because when CLL is detected at an early stage it is impossible to know whether the patient has indolent or progressive disease [90, 91].

Table 1.2: CLL classification and survival time according to Rai and Binet staging system[92].

System	Staging and risk	Definition	Survival time (Years)
Rai System			
	0 (Low risk)	Lymphocytosis only	>10.0
	I (Intermediate risk)	Lymphocytosis and lymphadenopathy	9.0
	II (Intermediate risk)	Lymphocytosis in blood and bone marrow with splenomegaly/or hepatomegaly (with/without lymphadenopathy)	7.0
	III (high risk)	Lymphocytosis and anaemia	5.0
	IV(high risk)	Lymphocytosis and thrombocytopenia	5.0
Binet system			
	A (Low)	Enlargement lymphoid area of <3 (cervical, axillary, inguinal, spleen, liver)	>10.0
	B (Intermediate)	Enlargement lymphoid area of >3	7.0
	C (High)	Anaemia or thrombocytopenia	5.0

There have been many attempts at distinguishing indolent from progressive disease in CLL, and to this effect prognostic biomarkers such expression of zeta-chain associated protein kinase 70 (ZAP-70), CD38 and CD23, serum β2-microglobulin and thymidine kinase levels have all been related to disease prognosis [93]. However, none of these biomarkers give information regarding the factors which drive development/progression of the disease. With respect to this latter point, in 1999

somatic mutation of the immunoglobulin heavy chain variable region gene (*IgV_H*) was discovered to relate to CLL disease prognosis by two different groups [86]. Thus, patients where IgV_H sequences remained similar to germ line sequences were termed as unmutated CLL (UM-CLL), whilst those who had IgV_H sequences with significant deviation from germ line sequences were termed mutated or M-CLL [80]. Importantly, patients with UM-CLL disease had poorer disease prognosis than patients with M-CLL disease. The reason why this discovery is important to our understanding of CLL is because it led to insights regarding the role of the BCR in driving disease progression in CLL [94]. Thus, *in-vivo* BCR engagement is thought to drive proliferation and survival of the malignant clone in CLL, and this paradigm has recently been exploited for the therapy of CLL. Two new drugs, ibrutinib and idelalisib, which respectively target Bruton's tyrosine kinase (Btk) and phosphatidylinositol 3-kinase δ (PI3Kδ) are now approved for clinical application in the treatment of CLL [95-97].

Figure 1.5 illustrates the BCR pathway and shows a key role for PKCβ in potentiating signalling within this pathway. Importantly, targeted deletion of the gene coding for PKCβ does not affect adaptive immunity, rather the B cells responsible for innate antibody production (MZ and B-1 cells) are missing [98]. This suggests that PKCβ plays a role in fine modulation of the BCR signalling pathway, a notion that is supported by work in this Department showing that transgenic overexpression of PKCβII leads to favoured development of MZ and B-1 cells in mice [99]. With respect to CLL, work from this Department has shown that CLL cells overexpress PKCβII and that it affects BCR signalling strength when it is active [1]. Furthermore, work by others using a mouse model of CLL where transgenic expression of the gene product of TCL1 results in the development of a CLL-like disease shows that this does not happen in mice where PKCβ expression is knocked out [3]. Taken together, these observations justify a need to investigate the mechanisms regulating PKCβII overexpression in CLL.

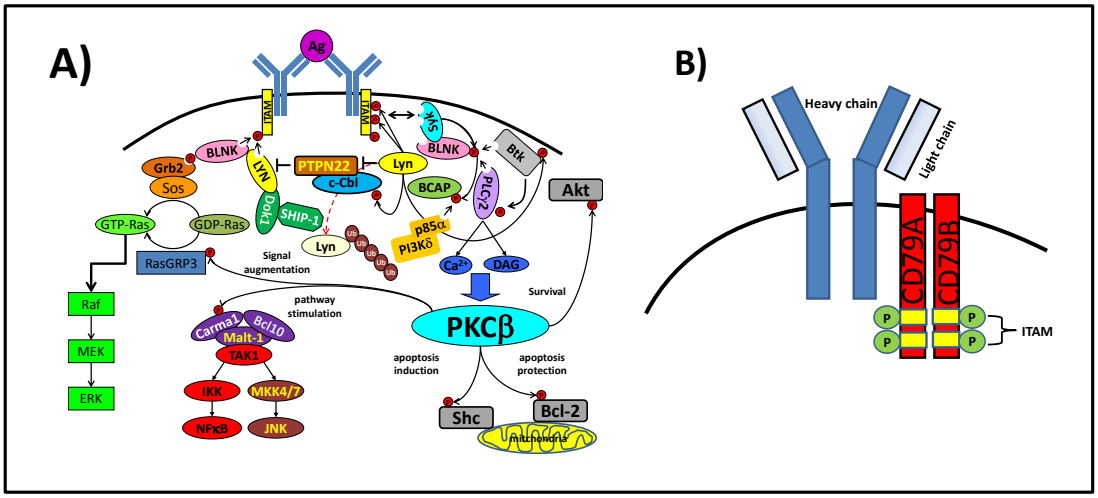


Figure 1.5: BCR signalling pathway and structure of BCR receptor. A) Antigen engagement triggers phosphorylation of the tyrosine residues within the ITAM (immunomodulatory tyrosine activation motifs) of CD79a/b by Lyn. This attracts Syk which phosphorylates BLNK and BCAP, the latter of which attracts and activates PI3Kδ. Generation of phosphatidylinositol trisphosphate at the plasma membrane attracts Btk and phospholipase Cγ2 (PLCγ2), which when bound to BLNK, are subsequently phosphorylated by Syk. This activates Btk, which, in turn, phosphorylates and activates PLCγ2 leading to the generation of diacylglycerol (DAG) and intracellular Ca2+ release (through the production of inositol trisphosphate). DAG and Ca2+ activate PKCβ which then acts to augment MAPK activation, stimulate NFκB and JNK pathway activation, and downregulate BCR signalling by phosphorylating Btk (not shown) and inducing its relocation away from the plasma membrane. (adapted from Slupsky, J.R., 2014[100]). **B)** Structure of BCR receptor. (adapted from Gold, 2002 [101]).

1.4 Protein kinase Cβ

1.4.1 Protein kinase C (PKC) family overview

Protein kinase C (PKC)s are a family of serine/threonine kinases [102, 103] that were initially described in extracts of rodent brain [104-106]. Further work after this initial discovery showed that this family of protein kinases is comprised of 11 isoforms which are distributed into three subgroups depending on their structure and activation requirement factors: Classical / conventional PKC isozymes include PKCα, PKCγ, PKCβI and PKCβII and are activated by the presence of diacylglycerol (DAG), Ca^{2+} and phosphatidylserine (PS) [107]. Novel PKC isozymes include PKCδ, PKCε, PKCη, and PKCθ which are activated by the presence of DAG [108]. Atypical

PKC isozymes include PKCι, PKCζ, and PKCλ and activation of these kinases is both DAG and Ca^{2+} independent [109, 110].

The PKC family of proteins is structurally conserved within eukaryotic organisms, and belong to a larger super family of protein kinases known as ABC (for protein kinase **A**, protein kinase **B** and protein kinase **C**) kinases [111]. These proteins all require phosphorylation of critical serine/threonine residues within a conserved domain known as the activation loop [111]. PKCs are composed of two main domains, the catalytic domain located in the C-terminus of the protein, and the regulatory domain located in the N-terminus. Each of these domains is further divided into functional regions. Within the catalytic domain there is the C3 region which is responsible for ATP binding, and the C4 region which contains the activation loop and is responsible for substrate binding [107, 111]. The regulatory domain contains the regions responsible for controlling activity; classical isoforms contain regions for binding DAG and Ca^{2+} whereas novel isoforms contain only a region for binding DAG [107, 111]. In contrast, atypical isoforms have neither of these DAG/Ca^{2+} binding domains. Importantly, the regulatory domain of all PKC isozymes contains a pseudosubstrate which acts to bind the catalytic domain and restrict its access to phosphorylation targets. Thus, upon binding of regulatory factors, such as DAG and Ca^{2+}, a structural change occurs in the regulatory domain that disengages the pseudosubstrate from the catalytic domain to allow it to hinge away and gain ability to recognise and phosphorylate target sequences [106, 110, 111] (Figure 1.6).

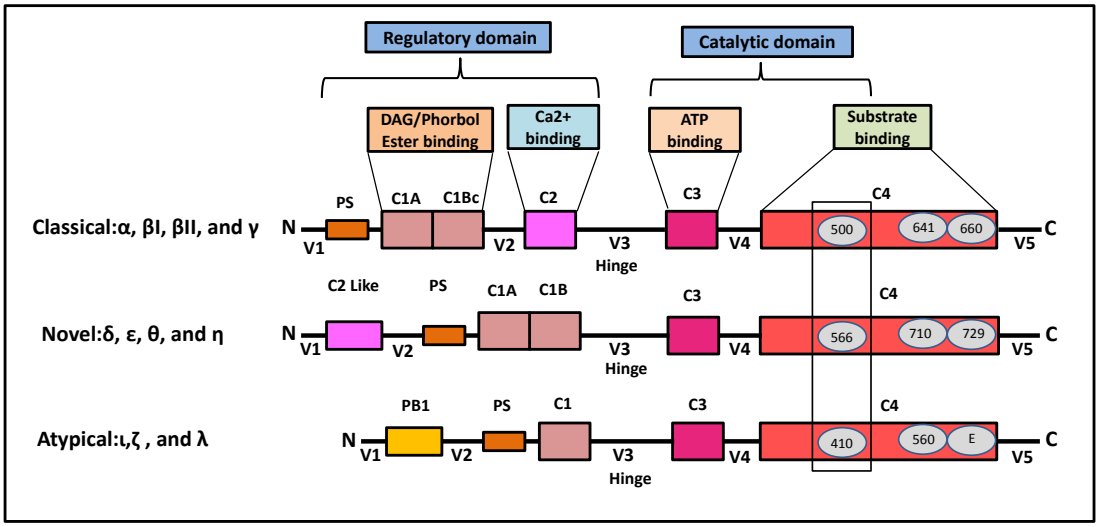


Figure 1.6: Schematic representation of PKC isoforms structure and their activation cofactors (adapted from Newton 2010[111] and Cosentino-Gomes *et al.,* 2012[112]). The catalytic domain contains conserved regions for ATP (C3) and substrate binding (C4). In particular, the C4 region contains the activation loop which requires phosphorylation of conserved T/S residues for full kinase activity (enclosed box). The regulatory domain of PKC contains regions required for DAG/membrane association (C1A and C1B) and Ca^{2+} binding (C2 region in classical isoforms). The variable regions (V1-V5) connect the conserved regions of PKC. The hinge V3 region allows protein folding so that the pseudosubstrate region (PS) interacts with the catalytic domain to block kinase activity. When PKCs come into contact with their agonists, structural changes within the regulatory domain force the PS away from the catalytic domain so that the active kinase is free to phosphorylate its target substrates.

PKC isoforms phosphorylate a wide variety of protein substrates within cells, and are involved in an array of cellular processes such as proliferation [113, 114] and differentiation [115], cell-death [114], regulation of gene expression [116], mediating intracellular signalling related to the immune response [117, 118], cell-cell adhesion and migration [110, 119, 120]. Remarkably, only one PKC isozyme, PKCλ, is important for embryogenesis [121].Targeted disruption in expression of the other isozymes in mice show mild effects on phenotype. Disruption of PKCα leads to hypercontractility of cardiac tissue [122] whereas disruption of PKCβ affects the development of MZ and B-1 B cells [98]. Knockout of PKCδ expression results in the development of a lupus like syndrome because B cells are able to emerge from anergy [123], whereas knockout of PKCε alters macrophage

functioning due to defects in early stage response to lipopolysaccharide [124]. Finally, disruption of PKCζ leads to defects in the development of Peyer's patches and spleen because of the role it plays in the NFκB pathway [125]. The mild phenotypes observed associated with mice having disruption of PKC isozymes suggest functional redundancy. However, PKC isoforms show specificity with respect to certain functions in particular contexts: PKCδ is key in cellular senescence [126],PKCζ is involved in artery constriction of the lung [127], and PKCβ plays a key role in mediating Toll-like receptor (TLRs) and BCR signalling in B cells [128, 129]. These functions depend on their cellular localization [111], their expression levels in certain cell contexts [111], the nature of the cellular stimuli and the nature of the substrate downstream of the activated isoform [111, 130].

1.4.2 Expression and functional role of PKCβ in CLL cells and other types of cancer cells

CLL cells express a unique profile of PKC isoforms, which likely plays a role in the pathogenesis of this disease. For example, CLL cells show low expression of PKCα, and this may lead to increased tumourgenicity because of a recent demonstration showing that disruption of PKCα function facilitates the development of a CLL-like disease within an *in-vitro* model of B cell differentiation [131]. Furthermore, inhibition of PKCδ with rottlerin results in CLL cell death possibly by affecting the expression of pro-survival proteins such as Mcl-1 and XIAP [132, 133]. Finally, work from this Department regarding the expression profile of PKCs in CLL cells showed that PKCβII is overexpressed in these cells, and that this overexpression could distinguish CLL cells from other types of haematological tumour cell [1].

PKCβII is a key mediator of BCR signalling in B cells as illustrated in figure 1.5. Overexpression of PKCβII in CLL cells is reported to enhance the survival of these cells by acting to phosphorylate S^{473} within protein kinase B (Akt) leading to increased expression of Mcl-1 [134] and other anti-apoptotic proteins [135]. In addition, PKCβII can translocate to the mitochondrial membrane where it mediates phosphorylation of Bcl-2 at S^{70} to allow sequestration of Bim [136]. This same report also showed that PKCβII catalysed increased degradation of Bim_{EL} by

targeting it for proteasomal degradation [136] (Figure 1.7). That PKCβII is highly expressed in CLL cells has therapeutic consequences because depletion of PKCβII expression in these cells by treatment with bryostatin enhances the cytotoxic effect of an antibody targeted against CD22 (BL22) [137]. This suggests that high levels of PKCβII protect CLL cells against CD22-directed immunological therapies.

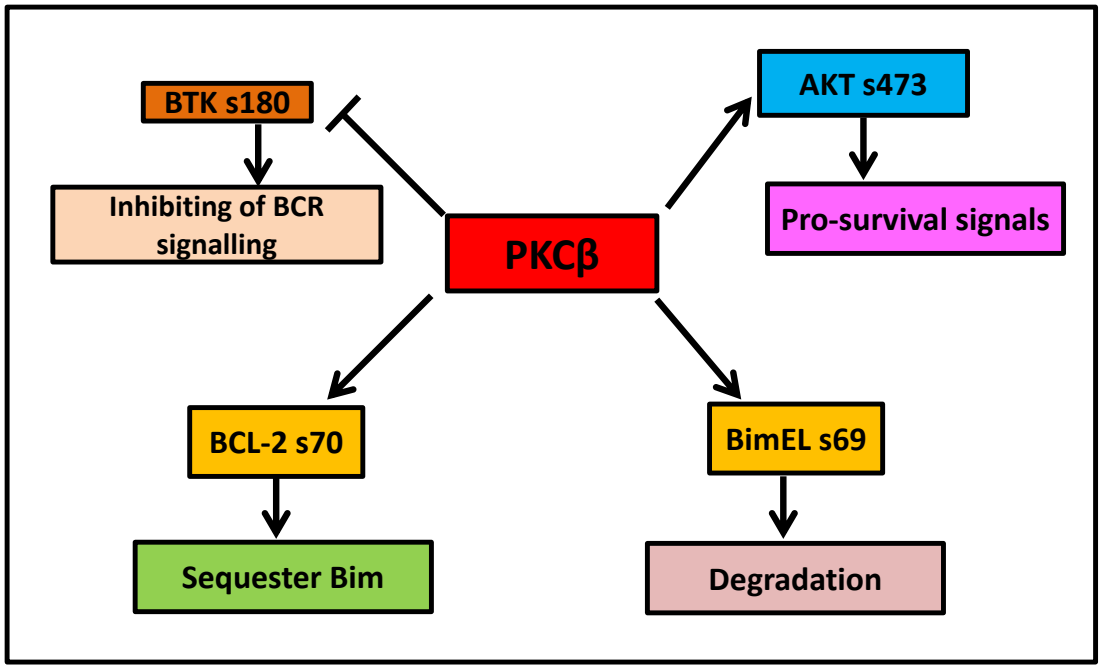


Figure 1.7: Role of PKCβ in CLL cells. PKCβII is overexpressed in CLL which inhibits BCR signalling by phosphorylation of Btk on S^{180} that prevents its activation. Additionally, PKCβII augments anti-apoptotic signalling by inducing S^{69} phosphorylation of Bim_{EL} and S^{70} phosphorylation of Bcl-2. Phosphorylation of these two residues results in Bim_{EL} proteasomal degradation, and sequestration of Bim_{EL} by Bcl-2 respectively. PKCβII can also activate Akt which is an important mediator of CLL cell survival.

There is a clear role for PKCβ within the BCR signalling pathway. As a facilitator of BCR signalling, PKCβ phosphorylates CARD-containing MAGUK protein 1 (CARMA1) within the CBM complex [(CARMA1/ B- cell lymphoma 10 (Bcl10)/ Mucosa Associated Lymphoid Tissue Lymphoma Translocation 1 (MALT1)], and this leads to stimulation of Transforming growth factor-beta-activated kinase 1 (TAK1) [138] [139] and eventually to NFκB pathway activation. Such NFκB pathway activation

then induces expression of anti-apoptotic survival proteins such as Bcl-2 and Bcl-xL. This is important with respect to CLL cells because overexpression of PKCβ and constitutive activation of the NFκB pathway are features of these malignant cells [140]. Active PKCβ as a facilitator of BCR signalling can also phosphorylate RasGRP3, which is a Ras-guanine exchange factor that can generate GTP-Ras in response to the presence of DAG [141]. Phosphorylation of RasGRP3 enhances its enzymatic function allowing GTP-Ras to augment the function of son-of-sevenless (SOS) [142] and lead to the activation of Extracellular Signal-Regulated kinases (ERK) [141].

In contrast to its role as a facilitator of BCR signalling, PKCβ can also act within a feedback mechanism to negatively regulate this process. This mechanism involves phosphorylation of Btk at serine 180, and this interferes with the PH (pleckstrin homology) domain within Btk so that it can no longer bind Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), and results in migration of this kinase away from the plasma membrane. Away from the plasma membrane Btk is unable to phosphorylate and activate its substrate PLCγ2 and this effectively shuts the BCR signalling pathway off [128]. How this negative feedback mechanism contributes to disease pathogenesis in CLL is unclear, but could possibly explain some aspects of BCR anergy that are observed with CLL cells. A previous report from this Department showed that VEGF stimulates PKCβ activity, and that this dampens the BCR signalling response in CLL cells [143].

Whether overexpressed PKCβII acts as facilitator, inhibitor or both of BCR signalling in CLL cells has not been clearly demonstrated. Nevertheless, there is evidence to indicate that overexpression of PKCβ plays a role in CLL development (Figure 1.8). Within the Tcl1 mouse model of CLL, expression of PKCβ is important in the pathogenesis of the malignant CLL-like cells [3]. In this model, the CLL-like disease does not develop in mice where the gene coding for PKCβ has been disrupted [3]. Although subsequent work has indicated that PKCβ expression within stromal cells aids their ability to support malignant cell establishment and growth [144], there is also a possibility that malignant cells do not form because the subgroup of B cells

from which the CLL-like disease would develop are missing due to the effect of disrupted PKCβ expression on MZ and B-1 cell development [3, 98].

A second model which indicates a role for PKCβ in CLL cell pathogenesis is suggested in a recent study by Nakagawa *et al.,* who show that PKCβII expression is upregulated in the malignant cells which form in their model of this disease. Importantly, the same study showed that targeting PKCβ activity with a specific inhibitor (enzastaurin) resulted in induced apoptosis and growth inhibition of the malignant cells [131]. Finally, work from this Department showed that B cell-specific overexpression of PKCβII leads to favoured development of MZ and B-1 cells while follicular B cell development is reduced [99]. Although disease was not observed to develop within this model, it may still be relevant because virtually all mouse models of CLL develop from a lymphocytosis of B-1 cells [145-147].

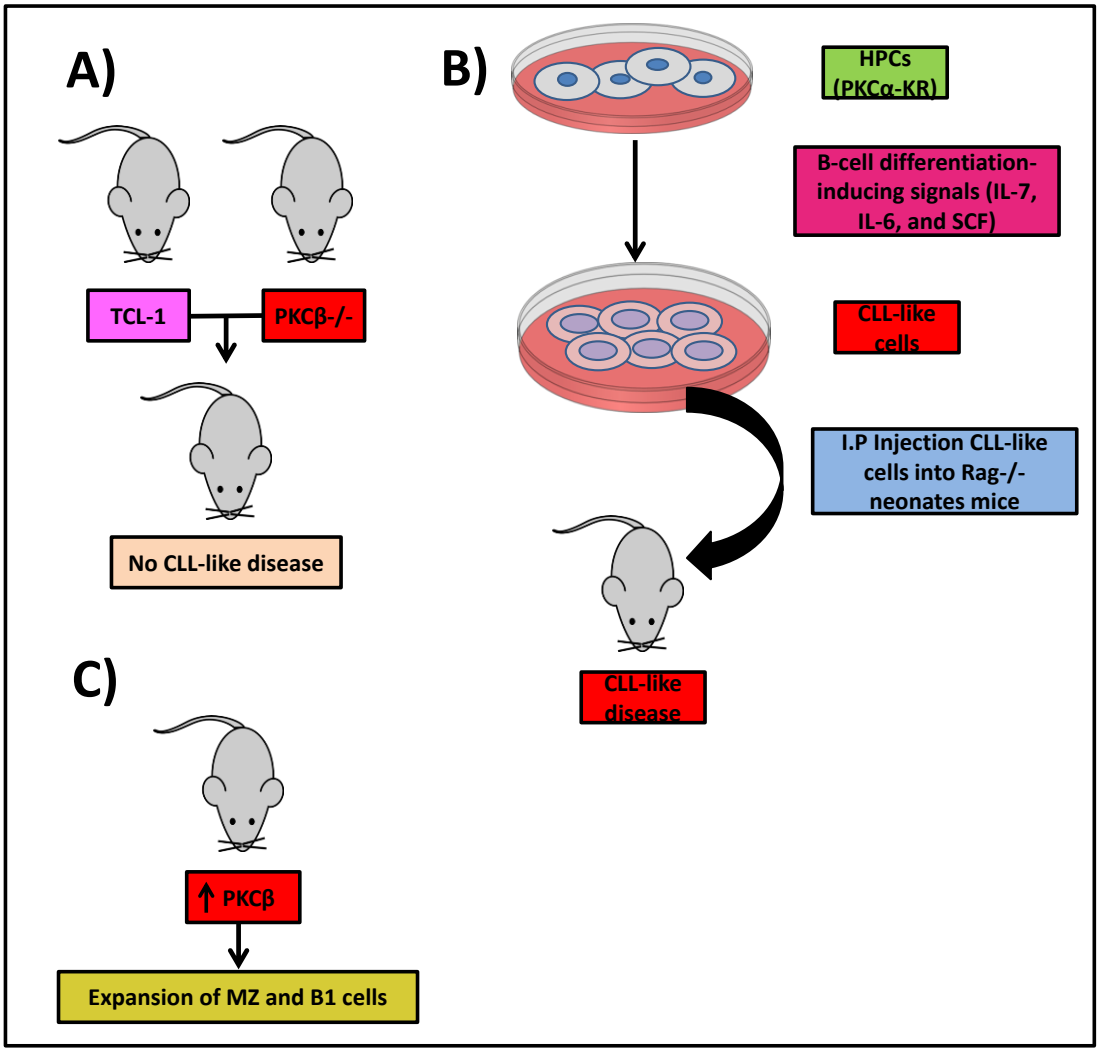


Figure 1.8: ***In vivo*** **studies support the key role of PKCβ in CLL.** Mouse models illustrate the importance of PKCβ in CLL cell development. **A)** CLL-like disease does not develop in TCL-1 transgenic mice that have been crossed with PKCβ knockout mice. (Adapted from Holler *et al.* 2009[3]). **B)** In this model fetal liver haematopoietic cells (HPCs) are transduced to express kinase-dead PKCα-KR and then induced to differentiate into B cells. The resultant cells become neoplastic and are phenotypically similar to human CLL cells (Adapted from Nakagawa *et al.* 2006[148]). Moreover, these cells behave similarly to CLL cells when injected into Rag-/- neonatal mice. **C)** B cell-specific transgenic overexpression of PKCβII shows favoured expansion of marginal zone (MZ) and B-1 cells (Adapted from Azar *et al.* 2011 [99]).

Although PKCβII overexpression is a phenotypic characteristic of CLL cells, overexpression of this PKCβ isoform is also observed in other B-lymphocyte malignancies such as diffuse large B cell [149] and mantle cell lymphomas [150]. Further, overexpression of PKCβII is observed in epithelial tumours such as

carcinoma of the colon where development of this disease is intrinsically linked with expression of this PKC isozyme [151], and in tumours of the breast where overexpression of PKCβII has been linked to disease progression [152, 153]. Therefore, understanding the regulatory mechanisms governing expression of PKCβII may give insight into the pathogenesis and the progression of CLL and other cancers.

1.4.3 Overview of the mechanisms regulating PKCβ gene expression within different cellular contexts

PKCβII and PKCβI are encoded by the *PRKCB* gene, which is located on the long arm of chromosome 16 at position 11.2. Thus, PKCβII and PKCβI are alternative splice variants of this gene which differ from each other within, respectively, the last 52 and 50 C-terminal amino acids [154, 155]. How this difference in the C-terminus of PKCβI and PKCβII is functionally manifested is unclear because systematic investigation into the individual roles of these isoforms has not been performed. Nevertheless, one early study has indicated that this region influences the Ca^{2+} requirement of each isoform for full activation; PKCβII requires Ca^{2+} in greater amounts for full activation than does PKCβI [156]. Moreover, the difference in C termini may influence the ability of PKCβI and PKCβII to associate with different target proteins. For example, PKCβI has been shown to associate with and affect the function of Btk in mast cells [157]. In CLL and other malignant cells it is predominantly PKCβII that is overexpressed [1, 149-152]. It is unclear why the splicing mechanism favours PKCβII over PKCβI in CLL and other malignant cells, but could be due to activated Akt2 as has been suggested in cardiac myocytes [158]. At a post-translational level it is thought that the protein phosphatases PHLPP1 and 2 (PH domain and Leucine rich repeat Protein Phosphatase) and protein phosphatase 2A (PP2A) play roles in mediating PKCβII deactivation and degradation following long term activation [159]. This may be important for PKCβII overexpression in CLL cells because these cells have been shown to have reduced expression of PHLPP1, and because PKCβII protein has a long half-life [160].

The purpose of this thesis is to discover the mechanism(s) governing PKCβII expression at the transcriptional level. In this respect, the promoter of *PRKCB* has been characterized by two groups who described the basal region responsible for driving gene expression [4, 161]. One of these studies identified a number of binding sites for potential transcription factors such as Sp1, Oct-1, and AP1 [4]. Later, Park *et al*., found that Microphthalmia-associated Transcription Factor (MITF) appeared to be involved in regulating the expression of PKCβ in melanocytes [7], while another study implicated a role for RUNX1 in U937 cells [6]. Recently, Farren *et al.* characterized a role for STAT3 as a suppressor of PKCβ gene expression in dendritic cells [8]. However, none of these studies described the major driver of *PRKCB* promoter activity within their cell system.

With respect to the role of epigenetic modifications in regulating the expression of *PRKCB*, a single recent study by Hagiwara *et al*. suggested that methylation of the *PRKCB* promoter played a role in transcription of this gene within a HeLa cell model [5]. However, the relevance of this observation to CLL is unclear because gene methylation studies of the malignant cells in this disease compared to normal B cells do not identify *PRKCB* as being differentially methylated [162].

Finally, work from this Department has shown that transcription of the *PRKCB* gene can be induced in CLL cells through a mechanism involving VEGF-induced stimulation of PKCβII activity [143]. This mechanism has also been reported in other cell systems [163, 164], but how this is mediated at the transcriptional level has not been described. Furthermore, the PKC agonist Bryostatin induces both differentiation of CLL cells [165] and reduction of PKCβII expression [137]. While it is clear that bryostatin is likely to catalyse PKCβII protein degradation through a mechanism involving PHLPP and PP2A [159], the mechanism inhibiting *PRKCB* transcription is still unclear.

Thus, the main aim of this thesis is to investigate the regulatory mechanisms governing overexpression of PKCβII in CLL cells at the transcriptional level. At the beginning of this thesis, the only known transcription factors involved were MITF [7]and RUNX1 [6]. Therefore, I began this thesis with the aim to describe the role of

these Transcription factors, as well as that of Sp1 whose potential role was identified in the early descriptions of the *PRKCB* basal promoter [4]. Subsequently, STAT3 was also identified [8], and I have included this within this this thesis as well.

1.5 Transcription factors regulating *PRKCB* expression.

In this section I give an overview of the transcription factors known to regulate *PRKCB* transcription (RUNX1, MITF and STAT3) and of Sp1/Sp3 whose role in driving expression of this gene was implicated the early studies characterising the basal promoter region.

1.5.1 Specificity protein 1 and 3 (Sp1)/(Sp3)

Specificity proteins 1 and 3 (Sp1)/(Sp3) are pleiotropic nuclear transcription factors which show a similar strong affinity to the GC-rich consensus binding sequence within regulatory regions of target genes [166-169]. These transcription factors are members of a family of proteins known as the Specificity protein (Sp)/Kruppel-like factor (KLF) family [170, 171], and are characterised by a structure that includes three zinc fingers which define their DNA binding domain [170] (Figure 1.9). There are 9 Sp type members of this family [172, 173], each one located on a different chromosome and contiguous with a homeobox (HOX) gene [174]. Sp1 and Sp3 stand out from this family of proteins by targeting highly similar consensus sequences (Table, 1.3) [172, 175].

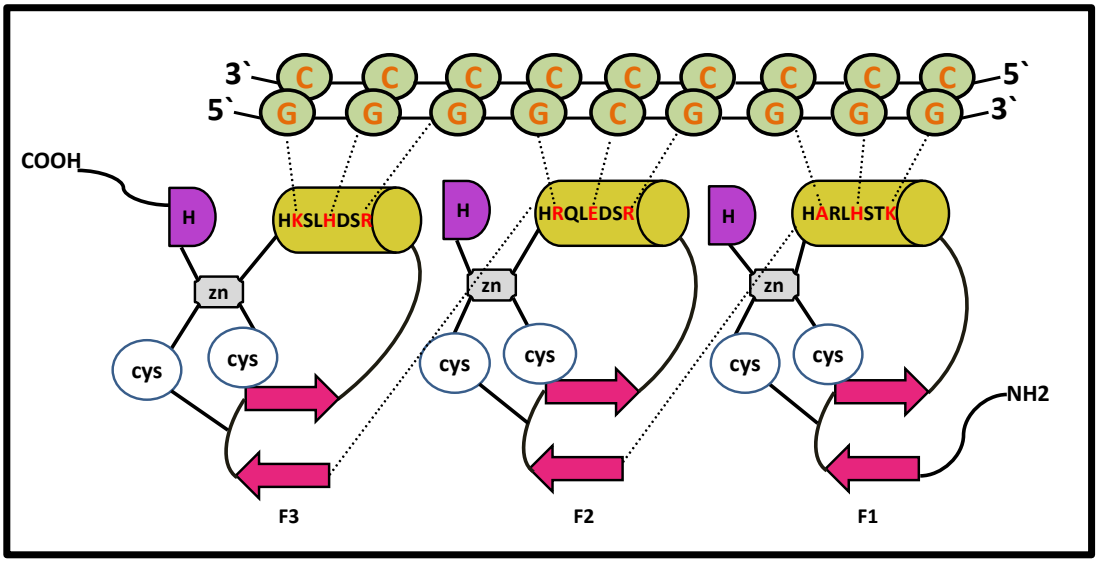


Figure 1.9: Recognition and binding of Sp1 to DNA. Three specific amino acids (highlighted in red) within in each of the 3 zinc finger regions (F1 – F3) are responsible for nucleotide recognition within DNA containing the cognate binding site of Sp1/Sp3 (adapted from Bouwman and Philipsen, 2002[172]).

Table 1.3: Sp protein expression and their DNA binding consensus sequences

Sp protein members and their expression	DNA binding consensus sequence
Sp1 and Sp3 (pleiotropic) / Sp4 (dominant in brain)[175]	GC-box/ GT-box
Sp2 (Various cell lines, tissue unidentified)[169, 170]	GT-box

Sp1/Sp3 are involved in regulating constitutive as well as inducible expression of different genes related to various cellular functions such as growth, differentiation, apoptosis, metabolism, and chromatin remodelling enzymes [176]. The binding of Sp1/Sp3 to the promoter of these genes is modulated by different factors (Figure 1.10). Firstly, the level of Sp1/Sp3 expression can be driven by the same transcription factors that modulate cell cycle phases and its related proteins such as E2F1 [177], and regulated post-transcriptionally by miRNAs such as miRNA29b that directly target Sp1 mRNA for degradation [30, 178, 179]. Secondly, direct

interactions with other proteins such as the transcription factors E2F1 [180, 181], or with chromatin-remodelling enzymes like p300, histone methyltransferases [182] and histone deacetylases [183] can alter the function of Sp1. Thirdly, post-translational modifications of Sp1 and Sp3 stabilize their expression, nuclear localization, and the interaction with other proteins [49, 176]. These modifications can influence the interaction of Sp1 with chromatin-remodelling enzymes and change the chromatin landscape structure from heterochromatin to euchromatin and affect the accessibility for other transcription factors [184, 185]. Finally, methylation of CpG sequences which contain the consensus binding sites for Sp1/Sp3 limits their accessibility to gene promoter regions to suppress transcription [186, 187] .

Sp1/Sp3 play a crucial role in different types of cancers through their role in modulating the expression of genes responsible for tumour cell behaviour. For example, it is known that Sp1 regulates expression of the genes controlling vascularization in prostate, gastric and pancreatic cancers [188-190]. Sp1 is also known to regulate the genes responsible for apoptosis resistance in T cell lymphomas [191, 192], and for controlling growth and progression of disease in breast, gastric and lung cancer [193-196]. Sp1 is found overexpressed in multiple myeloma and acute myeloid leukaemia cells where it enhances their proliferation [179, 197]. This role of Sp1/Sp3 can be mediated either through direct interaction of these transcription factors with promoter regions of genes, or indirectly through Sp1-mediated expression of genes coding for enzymes involved in DNA methylation/demethylation [30, 198, 199].

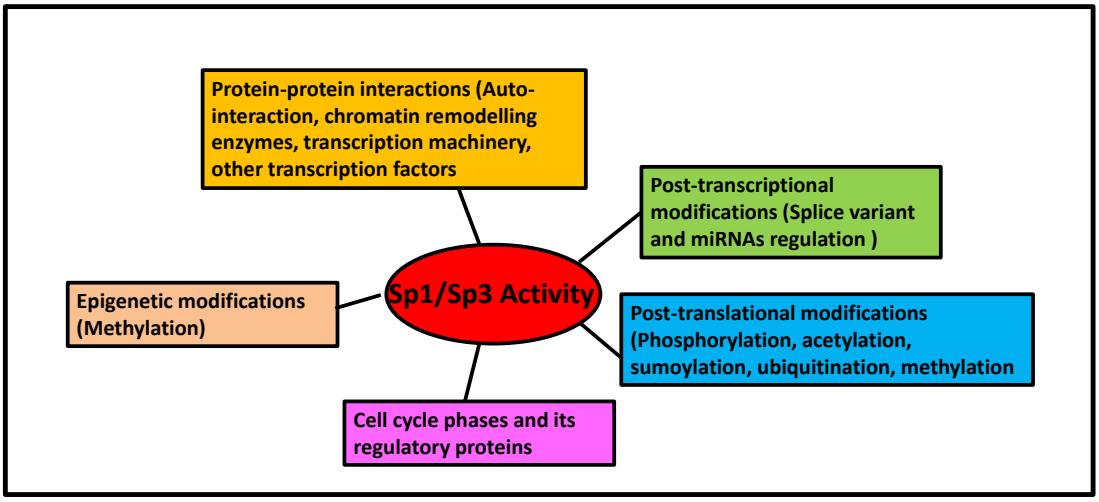


Figure 1.10: Factors influencing Sp1/ Sp3 transcriptional activity.

1.5.2 Runt-related transcription factor 1 (RUNX1)

Runt-related transcription factor 1 (RUNX1, also known as acute myeloid leukaemia 1 (AML 1)) and its isoforms RUNX2 and RUNX3 are encoded from a single gene but are regulated by different promoters and result from alternative splicing [200-202]. RUNX1, RUNX2 and RUNX3 are similar with respect to the runt-homology domain that is important for recognition and binding to their consensus sequence within DNA, but differ in their N-termini [200]. Interestingly, despite being coded by the same gene, knockout studies of the different promoters show clear function of these isoforms: RUNX1 is crucial for haematopoiesis [203], RUNX2 plays an important role in osteogenesis [204, 205], while RUNX3 is important in neurogenesis [206], development of cytotoxic T-lymphocytes [207] as well as development and differentiation of epithelial cells within the lung [208] and gastrointestinal system [209]. RUNX proteins have been found to play a role in carcinogenesis. It is well known that acute myeloid (AML) and B-acute lymphoblastic (B-ALL) leukaemias have high incidences of chromosomal translocations which create protein chimaeras of RUNX1 with ETO or TEL (ETV6) (respectively t(21;8) or t(21;12) translocations) [200]. Furthermore, the RUNX1 gene in these leukaemias is often mutated in such a way that its ability to bind DNA is affected [210, 211]. Together, these two types of genetic aberration within the RUNX1 gene contribute to the development and transformation of the malignant

cells in AML [212] and B-ALL [213]. With respect to CLL, there are no reports implicating RUNX1, RUNX2 or RUNX3 in the pathogenesis of this disease.

1.5.3 Microphthalmia-associated Transcription Factor (MITF)

Microphthalmia-associated Transcription Factors (MITF) are basic helix-loop-helix (bHLH) leucine zipper transcription factors that are encoded from the *MITF* gene located at chromosome 3 [214]. These transcription factors belong to the MYC superfamily, and recognize either enhancer box (E-box) or M-box sequences within the regulatory regions of their target genes [215]. The helix-loop-helix and leucine zipper motifs within MITFs are important for complex (dimer) formation either with itself, or with other bHLH-related proteins such as TFEB, TFEC, and TFE3 [214]. MITF has nine isoforms (A, B, C, D, E, H, J, and M) generated by alternate splicing from a single gene whose expression is driven by four different promoters [216]. MITF-M is the most common isoform, and its expression is linked to melanocyte growth and differentiation [215]. Alteration in the expression or activity of this transcription factor due to genetic aberration or other factors leads to the formation of melanoma [217], and, as such, expression of MITF in melanocytes is used as a biomarker of this disease [218]. It is unclear whether expression or mutation of MITF has a role to play in cancer cells other than melanoma. However, I included this transcription factor in this study because of its described role in regulating *PRKCB* transcription [7].

1.5.4 Signal transducer and activation of transcription 3 (STAT3)

Signal transducer and activation of transcription 3 (STAT3) is a cytoplasmic protein responsible for cell proliferation, differentiation and survival [219]. It belongs to a family of latent transcription factors that includes STAT1, STAT2, STAT4, STAT5a/b, and STAT6 [220]. The function of these transcription factors is stimulated by growth factors / cytokines which induce the phosphorylation of STATs either by Janus kinases (JAKs) [221] or Src-family kinases (SFKs) [222]. With respect to STAT3, it is activated by phosphorylation of tyrosine 705 / serine 727 which results in the self-dimerization necessary for nuclear transport where it is able to bind its target genes[223]. The process of STAT3 activation is receptor-dependent, and is

stimulated by cytokines such as IL-6 and IL-21 [224], by growth factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) [225, 226], and by steroids [227]. Interestingly, STAT3 can also complex with NFκB and become imported into the nucleus as an unphosphorylated protein to bind its consensus sequences within the regulatory regions of target genes [228].

Constitutive activation of STAT3 is a phenotypic feature of solid tumours and leukaemias. Within this setting, constitutively active STAT3 can act as an oncogene where it controls transcription of genes involved in angiogenesis (VEGF) [229, 230], resistance to apoptosis (Bcl-family proteins and survivin) [231], invasion and metastasis (Matrix metalloproteinase 1/ 2 (MMP1 /2) [232, 233] and proliferation (Cyclin D1 [234], c-Myc [235], and pim-1/2 [236]). STAT3 activation in haematological cancers shows a correlation with disease stage. For example, in diffuse large B cell lymphoma (DLBCL) STAT3 activation is highly associated with the progressive activated B cell subtype [237]. Regarding CLL, the malignant cells in this disease are distinct in having a constitutive phosphorylation of STAT3 at serine 727 [238]. The mechanism of this phosphorylation is unclear, but its presence seems important for the survival of the malignant clone in this disease because the extent of pS^{727}-STAT3 in CLL cases correlates with cell resistance to apoptosis [239]. With respect to *PRKCB*, it was recently shown that active STAT3 acts as a repressor for transcription of this gene [8].

1.6 Hypothesis and aims

PKCβII is overexpressed in CLL cells as well as other haematological and solid tumor cells. This overexpression plays a key role in the pathogenesis of the malignant clone in CLL. Understanding the mechanisms regulating the expression of the gene coding for PKCβII may provide insight into the pathogenesis of CLL cells, because it may provide a foundation for understanding how other genes important for the malignant phenotype of these cells may become deregulated.

In the early reports by Obeid *et al.,* [4] and Niino *et al*. [161] the basal promoter region of *PRKCB* was shown to be dependent on a section of DNA containing binding sites for Sp1. Thus, we hypothesised that Sp1 is the major driver for the overexpression of PKCβII in CLL cells. To test this hypothesis I aimed to investigate the role of the following elements in regulating the expression of *PRKCB* expression in CLL cells: Sp1, other transcription factors (Sp3, RUNX1, MITF, and STAT3), epigenetic modifications (DNA methylation and histone modifications). Finally, I tested these findings within a functional setting to understand how VEGF and Bryostatin stimulated/inhibited *PRKCB* expression at the transcriptional level.

❖ Chapter Two: Materials and Methods

This chapter principally is divided into five main sections of the methodology that generally have been performed during this study. The specific or optimized materials and methods for these techniques will be mentioned in each relevant section of the results in each chapter.

2.1 Tissue culturing techniques

2.1.1 Isolation, Purification, and storage of CLL and normal B cells

2.1.1.1 Isolation and storage of CLL and normal B cells

Peripheral blood samples were collected from CLL patients with informed consent, and with the approval of the Liverpool Research Ethics Committee (#06/Q1505/82). Blood samples were processed according to a standard operating procedure (SOP) developed by the Liverpool Leukaemia Biobank, University of Liverpool. Briefly, the whole blood sample was layered over Lymphoprep™ (Alis-Shield PoC AS, Oslo, Norway) with a volumetric relationship of 1 part Lymphoprep™: 2 parts blood, and then centrifuged at 800x*g* for 30 mins. Lymphocytes were collected from the plasma/Lymphoprep™ interface and then diluted to 50 ml with complete medium [RPMI-1640 + 10% Fetal calf serum (FCS), 100 U/ml penicillin, 100µg/ml streptomycin, 0.29 mg/ml L-glutamine (all from Sigma Aldrich, Gillingham, UK)] followed by centrifugation at 550x*g* for 5 mins. The cell pellet was resuspended with pre-chilled RPMI-1640 medium containing 10% FCS, and this was followed by a drop wise addition of an equal volume of pre-chilled RPMI-1640 medium containing 20% Dimethylsulfoxide (DMSO) (Sigma Aldrich, Gillingham, UK). The volumes of FCS and DMSO containing media to be used in the previous step was determined using the total white cell count of the blood sample, and calculating an end concentration of cells of $2x10^7$/ml. Finally, 1 ml of this suspension of cells was distributed to labelled cryovials held within a polystyrene holder, and then transferred to a -80^oC freezer for short-term storage, followed by long term storage at -150^oC.

2.1.1.2 Isolation, purification and storage of normal B lymphocytes from buffy coats

Buffy coats were obtained from the British Transfusion Service (Liverpool, UK). Lymphocytes were first isolated over Lymphoprep™ using the above procedure (2.1.1). B cells were purified from mixed lymphocyte populations by negative selection using magnetic cell separation according to the manufacturer's protocol (MACS®, Miltenyi Biotec Ltd, Surrey, UK). Purified B cells were stored frozen in RPMI-1640 + 10% FCS, 10% DMSO as above.

2.1.2 Thawing, purification, and culturing of cells

2.1.2.1 Thawing cryopreserved CLL cells

The cryopreserved CLL samples vials were taken from the Liverpool Leukaemia Biobank freezer and were quick thawed at 37^{o}C. The thawed cells were then transferred into pre-chilled universal tubes placed in ice. Pre-chilled thawing medium (RPMI-1640 medium containing 1% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.29 mg/ml L- glutamine) was added in a drop wise fashion over the course of 30 mins with constant swirling of the tubes. Afterwards the suspended cells were centrifuged at 550x*g* for 5 mins at 4^{o}C. The cell pellet was further washed twice with 5 ml of complete RPMI-1640 medium. Finally, the cell pellets were resuspended with 5 ml of complete RPMI-1640 medium ready for cell counting. Cell concentration was adjusted to 1x10^{7}/ml with further addition of complete RPMI-1640 medium.

Following the thawing procedure cells were left in an incubator with an atmosphere containing 5% CO_2 at 37 ^{o}C for 1h for recovery. Only samples which showed more than 75% viable cells were included in further experimentation.

Clinical information regarding the samples that have been used in this study is mentioned in appendix A.

2.1.2.2 Analysis of CLL cell purity

CLL cells were analysed for purity using flow cytometry. Thawed CLL cells ($1x10^6$) were incubated with PE-conjugated CD3 and non-specific control IgG antibodies (Becton Dickinson, Mountain View, CA) in order to identify T cells. When CLL cell samples contained greater than 5% T cells within the entire population they were excluded from study.

2.1.2.3 Culturing of CLL cells

Tissue culture plates were coated with non-toxic poly 2-hydroxyethyl methacrylate (poly-HEMA) (Sigma Aldrich, Gillingham, UK). 200 µl of 12 mg/ml poly-HEMA in 95% ethanol was added to each well of a 24 well tissue culture plate (Falcon, BD Biosciences, UK) that was then left to dry at 37 ^oC overnight. This was done to avoid the potential effects of cell adhesion to the plate surface during culture of CLL cells [240].

CLL cells ($1x10^7$/ml) were seeded into prepared culture plates and kept incubated at 37^oC in an atmosphere of 5% CO_2 until used. For most experiments cells were cultured for at least an hour following the thawing procedure.

2.1.3 Thawing, culturing, passaging and cryopreservation of human B lymphocytes cell lines MEC1 and Daudi cells

2.1.3.1 Thawing and culturing of human B lymphocyte cell lines MEC1 and Daudi cells

Cell lines were used in this thesis for functional experiments. Therefore, controlling the factors such as thawing, culturing, passaging, and cryopreserving that may affect their quality are fundamental.

The MEC1 CLL cell line is derived from a 61 year old man with CLL that was undergoing prolymphocytoid transformation [241]. These cells were obtained from the Leibniz Institute (DSMZ) German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were used a low passage number.

The Daudi cell line is a B lymphoblastoid cell line derived from a sixteen year old boy with Burkitt's lymphoma [242]. This cell line was obtained from ATCC (LGC Standards, Middlesex, UK).

Cryopreserved vials of each cell line were rapidly thawed by immersion in a 37^{o}C water bath. Thawed cells were then transferred to pre-chilled universal tubes and treated in the same way as for CLL cells (section 2.1.2.1) with the exception that MEC1 cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Gillingham, UK) and Daudi cells were cultured in complete RPMI-1640 medium. Final cell culture was maintained in vented Nunc (T25) culture flasks (Fisher Scientific, UK) under standard conditions (37 ^{o}C and 5% CO_2). The cultured cell lines were tested regularly for mycoplasma infection by our department technician using an established protocol (see Appendix B).

2.1.3.2 Passaging of human B lymphocyte cell lines MEC1 and Daudi cells

Cell lines were passaged every three days to maintain optimal growth. MEC1 and Daudi cell lines were split using a 1:3 ratio of cell suspension to new appropriate media (see section 2.1.3.1). The culture of cell lines was maintained until they were used in experiments, or the total number of passages exceeded 10. Cell lines were not used beyond 10 passages in culture.

2.1.3.3 Cryopreservation of human B lymphocyte cell lines MEC1 and Daudi cells

To minimize genetic and phenotypic changes of the cell lines I used in this thesis, and to ensure access to cell lines free from microbial infection it was necessary to cryopreserve stock cell lines with minimal number of passages.

At the beginning of this thesis 1 vial each of MEC1 and Daudi cells was grown and tested to be free of mycoplasma. The cultured cells were then expanded to a density and volume that would achieve at least 20 vials each of $2x10^6$ cells. The required number of cells was centrifuged at 550x*g* for 5 mins, and the cell pellet resuspended into ice cold appropriate media containing 10% FCS. An equal volume

of ice cold appropriate media containing 20% DMSO was gradually added in a drop wise fashion over the course of 30 mins. 1 ml aliquots of cell suspension ($2x10^6$ cells) was placed into cryovials and frozen at -80^oC overnight in a polystyrene holder to ensure constant temperature change for each vial. The next day, or as soon as possible afterwards, the vials were deposited in a -150^oC freezer until they were needed.

2.1.4 Preparing growth-arrested MEC1 and Daudi cells

Some experiments required the use of growth-arrested cells in order to mimic CLL, which are arrested in G_0/G_1 phase of the cell cycle. Twenty four hours prior to starting such an experiment the cultured cell lines were placed into appropriate serum free medium (section 2.1.3.1) by first washing the cells with phosphate buffer saline (PBS) twice, and resuspending the cells in serum free medium. Viability and growth arrest was assessed by cell counting before and after this procedure.

2.2 *In vitro* Treatment

2.2.1 Cell Counting

CLL cells and B cell lines were counted for experimental purposes by using a haemocytometer after mixing the cells 1:10 with a 0.1% Trypan blue dye solution. The following equation was used to calculate the volume of cell suspension required to obtain desired cell density for each experiment as indicated in the relevant sections.

$$V = \frac{CD_{des}}{CD_{obt}} \times n$$

Equation 2.1: Where V = total volume of cell suspension, CD_{des} = desired cell density of suspension, CD_{obt} = obtained cell density of suspension and n = number of treatments.

2.2.2 Different treatments of CLL and B lymphocyte cell lines

2.2.2.1 Determination of the effective concentration of mithramycin to use in experiments with human B lymphocyte cell lines and CLL cells

CLL cells were used at a cell density of $1x10^7$/ml (section 2.1.2.3). The cells were incubated with different concentrations (50, 100, 200, 400 nM) of mithramycin (Sigma Aldrich, Gillingham, UK) dissolved in methanol. As an untreated control cells were treated with the equivalent volume of methanol needed to add the maximum concentration of mithramycin. Cells were harvested following for 24h culture.

For the MEC1 and Daudi cell lines a cell density of $2x10^6$/ml was used. Culture conditions were varied between serum free (section 2.1.4) and 10% FCS within DMEM and RPMI1640, respectively. Mithramycin or methanol was added in the same amounts as above, and the cells cultured for 24 h.

2.2.2.2 Determination of the optimal time point for cell harvesting following treatment of human B lymphocyte cell lines and CLL cells with 200 nM mithramycin

Cultures of CLL cells and growth-arrested MEC1 and Daudi cells were treated with 200 nM of mithramycin or with an equivalent volume of methanol. The cells were then harvested at the following time points; 3, 6, 9, 12, 15, 18, 21, and 24 h. The initial time (T0) sample is cells untreated in any way, and harvested at the beginning of the experiment.

2.2.2.3 Treatment of CLL cells with Vascular Endothelial Growth Factor (VEGF)

CLL cells ($1x10^7$/ml) were cultured under standard conditions (section 2.1.2.3) in the presence or absence of 100 ng/ml vascular endothelial factor (VEGF) (Calbiochem, UK) for 24 h. This concentration of VEGF and incubation was chosen based on a previous report [143].

2.2.2.4 Treatment of CLL cells with Bryostatin

CLL cells ($1x10^7$/ml) were cultured under standard conditions (section 2.1.2.3) in the presence of 50 nM Bryostatin (Sigma Aldrich, Gillingham, UK), a PKC agonist, or an equivalent volume of DMSO (as control). Following 24 h the cells were harvested. 50nM of Bryostatin was chosen as optimal based on previous reports using this compound with CLL cells [243] and [137].

2.2.2.5 Treatment CLL and MEC1 cells with c-Myc inhibitor (10058-F4)

CLL cells ($1x10^7$/ml) were seeded under standard conditions (section 2.1.2.3) either in the presence of 60 μM of the c-Myc-specific inhibitor 10058-F4 (Sigma Aldrich, Gillingham, UK)[244], or an equivalent volume of DMSO as control. For experiments involving MEC1 and Daudi cells, $2x10^6$ cells/ml were seeded in serum free or 10% FCS media (section 2.1.4) and then cultured in the presence or absence of 60 μM 10058-F4 for 24h.

2.2.2.6 Treatment of CLL and MEC1 cells with chromatin-remodelling enzyme inhibitors (Romidepsin and C646).

Romidepsin is histone deacetylase inhibitors (HDACi), while C646 is a histone acetyltransferase inhibitor (HATi) specific for p300 [245]. Histone deacetylases and transferases are responsible for removal or addition of acetyl groups to histones, allowing change in the chromatin structure and landscape [246].

CLL ($1x10^7$/ml) and MEC1 ($2x10^6$/ml) cells were seeded according to their optimal culture conditions. For romidepsin [(Celgene, San Francisco, CA United States of America (USA)], CLL and MEC1 cells were cultured in the presence of 10 nM of this compound, or an equivalent volume of DMSO. For C646 (Calbiochem, UK), a concentration of 10 μM was used. In these experiments, CLL and MEC1 cells were cultured to 24 h with these inhibitors prior to further analysis.

2.2.3 Cell Viability

Cell viability for both CLL and B lymphoid cell lines was checked before and after the different treatments using trypan-blue exclusion. Using this method live and dead

cells can be distinguished; dead cells with disrupted plasma membranes will take up the dark blue dye, while live cells will appear shiny bright. As with the procedure for cell counting, cell suspensions were diluted 1:10 with 0.1% trypan blue dye solution, and dead and live cells were counted using a haemocytometer. Both live and dead cells were counted, and results reported as percent live cells within the total cell count.

2.3 Molecular Biology Techniques

2.3.1 Nucleotide Studies

2.3.1.1 Total RNA Extraction

Cells harvested from experiments were collected in 1.5 ml nuclease-free eppendorf tubes (Anachem Ltd, UK). Total RNA was isolated from these cells using a ZR RNA Midiprep™ kit (Zymo Research, UK) following the manufacturer's instructions. In brief, the cell pellet was lysed with 400 µl RNA lysis buffer and then centrifuged at 12,000x*g* for 1 min. The supernatant was transferred to a Zymo-Spin IIIC column, and this was centrifuged for 30 s at 8000x*g*. The flow-through was mixed with 320 µl of 100% ethanol, and then transferred to a Zymo-Spin IIC column where it was centrifuged at 12000x*g* for 1 min. The column with attached RNA was washed once with 400 µl RNA prep buffer, once with 800 µl and then with 400 µl RNA wash buffer using 30 s pulses of centrifugation at 12000x*g*. An additional centrifugation step involved 2 mins at 12000x*g* in order to completely remove residual wash buffer. As a final step RNA was eluted from the column with 30 µl of elution buffer and centrifugation for 30 s at 10000x*g*. Isolated RNA was immediately stored at -80 ^{o}C following assessment for purity and quantity.

2.3.1.2 Assessment of RNA purity and quantity

The assessment of isolated RNA quality and quantity is essential for further molecular biology work. Quality of the isolated RNA from section 2.3.1.1 was assessed using a Nandrop 2000 spectrophotometer (Thermo scientific, UK). The isolated RNA was considered to be pure if the ratio light absorbance at 260 nm and

280 nm fell within the range 1.6-2; a value below 1.6 indicates extensive protein contamination. An additional ratio of light absorbance was taken, A_{260nm}/A_{230nm} and values between 2-2.2 were taken a pure; values outside this range indicate possible peptide or other contamination.

Quantitation of RNA within the isolation preps was also performed with the Nanodrop 2000. To do this, the absorbance value at 260nm was taken and divided by 0.025 (the extinction coefficient for single stranded RNA) to obtain the concentration in ng/µl.

2.3.1.3 Synthesis of complementary DNA (cDNA) from RNA

To synthesize cDNA from isolated RNA, 1µg of RNA was mixed with 1 µl of Oligo (dT) primer [500 ng/µl (Eurofins MWG Operon, Ebensburg, Germany)], and this was topped up with the addition of nuclease-free distilled water to a final volume of 14 µl. This was incubated for 5 min at 37^{o}C, and afterwards chilled on ice. Meanwhile, the reverse transcription master mix was prepared; per reaction 5 µl 5xRT Buffer, 1 µl (10,000U) Moloney murine leukaemia virus reverse transcriptase, 1 µl (10 mM) dNTP mix, 1 µl (2,500 U) RNase plus Rnase inhibitor (all from Promega, Southampton, UK) and 3 µl nuclease free water were mixed. To start the cDNA synthesis 11 µl of master mix was added to the 14 µl of RNA/oligo dT mix. Synthesis was complete following incubation of the mixture at 42^{o}C for 1 h. Synthesized cDNA was kept in -20^{o}C until further used.

2.3.1.4 Polymerase Chain Reaction (PCR)

The basic RT-PCR reaction contained 4 µL of Hot Fire pol EvaGreen qPCR master mix (newmarketScientific, Kennet, UK), 1 µl DNA (cDNA (prepared in section 2.3.1.3) or chromatin (prepared in section 2.3.6), 1 µl each (5 pmol/µl) of the forward and reverse primers (Eurofins MWG Operon, Ebensburg, Germany) needed to amplify the gene of interest, and 13 µl nuclease-free water to make a final reaction of 20 µl. Amplification was performed using a DNA Engine® RPTC-200 Peltier thermal cycler (MJ Research, Watertown, Massachusetts, USA), and the following thermal profile: An initial step of heating to 95^{o}C for 1 min. This was

followed by 40 cycles of denaturation at 95^{o}C for 30 s, annealing for 30 s at a temperature specific for the primers being used (this information is tabulated for each primer pair in Appendix C), and extension at 72^{o}C for 30 s. A final step consisted of extension at 68^{o}C for 5 min. With each PCR reaction a control experiment was included where nuclease-free water was added in place of DNA. Each PCR reaction was verified to amplify a single product of the correct size by agarose gel electrophoresis.

2.3.1.5 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative, or real time, polymerase chain reaction is a powerful molecular technique that allows either absolute or relative quantification of specific sequences of DNA. Generally, quantification of amplified sequences is achieved by measuring, in real time, the fluorescence signal that is released from a dye, such as SYBR green dye, as it binds to the minor groove of newly synthesised DNA double strands during the elongation phase of the PCR reaction. Relative quantification is performed using a reference gene, whereas absolute quantification is determined from a standard curve. This technique has been used in various applications such as gene expression analysis, gene amplification studies and chromatin immunoprecipitation (ChIP).

The basic PCR reaction in this study is basically the same as is listed in section 2.3.1.4. The exceptions are that amplification took place using a Stratagene MX3000P PCR machine (Agilent Technologies, Stockport, UK). The cycling conditions were slightly changed to include a fluorescence measurement step of 11 s following the elongation step, and following the last cycle a melting curve was generated consisting of a final heat cycle of heating to 95^{o}C for 1 min, cooling to 55^{o}C for 30 s and reheating to 95^{o}C for 30 s where fluorescence is measured. Generation of the melting curve assayed for purity and specificity of the amplified products by appearance of a narrow single peak.

All PCR reactions had the same optimized cycling conditions with the exception that the temperatures for primer annealing and where the fluorescence data was

collected were different used. This information is listed for specific primer pairs and genes of interest in Appendix C.

RNA polymerase II (For: 5’-CAAGACTGCTGAGACTGGATAC-3') and (Rev: 5'-CAAAGCGGAACTTCTTCTCAAAAG-3') was run as a reference gene for expression analysis of targeted genes in this study. RNA polymerase II was chosen because it showed a constant Ct value in qRT-PCR for all samples and different experimental conditions. As well, it is reported that RNA polymerase II expression is the most robust and constant reference gene within a comparison of all the classical reference genes (β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-Box binding protein (TBP), hypoxanthine–guanine phosphoribosyltransferase (HPRT), Peptidyl prolyl isomerase A (PPIA), glucose 6-phosphate dehydrogenase (G6PDH), ribosomal protein L13(L13), β2-microglobulin (β2M), phospholipase A2 (PLA), α-tubulin (Tub), albumin (Alb) and Porphobilinogen deaminase (PBGD); when measured in cells in different studies under diverse experimental conditions [247].

To normalize target gene expression to RPolII, the following equation was used:

$$\textbf{Target gene expression level} = \mathbf{A} = \mathbf{2}^{(-\Delta \mathbf{Ct})}$$

$$\Delta \mathbf{Ct} = (\mathbf{Ct\ target\ gene} - \mathbf{Ct\ reference\ gene})$$

Equation 2.2: Normalization of the target gene to the reference gene by ΔCt method.

Where Ct value represents the number of the cycles needed for the fluorescent signal to rise above baseline.

2.3.1.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used in molecular biology for separation, quantification and purification of DNA fragments based on their length as measured by base pairs. The procedure uses an electrical field to separate DNA fragments within agarose gels where high percentage gels are used for small DNA fragments, and low percentage gels for large DNA fragments. The DNA fragment size is determined by comparison to a DNA ladder which is composed of DNA fragments of known base pair length.

Agarose gels were prepared by weighing the desired amount of granular agarose (Web Scientific, UK) and adding 100 ml of Tris-borate- Ethyenediaminenetetraacetic acid (EDTA) (TBE) buffer (diluted from a 10X stock consisting of 0.445 M Tris borate, 0.01M EDTA pH=8.2-8.4). The agarose is then melted by heating in a microwave, and therefore requires that the holding flask be suitable for use in this manner. Normally, the flask is heated for an initial 1 min, and then again in short bursts until all the agarose is melted. The melted agarose solution was left to cool down but not solidify. At this point 1 µl of Ethidium Bromide (EtBr) dye (Promega, UK) was added to the agarose solution before slowly pouring it into a gel tray in order to avoid air bubble formation. EtBr is added so that DNA can be visualised under ultraviolet (UV) light. With the well comb in place, the agarose solution is then allowed to solidify for at least 1 h.

The solid agarose gel is placed in an electrophoresis tank. This tank is then filled with TBE buffer to which EtBr is added 1 µl for each 100 ml of TBE buffer) until the gel is covered. The comb is removed from the gel, and DNA samples, prepared by mixing DNA preparations with 6X DNA sample buffer (10 mM Tris-HCl pH 8.0, 50 mM EDTA, 15% Ficoll-400 and 0.5% orange G), and then carefully pipetted into the wells.

An electrical field was then applied (100V constant voltage) for 1 h, or sufficient movement of the tracking dye (orange G) was observed. At this point separated DNA fragments were able to be seen under UV light using a manual adjust system for exposure time (UVITEC, Alliance chroma system, Cambridge, UK) when obtaining images.

2.4 Plasmid DNA preparation, transformation, and validation

2.4.1 Plasmid DNA isolation and Transformation

The generously gifted DNA plasmid constructs pGL3-pkcβ-1.2(wt), and three pGL3-pkcβ-1.2 constructs containing mutated STAT3 binding sites used in this study were from Dr. Kelvin P Lee (Department of Immunology, Roswell Park Cancer Institute, Buffalo, USA). These constructs were provided blotted on to filter paper, and had to

be removed. Sterile scissors and forceps were used to carefully cut the circle of filter paper in which the DNA was blotted. DNA was then eluted from the filter paper with 100 – 500 µl nuclease-free water depending on the size of the circle. The concentration of the dissolved DNA was measured using Nandrop2000 spectrophotometry. 100 ng of the DNA plasmid was then used to transform MAX Efficiency® DH5α™ Competent Cells (Invitrogen™, Life Technologies, UK). Briefly, the competent cells were thawed on ice, and then 20 µl were transferred into a pri-chilled 1.5 ml eppendorf tube containing the DNA plasmid. The tube was gently mixed, and then left on ice for 30 min. This was followed by heat shocking the bacteria by placing the tubes in a water bath set at 42^{o}C for 45 s. The tubes were again incubated on ice for 2 min, and then 500 µl of Super optimal broth with catabolite repression (SOC) medium (Sigma Aldrich, Gillingham, UK) was added and tubes were then transferred to an incubator [C24 incubator shaker (New Brunswick Scientific, USA)] where they were left at 37^{o}C for 1 h with constant agitation 225 Revelations per minute (RPM). 50 and 100 µl aliquots of this bacterial culture were spread onto separate pre-warmed Luria agar (LB) plates which contained 100 µg/ml ampicillin. The plates were then incubated for overnight at 37^{o}C. The next day I picked a single colony and inoculated a 5 ml culture of LB medium containing 100 µg/ml ampicillin within a 15 ml universal tube. This tube was further incubated with a loosened cap for 12-16 h at 37^{o}C with 225 RPM constant agitation. In some cases, 500 µl of this final bacterial culture was added to a cryovial along with 500 µl of 50% sterile glycerol in order to make a glycerol stock for long term storage at -80^{o}C.

2.4.2 Plasmid DNA minimum-preparation (Miniprep) and Midi-preparation (Midiprep)

For mini-prep purification of plasmid DNA 3 ml of bacterial culture (like the one prepared above) was taken and processed following manufacturer's instructions for the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Cambridge Biosciences Ltd, Cambridge, UK).

For midi-prep purification of plasmid DNA, I inoculated a larger culture 500 ml of LB broth with 100 µg/ml ampicillin with 100 µl of small bacterial culture (like the one

prepared above). This larger culture was incubated for a further 18-21 h with constant shaking. The entire culture was then processed according to the manufacturer's instructions of the PureYield™ Plasmid Midiprep System (Promega, UK).

Purity and quantity of both miniprep and midiprep prepared DNA was checked by Nanodrop 2000 spectrophotometry.

2.4.3 Plasmid DNA digestion and validation

To verify the identity of the plasmids I used in this study, single and double digestions using specific restriction enzymes was performed. For example, 500 ng of miniprep-prepared pGL3-pkcβ-0.5 plasmid was mixed with 1 µl of *HindIII* and/or with 1 µL *XhoI* restriction enzymes, as required for single/double digestion of the plasmid. 4 µL of digestion buffer 3 was added, 0.2 µl of molecular biology grade BSA solution, and the total reaction volume was topped up to 20 µl with nuclease-free water. The digestion was allowed to proceed for 1 h at 37^{o}C, and then the DNA fragments separated by electrophoresis using a 0.8% agarose gel (Figure 2.1). All restriction enzymes and buffers used in this thesis are from New England Biolabs, UK.

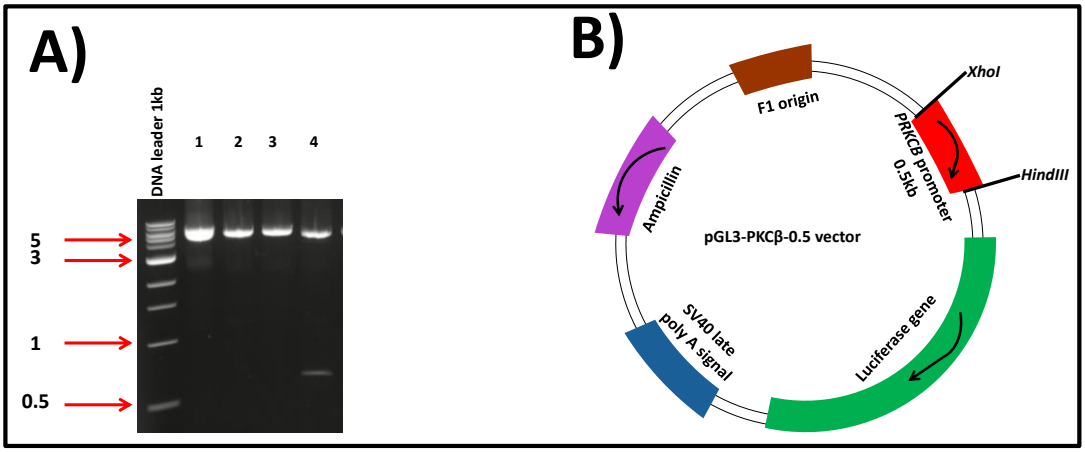


Figure 2.1: Validation of pGL3-pkcβ-0.5kb plasmid. The pGL3-pkcβ-0.5kb was validated for the *PRKCB* minimal promoter region (~0.5kb) insert in the basic pGL3 plasmid by digestion method. Figure 2.1 **A)** shows the plasmid pGL3-pkcβ-0.5kb wt (lane 1). This plasmid was digested as single by either HindIII restriction enzyme (lane 2) or by XhoI restriction enzyme (lane 3) or as a double digest using both of these restriction enzymes in the same reaction (Lane 4). B) PGL3-pkcβ-0.5kb plasmid map shows the inserted 0.5kb of *PRKCB* promoter into the PGL3-Basic vector which drives the luciferase gene transcription in this vector by cutting the plasmid using digestion enzymes *XhoI* and *HindIII*. Arrows within the luciferase and Ampicillin gene boxes indicates the direction of the transcription.

2.5 Loss of function Techniques

2.5.1 Nucleofection of CLL and B cell lines

Nucleofection is a technique that uses optimized voltage and an electrical conductive reagent to transfer nucleic acids into a target cell. To perform this procedure MEC1 and Daudi cells ($2x10^6$/transfection) or CLL cells ($1x10^7$/ transfection) were prepared by washing the cells twice with sterile pre-warmed PBS at 37^oC. The cells were finally suspended in 100 µl/transfection of nucleofection solution [nucleofection solution V (Lonza Group, Switzerland) for the CLL and MEC1 cells, or Ingenio Mirus electroporation solution (Geneflow, Lichfield, UK) for Daudi cells]. When cells were in these solutions, they were kept in ice. The transfection procedure itself consisted of mixing 100 µl of cells with different siRNA(s) or plasmids, transferring this mixture to a transfection cuvette and placing the cell within the Amaxa nucleofector (Lonza Biologics plc, UK). The following programs

were used for electroporation; U-13 for CLL cells, X-01 for MEC1 cells, and M-13 for Daudi cells. Directly following the electroporation the cells were transferred to 500 µl of pre-warmed media within a 1.5 ml eppendorf tube, and maintained at 37^{o}C until all transfection procedures were complete. This was followed by transfer of the cells to more formal culture conditions (24 well plate) where they could be kept for 24 h under optimal conditions. Next day, the media was changed, and the cells were cultured for another 48 h before performing analysis for mRNA and protein levels.

2.5.2 Knockdown studies by small interfering RNA (siRNA)

Knocking down specific proteins in a highly precise fashion is one of the tools that is used to investigate the functional significance of these proteins under certain physiological and cellular conditions. This aim can be accomplished using different techniques such as siRNA, short hairpin RNA (shRNA), and, most recently, genomic editing using clustered regularly interspaced short palindromic repeats (CRISPR) and expression of the enzyme Cas9.

For the purposes of this thesis siRNA was used. Table (2.1) lists the siRNA oligos used to target the genes of interest in this thesis. For many of the experiments in this thesis, pooled siRNA oligos were used. However, for some of the experiments single siRNA oligos were used. Where this was done is made explicit within the figure legends where illustrations of these experiments are presented. For specificity purposes, all experiments included a negative control where non-targeted siRNA oligos were used. The amount of siRNA oligo solution used for particular target genes is made explicit within the figure legends where illustrations of these experiments are presented. Oligonucleotides siRNA for P300 was from [Santa Cruz technologies, Inc, Heidelberg, Germany, catalogue number (SC-29431)] while for E2F1 and c-Myc they were from Thermo scientific, MA, USA.

Table 2.1: siRNA duplex sequences of Sp1, Sp3, and MITF transcription factors

Transcription factor	siRNA duplex sequence	Source
Sp1	D1 sense:5'-CCAAGGAAAUAAGGA-CAGUCUAGCT-3' α-sense:3'-AUGGUUCCUUUAUUCCUGUCAGAUCGA-5' D2 sense:5'-CCCUCAACCCUAUUC-AUUAGCAUTA-3' α-sense:3'-AUGGGAGUUGGGAUAAGUAAUCGUAAU-5' D3 sense:5'-GGUGCAAACCAACAGAUUAUCACAA-3' α-sense:3'-GUCCACGUUUGGUUGUCUAAUAGUGUU-5'	TriFECTaRNAi Integrated DNA Technologies, Glasgow, UK
Sp3	D1 sense:5'-GCAAUGAAGAAAGUAAGUAGUCUTG-3' α-sense:3'-UACGUUACUUCUUUCAUUCAUCAGAAC-5' D2 sense:5'-CCAGUAAAGUGUAACAUAUGCAAAC-3' α-sense:3'-AUGGUCAUUUCACAUUGUAUACGUUUG-5' D3 sense:5'-CCUAGAGUAAAUAAGAAUGAGCUTA-3' α-sense:3'-AUGGAUCUCAUUUAUUCUUACUCGAAU-5'	TriFECTaRNAi Integrated DNA Technologies, Glasgow, UK
MITF	D1 sense:5'-CCAAGUACCACAUACAGCAAGCCCA-3' α-sense:3'-GUGGUUCAUGGUGUAUGUCGUUCGGGU-5' D2 sense:5'-CCUAUGUAGACAAUAUAAGAGCUTC-3' α-sense:3'-ACGGAUACAUCUGUUAUAUUCUCGAAG-5' D3 sense:5'-GCCUAGAAUCAAGUUAUAAUGAGGA-3' α-sense:3'-AUCGGAUCUUAGUUCAAUAUUACUCCU-5'	TriFECTaRNAi Integrated DNA Technologies, Glasgow, UK

2.6 Promoter Functional Study Techniques

2.6.1. Dual Luciferase assay

The dual luciferase assay is a technique that allows study of the naked promoter region of a specific gene in order to identify the factors, such as transcription factors, that affect its function.

Promoter activity for the *PRKCB* gene was assessed using two different plasmid constructs. pGL3-pkcβ-0.5 is a 0.5kb construct consisting of the basal promoter region of the *PRKCB* gene upstream of the transcriptional start site inserted into the

pGL3 plasmid [A kind gift from Dr. A. P. Fields (Mayo Clinic College of Medicine, Jacksonville, Florida, USA]. pGL3-pkcβ-1.2 is a longer version (1.2kb) of the *PRKCB* containing the basal promoter region upstream of the transcription start site and including identified STAT3 binding sites within the promoter. This construct is cloned into the pGL3 plasmid backbone [a kind gift from Dr. K. P. Lee (Department of Immunology, Roswell Park Cancer Institute, Buffalo, USA)].

These plasmids were transfected into MEC1 and Daudi cells ($2x10^6$/experiment). Generally, 2 µg of either pGL3-pkcβ-0.5 or pGL3-pkcβ-1.2 was transfected with 100 ng of pRL (a reference plasmid where expression of Renilla luciferase is driven by the CMV promoter). Luciferase activity was assessed 48 h following transfection, and was performed using the Dual Luciferase kit (Promega, UK) following the manufacturer instructions.

In some experiments B cell lines transfected with pGL3-pkcβ-0.5 were treated with 200 nM of mithramycin. Where this was done, mithramycin was added 24 h following transfection, and luciferase analysis carried out after an additional 48 h. In other tests investigating pGL3-pkcβ-1.2, transfected cells were treated with 100 nM PKCβ inhibitor (LY333531) (Cayman chemical company, Michigan, USA), 0.5 µM STAT3 inhibitor VII (Calbiochem, UK), 150 ng/ml Interlukin-6 (IL-6) (InvitrogenTM, Life Technologies, UK), 50 ng/ml Interlukin-21 (IL-21) (Invitrogen^{TM}, Life Technologies, UK), or with 100 ng/ml VEGF. These reagents were added 2h following transfection, and analysis for promoter function performed after 48 h.

2.6.2 Site- directed mutagenesis of Sp1 binding sites within the *PRKCB* promoter region

Site-directed mutagenesis is a molecular technique whereby one or more alterations in a known nucleotide sequence of DNA is made. This methodology allows a precise understanding of the importance of certain nucleotide sequences on the activity of a gene promoter, or of an amino acid residue within a specific enzyme.

Alteration of the Sp1 binding sites within the *PRKCB* promoter was carried out by introducing mutations using a Stratagene QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Stockport, UK) following the manufacturer's instructions. This kit uses a PCR-based protocol to achieve this purpose. Briefly, 25 ng of pGL3-pkcβ-0.5 was used as a template in the PCR reaction. The PCR reaction consisted of pGL3-pkcβ-0.5 mixed with 5 µl of 10X reaction buffer, 1.25 pmol/µl each of forward and reverse primers (Table 2.2), 1 µl dNTP mix, and 10 µl of homemade 5X combinatorial enhancer solution (CES) (2.7 M betaine, 6.7 mM DTT, 6.7% DMSO, and 55 µg/ml BSA) which is used to solve the problem of PCR amplification of a GC-rich region (>85%) within the template plasmid. The total volume was made up to 50 µl with nuclease-free water. Finally, 1µl of PufoTurbo DNA polymerase (2.5 U/µL) was added. The PCR reaction tube was briefly centrifuged and then put into a PCR machine where the following specific thermal profile was used to introduce the desired mutation: an initial denaturation step lasting for 30 s at 95^{o}C, 18 cycles whereby denaturation at 95^{o}C for 30s was followed by annealing at 70^{o}C for 1min and extension at 68^{o}C for 7 min. The PCR reaction was terminated with a final extension step at 68^{o}C for 7 min. Following the PCR reaction the parental plasmid template was digested with 1 µl of DnpI at 37^{o}C for 1 h. This digestion step was repeated. Then, 5µL of the PCR product containing the mutated promoter was transformed into 50 µl of XL1-Blue competent cells (section 2.4.1). Single colonies were picked and grown for miniprep preparations of the new plasmid. Introduced mutations were verified by commercial sequencing (Source BioScience plc, Nottingham, UK).

Table 2.2: Sequence of the primers used to introduce mutations for Sp1 binding site in *PRKCB* promoter region.

Sp1 binding sites	Primers sequence that used to introduce the mutations
Sp1m1	Forward:5'-AGCAGCTGGCAGCGCTATGCTAGGCCTGGGCGCG-3' Reverse:5'-CGCGCCCAGGCCTAGCATAGCGCTGCCAGCTGCT-3'
Sp1m2	Forward:5'TGGGCGCGATGCAAATGAGGAATGCTAGGCTGGCCCGGG-3' Reverse:5'CCCGGGCCAGCCTAGCATTCCTCATTTGCATCGCGCCCA-3'

2.6.3 Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitation (ChIP) is a versatile tool in molecular biology because of its ability to define precise relationships between DNA and factors which regulate/affect gene expression/chromatin structure. Such factors can include transcription factors, chromatin remodelling proteins and histones, as well as DNA repair and replication proteins.

In this thesis CLL and MEC1 cells were used to investigate the association between transcription factors and the *PRKCB* promoter. For each cell type $1x10^7$ cells were washed twice with PBS and then treated with 0.5% formaldehyde solution (freshly prepared by dissolving paraformaldehyde in water with heating and in the presence of 7 mM KOH) for 5 min at R.T. to cross link proteins and DNA. The reaction was stopped with the addition of 1.35 M glycine to a final concentration of 135 mM, and then further kept at R.T. for 5 min. The fixed cells were washed twice with cold PBS, and then lysed with 1ml cold lysis buffer [(10 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.2% IGEPAL® CA-630, plus 1:100 diluted protease inhibitor (MerckMillipore, Watford, UK) and phosphatase inhibitor cocktails (200 mM Imidazole, 200 mM sodium fluoride, 100 mM Sodium orthrovanadate, 400 mM Sodium Tartrate, 100 mM β-glycerolphosphate, 100 Mm sodium pyrophosphate)] on ice for 15 min. Nuclei from the lysed cells were isolated by centrifugation at 500*xg* for 5 min at 4°C. These nuclei were washed once with 1 ml of cold MNase buffer [0.3 M sucrose, 10 mM Tris-HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 0.5 mM spermidine, 0.15 mM spermine] using the same centrifugation conditions. This was followed by resuspending the pellet in 500 µl of cold MNase buffer to which 125 U micrococcal nuclease (New England Biolabs, UK) was added, and incubated the suspended nuclei for 20 min at 37°C. The nuclease digestion reaction was stopped by with the addition of EDTA to a final concentration of 10 mM, and this was then sequentially followed by addition of IGEPAL® CA-630, sodium deoxycholate and SDS to final concentrations of 1%, 0.5% and 0.1%, respectively. Nuclei were disrupted by sonication using a MS73

sonication probe and two 30 s pulses at 40% power (Sonopuls ultrasonic homogenizer, BANDELIN GmbH & Co. KG, Berlin, Germany). This process released fragmented chromatin with an approximate size of 100-500bp (Figure 2.2). Finally, the tube was further by centrifuged at 12000*xg* for 5 min at 4^{o}C in order to remove insoluble material.

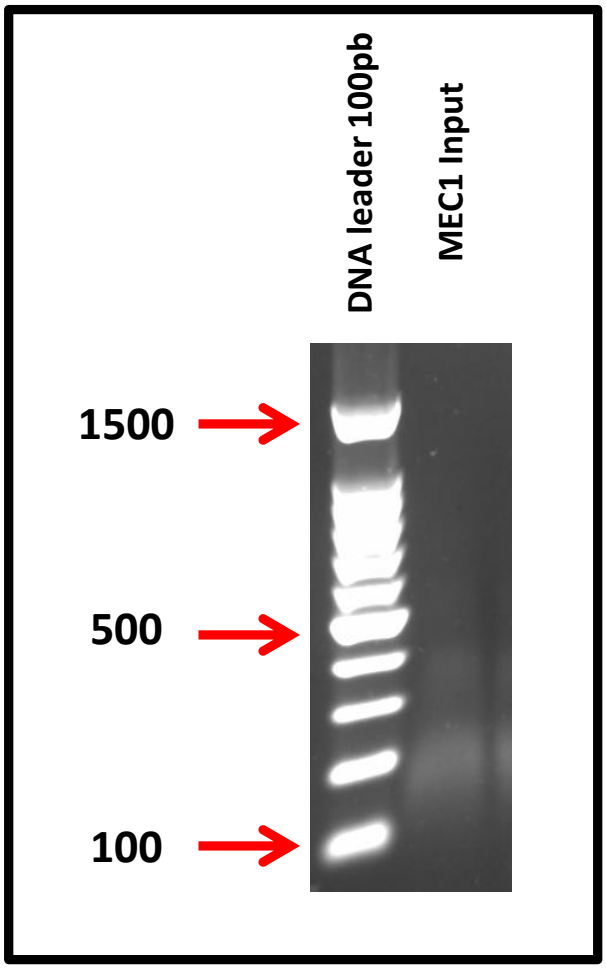


Figure 2.2: Sheared chromatin input sample from MEC1 cells. 1x10^{7} cells of MEC1 cell line were fixed with 0.5% paraformaldehyde to cross link DNA and associated proteins. The nucleus was disrupted by sonication for two 30sec pulses at 40% power. The cross link was reversed and the size of 10µl purified DNA was observed by running it in 1% agarose gel.

The supernatant resulting from the above procedure contains protein/DNA complexes. This supernatant was initially precleared for 1 h with 20 µl Magna ChIP protein A+G magnetic beads (Millipore, Watford, UK), and then incubated overnight at 4^{o}C with either indicated ChIP-grade antibodies (Appendix D), or non-specific IgG control antibodies (Merck Millipore, Watford, UK) together with 20 µl Magna ChIP protein A+G magnetic beads (Millipore, Watford, UK). The immunoprecipitated complexes were then sequentially washed with 500 µl each the following buffers in order: Low salt (0.1 %SDS, 1% Tritron-X 100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl pH=8.1), high salt (0.1 %SDS, 1 %Tritron-X 100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris-HCl pH (8.1), LiCL (0.25 M LiCL, 1% IGEPAL®CA-630, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl pH=8.1), and TE (10mM Tris-HCl pH=8.0 and 1Mm EDTA). Washed beads were then resuspended in 100 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$, 0.2 M NaCl) to which 1 µl of RNAase inhibitor [248] was added, and then kept at 37^{o}C for 30 min with constant shaking using an Eppendorff thermomixer comfort (Humberg, Germany). The beads were removed by magnetic separation, and the supernatant taken and incubated at 65°C for 6 h in the presence of 10 µg of proteinase K to reverse cross links. The DNA contained within this eluate was purified using a DNA Clean & Concentrator™-5 kit (Zymo Research, Cambridge Bioscience Ltd, UK) according to the manufacturer's instructions. DNA purified in this way was isolated in a total volume of 30 µl.

One microliter of purified DNA was used for PCR amplification using the following optimized thermal profile conditions: 20 s at 95^{o}C for denaturation, then annealing for 20 s and extension for 30 s at 72^{o}C, followed by an 11 s incubation to collect florescence data. The PCR reaction was performed using specific primers designed to cover the potential transcription factor binding sites within the *PRKCB* promoter (Figure 2.3) (Appendix C). Success of the immunoprecipitation step was confirmed by using primers for a gene known to be regulated by the transcription factor targeted by the antibodies used in this study (Appendix D).

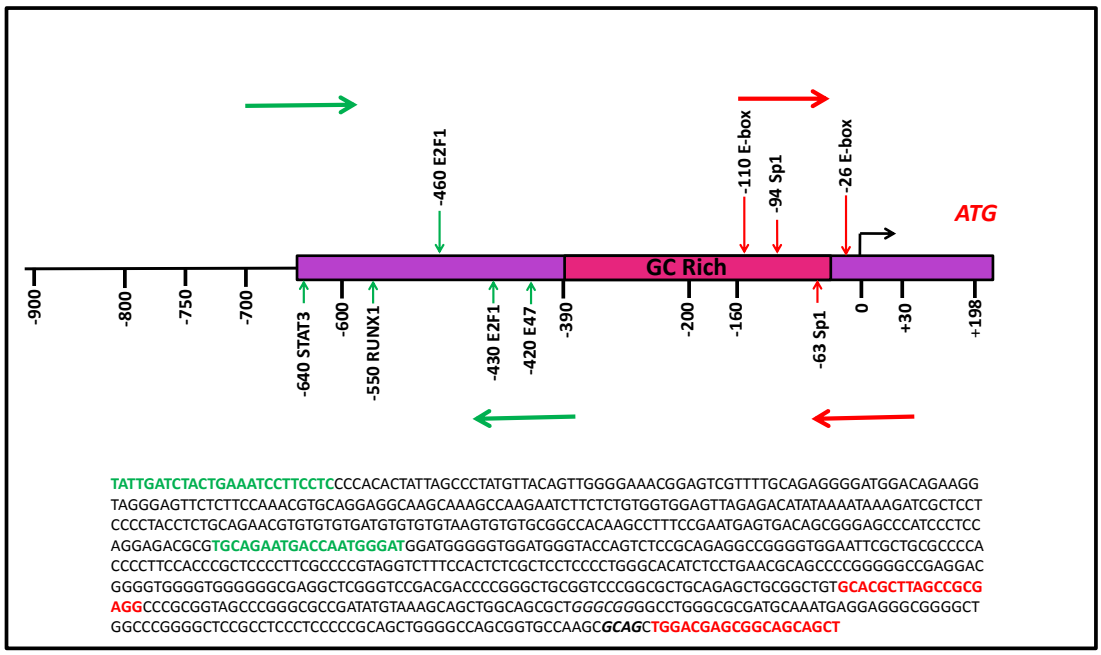


Figure 1.3: Schematic diagram of the *PRKCB* promoter showing the location and sequence of the ChIP primers. This schematic drawing shows the binding sites for Sp1, E-box, E2F1, E47, RUNX1, and STAT3 binding sites within the *PRKCB* promoter and the ChIP primers that were used in this study covering the proximal and distal regions of this promoter. The transcription binding sites and the arrow for the proximal region of the promoter labelled in red while, for the distal region labelled with green. Underneath the schematic diagram is the sequence of the ChIP primers and labelled with same colour for each region as previously mentioned.

2.6.4 *PRKCB* promoter methylation studies

Promoter methylation occurs in different regions of a gene, including the gene body and the promoter region. This process is driven by DNA methyltransferases which add a methyl group to carbon number 5 within the pyrimidine ring of cytosine that are contained in CpG islands of DNA. Understanding DNA methylation is important because this is one epigenetic mechanism controlling gene expression, particularly when methylation occurs within the promoter region of genes.

Study of the *PRKCB* promoter methylation status in this thesis was done using a EZ DNA Methylation-Gold™ Kit (Zymo Research, UK). This kit exploits the bisulfite conversion method which uses bisulfite to convert un-methylated cytosine within DNA sequences into uracil, and leaves methylated cytosine residues unchanged.

The product of this reaction is then studied following PCR amplification (in this thesis using primers specific for the *PRKCB* promoter) by sequencing. The percentage methylation in the CpG islands located within the DNA region of interest was measured relative to the ratio of T and C in the pyrogram.

The protocol in brief, 1 µg of genomic DNA was sodium bisulphite treated using a EZ-DNA methylation gold kit (Zymo Research, Irvine, CA, USA). A CpG-rich target region within the *PRKCB* promoter was selected for interrogation, and forward, reverse and pyrosequencing primers designed using Pyromark Assay Design 2.0 software (Qiagen, Valencia, CA, USA) and synthesized by Eurofins MWG Operon: PRKCBmeth_fwd: 5'-GTTTGGGTATATTTTTTGAA-3', PRKCBmeth_rev: 5'-Biotin- CCC TCCTCATTTACATC-3', PRKCBmeth_seq: 5'-TTGGGTATATTTTTTGAA-3'. PCR amplification was performed using 400 µM forward and reverse primers, 60 ng bisulphite-treated DNA, 200 µM dNTPs, 1 mM $MgCl_2$ and 1.25 u GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA). PCR cycling conditions were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s and an additional 72°C extension for 10 min. Specific PCR product quality and quantity were confirmed by agarose gel electrophoresis. PCR products were then immobilized on to Streptavidin Sepharose beads (GE Healthcare Biosciences, Pittsburgh, PA, USA) and sequentially washed in 70% ethanol, 0.2 M NaOH and 10 mM Tris acetate, pH 7.5, using a PSQ96 Vacuum Workstation (Qiagen, Valencia, CA, USA). PRCKBmeth_seq primer was then hybridized to the retained biotinylated DNA strand in annealing buffer and analysed using PSQ96 MA Pyrosequencer and PyroMark Gold Q96 reagents (Qiagen, Valencia, CA,USA). Results presented as % methylation represent the mean average methylation of 8 CpGs within the analysed sequence.

2.7 Protein Electrophoresis and Blotting Analysis

2.7.1 Preparation of cell lysate and protein determination

2.7.1.1 Preparation of cell lysate

Cell lines or CLL cells were harvested and washed once with cold PBS. Cells were lysed with 100 µl of 1% SDS lysis buffer (1% SDS, 125 mM Tris pH6.8, 5 mM EDTA, and 10% glycerol). The samples were kept in ice, and were sonicated for 30sec at 40% power to disrupt released DNA. The samples were then heated at 95^{o}C for 10 min, and finally centrifuged at high speed for 15 min to remove any debris. The lysates were kept at -20^{o}C until used.

2.7.1.2 Protein determination

The concentration of the total protein content for each sample was determined using the Bio-Rad DC protein assay kit (Bio-Rad laboratories Ltd, UK). Briefly, 5 µl of cell lysate was pipetted into wells of a 96 well plate in duplicate. In separate wells, a serial dilution of BSA standard (0-2 mg/ml) prepared in SDS lysis buffer was also applied. 1 ml of reagent A was freshly mixed with 20 µl of reagent S, and 25 µl of this mixture was then added to each well containing sample or standard. Then 200 µl of reagent B was added per well, and the plate kept in dark for 15 min at room temperature. Absorbance readings were taken at 650 nm, and protein concentration within the samples was determined by comparison to the readings taken for the protein standards. The measurement was considered valid if there was a doubling of absorbance with each doubling of protein standard concentration, and the correlation coefficient value for the standard curve was 0.99.

For application to SDS gels, 10 µg of protein within the prepared cell lysates was used. The volume of each sample applied was equalised with SDS lysis buffer, and then 5X loading sample buffer (5% SDS, 625 mM Tris pH6.8, and 50% glycerol, β-mercaptoethanol and bromophenol blue) was added prior to gel loading.

2.7.2 Western Blotting

2.7.2.1 Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS–PAGE)

SDS-PAGE is a chromatographic technique used to separate proteins based on size. Protein size in this technique is discriminated by changes in the percentage of acrylamide used to make the separating gels; low percentage acrylamide gels are better at resolving large molecular weight proteins, whereas high percentage gels are better at resolving low molecular weight proteins. Protein migration within gels is enabled by the application of an electrical field. So that all proteins migrate, the running pH of the separating gel is maintained at 8.8, and proteins are denatured in the presence of SDS which adds additional negative charge to facilitate protein migration. The final important feature of this system is the stacking gel, whose function is to focus (stack) proteins within samples by creating an ion gradient between faster running Cl- ions and slower running glycine by using a buffer whose ion strength is weaker than that used for the resolving gel, but has a pH of 6.8. The molecular weight for any protein of interest can be determined by comparison with the migration of proteins with known molecular weights that are applied to run alongside the protein sample.

The acrylamide composition of the gels used in this thesis is listed in the figure legends where this technique is used. For the purposes of this section I describe the preparation of a 10% acrylamide gel for a Bio-Rad minigel apparatus (Bio-Rad laboratories Ltd, UK): 4 ml resolving gel buffer (Geneflow, UK) was mixed with 5.3ml acrylamide solution (19:1 Acrylamide/Bisacrylamide, Geneflow, UK) and 6.7 H_2O. Polymerisation of the gel was initiated with the addition of 75 µl 10% Ammonium persulphate (APS) and 15 µl tetramethylethylenediamine (TEMED). The amount of resolving gel solution made is ideal for casting 2 X 1.5mm PAGE gels, which were left to polymerize at R.T. for 1 h. A 5% polyacrylamide stacking gel was prepared by mixing 1.5 ml stacking gel buffer (Geneflow, UK) with 1 ml acrylamide solution and 3.5 ml H_2O, and then adding 10% APS and TEMED. 10 or 15 well combs were used

to create sample cells in the stacking gel. The gels were used immediately upon polymerisation.

Samples were loaded into the prepared sample wells of the gels, and the electrophoresis apparatus was assembled and finally filled with 1X running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS pH=8.3). For protein size Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad laboratories Ltd, UK) were used. Protein separation was achieved with the application of 35V per gel constant voltage for 55 min.

2.7.2.2 Protein Transfer

Proteins separated by SDS-PAGE were transferred to polyvinylidene diflouride (PVDF) membranes for analysis by Western blot. PVDF membranes (0.45mm pore size, Roche Diagnostic Limited, UK) were cut to a 6X9 mm size, wetted with methanol, and placed into transfer buffer (25 mM Tris, 190 mM glycine, 0.1% SDS pH=8.3). A transfer sandwich was prepared, and was placed into the transfer apparatus along with chilled transfer buffer and a frozen cooling core. Finally, an electrical current 400mA (constant current) was applied for 1 h.

2.7.2.3 Membrane probing and development

To minimize non-specific interactions between membranes and probe antibody in Western blots the PVDF membrane was blocked using a blocking buffer consisting of T-TBS (25 mM Tris pH=7.6, 150 mM NaCl, 0.1% Tween-20) in which 5% (w/v) of advanced blocking agent (Fisher Scientific, UK) was dissolved for 1h with continues agitation at R.T. This blocking step was followed by probing the membranes for proteins of interest using primary antibodies; information regarding each of these including source and dilution is listed in Appendix D. Generally, membranes were incubated with primary antibodies diluted in blocking buffer overnight at $4^{o}C$ with continuous agitation. The next day the membranes were washed for 30 s, 5 min, and 10 min respectively with T-TBS, and then incubated with HRP (Horseradish peroxidase)-conjugated anti-rabbit/-mouse secondary antibodies diluted at either 1:5000 or 1:10000 in blocking buffer for 1 h at R.T. with continuous agitation.

One exception to the above procedure was when membranes were probed for β-actin. Here, membranes were probed with mouse monoclonal anti-β-actin antibody (Clone AC-74, product No. A 5316, Sigma Aldrich, Gillingham, UK) diluted 1:10000 in blocking buffer for at R.T. 30 min. The membrane was then incubated with HRP-conjugated anti-mouse secondary antibody (1:10000) at R.T. for 30 min.

Proteins of interest on the membranes were detected using either WESTAR® Supernova enhanced chemiluminescence (ECL) substrate reagent (Geneflow, UK) or Immobilon™ Western Chemiluminescent HRP Substrates (MerckMillipore, Watford, UK), and imaged using an LAS-1000 (Fujifilm, Japan). Densitometry was performed using AIDA image analyser software (v4.27.039).

2.8 Immunoprecipitation (IP)

Immunoprecipitation (IP) is a technique that allows isolation and concentration of a specific protein or a complex of proteins from a mixture, usually a cell lysate. This technique depends on the interaction of a specific antibody that recognizes a precise epitope on a protein of interest. For this study $1x10^7$ CLL cells were lysed with RIPA buffer (10% Glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH=7.6, 2 mM EDTA, 2mM EGTA, 25 mM sodium pyrophosphate, 50 mM sodium glycerolphosphate, 150 mM NaCl and 50 mM sodium fluoride) and centrifuged a 16,000xg for 15 min at 4°C. The supernatant was then incubated with Sp1 antibodies overnight, and then precipitated with protein A/G magnetic beads. The beads were washed twice with RIPA buffer, and the attached proteins solubilised with SDS-PAGE sample buffer.

2.9 Statistical Analysis

The data in this thesis were analysed for statistical significance using either a Student's t-test for paired data when comparisons between two connected variables were done, or a Mann-Whitney U-test for comparisons of data distributions such as expression of a particular gene between normal and CLL B cells

from different patients. The software used for these calculations was either Microsoft Excel™, or IBM–SPSS™ (v22).

Table 2.3: Sources for the chemicals that were used for the buffers preparations

Name of supplier	Chemical	Name of supplier	Chemical
Sigma Aldrich, Gillingham, UK	Betaine	Fisher Thermo Scientific, UK	$CaCl_2$
	β-glycerolphosphate		Glycine
	β-mercaptoethanol		KCl
	bromophenol blue dye		KOH
	BSA		$MgCl_2$
	DTT		NaCl
	EDTA		NaOH
	Ethanol		SDS
	Ficoll-400		sodium deoxycholate
	Glycerol		Tris acetate
	IGEPAL® CA-630		Tris borate
	Imidazole		Tris-HCl
	LiCL		Tween20
	$NaHCO_3$		paraformaldehyde
	Orange G		
	Sodium fluoride		
	Sodium orthrovanadate		
	Sodium pyrophosphate		
	Sodium Tartrate		
	Spermine		
	spermidine		
	Tritron-X 100		

❖ Chapter Three: The key role of Sp1 in regulating *PRKCB* gene transcription

3.1 Introduction and aim

In 1992 the 5´untranslated region of *PRKCB*, a region which includes the gene promoter, was cloned and characterized by Obied *et al.* [4]. They found that the PKCβ gene has a TATA-less promoter type with a high GC content. In addition, they showed that the 600bp region upstream of the transcriptional start site contained a number of potential binding sites for transcription factors (Figure 3.1). Interestingly, they found that a 100bp section immediately before the transcriptional start site was vital for basal promoter activity. Likewise, similar results were found in a different cellular system described by Niino *et al.* [161]. This 100bp section was noted to contain putative Sp1 binding sites.

CLL cells are distinctive from normal B cells and other B cell malignancies by their ability to overexpress PKCβII [1]. Overexpression of this PKC isoform is regulated at the transcriptional level, and, as such, is markedly inhibited by mithramycin [143], a drug that inhibits gene transcription by intercalating into GC-rich areas of DNA particularly at Sp1 binding sites.

Prior to beginning this thesis any role for Sp1 in PKCβ gene transcription was unknown. Therefore, a principle aim of this chapter was to investigate whether Sp1 drove promoter activity and transcription of *PRKCB* in CLL cells. Secondary to this aim, was to determine the relationship between Sp1 and PKCβII expression in CLL cells in order to understand whether Sp1 contributes to disease pathogenesis. However, using CLL cells in genetic manipulation studies is difficult. Therefore, a cell line model was used to develop these studies, which were then confirmed in CLL cells.

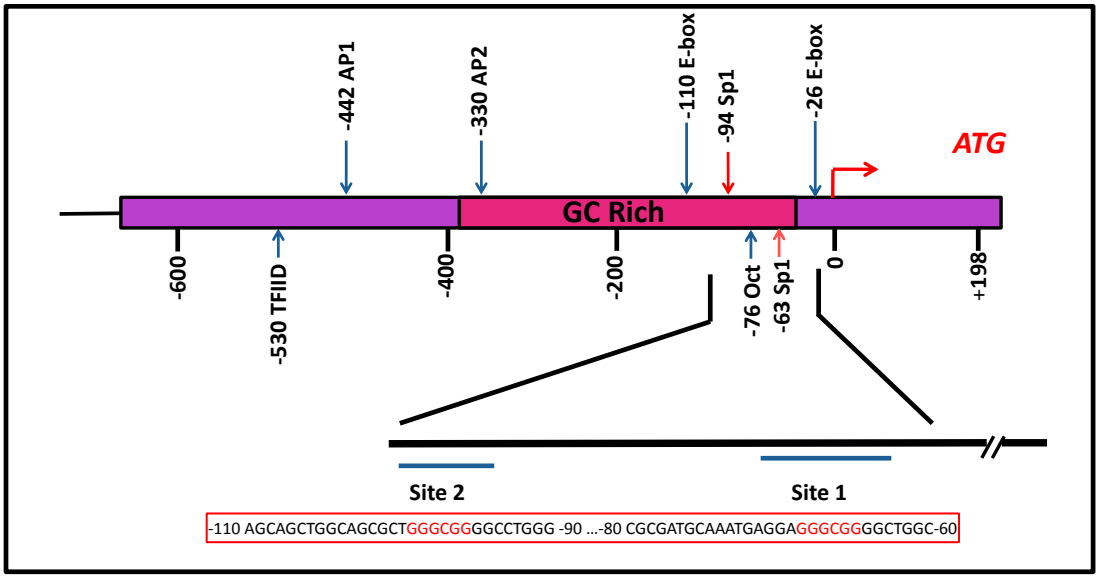


Figure 3.1: Schematic drawing of the basal *PRKCB* promoter. This schematic drawing shows the CG-rich region of the *PRKCB* promoter in pink, and lists potential transcription factor binding sites. The transcription start site is labelled by the red arrow and the translational site labelled by ATG in red.

3.2 Results

3.2.1 PKCβII mRNA levels are overexpressed and correlated with PKCβII protein levels in CLL cells

Previously, Abrams *et al.,* 2007 reported that PKCβII protein is overexpressed in CLL cells compared to normal B and other types of mature B lymphoid malignant cells [1]. In addition, they showed that overexpression of this protein is due to the dominant transcript of PKCβII mRNA from the *PRKCB* gene. Moreover, they found a strong positive correlation between the mRNA of this isoform and its protein expression in these malignant cells. Therefore, I have started my investigations to understand the molecular regulatory mechanism(s) that control expression of the *PRKCB* gene in CLL cells by confirming these results.

Using qRT-PCR and specific primers covering the coding region of PKCβII, I compared the relative expression of PKCβII mRNA in normal B and in CLL cells. I found that PKCβII mRNA was expressed at significantly higher levels in CLL cells

compared to normal B cells (Figure 3.2A). Western blot analysis of PKCβII protein levels in CLL and normal B cells showed that this PKC isozyme is overexpressed in the former (Figure 3.2B and C). In these experiments β-actin was used to indicate equal protein loading. Furthermore, as positive and negative controls I used Bcl2 and ERK. It is well known that CLL cells overexpress Bcl2 protein compared to normal B cells, and Figure 3.2B confirms this in my results. In contrast, normal B cells and CLL cells showed equal amounts of total ERK protein (Figure 3.2B). Taken together, these results indicate that PKCβII is overexpressed in CLL cells at both the transcriptional and protein levels, allowing reconfirmation of the strong correlation between PKCβII mRNA and protein levels in CLL cells (Figure 3.2D).

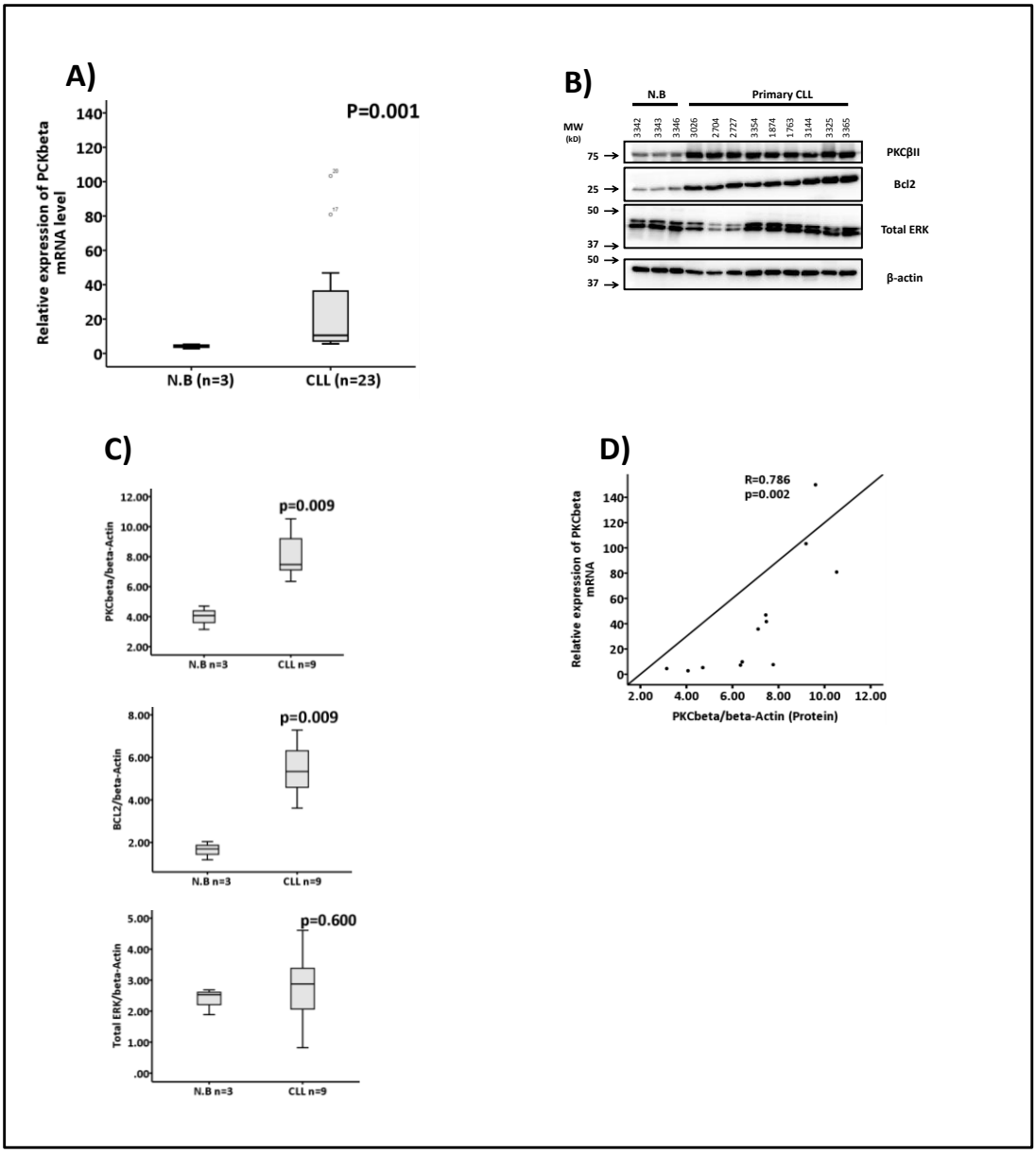


Figure 3.2: PKCβII is overexpressed in CLL compared to normal B cells. CLL and normal B cells were purified by positive selection and analysed for expression of PKCβII mRNA by qRT-PCR and protein by Western blot. **A)** PKCβII mRNA levels in CLL (n=23) and normal B (n=3) cells. Levels are reported relative to those of RNA Polymerase II, and are expressed as arbitrary units. **B)** PKCβII protein expression in CLL and normal B cells. Lysates of CLL and normal B cells were prepared and 10 µg protein was separated by SDS-PAGE, transferred to PVDF membrane, and probed for the indicated antibodies. **C)** Quantitative analysis of PKCβII, Bcl2, and total ERK protein expression in CLL and normal B cells depicted in part B. The Western blots prepared in part B were analysed by densitometry, and respective protein levels are reported relative to β-actin. Statistical significance was determined using a Mann-Whitney U-test. **D)** Correlation analysis of PKCβII mRNA and protein in CLL cells.

3.2.2 Different leukaemia and lymphoma cell lines show different levels of PKCβII mRNA and protein expression

Due to difficulty of using CLL cells in some of the functional experiments, it was important to determine and characterise a cell line for this purpose. Thus, I investigated the expression of PKCβII mRNA and protein levels in different leukaemic and lymphoma cell lines. qRT-PCR analysis showed variation in the relative expression of PKCβII mRNA level between the cell lines that were screened (Figure 3.3.A). Also, PKCβII protein level in these cell lines showed dissimilarity in the expression (Figure 3.3.B). For example, PKCβII protein was completely un-expressed in KCL22 cells, a cell line derived from a chronic myelogenous leukaemia patient who is in blast crisis stage [249]. This is in contrast to the K562 cells which are also derived from a chronic myelogenous leukaemia patient in blast crisis stage [250]. The mRNA and protein levels of PKCβII expression in MEC1 cell line that represents a model of CLL cells showed the highest expression between these different malignant cells. In addition, Daudi cells showed a slightly lower level of PKCβII protein and this was in accord with its mRNA level in these cells. These results show that MEC1 and Daudi cells are the most suitable cells that I could use later in my functional experiments.

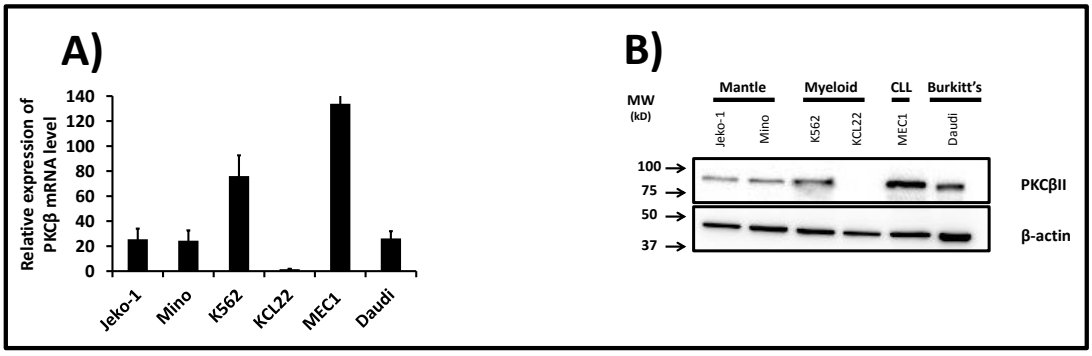


Figure 3.3: Different cell lines show different levels of PKCβII mRNA and protein expression. Various leukaemia and lymphoma cell lines were screened for PKCβII mRNA and protein expression. **A)** qRT-PCR analysis of PKCβII mRNA expression in the indicated cell lines. PKCβII mRNA levels are reported relative to RNA Polymerase II and are presented as mean ± SE of n=3 separate measurements. **B)** Western blot analysis of cell lysates prepared from the indicated cell lines. 10 µg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to PKCβII and β-actin.

3.2.3 Sp1 mRNA is overexpressed and correlates with protein level in CLL cells

As previously mentioned, overexpression of PKCβII is a distinctive feature of CLL cells where it likely contributes to the pathophysiology of this disease. Early studies of *PRKCB* promoter function suggested a potential role for Sp1 in transcription of the gene [4, 161]. To investigate this potential role it seemed logical to determine the level of expression of Sp1 in CLL cells.

To study Sp1 mRNA expression three sets of primer pairs covering the entire coding region of Sp1 were designed (Table 3.1). First, a temperature gradient PCR for the primer sets was performed in order to optimize the suitable annealing temperature. I found that primer set number 3 amplified a single band without any formation of primer dimers at an annealing temperature of 64^{o}C (Figure 3.4A). qRT-PCR was then used to amplify Sp1 from CLL samples, and this showed exponential amplification starting at cycle 26 (Figure 3.4B), with a single product as detected with a melting curve (Figure 3.4C). Thus, primer pair 3 was used to analyse Sp1 mRNA expression in the cells used in this thesis.

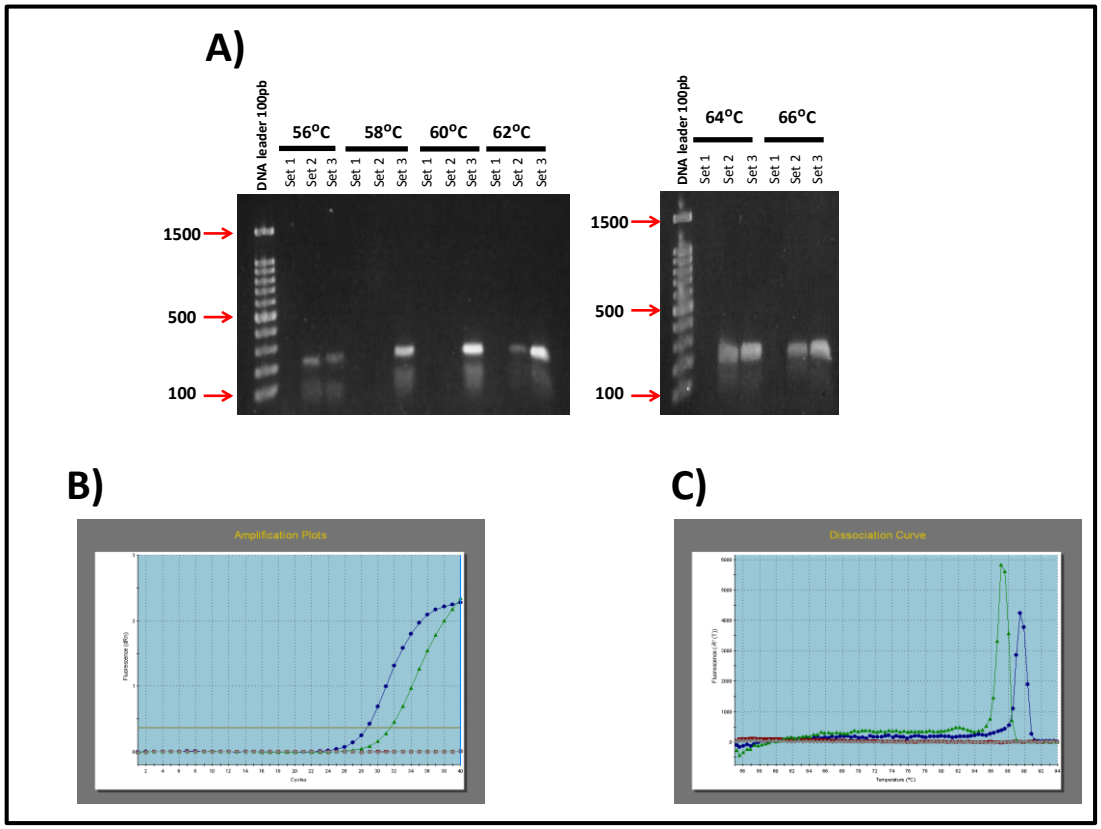


Figure 3.4: Selection and optimization of PCR conditions for Sp1 primer pairs. A) An annealing temperature gradient of the three different primer pairs used to amplify Sp1. MEC1 cells were used as a source of cDNA, and the listed annealing temperatures were used as part of the PCR cycle. The cDNA template was amplified for 40 cycles using a DNA Engine RPTC-200 Peltier thermal cycler, and the products were separated using a 1% agarose gel. **B)** Amplification curve of the Sp1 amplicon using primer set 3 (blue) relative to that of the RNA Polymerase II amplicon reference gene (green). PCR was performed to 40 cycles using a Stratagene qRT-PCR machine. Nuclease free water was used as a negative control (Red and Grey). **C)** Melting curve of the final qRT-PCR products produced in part B. Single PCR products for the Sp1 (Blue) and RNA Polymerase II (green) amplicons were observed after 40 cycles of amplification**.**

Table 3.1: Sequence of primer sets for measuring Sp1 mRNA

Name of the primer	Sequence of the primers
Primer set 1	For: 5'-ACCACCTGTCAAAACATATCAAGACCCAC-3' Rev: 3'-TGCCACCTGCATGACGTTGATGCC-5’
Primer set 2	For: 5'-TTTGCCTGCCCTGAGTGTCCTAAG-3' Rev: 3'-GGTGGTAATAAGGGCTGAAGGAGTG-5’
Primer set 3	For: 5'-TCAAGACCCACCAGAATAAGAAGGGAG-3' Rev: 3'-GACGTTGATGCCACTGTTGGCAAG-5’

I found that Sp1 mRNA levels were significantly overexpressed in CLL compared to normal B cells (Figure 3.5.A). Analysis of Sp1 protein levels showed a similar result, this protein being overexpressed in CLL compared to normal B cells (Figure 3.5.B and C). Finally, I showed that levels of Sp1 mRNA correlated with protein levels (Figure 3.5.D) indicating a potential relationship between Sp1 and PKCβII that is investigated in section 3.2.5.

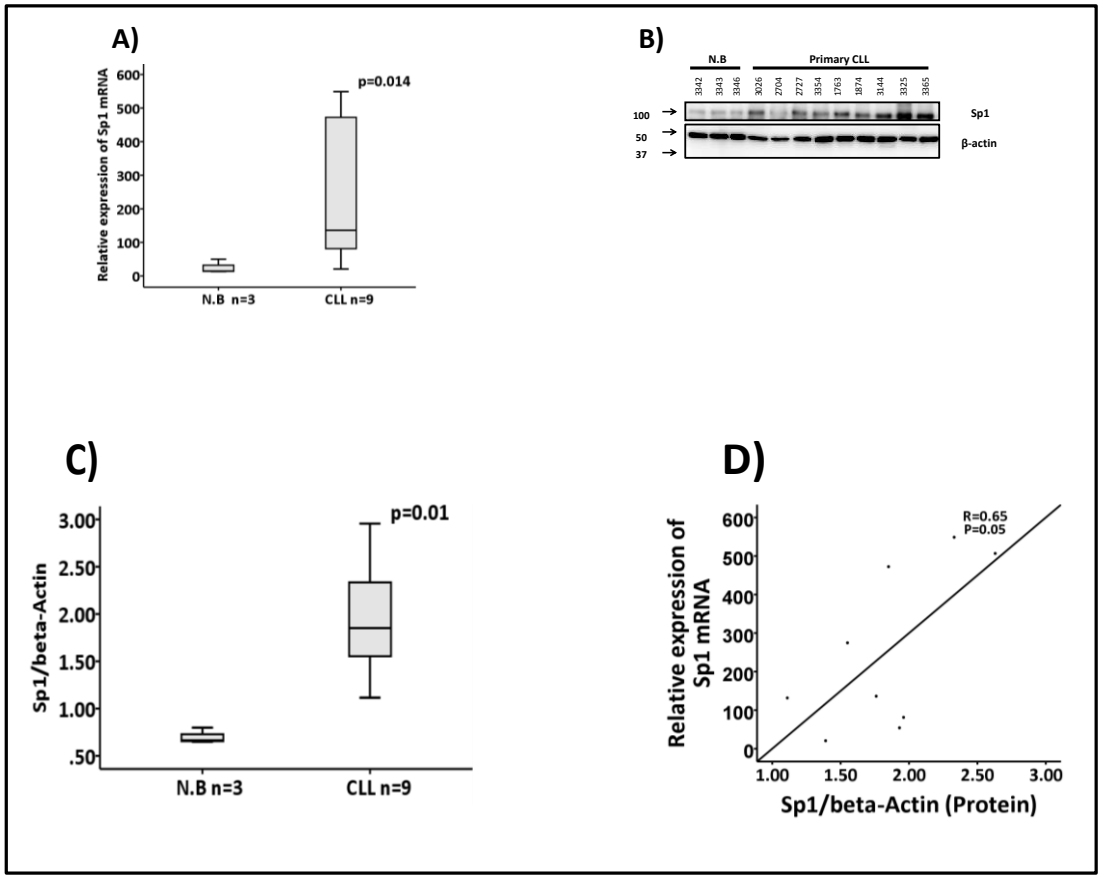


Figure 3.5: Sp1 mRNA and protein is overexpressed in CLL compared to normal B cells. Purified CLL and normal B cells were analysed for expression of Sp1 by qRT-PCR and Western blot. **A)** qRT-PCR analysis of Sp1 mRNA levels in CLL (n= 9) and normal B cells (n=3). Sp1 mRNA expression is reported as arbitrary units relative to RNA Polymerase II as reference gene. Statistical analysis was performed using a Mann-Whitney U-test (p=0.014). **B)** Differential expression of Sp1 protein between normal B (n=3) and CLL cells (n=9). 10 µg protein from normal B and CLL cell lysates was separated by SDS-PAGE (10% acrylamide gel) and transferred to PVDF membrane, which was then probed with the indicated antibodies. **C)** Quantitative analysis of Sp1 protein expression relative to β-actin. Gel densitometry was used to analyse Sp1 and β-actin expression in the gels illustrated in part B. Statistical analysis was performed using a Mann-Whitney U-test. **D)** Correlation analysis of Sp1 mRNA with protein levels in primary CLL cells. Pearson coefficient was calculated using SPSS version 22 (R=0.65) and the correlation is significant at the 0.05 level (p=0.05).

3.2.4 Differential expression of Sp1 mRNA and protein in different leukaemia and lymphoma cell lines

Given that cell lines were to be used in some of the experiments that relate to the functional studies, it was reasonable next to investigate Sp1 mRNA and protein expression levels in these different leukaemia and lymphoma cell lines. I found that the Sp1 mRNA levels are similar in MEC1, K652, Jeko-1 and Mino cell lines. Conversely, it was considerably lower in both KCL22 and Daudi cells (Figure 3.6A). Also, I found that Sp1 protein levels were similar in all of the cell lines examined (Figure 3.6B). Accordingly, I decided to use MEC1 cell line in the future studies because as mentioned earlier it is derived from CLL patient. In addition, I used Daudi cell lines because they showed reasonable level of Sp1 mRNA and protein. Also, previous work in our lab showed that these cell lines are easy to be transfected.

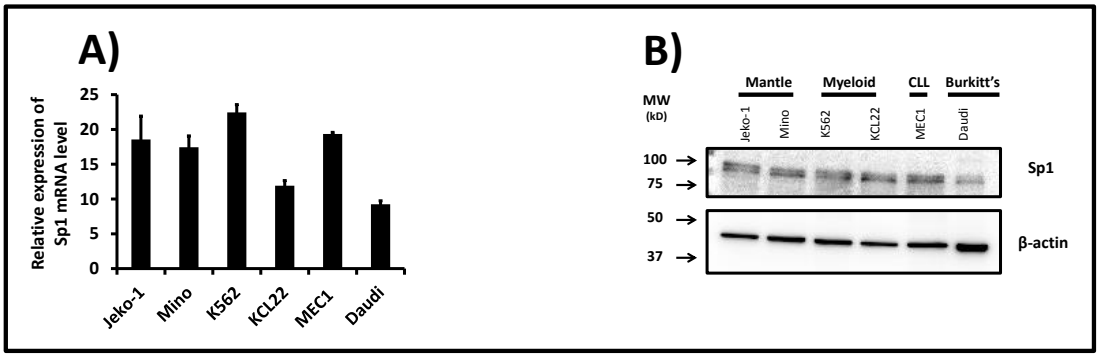


Figure 3.6: Different cell lines show different levels of Sp1 mRNA and protein expression. Various leukaemia and lymphoma cell lines were screened for PKCβII mRNA and protein expression. **A)** qRT-PCR analysis of Sp1 expression in the indicated cell lines. Sp1 mRNA levels are reported relative to RNA Polymerase II and presented as mean ± SE of n=3 separate measurements. **B)** Western blot analysis of cell lysates prepared from the indicated cell lines. 10 µg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to Sp1 and β-actin.

3.2.5 Sp1 protein levels correlate with PKCβII mRNA in CLL cells

The data in section 3.2.3 demonstrates overexpression of Sp1 mRNA and protein levels in CLL cells. This observation suggests a correlation between PKCβII mRNA and Sp1 protein levels in CLL cells. Indeed, Figure 3.7 shows significant correlation between PKCβII mRNA and Sp1 protein levels in these cells, indicating that Sp1 potentially plays a role in PKCβ gene transcription. The rest of this chapter is devoted to characterising this role.

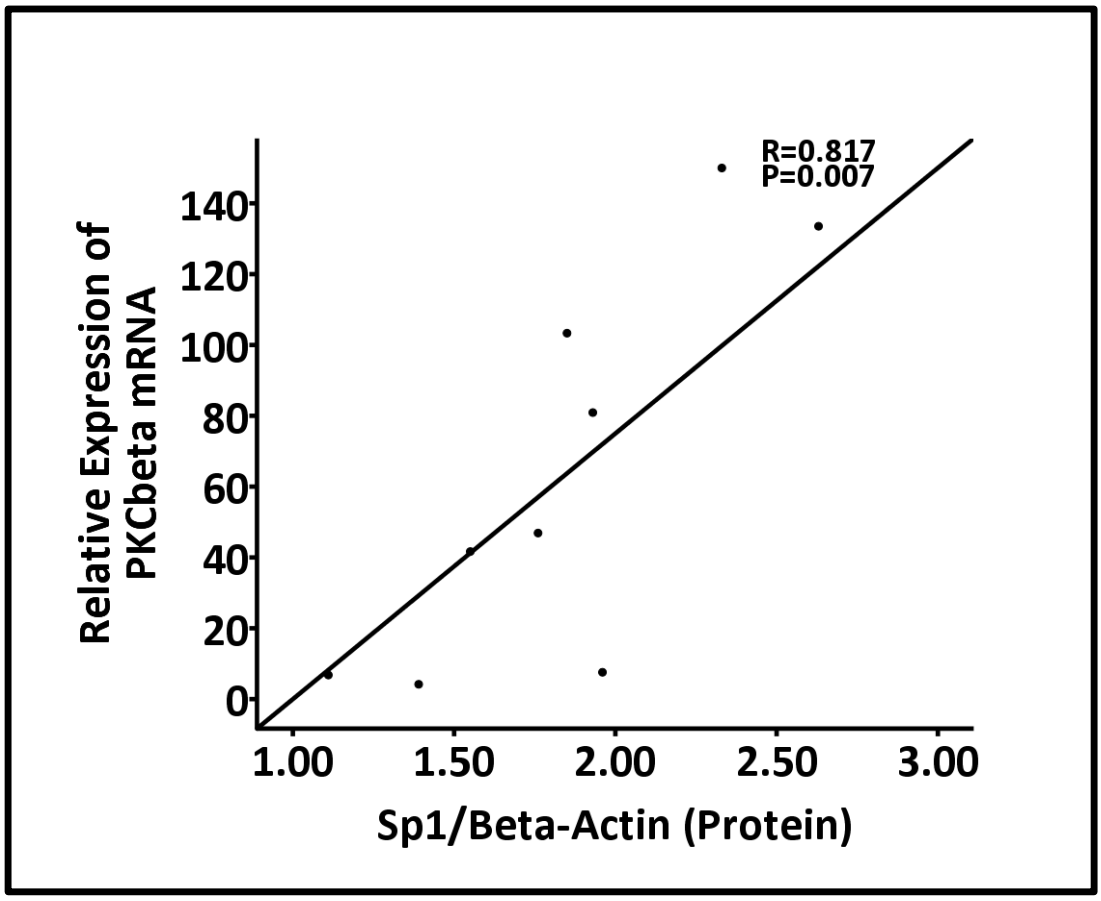


Figure 3.7: Sp1 protein correlates with PKCβII mRNA in CLL cells. Sp1 protein levels were determined by Western blot and PKCβII mRNA expression was determined by qRT-PCR in purified CLL cells (n=9). The points associated with each individual case were plotted and linear regression applied. Pearson coefficient (R=0.817) was calculated by using SPSS version 22 software, and the correlation was considered significant at the 0.01 level (p=0.007).

3.2.6 Mithramycin inhibits PKCβ gene expression in B lymphoid cell lines and CLL cells in a concentration-dependent manner

As a first step in elucidating the role of Sp1 in regulating PKCβ gene transcription in CLL cells I used mithramycin. Mithramycin is one of a family of antibiotics whose structure is based on aureolic acid. Mithramycin was isolated and characterized in the early 1950s, and is derived from fungus like bacteria such as *Streptomyces plicatus* that belong to the phylum actinobacteria. Accordingly mithramycin has also been named plicamycin owing to the organism from it was first isolated [251]. Mithramycin has a chemically distinctive feature that allows selective binding to GC-rich regions in the minor groove of DNA [252, 253]. These GC-rich regions of DNA are often found within the regulatory regions of genes and contain binding sites particularly for Sp group transcription factor members. Interaction of mithramycin with GC-rich regions of DNA can therefore block the binding of Sp group transcription factors, and this can lead either to down-regulation, or to up-regulation of gene expression. An important member of the Sp group of transcription factors is Sp1, and many cancers, including CLL, show high expression of this protein. Thus, mithramycin was investigated as a therapeutic strategy to target Sp1 in different types of cancer cells [254, 255], and is recently approved in phase II clinical trials for the treatment of osteosarcoma [256].

I first established the optimal concentration of mithramycin to use. This was done by treating CLL as well as MEC1 and Daudi cells with different concentrations (0, 50, 200, and 400 nM) of mithramycin. Figure 3.8 show that the presence of mithramycin reduced PKCβII mRNA levels in these cells in a concentration-dependent fashion. As previously shown by our group [143], the presence of mithramycin with CLL cells had no effect on their viability. The same was true for MEC1 and Daudi cells, regardless of whether the cells were cultured under serum-free or serum-rich conditions. This latter experiment was necessary because Sp1 transcribes many genes involved in the cell cycle [176], and is itself regulated by cell cycle genes such as E2F1 and CDK4, among others [257]. Thus, we observed that maximal reduction of PKCβII mRNA levels in both cell lines and in CLL cells were achieved using a concentration of 200 nM mithramycin. This similarity in response

suggested that MEC1 and Daudi cells could be used to model the behaviour of *in vitro*-cultured CLL cells.

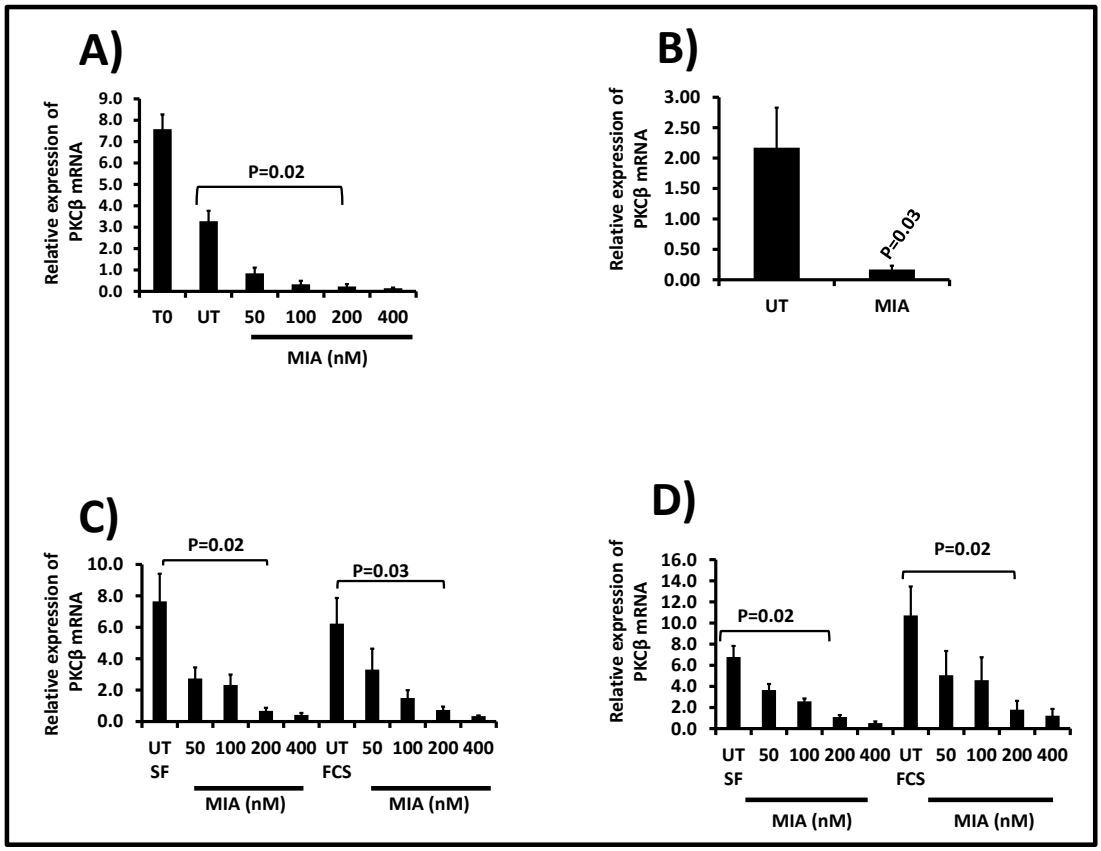


Figure 3.8: Mithramycin inhibits PKCβII mRNA levels in B lymphoid cell lines and in CLL cells in a dose dependent manner. CLL cells ($1x10^7$/ml) or B lymphoid cell lines (MEC1 and Daudi) ($2x10^6$/ml) were cultured for 24 h in the absence (UT) or presence (MIA) of the indicated concentrations of mithramycin (nM). **A)** PKCβII mRNA levels in CLL cells taken from different six patients (mean±SE). **B)** Effect of Mithramycin (200 nM) on PKCβII mRNA levels in CLL cells from a single CLL patient (n=3 separate biological replicates) (mean±SE). **C)** and **D)** PKCβII mRNA levels in MEC1 and Daudi cell lines, respectively, cultured under serum free (SF) or serum rich (FCS) medium conditions. This experiment illustrates (n=3) separate biological replicates (mean±SE) and the relative mRNA to RNA Polymerase II for each replicate was measured by qRT-PCR as triplicates. Statistical analysis for all parts of this figure was performed using a student's t-test for paired data.

3.2.7 Mithramycin inhibition of PKCβ gene transcription in MEC1 and CLL cells results in a time-dependent decrease in PKCβII mRNA levels

200 nM of mithramycin was determined optimal for the inhibition of PKCβ gene expression because PKCβII mRNA levels were quantitatively reduced in treated cells without affecting cell viability. I next used this concentration to investigate the kinetics of this reduction. Figure 3.9 shows that PKCβII mRNA levels began to drop in MEC1 and CLL cells after 3 h incubation with mithramycin, and maximal effect was observed after 12 h. The half-life of PKCβII mRNA in these cells is estimated to be between 6-9 h. Because mithramycin does not affect cell viability under these conditions, I used a 24 h incubation period for all subsequent experiments.

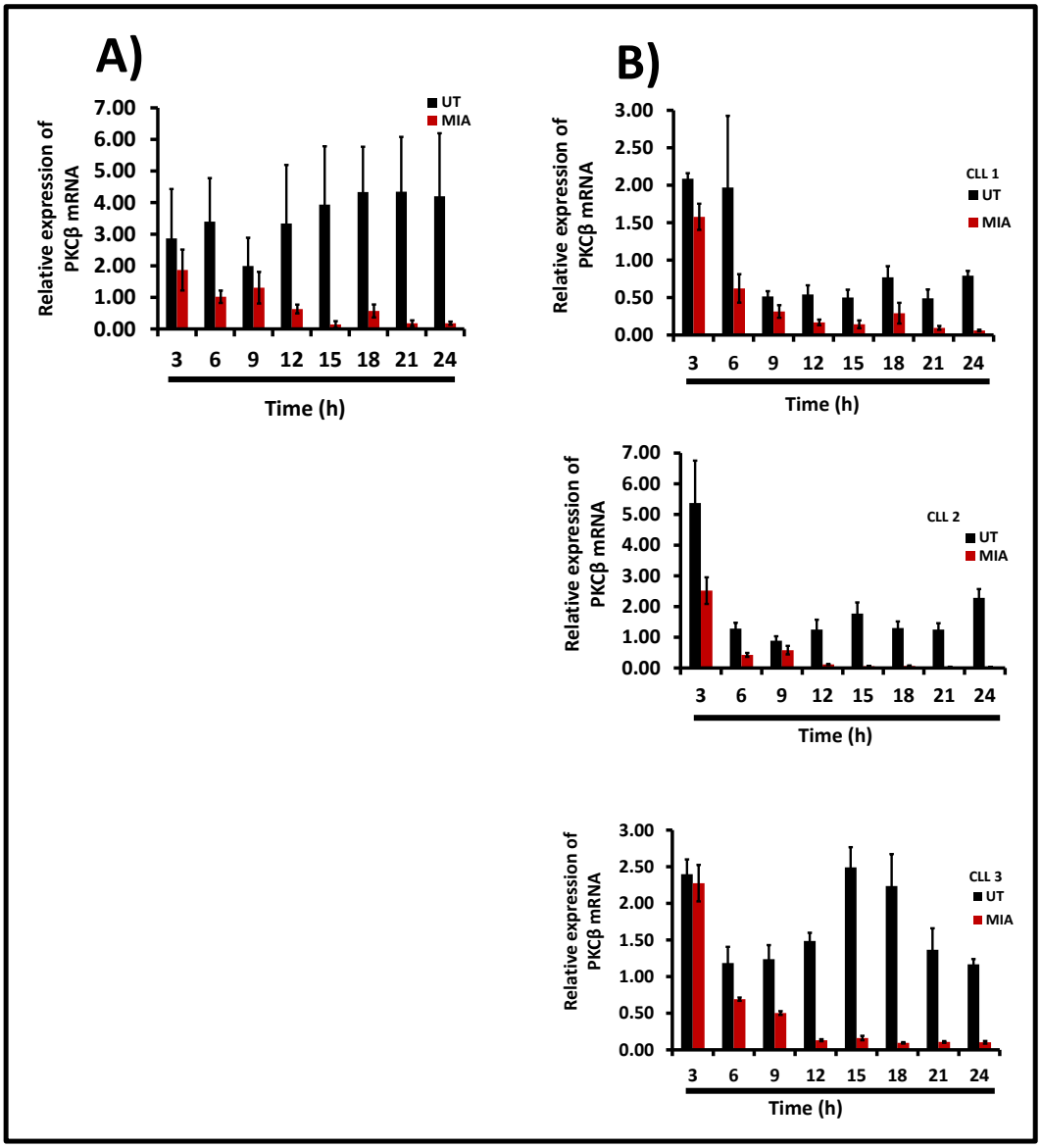


Figure 3.9: Change in PKCβII mRNA levels in MEC1 and CLL cells treated with mithramycin. MEC1 cells ($2x10^6$/ml) or CLL cells ($1x10^7$/ml) were cultured in the absence (UT) or presence of 200 nM mithramycin (MIA) for 24 h. Every 3 h a sample of cells was taken and the expression of PKCβ mRNA relative to RNA Polymerase II was measured by qRT-PCR. **A)** PKCβ mRNA levels in MEC1 cells. **B)** PKCβ mRNA levels in CLL cells from three different patients. All the experiments in this figure illustrate (n=3) separate biological replicates, and values are presented as mean±SE.

3.2.8 Mithramycin inhibits Sp1 gene expression in MEC1, Daudi and CLL cells

It was reported that Sp1 gene expression is auto-regulated by Sp1 binding to its promoter [257]. I next asked the question whether mithramycin treatment of the cells used in the present study also affected Sp1 mRNA levels. Indeed, similar to the effect of mithramycin on PKCβII mRNA levels, 200 nM of mithramycin also caused marked inhibition of Sp1 mRNA expression in MEC1, Daudi and CLL cells after 24 h treatment (Figure 3.10). This result suggested two questions for investigation. The first question is that mithramycin can affect many genes, so it should be determined whether there are specific effects on other genes. Secondly, my earlier observations indicated a close correlation between expression of Sp1 and that of PKCβII, indicating that a reduction in Sp1 expression potentially affects PKCβ gene transcription. Therefore, clarification of a direct role for Sp1 in PKCβ gene transcription needs to be established.

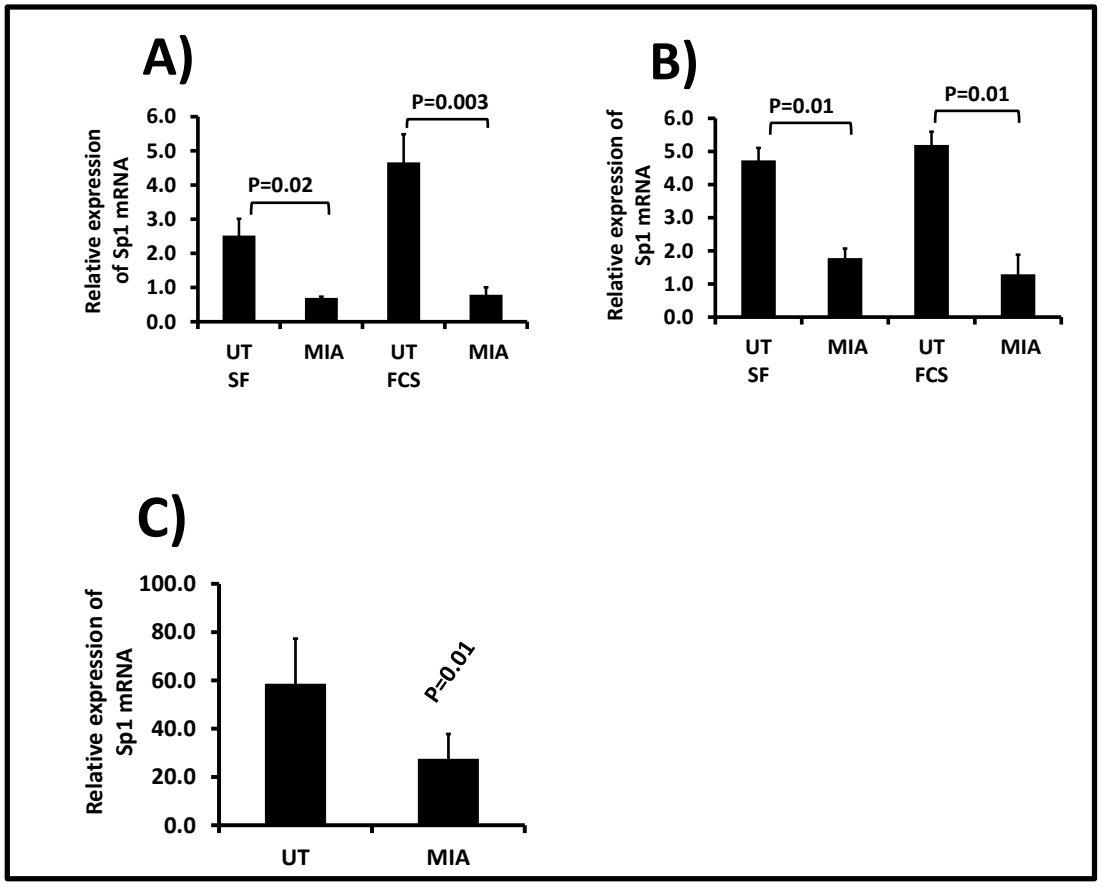


Figure 3.10: Effect of mithramycin on Sp1 mRNA levels in MEC1, Daudi and CLL cells. A) MEC1, **B)** Daudi (each $2x10^6$/ml) and **C)** CLL ($1x10^7$/ml) cells were cultured for 24 h in the absence (UT) or presence of 200 nM mithramycin (MIA). MEC1 and Daudi cells were cultured under serum free (SF) or serum rich (FCS) conditions. Sp1 mRNA levels in each cell type was determined by qRT-PCR and is expressed relative to RNA Polymerase II. Each illustration represents 3 separate biological replicates (mean±SE). In part C, CLL cells from 3 different patients were used. Statistical analysis for all parts of this figure was performed using a student's t test for paired data.

3.2.9 Mithramycin treatment does not change BCL10 mRNA levels in MEC1 of CLL cells

To investigate the specificity of mithramycin I used BCL10 as a negative control. Use of the Encyclopaedia of DNA Elements (ENCODE) reveals that this gene contains CpG islands and a single Sp1 binding site. This suggests that expression of BCL10 is potentially affected by mithramycin. Figure 3.11 shows that treatment of either MEC1 or CLL cells with 200 nM mithramycin do not affect the expression of BCL10. This result indicates that mithramycin selectively affects the expression of some genes, and, therefore, suggests that the drop in Sp1 and PKCβ mRNA levels in treated MEC1 and CLL cells is because of targeted disruption of gene expression by this drug.

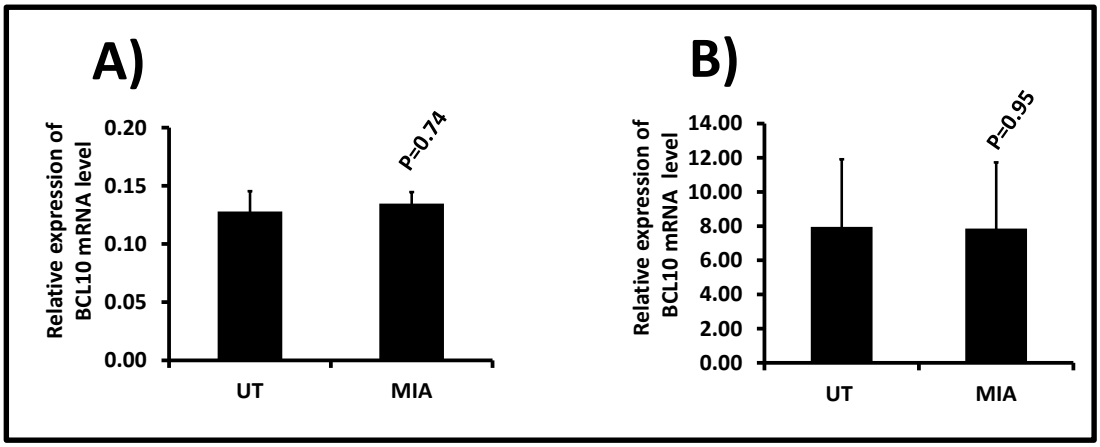


Figure 3.11: Effect of mithramycin on BCL10 mRNA levels in MEC1 and CLL cells. BCL10 mRNA levels were determined by qRT-PCR and are shown relative to RNA Polymerase II. **A)** MEC1 cells ($2x10^6$/ml) and **B)** CLL cells ($1x10^7$/ml) were incubated for 24 h in the absence (UT) or presence of 200 nM mithramycin (MIA). This experiment illustrates n=3 separate biological replicates (mean±SE). In the case of CLL, cells from 3 different patients were used. Statistical analysis for all part of this figure was performed by using a student's t-test for paired data.

3.2.10 Sp1-specific siRNA reduces PKCβII mRNA levels in MEC1, Daudi cell lines and CLL cells

The results from sections 3.2.5 and 3.2.6 suggest that Sp1 has a potential role in regulating the expression of *PRKCB* in CLL cells. Thus, to clarify this role I used 3 different Sp1-specific siRNA oligonucleotides to reduce the levels of Sp1 protein in MEC1, Daudi, and CLL cells and investigated whether this also affected PKCβII expression (Figure 3.12). The use of each specific oligonucleotide affected Sp1 expression to a different extent in MEC1 cells, an effect that was not observed with the control (non-specific) oligonucleotide. It was determined that the mix of all 3 oligos was most effective at reducing mRNA and protein levels of Sp1 in MEC1 and cells. Furthermore, the reduction in Sp1 protein levels was accompanied by a concomitant reduction in PKCβII transcript and protein levels in these cells.

In CLL and Daudi cells, similar results were observed. Use of the siRNA mix showed reduction in Sp1 mRNA and protein levels, and this was accompanied by reduction in PKCβII mRNA and protein levels (Figure 3.12G-I). Taken together, these results demonstrate that reduced Sp1 expression results in a reduction of PKCβII gene and protein expression, and suggests that the effect of mithramycin is due to inhibition of Sp1. However, whether Sp1 directly drives the transcription of *PRKCB* still needed to be determined.

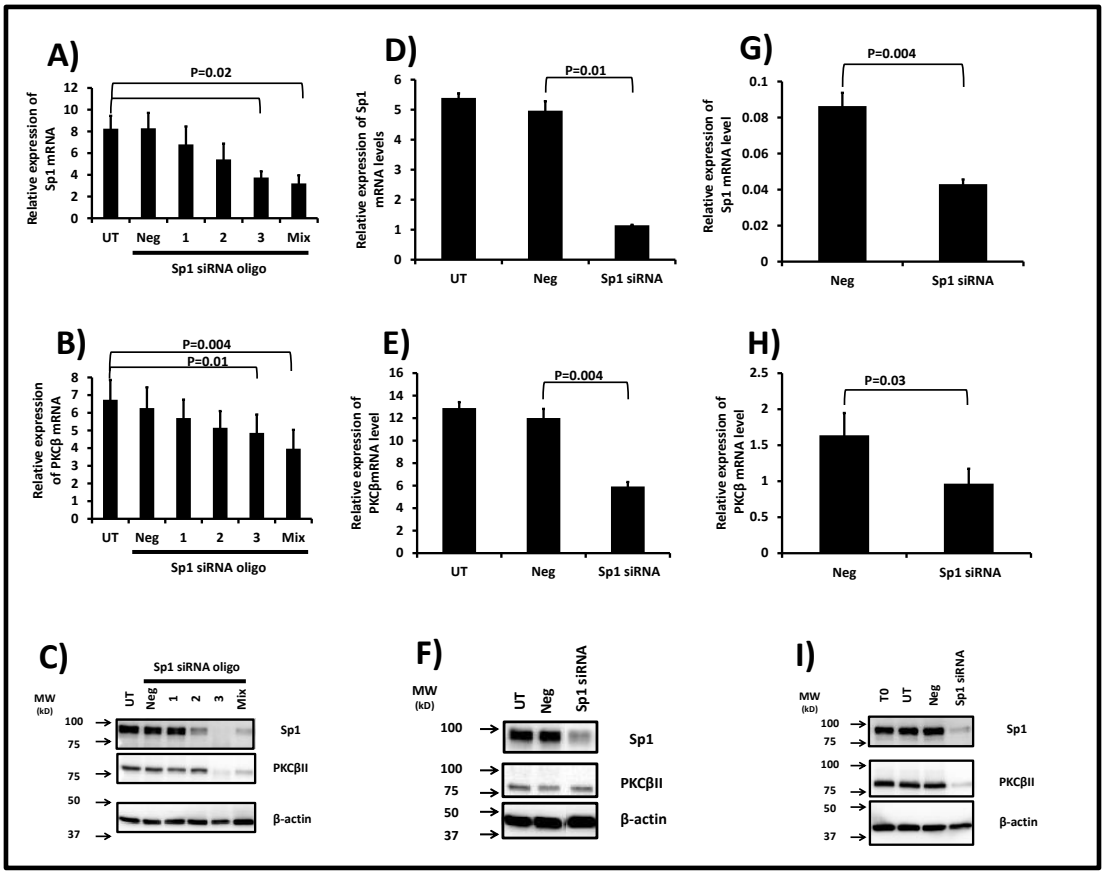


Figure 3.12: Sp1-specific siRNA reduces PKCβII mRNA and protein levels in MEC1, Daudi and CLL cells. MEC1 and Daudi cells ($2x10^6$ each) and CLL cells ($1x10^7$) were nucleofected with Sp1-specific or control siRNA oligonucleotides (2 μM), and then cultured under the conditions specific for each cell type for 72 h. Harvested cells were analysed for protein expression by Western blot and for mRNA levels by qRT-PCR measured relative to the reference gene RNA Polymerase II. **A)** Sp1 mRNA levels in MEC1 cells. **B)** PKCβII mRNA levels in MEC1 cells. **C)** Western blot analysis of MEC1 cell lysates for the indicated proteins. **D)** Sp1 mRNA levels in Daudi cells. **E)** PKCβII mRNA levels in Daudi cells. **F)** Western blot analysis of Daudi cell lysates for the indicated proteins. **G)** Sp1 mRNA levels in CLL cells. **H)** PKCβII mRNA levels in CLL cells. **I)** Western blot analysis of CLL cell lysates for the indicated proteins. Graphical data are presented as arbitrary units where each graph represents mean±SE of n=3 separate experiments. Where CLL cells were used, experiments were performed using material from different patients. β-actin is used as a loading control for Western blots. Statistical analysis for all parts in this figure was performed using a student's t-test for paired data.

3.2.11 Sp1 drives *PRKCB* promoter function

I next performed a functional assay to investigate the role of Sp1 in driving expression of the PKCβ gene. This was done using the basal *PRKCB* promoter region to drive expression of luciferase (from the pGL3-pkcβ0.5 plasmid) in Daudi and MEC1 cells. Figure 3.13A shows that Daudi (top panel) and MEC1 cells (bottom panel) have considerable luciferase activity when transfected with the pGL3-pkcβ0.5 plasmid compared to those transfected with pGL3 alone. This figure also shows that the presence of 200 nM mithramycin quantitatively reduces promoter activity in pGL3-pkcβ0.5-transfected Daudi and MEC1 cells to background levels. Thus, similar to its effect in reducing *PRKCB* transcription, mithramycin also suppresses *PRKCB* promoter function.

Reduction of Sp1 expression using specific siRNA also blocked *PRKCB* promoter function in pGL3-pkcβ0.5-transfected Daudi and MEC1 cells, an effect that did not occur in cells treated with either control siRNA or had no siRNA added (Figure 3.13B). These results clearly show a role for Sp1 in driving *PRKCB* promoter function, and, taken together with the data showing the effect of Sp1 knockdown on PKCβII mRNA levels, strongly suggest that Sp1 may drive PKCβ expression *in vivo*.

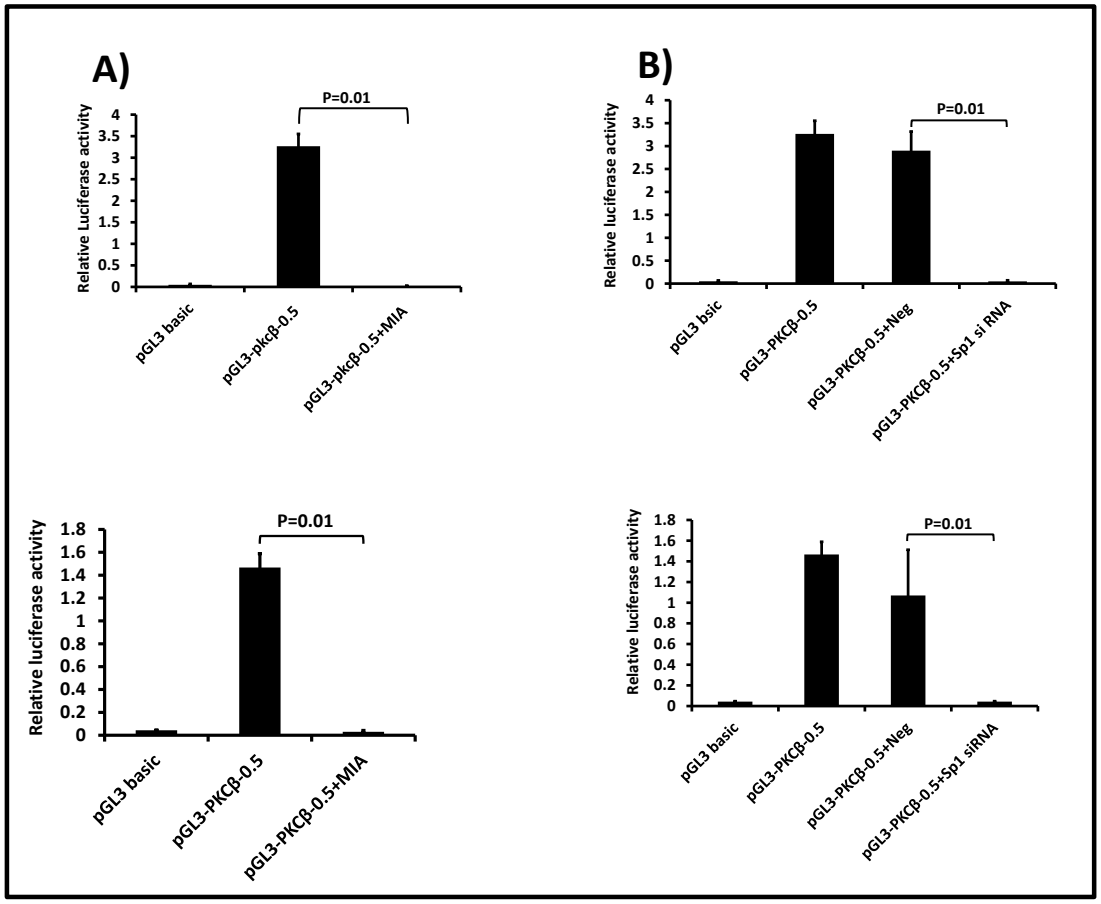
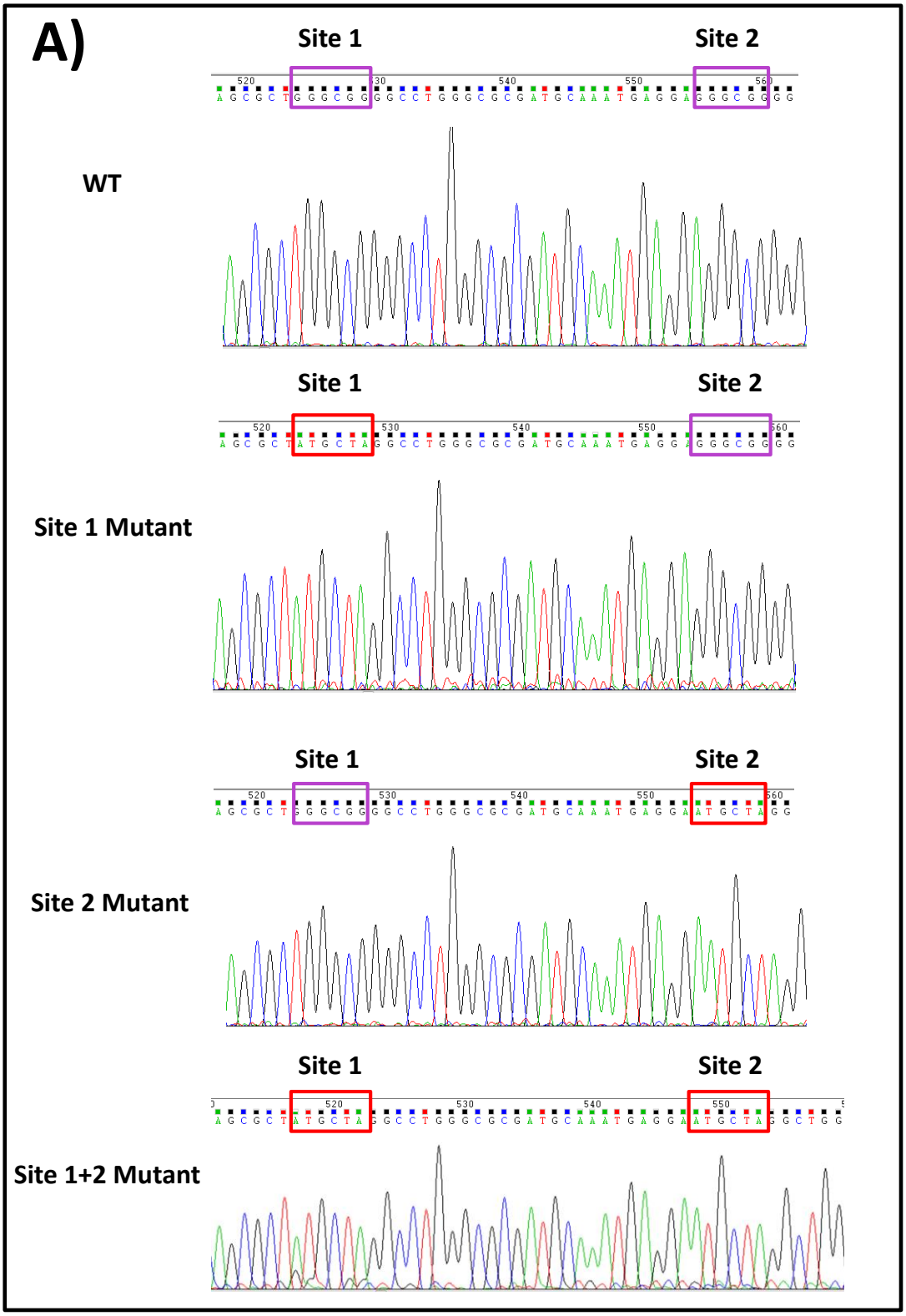


Figure 3.13: Sp1 drives *PRKCB* promoter activity. 2x10^6 Daudi (top panels) and MEC1 (bottom panels) cells were transfected with pGL3-pkcβ-0.5 and pRL according to the procedure outlined in the materials and methods. **A)** Effect of mithramycin. Following transfection, cells were cultured for 24 h under serum-rich conditions, and then transferred into serum-free for a further 48 h. For the final 24 h, 200 nM mithramycin was added where indicated. **B)** Effect of Sp1 knockdown using specific siRNA. Daudi and MEC1 cells were co-transfected with either control or Sp1-specific siRNA as indicated, and then further cultured for 72 h. Luciferase assays were performed and results are presented as activity mean±SE of firefly (promoter specific) relative to renilla (reference) of n=3 experiments. Statistical analysis was performed using a student's t-test for paired data.

3.2.12 Both Sp1 binding sites within the *PRKCB* promoter are important to its function

The promoter of *PRKCB* has two binding sites for Sp1, one at position -94 (site 1) and a second a -63 (site 2) [4]. To clarify which of these binding sites is important for driving *PRKCB* promoter activity I used site-directed mutagenesis. Thus, I created mutations within the Sp1 binding sites at site 1, site 2, or both site 1 and 2 within pGL3-pkcβ0.5 (Figure 3.14A). Sanger sequencing of the pGL3-pkcβ0.5 plasmid containing the various mutations confirmed that they had been introduced (Figure 3.14A). Transfection of these pGL3-pkcβ0.5 plasmid mutants into Daudi cells showed that mutations within either of the binding sites for Sp1, or both, within the *PRKCB* promoter resulted in a profound reduction in luciferase expression (Figure 3.14B). This finding is supported by studies of other gene promoters which show a requirement for multiple Sp1 binding sites for full promoter activity [258-260]. Importantly, this experiment demonstrates that Sp1 binding to both sites within the basal *PRKCB* promoter is required to drive activity. Because gene expression is reduced to background levels when Sp1 sites were mutated in the basal *PRKCB* promoter thus, this result implies that no other transcription factor is involved in basal promoter activity and that Sp1 is likely the main driver of PKCβ gene expression *in vivo*.


A)
Site 1
Site 2
WT
Site 1
Site 2
Site 1 Mutant
Site 1
Site 2
Site 2 Mutant
Site 1
Site 2
Site 1+2 Mutant

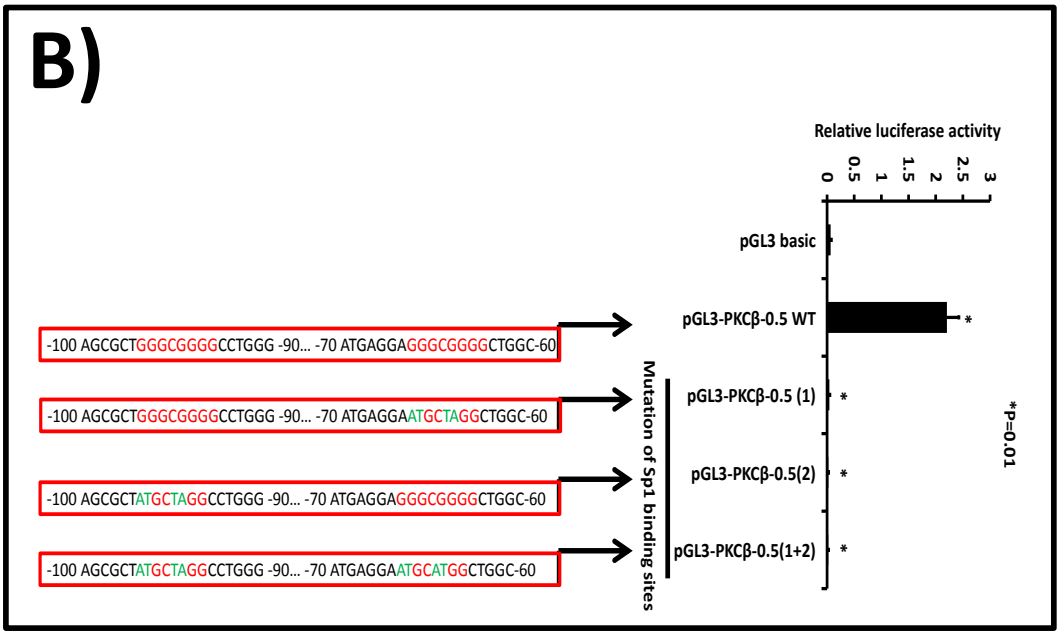


Figure 3.14: Effect of Sp1 binding site mutation on *PRKCB* promoter-driven luciferase expression. The Sp1 binding sites within the basal *PRKCB* promoter of pGL3-pkcβ0.5 were mutated by site-directed mutagenesis and validated by Sanger sequencing. **A)** Sanger sequences of pGL3-pkcβ0.5 comparing the basal *PRKCB* promoter region containing the Sp1 binding sites. The sequence enclosed by the red box shows those with mutations compared to unaltered sequences enclosed within the purple box. **B)** Daudi cells **(**$2x10^6$) were transfected with wt pGL3-pkcβ-0.5, or with pGL3-pkcβ-0.5 containing mutations within Sp1 binding site 1, site 2, or site 1 and 2 (sequence changes are denoted in green). Luciferase assays were performed following 72 h culture of the cells under serum-rich conditions, and are reported relative to renilla expression. The data are shown as mean±SE of three independent experiments. Statistical analysis was performed using a student's t-test for paired data.

3.2.13 Sp1 binds directly to the *PRKCB* promoter in CLL and MEC1 cells

To further investigate the direct role of Sp1 in regulating PKCβII gene expression I used Chromatin immunoprecipitation (ChIP). Here, I compared the binding of this transcription factor to the *PRKCB* promoter in both CLL cells and normal B cells. Figures 3.15A and B show that Sp1 binds the *PRKCB* promoter in CLL cells to a significantly greater extent than in normal B cells. In addition, I found that Sp1 is also associated with the *PRKCB* promoter sequence in MEC1 cells (Figure 3.15C). All these experiments included a negative control where a non-specific antibody was

used. Moreover, as a positive control the DHFR gene promoter was used because this gene is known to be controlled by Sp1 [261] (Figure 3.15D). Thus, Sp1 binds directly to the *PRKCB* promoter sequence.

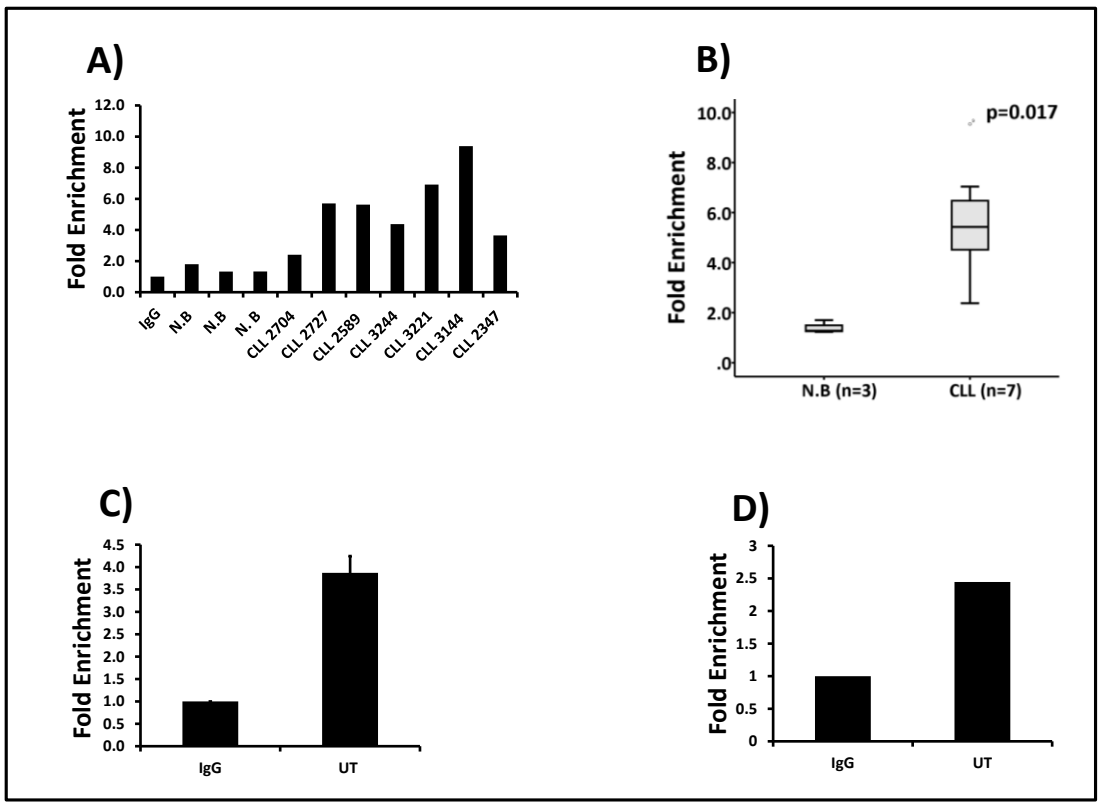


Figure 3.15: Sp1 binds to the *PRKCB* promoter sequence in CLL and MEC1 cells. CLL and normal B cell extracts were prepared for ChIP analysis, and Sp1, or a non-specific antibody was used for immunoprecipitation. The *PRKCB* promoter sequences associated with Sp1 were detected by qPCR, and are presented as fold enrichment compared to the *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. **A)** Shows the results of each single experiment for different CLL patient's samples and three samples of purified normal B cells. **B)** Shows the summary (mean±SE) of the experiments displayed in part A. Statistical analysis was performed using a Mann-Whitney U-test. **C)** Shows Sp1 association with the *PRKCB* promoter sequence in MEC1 cells. The data represents mean±SE of three independent experiments. **D)** Shows Sp1 binding to the DHFR gene promoter, a positive control for amplification of Sp1 association with a specific sequence in a promoter region of a gene.

3.2.14 Mithramycin displaces Sp1 from its cognate binding sequence within the *PRKCB* promoter in CLL and MEC1 cells

I next employed ChIP to investigate the mechanism mithramycin employs to suppress PKCβ expression in CLL and MEC1 cells. Figure 3.16 shows that Sp1 association with the *PRKCB* promoter sequence in both CLL and MEC1 cells is eliminated by the presence of 200 nM mithramycin. This result, taken together with my experiments demonstrating the role of Sp1 in driving *PRKCB* promoter function, show that mithramycin inhibits PKCβ gene expression by limiting the access of Sp1 to its binding sites. These results also suggest that Sp1 is the main driver of *PRKCB* transcription in CLL cells.

Interestingly, my experiments with CLL cells showed that Sp1 association with the *PRKCB* promoter specific sequence decreased in CLL cells after 24 h culture (Figure 3.16A). This reveals a potential regulation of PKCβ gene expression through control of Sp1 association with the promoter. This aspect is further investigated in a subsequent chapter of this thesis.

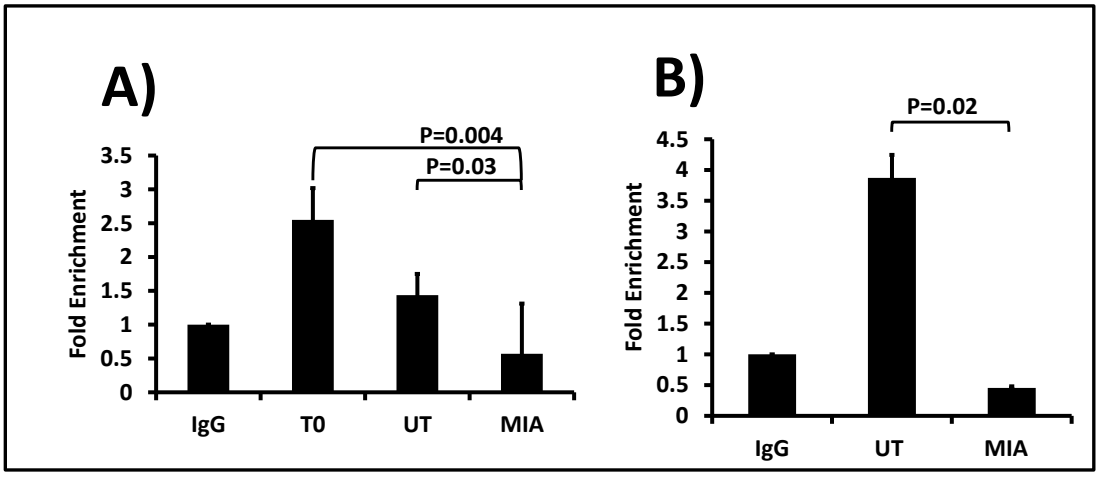


Figure 3.16: Mithramycin displaces Sp1 from the *PRKCB* promoter sequence. CLL cells ($1x10^7$) and MEC1 cells ($2x10^6$) were incubated for 24 h in the absence (UT) or presence of 200 nM mithramycin (MIA). Sp1 was immunoprecipitated from prepared extracts, and the presence of Sp1 binding to the *PRKCB* promoter was detected using qPCR. The results are presented as fold enrichment of the *PRKCB* promoter sequences associated with Sp1 compared to the IgG immunoprecipitation control. **A)** Data derived using CLL cells from (n=3) different patients. T0 represents CLL cells used directly after thawing. **B)** Data derived from MEC1 cells and 3 biological replicates. The data presented illustrates the mean±SE of the replicates used. Statistical analysis was performed using a student's t-test for paired data.

3.1.3 Discussion

At the beginning of this work it was unclear how PKCβ gene expression was regulated. Two early studies characterised the basal promoter region of *PRKCB*, and showed that they had potential binding sites for a number of transcription factors [4, 161]. However, these studies did not investigate the individual contribution of these factors in regulating PKCβ gene expression, or whether their role is direct or not. Later studies showed that PKCβ gene expression could be regulated by mechanisms involving PKCβII activity [1, 164, 262], but these studies also did not investigate the role of individual transcription factors. Therefore, in this chapter I investigated the role of Sp1. I present data clearly showing that overexpressed PKCβ gene expression in CLL cells is regulated by this transcription factor. This finding is consistent with the function of Sp1 because the promoter region of *PRKCB*

is reported to be TATA-less [4, 161], and genes regulated by Sp1 are mostly of this type [176, 263]. Thus, I provide insight into the regulation of PKCβ gene expression, insight which is important to our understanding of the pathobiology of CLL cells, and also of the malignant cells of other neoplasms where PKCβII is overexpressed [149-152, 264]. Furthermore, this finding also provides insight into the effect of malignant B cells on stromal microenvironment because of a recent paper by Lutzny *et al*., [144] showing that CLL cells induce the expression of PKCβII in adjacent microenvironmental stromal cells.

The results I present in this chapter strongly suggest that overexpression of Sp1 plays a major role in overexpression of PKCβII in CLL cells. My finding that Sp1 is overexpressed in CLL cells compared to normal B cells agrees with those of another study which used an RNA microarray to identify overexpressed Sp1 in CLL cells [265]. Thus, I demonstrate that Sp1 protein levels strongly correlate with PKCβII mRNA levels in CLL cells, and that Sp1 association with the *PRKCB* promoter drives expression of the gene. This finding is important because it suggests that overexpression of Sp1 in CLL cells may be linked to their pathophysiology. Sp1 is a pleiotropic transcription factor regulating the expression of many genes [176]. Overexpression of PKCβII is a phenotypic feature that distinguishes CLL cells from other B cell malignancies [1]. The relationship between Sp1 and PKCβII overexpression therefore suggests that Sp1 may be regulating the overexpression of other genes important to the phenotype of CLL cells. For example, TCL1, Lyn and Syk are all overexpressed in CLL cells, particularly in patients with progressive disease, and with disease at a late stage [266-268]. The promoter regions of these genes all have Sp1 binding sites, and, in particular, TCL1 and Syk are known to be regulated by Sp1 [269]. This suggests that regulation of *PRKCB* may be taken as a model to understand the phenotype of CLL cells, and possibly other tumours types, such as those associated with the lung, where increased Sp1 expression has been shown to contribute to disease progression [194].

Many of the experiments I present in this chapter use B cell lines to model the behaviour of CLL cells. MEC1 cells were derived from a patient with CLL where the malignant lymphocytes were undergoing prolymphocytic transformation [241].

MEC1 cells retain many of the phenotypic features of CLL cells; for example, these cells are CD5 and CD19 positive, and have been used to model certain aspects of CLL cell behaviour [270-272]. Importantly, for the purposes of this Chapter, PKCβII is highly expressed in this cell line. In contrast, Daudi cells were derived from a patient with Burkitt's lymphoma and maintain a different phenotype to CLL and MEC1 cells. These cells express less PKCβII than do MEC1 cells, however, they are still useful because they are easily transfected. In terms of the experiments I perform in this Chapter, these cell lines behave in a highly similar way to CLL cells. Thus, PKCβ gene expression is suppressed in MEC1, Daudi and CLL cells by mithramycin and Sp1-specific siRNA. Increasing concentrations of mithramycin in cultures of CLL, Daudi and MEC1 cells showed similar and proportional reductions in Sp1 and PKCβII mRNA levels in these cells. Mixed pool of siRNA nucleotides used to knockdown Sp1 expression also showed similar and proportional reductions in Sp1 and PKCβII mRNA levels in MEC1 cells, justifying the use of the mixed pool of siRNA oligos to knockdown Sp1 expression in Daudi and CLL cells. Further evidence of the similarity of MEC1 and CLL cells was gained from ChIP analysis of Sp1 association with the promoter region of *PRKCB*. These experiments showed that Sp1 is associated with this promoter, and can be displaced by treatment of these cells with mithramycin. Importantly, MEC1 and Daudi cells were highly useful for studying *PRKCB* promoter function within a luciferase assay. This type of experiment cannot be easily done using CLL cells. Taken together, these data provide a strong foundation for using MEC1 and Daudi cells to model PKCβ regulation in primary CLL cells. This is important for the results presented in subsequent chapters of this thesis.

The results presented in this Chapter show that Sp1 is likely to be the major driver of *PRKCB* transcription in CLL cells, and thereby brings insight to previous studies characterising the basal promoter region of *PRKCB* [4, 161]. During the preparation of the data for this Chapter, a paper by Hagiwara *et al.* investigating the regulation of PKCβ gene expression in HeLa cells was published. This paper used mithramycin, Sp1 siRNA as well as ChIP to show a role for Sp1 in regulating PKCβII expression in these cells [5]. Thus, many of the findings I present in this Chapter are confirmed by

Hagiwara study. However, I additionally show a more direct role for Sp1 in regulating *PRKCB* promoter function. Mutation of the Sp1 binding sites results in complete suppression of promoter function, suggesting a dominant role for this transcription factor in regulating PKCβ gene expression. Similar dominant roles for Sp1 in regulating gene expression are reported for other genes in different cellular contexts [273, 274]. In particular, I demonstrate the importance of both Sp1 binding sites for *PRKCB* promoter function, a finding which is similar to that reported for the control of cholesterol acetyltransferase gene expression in HepG2 cells [275]. Finally, my study of PKCβ gene regulation is the first to demonstrate linkage between Sp1 and PKCβII expression in primary cells. The study by Hagiwara *et al*. was performed only using a cell line.

❖ Chapter Four: Investigating the potential roles of other transcription factors in regulating the expression of PKCβ in CLL cells

4.1 Introduction and aims

The results of Chapter 3 clearly show a role for Sp1 in regulating PKCβ gene expression in CLL cells. However, other transcription factors such as Sp3, MITF, RUNX1, and STAT3 have been reported to be involved in regulating the expression of the PKCβ gene [5-8]. In addition, the basal promoter region also contains two E-box motifs [4], and therefore could be regulated by transcription factors involved in mitosis such as c-Myc. This has basis in a study by Weidong *et al.,* showing that MAPK pathway activation stimulated PKCβ expression in a colon cancer cell line [164]. Therefore, the aim of this chapter is to investigate the role of these factors in regulating the transcription of *PRKCB* in CLL cells.

4.2 Results

4.2.1 The role of Sp3

4.2.1.1 Sp3 protein levels in CLL and normal B cells

Sp3 can compete with Sp1 for binding to GC-regions of target genes and repress transcription [176]. Considering that CLL cells overexpress Sp1, it seemed logical that Sp3 expression might be underexpressed. Thus, to investigate this notion, I examined normal B and CLL cells for Sp3 expression by Western blot. Figure 4.1 shows that Sp3 protein is expressed at higher levels in CLL compared to normal B cells. This is an unexpected result, and suggests that Sp3 is not acting to suppress PKCβ gene expression. I next investigated the potential role of Sp3 by manipulating its expression with siRNA.

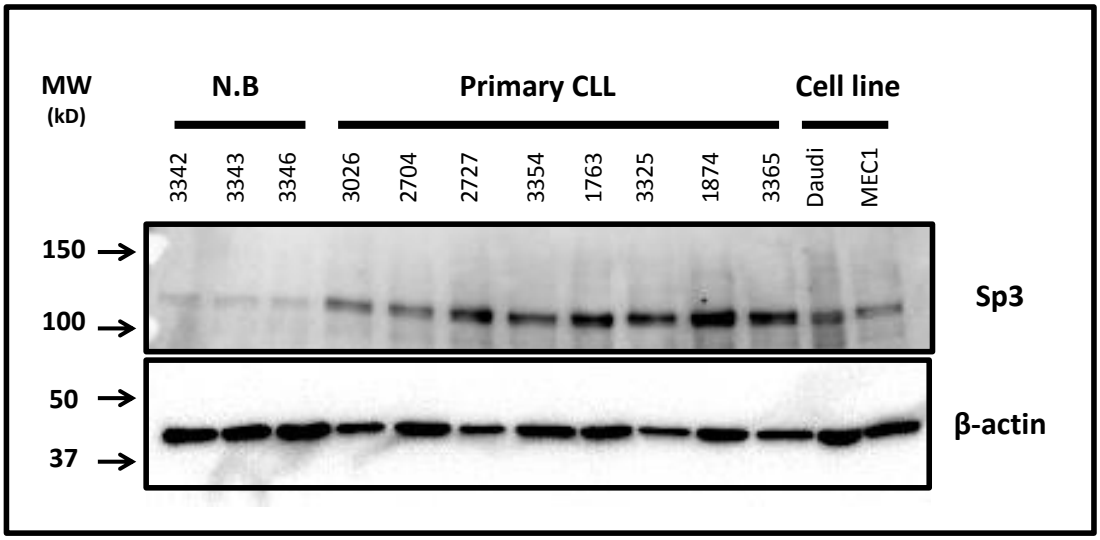


Figure 4.1: Sp3 protein levels in normal B and CLL cells. CLL and normal B cells were purified by positive selection and analysed for Sp3 protein expression by Western blot. Lysates of CLL and normal B cells were prepared and 10 μg protein was separated by SDS-PAGE (10% acrylamide gel) and transferred to PVDF membrane. The membranes were probed for the indicated antibodies. β-actin was used as a loading control.

4.2.1.2 Sp3-specific siRNA reduces PKCβII mRNA and protein levels in Daudi cells

In this section I used Daudi cells to investigate the role of Sp3 in PKCβ gene expression. Figure 4.2A shows that Sp3 mRNA and protein levels are reduced when cells are treated with Sp3-specific siRNA. Examination of PKCβ mRNA and protein levels in these treated cells also showed reduction (Figure 4.2B). These results suggest a potential role of Sp3 in promoting transcription of *PRKCB*.

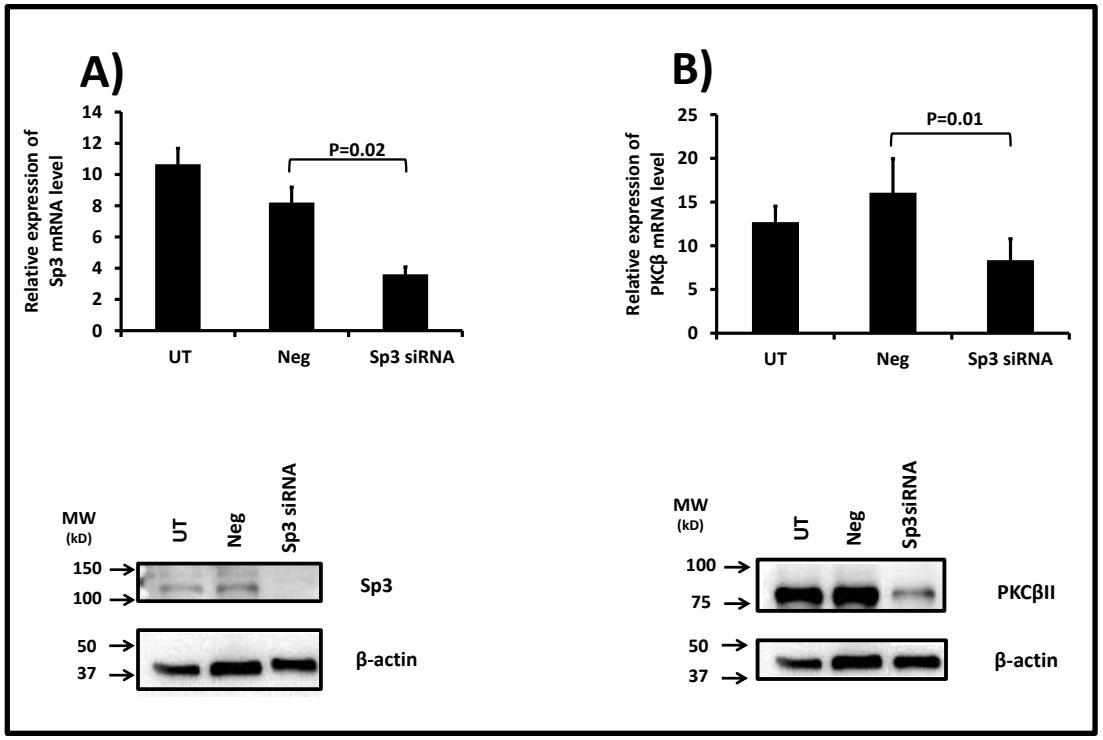


Figure 4.2: Sp3-specific siRNA reduces PKCβII mRNA and protein levels in Daudi cells. 2x10^6 Daudi cells were nucleofected with 2 μM Sp3-specific or control siRNA, and then cultured for 72 h. Harvested cells were analysed for protein expression by Western blot, and for mRNA levels by qRT-PCR. The mRNA levels were measured relative to the reference gene RNA Polymerase II and expressed as arbitrary units. Whole cell lysates were prepared and 10 μg protein separated by SDS-PAGE and transferred to PVDF membrane for detection with the indicated antibodies**. A)** Sp3 mRNA levels (*top panel*) and protein levels (*bottom panel*). **B)** PKCβII mRNA levels (*top panel*) and protein levels (*bottom panel*). Results for mRNA analysis are presented as mean±SE of n=3 separate replicates. Statistical analysis was performed using a student's t-test for paired data.

4.2.1.3 Sp3 reduces *PRKCB* promoter activity in Daudi cells

I next investigated a more direct role for Sp3 in driving *PRKCB* promoter function using a luciferase assay. Daudi cells were transfected with pGL3-PKCβ-0.5 (the minimal promoter region of *PRKCB* coupled to a luciferase gene) and with Sp3-specific siRNA. Figure 4.3 shows that knockdown of Sp3 results in profound reduction in *PRKCB* promoter activity. Taken together with the results present in section 4.2.1.2, these results support a direct role for Sp3 in driving PKCβ gene expression.

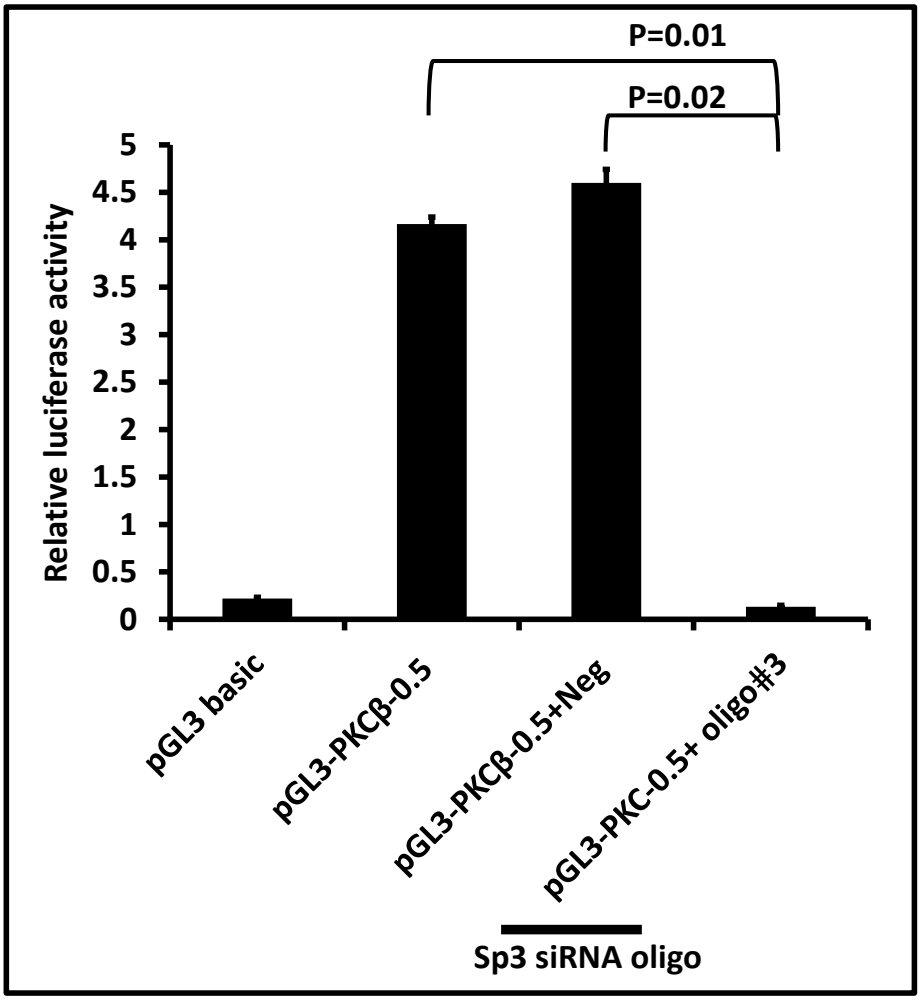


Figure 4.3: Knockdown of Sp3 expression with siRNA inhibits *PRKCB* promoter activity. 2x10^6 Daudi cells were transfected with pGL3-pkcβ-0.5 (2 μg) and pRL (0.1 μg) according to the procedure outlined in the materials and methods. Cells were cultured for 24 h under serum-rich conditions, and then transferred into serum-free for a further 48 h. In addition, the cells were co-transfected with either Sp3-specific or control siRNA (2 μM each). Luciferase assays were performed on harvested cells. The data are presented as mean±SE of three independent experiments. Statistical analysis was performed using a student's t-test for paired data.

4.2.1.4 Sp3 does not bind to the *PRKCB* promoter in CLL cells

To confirm a direct role of Sp3 in regulating *PRKCB* transcription I performed ChIP analysis for its binding to the GC-region of the *PRKCB* promoter where Sp1 binds. Figure 4.4 shows that Sp3 does not bind the *PRKCB* promoter in either normal B, CLL cells, or MEC1 cells. In contrast, a positive control shows direct binding of Sp3 to the DHFR gene promoter [276]. Thus, although gene expression analysis and promoter function assays suggest a clear role of Sp3 in driving PKCβ gene expression, my results using ChIP indicate that this role is not direct.

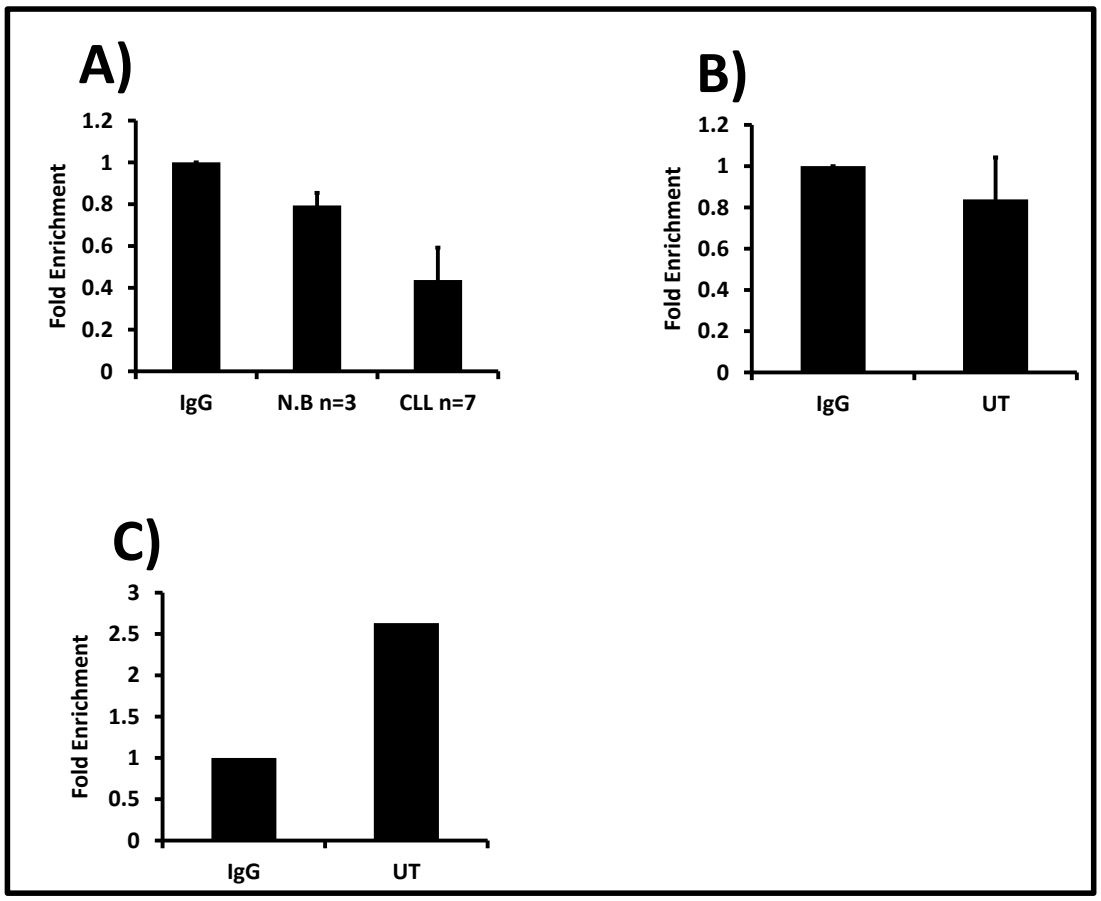


Figure 4.4: Sp3 does not bind to the *PRKCB* promoter sequence in normal B, CLL and MEC1 cells. Chromatin was prepared from $2x10^6$ MEC1 cells or from $1x10^7$ CLL and normal B cells. Sp3 was immunoprecipitated using a specific antibody, and DNA sequences associated with Sp3 were detected by qPCR amplification of the *PRKCB* or *DHFR* promoter. Results are presented as fold enrichment within Sp3 immunoprecipitates compared to non-specific IgG immunoprecipitation controls. **A)** Shows the summary (mean±SE) of Sp3 ChIP analysis with the *PRKCB* promoter in CLL cells (n=7) and normal B cells (n=3). **B)** Shows the summary (mean±SE) of Sp3 ChIP analysis with the *PRKCB* promoter in MEC1 cells (n=3). **C)** Shows Sp3 ChIP analysis with the *DHFR* promoter in MEC1 cells (n=1, positive control).

4.2.1.5 Reverse effect of Sp3/Sp1-specific siRNAs on Sp3 and Sp1 mRNAs levels in Daudi cells

It has been reported that Sp1 and Sp3 are involved in regulating the expression of each other [277, 278], and that Sp3 can associate with the Sp1 gene promoter [278]. I next investigated whether Sp3 could influence Sp1 expression within my system, thereby explaining an indirect role of Sp3 in driving PKCβ gene expression. Reduction of Sp3 with siRNA resulted in a reduction of Sp1 mRNA and protein (Figure 4.5A), indicating that Sp3 is involved in regulating expression of Sp1 in B cells. I also investigated the effect of Sp1 knockdown on Sp3 expression. Figure 4.5B shows that treatment of Daudi cells with siRNA targeting Sp1 results in increased Sp3 mRNA expression and protein expression. Thus, it is likely that Sp3 regulates *PRKCB* transcription in CLL cells by modulating the expression of Sp1.

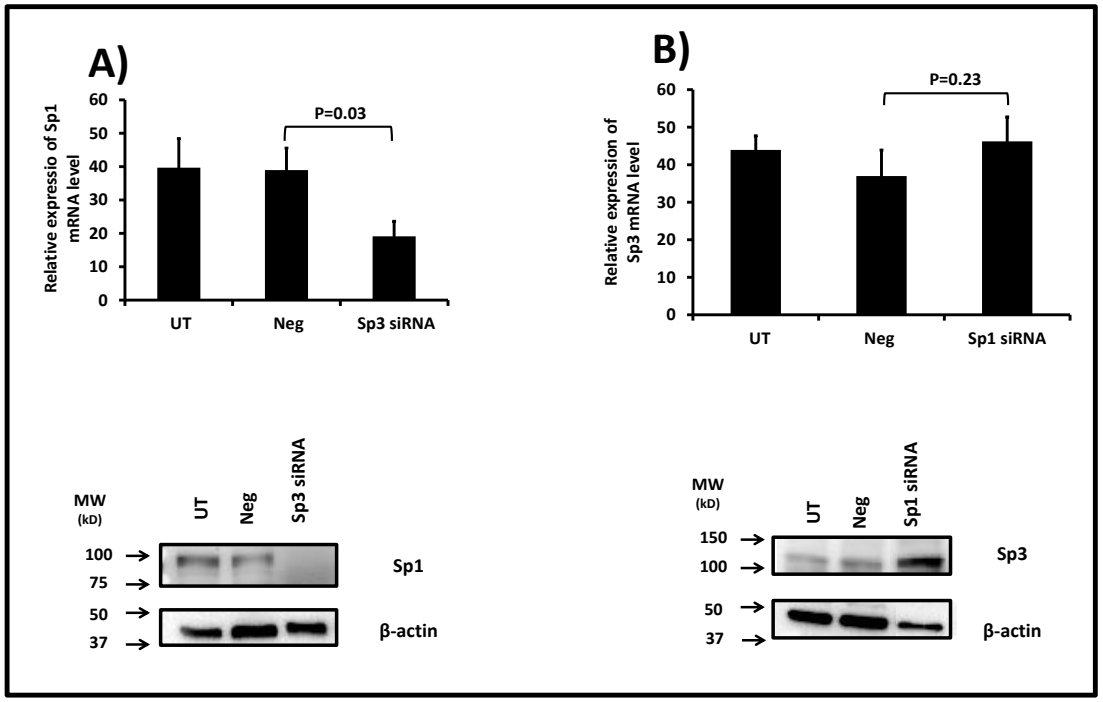


Figure 4.5: Reverse effect of Sp3/Sp1-specific siRNA on Sp1/Sp3 mRNA levels in Daudi cells. 2x10^6 Daudi cells were nucleofected with 2 μM Sp3-specific, Sp1-specific or control siRNA, and then cultured for 72 h. Harvested cells were analysed for protein expression by Western blot and mRNA levels by qRT-PCR. The mRNA levels were measured relative to the reference gene RNA Polymerase II and expressed as arbitrary units. Whole cell lysates were prepared and 10 μg protein separated by SDS-PAGE and transferred to PVDF membrane for detection with the indicated antibodies**. A**) Sp1 mRNA levels (*top panel*) and protein levels (*bottom panel*) in Daudi cells transfected with Sp3 siRNA. **B**) Sp3 mRNA levels (*top panel*) and protein levels (*bottom panel*) in Daudi cells transfected with Sp1 siRNA. In this figure the results are presented as mean±SE of n=3 separate replicates. Statistical analysis for all parts in this figure was performed using a student's t-test for paired data.

4.2.2 The Role of RUNX1

4.2.2.1 RUNX1 does not associate with the *PRKCB* promoter sequence in CLL cells

A previous paper showed that RUNX1 regulates *PRKCB* transcription in U937 cells by directly binding to the promoter of this gene [6]. In order to investigate whether this finding is similar within a CLL cell context, I performed ChIP analysis for RUNX1 binding to the *PRKCB* promoter in CLL, normal B, and MEC1 cells. Within this analysis, I used primers that amplified the section of DNA containing the RUNX1

binding site described in Hug *et al*., [6]. I found that RUNX1 does not bind to the PKCβ gene promoter in normal B, CLL, or MEC1 cells (Figure 4.6A and B). As a positive control I used RUNX1 binding to the *EVI1* promoter [279], and Figure 4.6C shows clear association of this transcription factor to this promoter in MEC1 cells. This indicates that RUNX1 likely does not play a role in the regulation of *PRKCB* transcription in CLL.

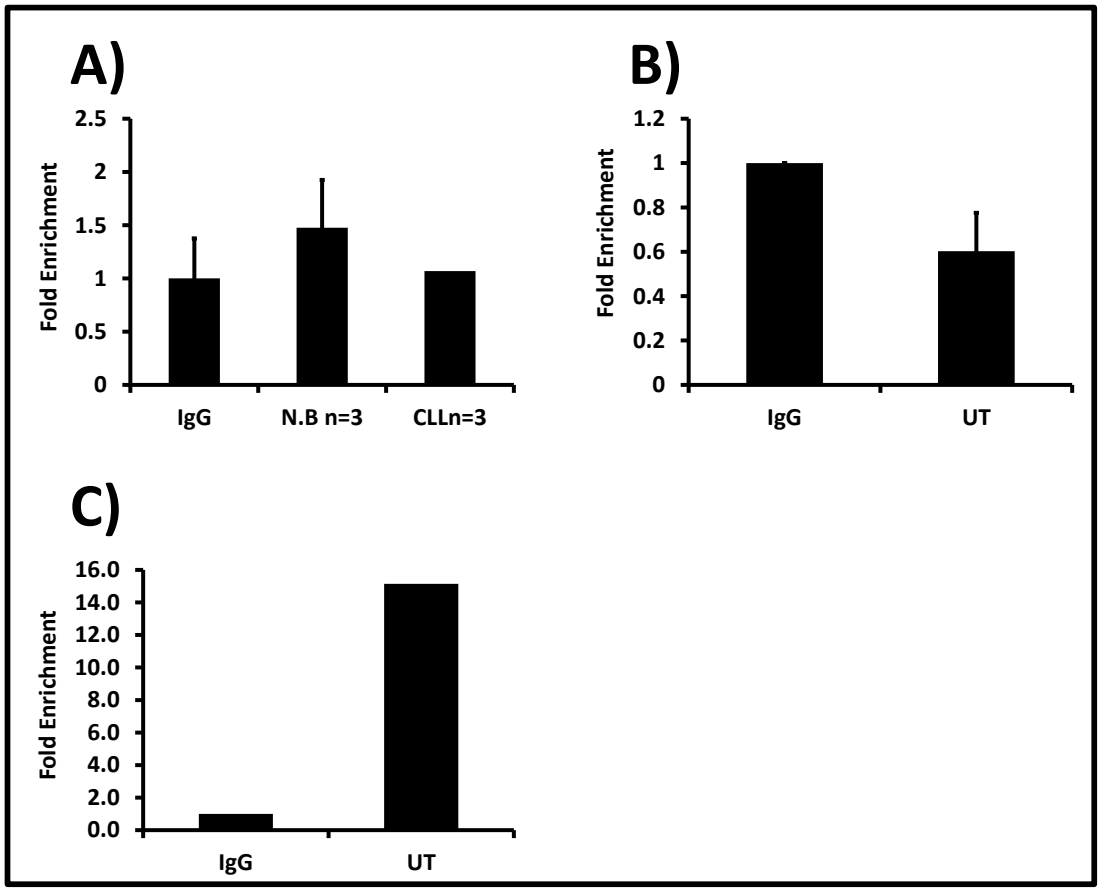


Figure 4.6: RUNX1 does not bind to the *PRKCB* promoter sequence in normal B, CLL and MEC1 cells. Chromatin was prepared from $2x10^6$ MEC1 cells or from $1x10^7$ of purified CLL and normal B cells. RUNX1 was immunoprecipitated using a specific antibody, and DNA sequences associated with RUNX1 were detected by qPCR amplification of the *PRKCB* or *EVI1* promoter. Results are presented as fold enrichment within RUNX1 immunoprecipitates compared to non-specific IgG immunoprecipitation controls. **A)** Shows the summary (mean±SE) of RUNX1 ChIP analysis within the *PRKCB* promoter in CLL cells (n=7) and normal B cells (n=3). **B)** Shows the summary (mean±SE) of RUNX1 ChIP analysis within the *PRKCB* promoter in MEC1 cells (n=3). **C)** Shows RUNX1 ChIP analysis within the EVI1 promoter in MEC1 cells (n=1, positive control).

4.2.3 The Role of MITF

4.2.3.1 Protein expression of MITF in normal B, CLL, and cell lines

In 2006 Park *et al*., suggested that MITF is involved in regulating the expression of *PRKCB* [7]. However, this observation may be cell type-specific because MITF is primarily found in melanocytes [7]. Thus, I first examined my cell system (normal B, CLL, MEC1 and Daudi cells) for MITF expression. Figure 4.7 shows Western blot analysis of MITF protein expression in these cells compared to a melanoma cell line used as a positive control. A dominant band is observed at approximately 52kD in all lanes, and is consistent with the predicted molecular weight of MITF. A weaker secondary band was also observed in most of the lanes, including the positive control, and could potentially be a proteolytic cleavage product [280]. In general, these results show that there is relatively little difference in MITF protein expression level between normal B and CLL cells. Thus, MITF is expressed in CLL cells, and could potentially play a role in regulating transcription of *PRKCB* in these cells.

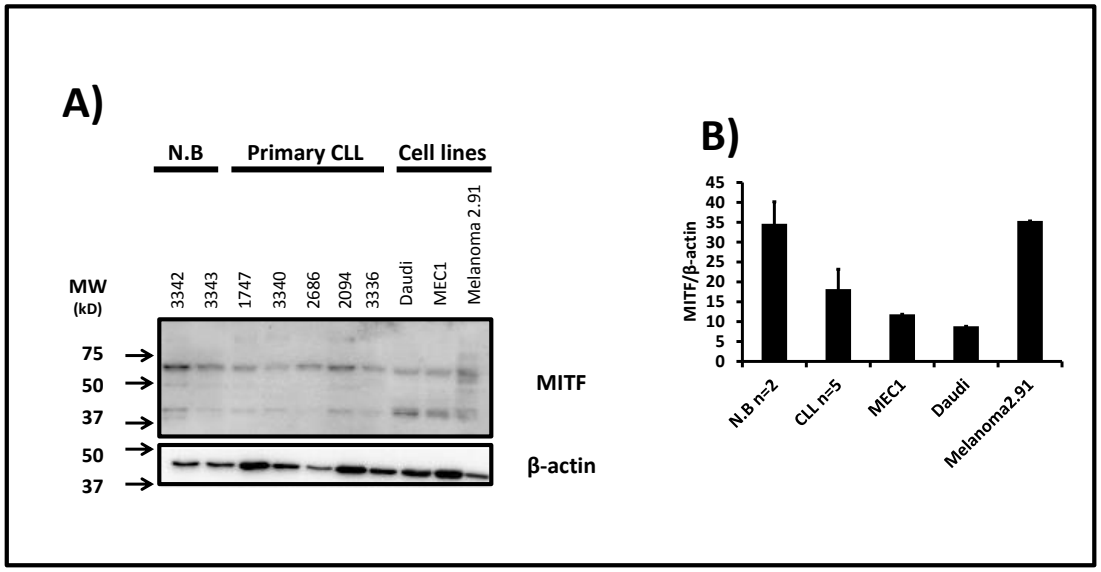


Figure 4.7: MITF protein expression in CLL, normal B, MEC1 and Daudi cells. A) Western blot analysis of cell lysates prepared from purified CLL and normal B cells, as well as from Daudi and MEC1 cells, and the melanoma 2.91 cell line used as a positive control. 10 μg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to MITF and β-actin. **B**) Graphical representation of the data presented in part A.

4.2.3.2 MITF binds within the *PRKCB* promoter sequence in CLL cells but not in normal B cells

MITF belongs to the basic helix-loop-helix/leucine zipper transcription factor group that recognizes and binds to E-box sequences within target promoters [214]. There are two potential E-box sequences within the minimal region of the *PRKCB* promoter (Figure 4.8A) [4]. To investigate whether MITF could interact with these sequences, I performed ChIP analysis. Figure 4.8B compares MITF binding to the minimal region of the *PRKCB* promoter in CLL or normal B cells. There appeared to be greater association of MITF to this region in CLL cells, but this was not significant. Nevertheless, it was considered that any MITF binding to this region was not responsible for driving PKCβ gene expression because mutation of the Sp1 binding sites within the basal promoter region eliminated promoter activity (Figure 3.14B). The notion that MITF is not responsible for driving PKCβ gene expression is in

agreement with the findings of Park *et al.,* [7] who show that overexpressed MITF does not result in enhanced basal promoter function.

However, the paper by Park *et al.,* suggested that overexpressed MITF could drive expression of a larger *PRKCB* promoter sequence [7]. To identify potential binding sites for MITF within this larger sequence, I used the PROMO3 web tool to analyse a 1kB region of the *PRKCB* promoter upstream of the transcriptional start site (TSS). I found that there is an E47 sequence that could also be recognized by MITF (Figure 4.8A). I performed ChIP for MITF targeting this region of the *PRKCB* promoter and found that MITF is strongly and significantly associated with this distal region of the PKCβ gene promoter in CLL cells compared to that in normal B cells (Figure 4.8C). This shows that MITF binds directly to the *PRKCB* promoter, but whether MITF has a direct role in regulating PKCβ gene expression in CLL cells remains undetermined.

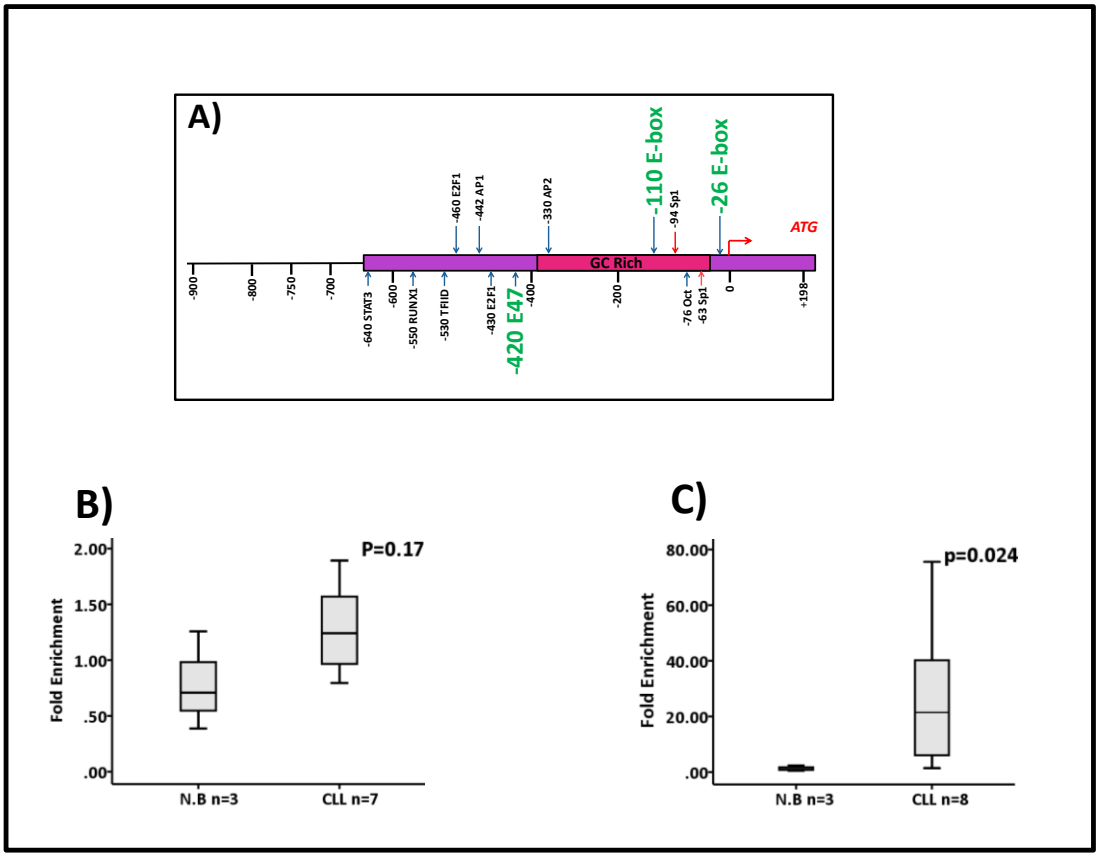


Figure 4.8: Comparison of MITF binding in distal and proximal region within *PRKCB* promoter sequence in CLL and normal B cells. 1x10^7 CLL or normal B cell extracts were prepared and MITF was immunoprecipitated using a specific MITF ChIP-grade antibody. The *PRKCB* promoter sequences associated with MITF were detected by qPCR and are presented as fold enrichment compared to the *PRKCB* promoter sequences associated with a non-specific IgG immunoprecipitation control. A) Schematic diagram of the *PRKCB* promoter 1kB upstream of the transcriptional start site. The E box and E47 sequences are highlighted in green colour to show their relationship to other potential drivers of transcription. B) Shows the results of MITF ChIP using primers to detect the E box sequences within the proximal region of the *PRKCB* promoter. C) Shows the results of MITF ChIP using primers to detect the E47 sequences within the distal region of the *PRKCB* promoter. Statistical analysis for parts B and C was performed using a Mann-Whitney U-test.

4.2.3.3 MITF-specific siRNA does not reduce PKCβ mRNA levels in MEC1 cells

To elucidate the specific role of MITF in regulating PKCβ gene expression, I used specific siRNA to examine how knockdown of MITF expression affected PKCβ mRNA levels in MEC1 cells. Figure 4.9 shows that reduction of MITF expression in these cells did not result in reduction of PKCβ mRNA levels. Interestingly, and seemingly in contrast to the observations of Park et al, a reduction in MITF expression in my system resulted in an increase in PKCβ mRNA. The degree to which PKCβ mRNA levels where changed, seemed related to the ability of the different oligos I used to knockdown MITF expression. Thus, Oligo 3 > Oligo 1 > Oligo 2 in ability to knockdown MITF expression, and Oligo 3 > Oligo 1 > Oligo 2 in ability to increase PKCβ mRNA levels. I also used a mixture of oligos 1, 2 and 3 to knockdown MITF expression, and although this was effective in reducing MITF expression, PKCβ mRNA levels remained similar to those in the control sample. The reason for this discrepancy is unclear, but could be the result of off-target effects. Nevertheless, these results indicate that MITF likely plays no role in promoting PKCβ gene expression.

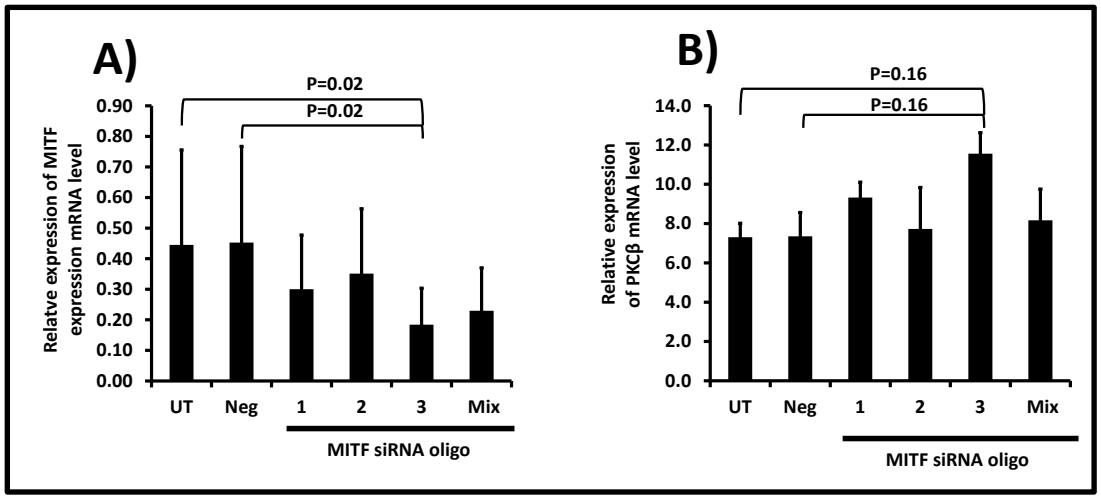


Figure 4.9: MITF-specific siRNA does not reduce the expression of PKCβ mRNA levels in MEC1 cells. $2x10^6$ MEC1 cells were transfected with 500 nM MITF-specific siRNA oligonucleotides or siRNA-negative control. Following 72 h culture the cells were harvested and analysed for mRNA levels by qRT-PCR. **A)** MITF mRNA levels measured by qRT-PCR. **B)** PKCβ mRNA levels measure by qRT-PCR. The mRNA levels for MITF and PKCβ were measured relative to the reference gene RNA Polymerase II, and expressed in arbitrary units as mean±SE of n=3 separate replicates. Statistical analysis for all parts in this figure was performed using a student's t-test for paired data.

4.2.3.4 MITF associates with Sp1 in MEC1 cells

Park *et al.,* [7] suggested that MITF may work with other factors to modulate expression of PKCβ within their system. Since my work has so far demonstrated a clear role for Sp1 in regulating PKCβ gene expression, it seemed logical to investigate whether MITF co-associated with this latter transcription factor. Figure 4.10 shows that immunoprecipitation of MITF from MEC1 cell lysates resulted in co-immunoprecipitation of Sp1. However, the ChIP data presented in Figure 4.8A indicate no significant difference in MITF association with the basal promoter region of *PRKCB* in CLL and normal B cells despite the much higher levels of Sp1 associated with this region (Figure 3.15B). These data indicate that although MITF may co-associate with Sp1, this association likely does not take place in the context of the *PRKCB* promoter, or that the *PRKCB* promoter is organised in a non-linearized fashion to promote MITF association with Sp1.

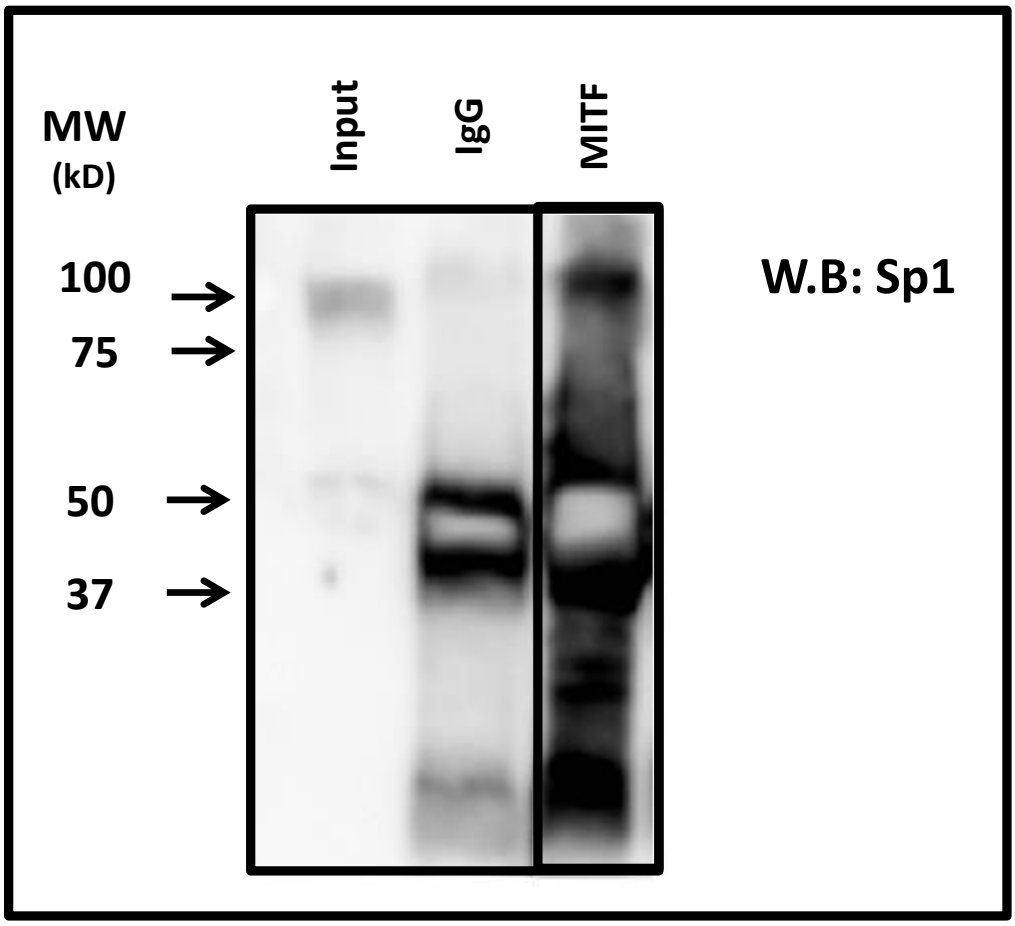


Figure 4.10: MITF associates with Sp1 in MEC1 cells. $1x10^7$ MEC1 cells were lysed with RIPA buffer, and lysates were immunoprecipitated with IgG control or MITF-specific antibodies. Immunoprecipitates were separated by SDS-PAGE alongside 10μg whole cell lysate used as an input control. Western blots were developed using Sp1 antibodies.

4.2.4 The Role of c-Myc

4.2.4.1 Expression of c-Myc in normal B and CLL cells

E-box sequences within gene promoters can bind c-Myc and become activated for transcription [281]. Moreover, it is known that c-Myc can contribute to the pathobiology of CLL cells, particularly those cases experiencing BCR engagement [282]. Finally, work by Cejas *et al*., has demonstrated a role for mitogenic signalling in the regulation of PKCβ gene expression [262]. To investigate whether c-Myc played a role in regulating PKCβ expression I first compared protein expression of this transcription factor in B and CLL cells. Figure 4.11 shows, as expected, that CLL cells generally expressed more c-Myc protein than did normal B cells. Therefore, c-Myc may have a potential role in regulating *PRKCB* transcription in CLL cells.

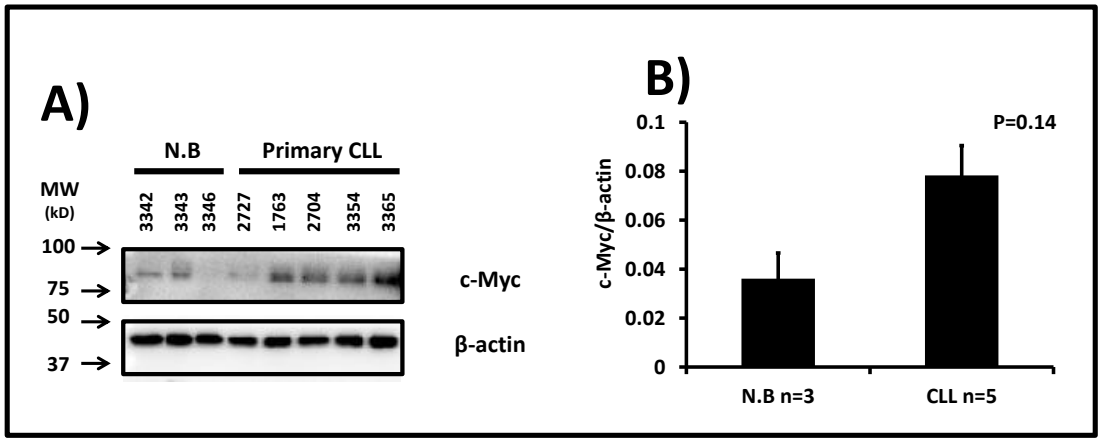


Figure 4.11: c-Myc protein expression in normal B and CLL cells. Western blot analysis of cell lysates prepared from purified CLL and normal B cells. **A)** 10 μg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to c-Myc and β-actin as loading control. **B)** Graphical representation of the data presented in part A.

4.2.4.2 The c-Myc inhibitor 10058-F4 reduces PKCβ mRNA levels in MEC1 and Daudi cells

To characterise the potential role of c-Myc in regulating *PRKCB* transcription, I initially used the specific c-Myc inhibitor 10058-F4. Figures 4.12A and B show PKCβ mRNA levels were reduced following treatment of MEC1 and Daudi cells with 10058-F4 regardless of whether the cells were cultured under serum rich or serum free conditions. These results indicate that c-Myc has a potential role in regulating PKCβ gene expression, but two issues need to be clarified: Firstly, whether the effect of 10058-F4 is specific or not, and, secondly, whether the role of c-Myc is direct or indirect.

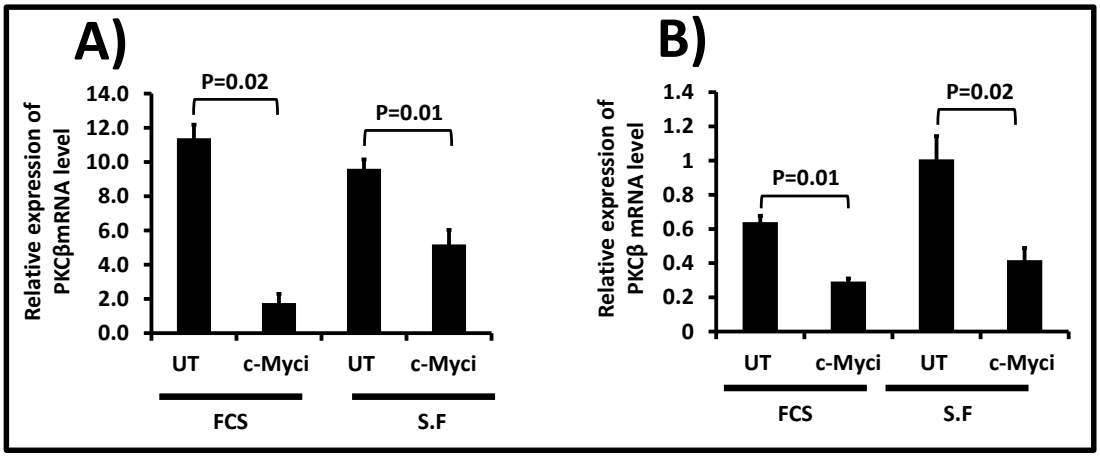


Figure 4.12: c-Myc inhibitor inhibits PKCβII mRNA levels in MEC1 and Daudi cells. $2x10^6$ were cultured under serum free (SF) or serum rich (FCS) medium conditions for 24h in absence (UT) or presence of 60 nM c-Myc inhibitor(c-Myci). A) PKCβII mRNA levels in MEC1 cells. B) PKCβII mRNA levels in Daudi cells. This experiment illustrates the mean ± SE of three separate biological replicates. The PKCβ mRNA level was measured relative to RNA Polymerase II by qRT-PCR and is represented as arbitrary units.

4.2.4.3 c-Myc-Specific siRNA decreases PKCβII mRNA levels in MEC1 and Daudi cells

To investigate the specificity of 10058-F4 in targeting c-Myc I used siRNA to knockdown expression of the transcription factor in MEC1 and Daudi cells. Figure 4.13 shows that knockdown of c-Myc expression results in reduction of PKCβ mRNA levels whereas treatment of MEC1 or Daudi cells with control siRNA has no effect. In addition, Figures 4.13C and D show that knockdown of c-Myc with siRNA reduced Sp1 protein expression. Taken together with the data generated using 10058-F4, these results suggest that c-Myc regulates *PRKCB* transcription, but whether the regulatory mechanism is direct or not still needs to be examined.

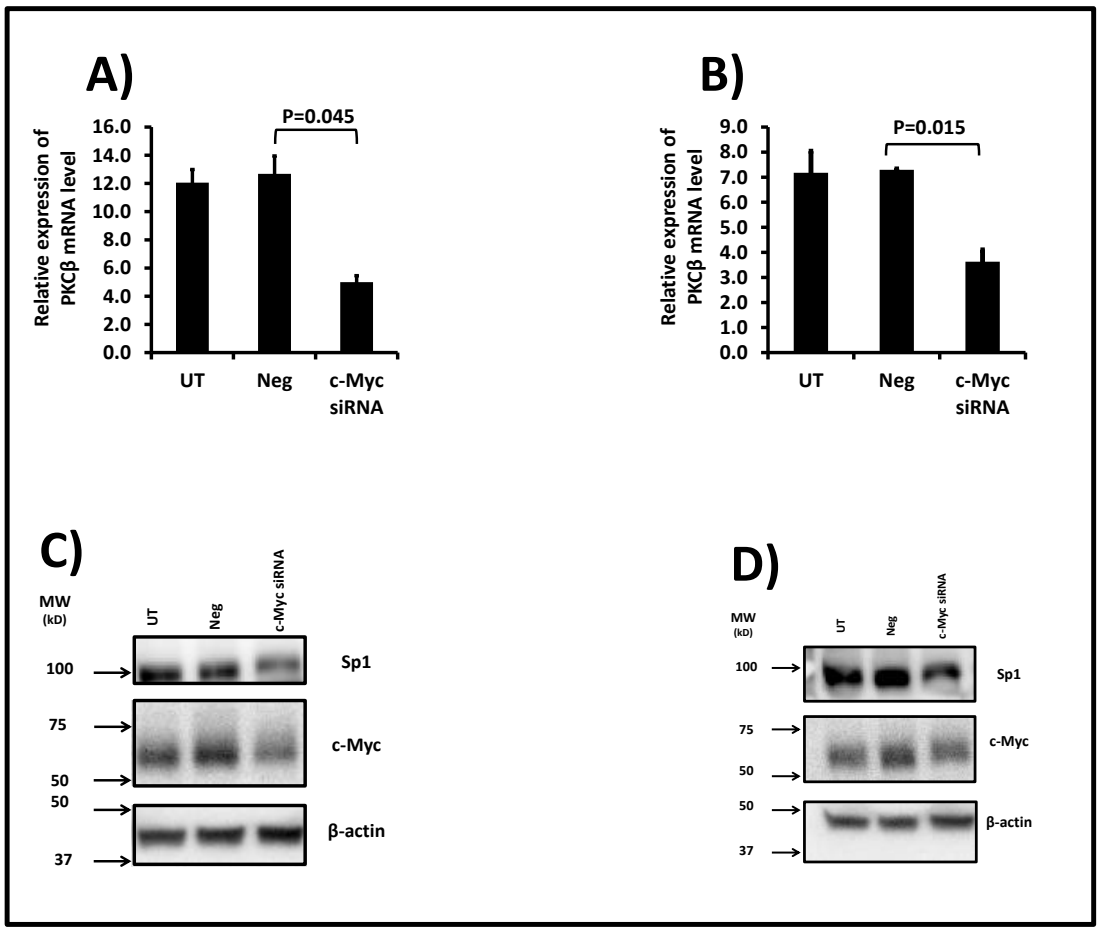


Figure 4.13: c-Myc-specific siRNA reduces PKCβII mRNA in MEC1 and Daudi cells. MEC1 and Daudi cells ($2x10^6$) were nucleofected with 100 nM of c-Myc-specific or control siRNA oligonucleotides. The cultured cells were harvested after 72 h and were analysed for protein expression by Western blot, and for mRNA levels by qRT-PCR. The PKCβ mRNA levels were measured relative to the reference gene RNA Polymerase II and presented as arbitrary units. **A)** PKCβII mRNA levels in MEC1 cells. **B)** PKCβII mRNA levels in Daudi cells. **C)** and **D)** Western blot analysis of MEC1 and Daudi cell lysates for the indicated proteins, respectively. Graphical data are presented as arbitrary units where each graph represents mean±SE of n=3 separate experiments. β-actin is used as a loading control for Western blots. Statistical analysis for parts A and B was performed using a student's t-test for paired data.

4.2.4.4 c-Myc does not bind to the *PRKCB* promoter in MEC1 cells

c-Myc is a member of the helix-loop-helix/leucine zipper family of nuclear transcription factors that recognize and bind to E-box sequences in target gene promoters [283]. The *PRKCB* promoter has two potential E-box binding sites in its minimal region (Figure 4.9A) [4]. Thus, to examine whether c-Myc binds to these E-

box sequences within the *PRKCB* promoter, I performed ChIP analysis. I found that c-Myc does not associate with the E-box sequences within the basal promoter of *PRKCB* (Figure 4.14A) in MEC1 cells. To ensure that my assay was working I analysed c-Myc binding to the Nucleolin gene promoter which also contains E-box sequences [284]. This experiment showed clear association of c-Myc with this gene promoter (Figure 4.14B). My analysis of the *PRKCB* promoter with the PROMO3 web tool showed that c-Myc had no other potential binding sites within the proximal or distal regions. Therefore, it is likely that this transcription factor does not have a direct role in regulating transcription of *PRKCB*. However, the results using the c-Myc inhibitor and c-Myc-specific siRNA suggests an indirect role for c-Myc, which is investigated in the next section.

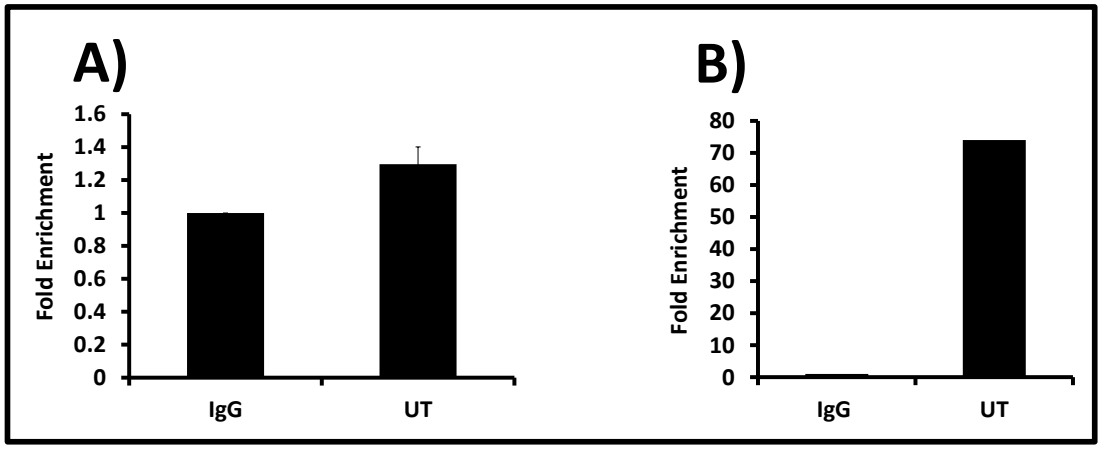


Figure 4.14: c-Myc does not associate with the *PRKCB* promoter in MEC1 cells. Chromatin extracts were prepared from $2x10^6$ MEC1 cells, and were immunoprecipitated using a specific antibody for c-Myc. c-Myc association to the E-box sequences within the *PRKCB* promoter was detected using qPCR. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with a non-specific IgG immunoprecipitation control. **A)** Shows the results of c-Myc ChIP within *PRKCB* promoter. **B)** Shows the results of c-Myc ChIP within the Nucleolin promoter region covering the c-Myc binding site.

4.2.5 The Role of E2F1

4.2.5.1 Protein expression of E2F1 in normal B and CLL cells

The PROMO3 web tool I used to analyse the promoter region of *PRKCB* identified two E2F1 binding sites within the 1kB region upstream of the transcription start site. Expression of E2F1 can be regulated by c-Myc [285], and this warranted further investigation of its potential role in regulating PKCβ gene expression. Therefore, to investigate this role I began by examining the expression of E2F1 protein levels in normal B and CLL cells by Western blot analysis. Figure 4.15 shows that E2F1 protein is expressed in CLL, but is barely detected in normal B cells.

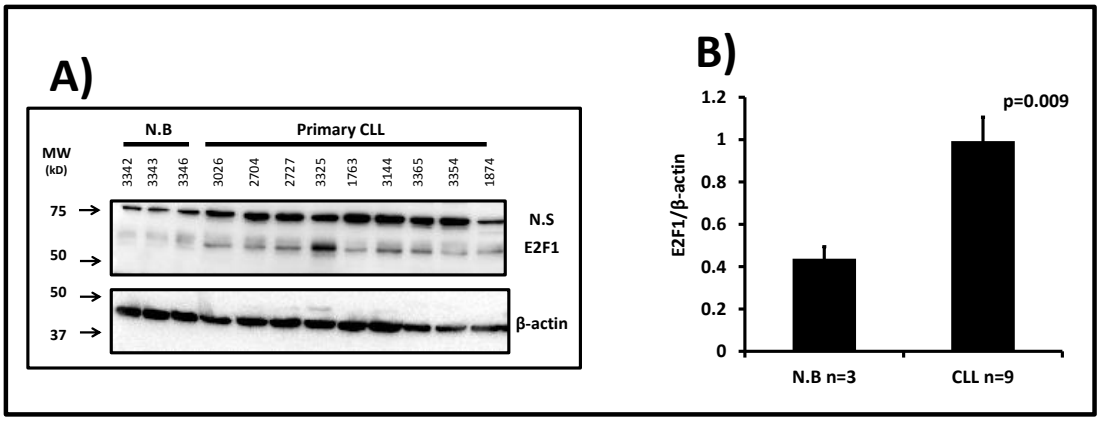


Figure 4.15: E2F1 protein expression in normal B and CLL cells. Western blot analysis of the cell lysate prepared from purified CLL and normal B cells. **A)** 10 μg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to E2F1 and β-actin. A nonspecific band appeared above E2F1 and is labelled as N.S **B)** Graphical representation of the data presented in part A.

4.2.5.2 E2F1-Specific siRNA inhibits the expression of Sp1 and PKCβII mRNA and protein levels in MEC1 and CLL cells

To investigate the specific role of E2F1 in regulating the expression of the PKCβ gene I used E2F1-specific siRNA oligonucleotides to decrease E2F1 protein levels in MEC1 and CLL cells. Figures 4.16A and D show that PKCβ mRNA levels are reduced in, respectively MEC1 and CLL cells, treated with E2F1 siRNA in comparison to those

treated with control siRNA. This suggested a role for E2F1 in regulating PKCβ gene expression. However, E2F1 is also known to regulate Sp1 expression[257] and it was therefore necessary to investigate whether this was happening in my system. Figures 4.16B and E show that knockdown of E2F1 resulted in reduction of Sp1 mRNA in MEC1 and CLL cells. For completeness, I also analysed protein expression of E2F1, Sp1 and PKCβII in the treated cells. Figures 4.16C and F show that E2F1 siRNA eliminated E2F1 protein expression, and also of Sp1 protein. PKCβII protein expression was also reduced, with a greater effect being observed in MEC1 cells than in CLL cells. These results strongly suggest that E2F1 has a role in regulating *PRKCB* transcription in CLL cells, but it is unclear whether this role is direct owing to the role E2F1 may in regulating Sp1 expression. Nevertheless, figures 4.13C and D show that knockdown of c-Myc with siRNA also reduces Sp1 protein expression [285]; an observation that, taken together with the data presented in this section with the known role of c-Myc in regulating E2F1 expression, provides a possible explanation of why knockdown of c-Myc reduces PKCβ mRNA levels.

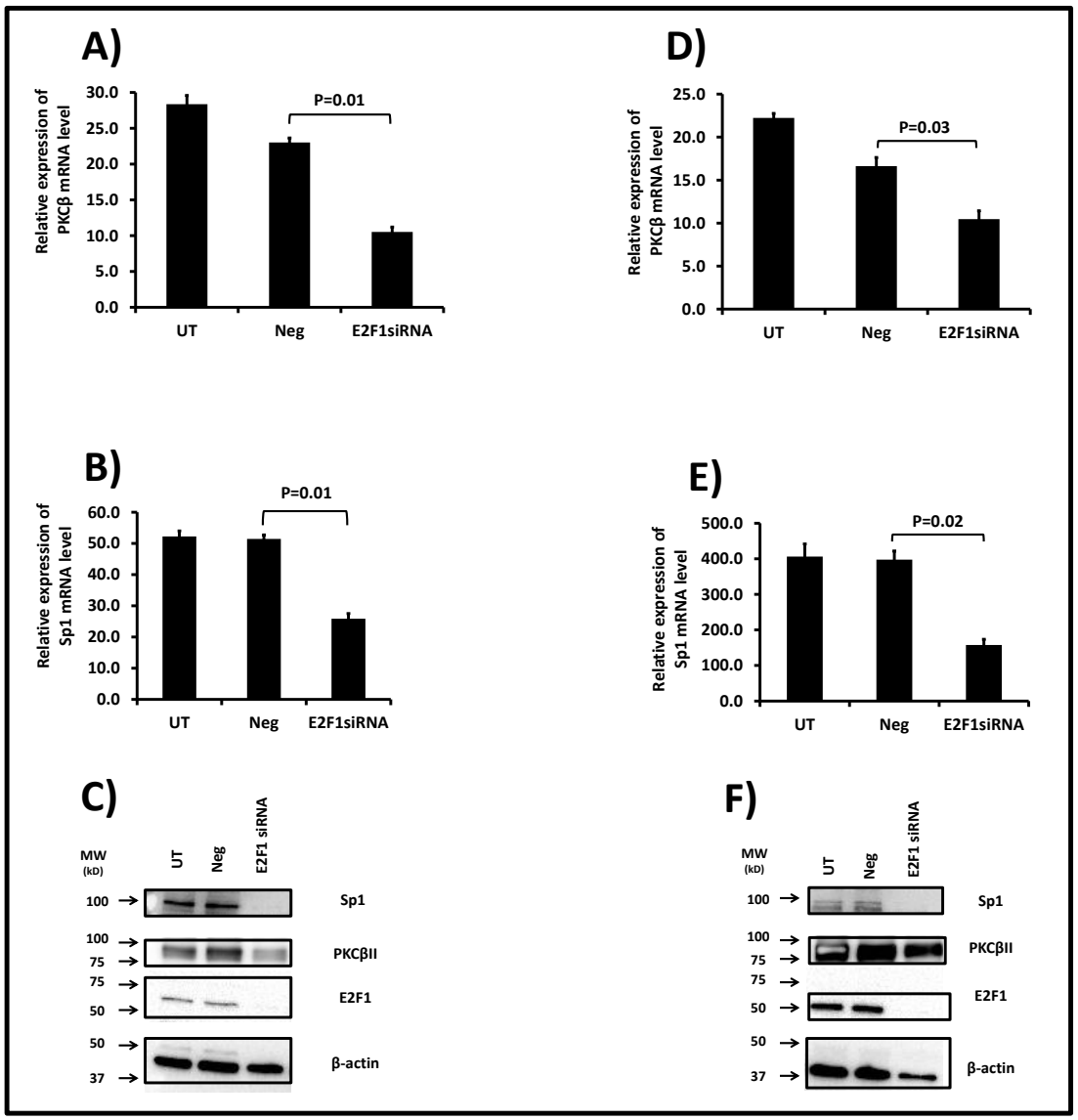


Figure 4.16: E2F1-specific siRNA reduces Sp1 and PKCβII mRNA and protein levels in MEC1 and CLL cells. $2x10^6$ MEC1 or $1x10^7$ CLL cells were nucleofected with 100 nM E2F1-specific or control siRNA oligonucleotides. The cultured cells were harvested after 72 h and were analysed for protein expression by Western blot and for mRNA levels by qRT-PCR. Measured mRNA levels are relative to RNA Polymerase II, reference gene, and presented as arbitrary units. **A)** PKCβ mRNA levels in MEC1 cells. **B)** Sp1 mRNA levels in MEC1. **D)** PKCβ mRNA levels in CLL cells. **E)** Sp1 mRNA levels in CLL cells. **C)** and **F)** Western blot analysis of MEC1 and CLL cell lysates for the indicated proteins. Graphical data are presented as arbitrary units where each graph represents mean±SE of n=3 separate experiments. β-actin is used as a loading control for Western blots. Statistical analysis for parts A, B, D and E was performed using a student's t-test for paired data.

4.2.5.3 E2F1 binds to the *PRKCB* promoter sequence in CLL cells

I next investigated whether E2F1 could bind directly to the *PRKCB* promoter using ChIP. Figure 4.17 shows that E2F1 associates strongly with the *PRKCB* promoter in MEC1 cells as well as in CLL cells, and that this binding in CLL cells was significantly higher than that observed in normal B cells. As a positive control E2F1 binding to the *DHFR* promoter was used [286].

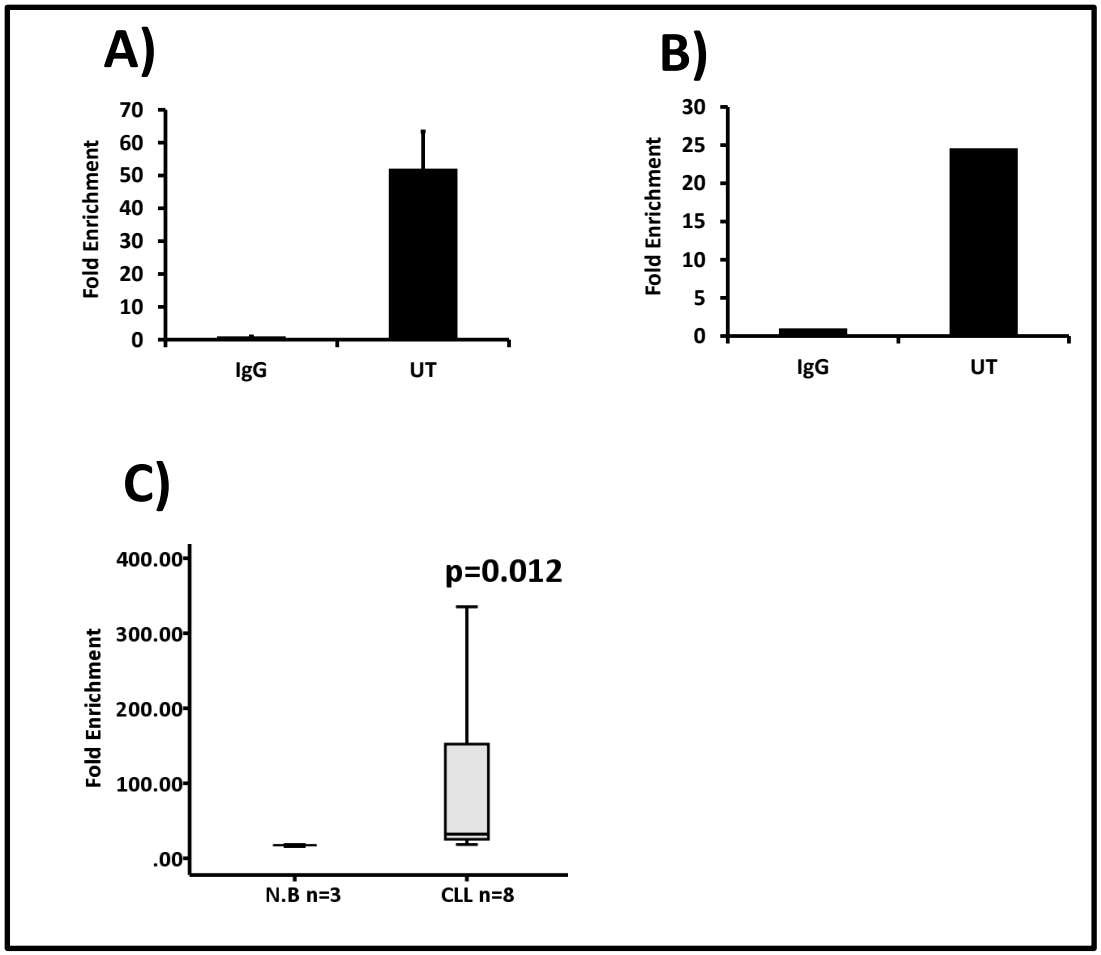


Figure 4.17: E2F1 associates with the distal region of the *PRKCB* promoter sequence. Chromatin extracts from MEC1 cells ($2x10^6$) or from purified CLL and normal B cells ($1x10^7$ each) were prepared, and E2F1 was immunoprecipitated using a specific antibody. E2F1 association within the distal region of *PRKCB* promoter was detected by using qPCR and primers that covered the identified E2F1 binding sequences within the promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with a non-specific IgG immunoprecipitation control. **A)** E2F1 association with the *PRKCB* promoter in MEC1 cells. **B)** E2F1 association with the DHFR promoter region in MEC1 cells, used as a positive control. **C)** Comparison of E2F1 association with the *PRKCB* promoter in eight different cases of CLL cells and three cases of normal B cells. Statistical analysis was performed using a Mann-Whitney U-test.

4.2.6 The Role of total STAT3

4.2.6.1 STAT3 represses *PRKCB* promoter activity in MEC1 cells

The final transcription factor I consider in this Chapter is STAT3. As a driver for the expression of many genes that play key roles in the pathophysiology of CLL cells [243, 287, 288], STAT3, therefore, deserves consideration. This is particularly relevant because a recent study identified four potential binding sites for STAT3 within the distal promoter region of *PRKCB* ([8] and Figure 4.18), and also showed that one of these sites (site 4), was important for the repression of gene expression during monocyte differentiation. Thus, to understand the role of STAT3 in regulating PKCβ gene expression in CLL cells, I first compared the activity associated with the basal *PRKCB* promoter (pGL3-pkcβ0.5kb) with that associated with larger promoter containing the STAT3 binding sites (pGL3-pkcβ1.2kb) in MEC1 cells. Figure 4.19A shows that the basal *PRKCB* promoter was significantly more active than the pGL3-pkcβ1.2kb construct containing the STAT3 binding sites. This finding agreed with those of previous studies [4, 8, 161], and suggested a potential of STAT3 binding to the *PRKCB* promoter in CLL cells. I next compared the role of each STAT3 binding site within the *PRKCB* promoter by site-directed mutagenesis to alter these sites so that STAT3 could no longer bind. Thus, mutation of sites 1 or sites 2 plus 3 had no effect on promoter activity, whereas mutation of site 4 resulted in significantly increased promoter activity (Figure 4.19B). However, this increase did not reach the activity of the basal promoter (Figure 4.19A), suggesting that other repressive elements are present. Taken together, these results indicate that STAT3 acts as a repressor for the transcription of *PRKCB* by binding to its gene promoter.

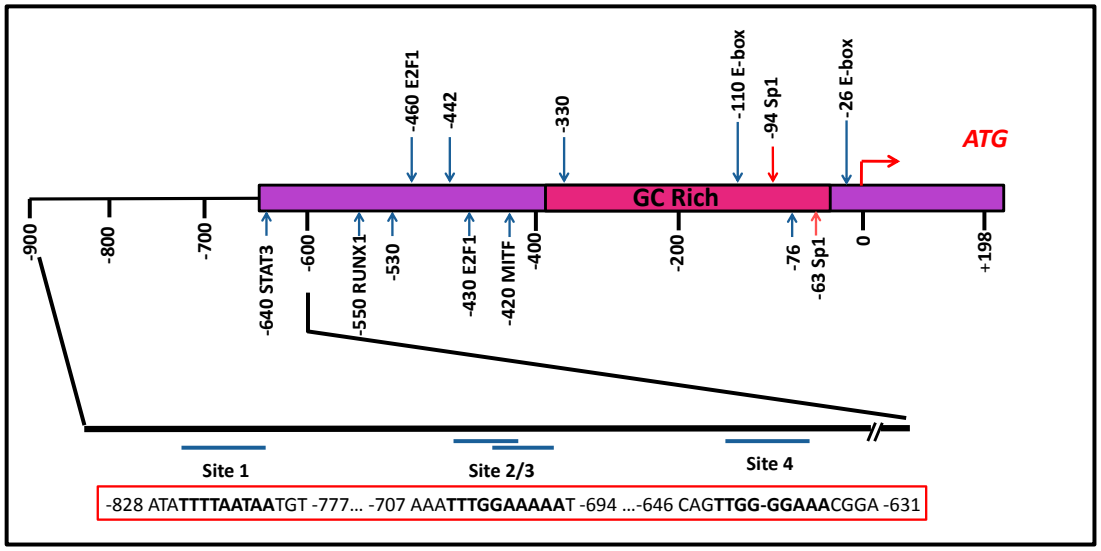


Figure 4.18: Schematic diagram of the *PRKCB* promoter showing the STAT3 binding sites. This schematic drawing shows the binding sites for STAT3 within the *PRKCB* promoter in relation to the binding sites of the other transcription factors discussed in this Chapter. The transcription start site is labelled by red arrow and the translational start site labelled as ATG in red. The sequences for the STAT3 binding sites within the *PRKCB* promoter are shown within the inset red box.

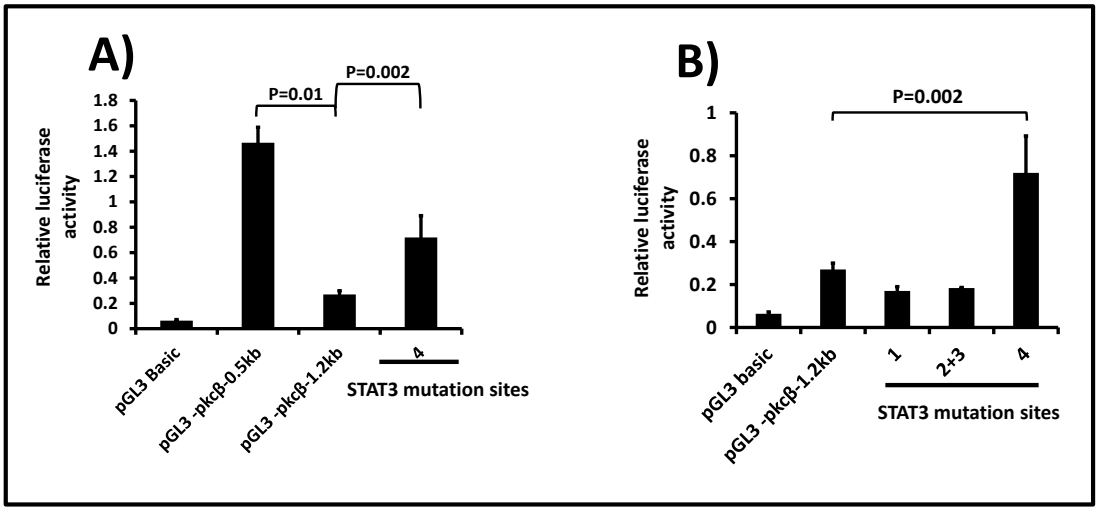


Figure 4.19: STAT3 suppresses *PRKCB* promoter activity. MEC1 cells $2x10^6$ were co-transfected with (0.1 µg) pRL and (2 µg each) of wt pGL3-pkcβ-1.2kb, wt PGL3-pkcβ-0.5kb, or different mutants of STAT3 binding sites within pGL3-pkcβ-1.2kb constructs. STAT3 binding sites within *PRKCB* promoter were altered by site-directed mutagenesis. **A)** Shows the constructs activities of wt pGL3-pkcβ-0.5, wt pGL3-pkcβ-1.2kb, or pGL3-pkcβ-1.2kb containing mutations within STAT3 binding site 4. **B)** Shows the constructs activities of wt pGL3-pkcβ-1.2 kb or mutant for STAT3 binding; sites 1, site 2+3, or site 4; within pGL3-pkcβ-1.2kb.Luciferase assays were performed following 72 h culture of the cells under serum-rich conditions, and are reported relative to renilla expression. The data represented mean±SE of three independent experiments. Statistical analysis was performed using a student's t-test for paired data.

4.2.6.2 STAT3 binds to *PRKCB* promoter in normal B and CLL cells

I next examined STAT3 binding to the *PRKCB* promoter in primary CLL cells by ChIP. Here, I examined STAT3 binding specifically to site # 4 within the promoter region isolated from CLL cells, and compared this to the binding observed for the same region isolated from B cells. Figure 4.20A shows that STAT3 binds to the distal region of the *PRKCB* promoter in normal B and CLL cells. The binding of STAT3 to this region in normal B cells appeared similar in the three cases tested, however, variability was observed with respect to the CLL cell samples. Some cases showed high levels of STAT3 association with the *PRKCB* promoter, whereas others showed little or no association. To ensure that this observation was not a technical artefact I used STAT3 association to the c-Fos gene promoter as a positive control [289] (Figure 4.20C). Those cases of CLL where STAT3 binding to the *PRKCB* promoter was

observed to be low or absent, showed STAT3 binding to the c-Fos gene promoter. This indicates that the immunoprecipitation reaction worked, and validates the variability of STAT3 binding to the *PRKCB* promoter in CLL cells. Nevertheless, comparison of STAT3 binding to this region in CLL with normal B cells showed no significant difference when analysed either with Mann-Whitney U-test, or Fischer's exact test (Figure 4.20B). Thus, these results indicate that STAT3 binds to the *PRKCB* promoter in CLL cells, but raises the question of the cause for the observed variability in binding. This I partially address in the next section.

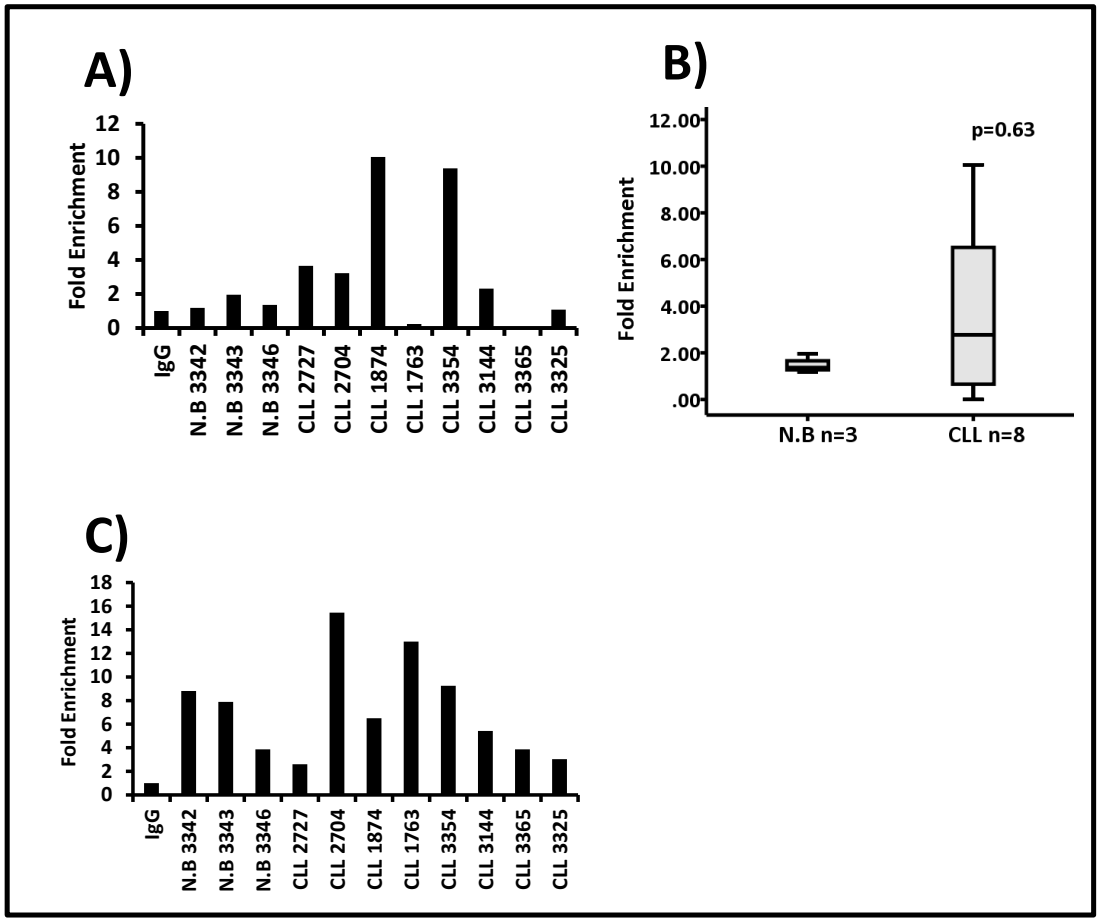


Figure 4.20: STAT3 associates with the *PRKCB* promoter in CLL and normal B cells. Chromatin extracts from $1x10^7$ purified CLL or normal B cells were prepared, and STAT3 was immunoprecipitated using a specific antibody. STAT3 association within the *PRKCB* promoter distal region was detected by using qPCR and primers covering the STAT3 binding site. The results are presented as fold enrichment compared to the same *PRKCB* promoter sequences associated with a non-specific IgG immunoprecipitation control. **A)** Shows the results of each single experiment for eight different CLL patient's samples, and three samples of purified normal B cells. **B)** Shows the summary (mean±SE) of the experiments displayed in part A. Statistical analysis was performed using a Mann-Whitney U-test. **C)** Shows STAT3 ChIP within the c-Fos gene promoter region, use as a positive control for the results presented in part A.

4.2.6.3 STAT3 association with the *PRKCB* promoter of CLL cells increases after 24 hours culture

Previous work from this Department showed that PKCβ mRNA levels decrease in CLL cells that are cultured overnight [143]. Other work showed that such culture resulted in an increase in phospho-STAT3 [243]. Therefore, it seemed plausible that any repressive role for STAT3 in regulating PKCβ gene expression might be due to its activation. I next examined the effect of overnight culture of CLL cells on the association of STAT3 to the *PRKCB* promoter. Figure 4.21 shows that such overnight culture of CLL cells results in significantly increased STAT3 binding to the *PRKCB* promoter. This observation potentially explains why PKCβ mRNA levels decrease in CLL cells that are cultured overnight, and this mechanism will be investigated in a subsequent Chapter of this thesis.

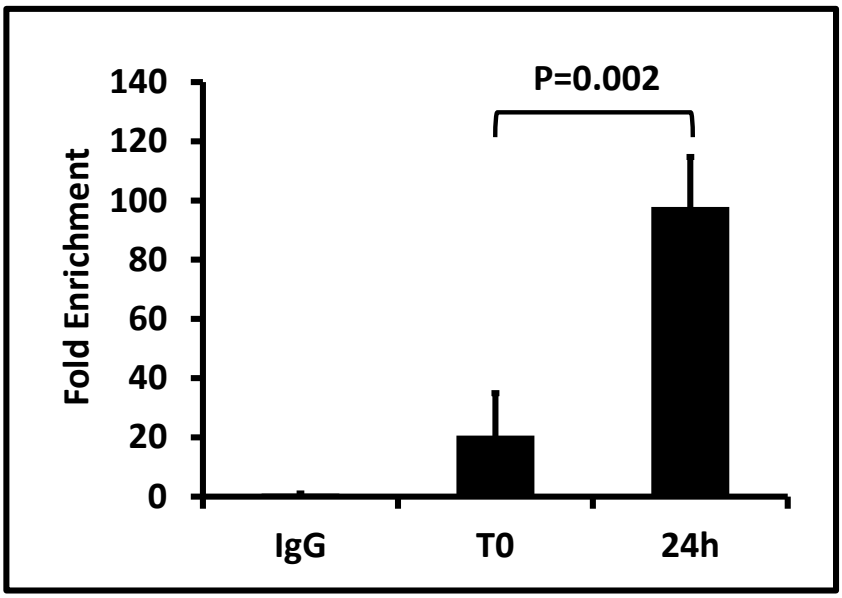


Figure 4.21: Increased STAT3 association with the *PRKCB* promoter of CLL cells after culturing overnight. Chromatin extracts from $1x10^7$ CLL cells at T0 (time zero, just after thawing) and after culturing for 24 h were prepared, and STAT3 was immunoprecipitated using a specific antibody. STAT3 association within the distal region of *PRKCB* promoter was detected by using qPCR and primers covered STAT3 binding site within *PRKCB* promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. Results show a summary of 4 experiments (mean±SE) using four different CLL patient samples. Statistical analysis was performed using a student's t-test for paired data.

4.8 Discussion

In chapter 3 I clearly showed that Sp1 is the main driver for the transcription of *PRKCB* in CLL cells. However, other studies have shown roles for Sp3, MITF, RUNX1, and STAT3 in regulating the expression of this gene in different cellular contexts [5-8]. Therefore, it was necessary to investigate the role of these factors in CLL cells. I also investigated the involvement of c-Myc and E2F1 in regulating the expression of PKCβ. With the exception of STAT3, none of the transcription factors I investigate in this Chapter play direct roles in regulating the transcription of *PRKCB* in CLL cells. These data are important because they support the main regulatory role of Sp1 in driving overexpression of PKCβII in CLL.

Transcription factors interact with DNA to generate three potential outcomes, activation or repression of gene expression, or stasis where transcription factor binding neither stimulates nor suppresses gene expression. Such static interaction of transcription factors with DNA is often observed within intronic sequences of genes, or, in some cases, also within the regulatory regions important for gene expression [290].

With regard to gene activation/repression, this could potentially be influenced by expression level of a particular transcription factor, and this has direct relevance to the current study because I show a correlation between Sp1 protein and PKCβ gene expression. A potential competitor of Sp1 function is Sp3 because of the similarity of the zinc finger domains which comprise the binding sites for the cognate regions of DNA they recognise [170]. Thus, many of the genes driven by Sp1 could also be affected by Sp3 [291]. In many respects Sp3 is described as a repressor of Sp1 function [292-295], and because Sp1 is overexpressed in CLL cells, I hypothesized that Sp3 may be underexpressed in order to generate the high expression levels of PKCβII. However, I found that Sp3 is overexpressed in CLL compared to normal B cells, indicating that Sp3 is likely not acting to repress PKCβ expression within this context. An alternative explanation could be that Sp3 can compete with Sp1 to

drive promoter function as is described for other genes [296, 297], particularly if, like the promoter region of *PRKCB*, there are multiple Sp1 binding sites [174, 296]. My experiments using Sp3 knockdown with siRNA suggested that this transcription factor may be driving *PRKCB* promoter activity in CLL cells, but were not supported by ChIP analysis which indicated that Sp3 did not interact directly with the *PRKCB* promoter. Instead, my results indicated that Sp3 played an indirect role in PKCβ gene expression by regulating the expression of Sp1 because knockdown of Sp3 resulted in decreased expression of Sp1 mRNA and protein. This conclusion is supported by a previous report which showed that Sp3 binds to the Sp1 promoter to regulate its expression [278]. My finding is important because it suggests that overexpression of Sp1 in CLL cells is potentially be due to the overexpression of Sp3.

Previous to this thesis two transcription factors that bind to the *PRKCB* promoter were recognised; RUNX1 [6] and STAT3 [8]. With respect to RUNX1, Hug *et al*. used ChIP analysis to demonstrate interaction of this transcription factor with the promoter region of *PRKCB* in U937 cells, a myeloid cell line [6]. However, I could not demonstrate this in CLL cells despite clear evidence of RUNX1 association with the EVI1 promoter, which is a known direct target for RUNX1 [279] and the positive control for my experiments with CLL. A possible explanation for these contradictory results could be related to the cellular context; RUNX1 is reported overexpressed in U937 cells, which are, in turn, used as a cell model for studying RUNX1 functions [298, 299]. It is not clear whether RUNX1 is overexpressed in CLL cells, the antibody I used for ChIP analysis was not efficient at detecting endogenous levels of RUNX1 by Western blot. It could be that RUNX1 expression levels are important for interaction with the *PRKCB* promoter. If this notion is true, the relative role of this transcription in CLL cells is less important than Sp1 because elimination of the RUNX1 binding within the *PRKCB* promoter does not eliminate activity of this promoter [6], whereas elimination of the Sp1 binding site does.

With respect to STAT3, this transcription factor plays a key pathophysiological role in CLL cells as a driver of genes such as MCL1, among others, which are important for their survival [243, 288, 300]. A recent study identified 4 potential STAT3 binding sites within the *PRKCB* promoter, of which one, site 4, is particularly

important in suppressing PKCβ gene expression in myeloid cells undergoing differentiation [8]. The results I present in this Chapter show that there is no significant collective difference in STAT3 association with the *PRKCB* promoter between normal B and CLL cells, but do identify individual CLL cases where association is clearly observable. This could be due to variation in STAT3 activation between CLL cases [243, 288]. Importantly, my data show that overnight culture of CLL cells results in significant increase in association of STAT3 to the *PRKCB* promoter, and this corresponds to a decrease in PKCβ mRNA levels within CLL cells. In previous work from this Department, overnight culture of CLL cells had the effect of inducing activating phosphorylation of STAT3 through a mechanism involving release of autocrine IL-6 and activation of PKC [243]. This indicates a potential regulatory role for STAT3 in CLL cells in suppressing PKCβ gene expression, the nature of which is more fully explored in Chapter 6.

An early study of melanocytes showed a potential role for MITF in regulating *PRKCB* transcription [7]. However, this study did not show direct binding of this transcription factor to the promoter region of *PRKCB*, and relied on luciferase assays where MITF was co-transfected with luciferase genes which were under the control of a 1.4kB region of the *PRKCB* promoter. Expression of MITF is highest in melanocytes [301], but expression in tissues such as lymphocytes has not been characterised. Therefore, I first determined that MITF is expressed in CLL, normal B, MEC1 and Daudi cells, which suggested that it may have a role in regulating the expression of *PRKCB* in these cells. Typically, MITF binds to E-box and E47 sequences of gene promoters, and the promoter of PRKCB has these sequences; an E-box within the basal promoter region and an E47 sequence within the more distal region. ChIP analysis of these regions showed that MITF binds strongly to the E47 sequence within the distal region, but not to the E-box sequences within the basal promoter. As a potential driver of *PRKCB* transcription, this binding of MITF to the PRKCB promoter is consistent with the results of Park *et al*., who show that co-transfection of MITF stimulates the activity of a 1.4kB *PRKCB* promoter, but not a 0.5kB basal *PRKCB* promoter construct [7]. However, knockdown of MITF expression within my system does not result in decreased levels of PKCβ mRNA;

rather, levels of this mRNA species are slightly increased. This suggests that MITF could be operating as a transcriptional repressor, a role that has been described when MITF is in association with the STAT3 inhibitor PIAS3 [302] and is consistent with the suggestion made by Niino *et al.,* who implicated repressive factors in regulating *PRKCB* [161].

I next investigated a role for c-Myc in regulating PKCβ gene expression. This is because overexpressed c-Myc and activation of the MAPK pathway can drive expression of the PKCβ gene [262, 303, 304]. However, these studies have not shown the mechanism behind this relationship, and this may be important within a CLL cell context because activated c-Myc is often observed in the malignant cells associated with progressive disease [305, 306]. Like MITF, c-Myc binds to E-Box sequences to regulate transcription of target genes, and ChIP analysis of c-Myc binding to the E box sequences within the basal promoter of *PRKCB* in CLL and MEC1 cells shows no association despite clear association with a positive control. This would indicate that c-Myc does not drive *PRKCB* promoter activity, but inhibition of c-Myc either by knockdown with specific siRNA or using an inhibitor compound (10058-F4) causes a profound reduction in PKCβ gene expression. This suggests an indirect role of c-Myc, possibly through its role in regulating expression of a protein involved in *PRKCB* transcription. Thus, analysis of the effects of c-Myc inhibition on Sp1 expression revealed a profound reduction in the levels of this latter protein. Importantly, there is no obvious binding site within the promoter region of the Sp1 gene for c-Myc to influence transcription, suggesting that a gene which c-Myc regulates must come in between. It is known that Sp1 expression can be regulated by transcription factors involved in the cell cycle, and an important example is E2F1 [138, 175]. My data show that E2F1 is overexpressed in CLL compared to normal B cells, and ChIP analysis indicates that this transcription factor binds the *PRKCB* promoter at higher levels in CLL than in normal B cells. However, a direct role for E2F1 could not be demonstrated because siRNA knockdown of E2F1 in CLL and MEC1 cells caused down-regulation of Sp1 expression, a result consistent with a report that shows Sp1 expression is regulated by E2F1 [180]. Ultimately, this question can only be answered in luciferase assays where the E2F1

binding site within the *PRKCB* promoter is mutated to eliminate binding. Considering the dominant role of Sp1 in regulating *PRKCB* transcription, it is likely that E2F1 plays only a minor role.

Taken together, these results from this chapter suggest that many of the additional transcription factors that bind the *PRKCB* promoter in CLL cells likely have a static function, neither directly promoting nor suppressing transcription of this gene. I have identified potential repressor functions for STAT3 and MITF, the former of which is further investigated in Chapter 6.

❖ Chapter Five: Role of epigenetic modifications in regulating *PRKCB* gene expression in CLL cells

5.1 Introduction and aims

In this thesis I have so far described the role of transcription factors in regulating gene expression of PKCβII. However, factors which influence transcription factor access to gene promoters also play roles in gene expression [307-309]. The chromatin landscape and its structural alterations, which are known as epigenetic modifications, are important for tissue-specific expression of particular genes[310]. These epigenetic modifications are under enzymatic control of chromatin-remodelling enzymes, thereby creating a second tier through which heritable gene expression is maintained in a tissue-specific way [37]. Importantly, epigenetic control of gene expression is a key to cellular differentiation, and in cancer cells such epigenetic control is often lost [311-313]. CLL cells are no different from other cancer cells in this respect, thus, the aim of this Chapter is to describe the role of epigenetics in the regulation of PKCβ gene expression in these cells.

Epigenetic modifications can silence gene transcription by different mechanisms [314]; miRNA disruption of translation or destabilisation of mRNA[20], histone modifications controlling chromatin condensation [18] and gene methylation [17]. miRNAs expression in CLL has so far not identified species involved in regulating PKCβ gene expression. Therefore, I focussed on the latter two epigenetic phenomenons.

5.2 Results

5.2.1 The *PRKCB* promoter is unmethylated in CLL cells

CpG islands within DNA sequences are targets for DNA methyltransferases, which target the cytosine residues within the CpG dinucleotides at these islands [315]. Gene expression is silenced when these islands are located within regulatory

regions of genes, and the methylation of cytosine within the CpG islands either interferes with the binding of transcription factors to their consensus sequences, or by recruiting chromatin silencing complexes [21]. Previous characterization of the *PRKCB* promoter showed that it is enriched with CpG dinucleotides; more than 70% of the 1kB region upstream of the transcription start site contains such motifs [4]. Importantly, gene methylation catalysed by *PROX1* has been shown to influence *PRKCB* transcription in a HeLa cell model system [5]. Therefore, it was necessary to examine the methylation status of the *PRKCB* promoter in CLL cells and compare this to that in normal B cells. These experiments assessed the methylation status of the CpG Island located near the Sp1 binding sites of the promoter in normal B and CLL cells (Figure 5.1). We found that this region was virtually unmethylated and markedly similar in CLL and normal B cells (p=0.84, Mann-Whitney U-test, Table 5.1). Therefore, these results show clearly that methylation status does not play a role in influencing Sp1 binding to the *PRKCB* promoter in CLL cells.

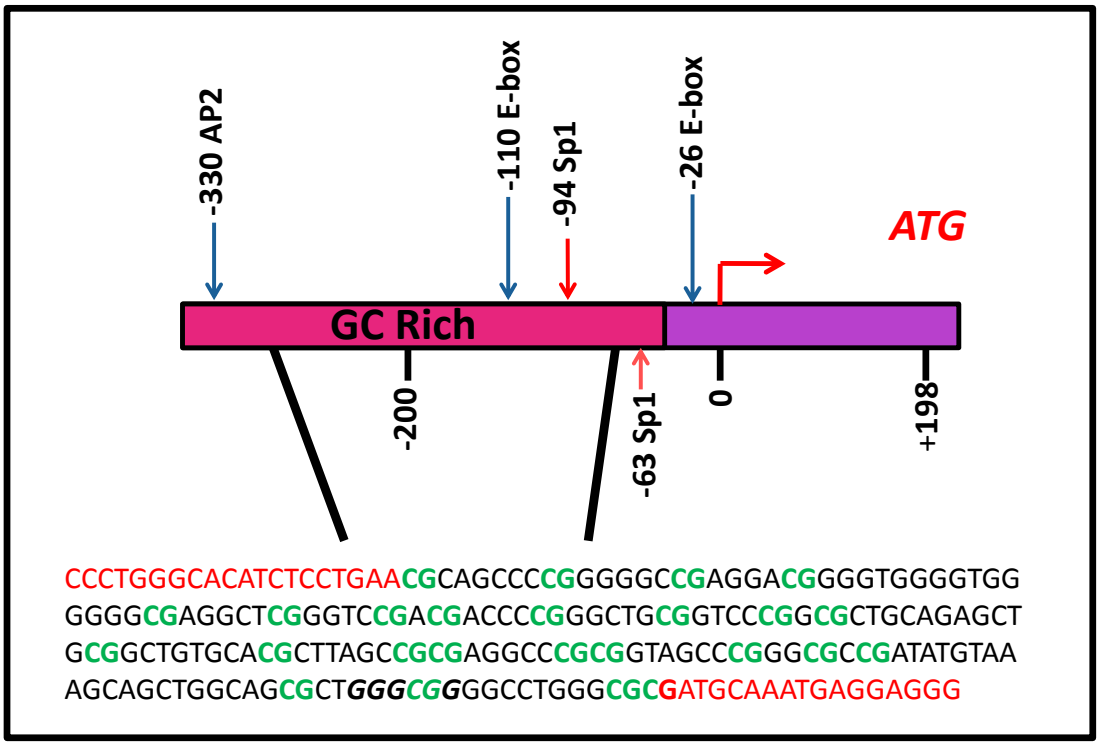


Figure 5.1: GC-rich region in *PRKCB* promoter. Schematic drawing of *PRKCB* promoter show the GC-rich region adjacent to Sp1 binding sites. The sequence underneath the drawing shows the CpG dinucleotides (green) adjacent to Sp1 binding site in italic font and the primers that used for methylation studies (red).

Table 5.1: *PRKCB* promoter methylation in normal B and CLL cells. Normal B and CLL cells were each purified from 5 healthy donors or CLL patients, respectively. Genomic DNA was isolated from these cells, and methylation of the *PRKCB* promoter was determined as described in the materials and methods. % methylation is reported as the mean average methylation of 8 CpGs within the analysed sequence. A dash within the % purity of the cells indicates the cell purity was not assessed following purification.

Sample ID	B-CLL / Normal B	% purity	Mean % methylation
2649	Normal B	96.12	**3.49**
2667		97.12	**2.22**
2668		93.15	**2.3**
2675		-	**1.73**
2063		99.79	**3.32**
2064	B-CLL	-	**4.51**
2226		-	**2.31**
2262		93.03	**1.05**
2458		91.09	**0.8**
2536		99.33	**3.16**

5.2.2 *PRKCB* chromatin is enriched with permissive histone marks in CLL cells

Acetylation of lysine 9 and 14 within histone H3 (collectively known as H3Ac), as well as tri-methylation of lysine 4 residues (H3K4me3), are modifications associated with transcriptionally active promoters and are therefore known as permissive histone marks [316]. I used ChIP to compare the status of these permissive marks approximately 500bp upstream and downstream of the transcription start site of *PRKCB* in CLL and normal B cells. These regions were chosen as it has been demonstrated that transcriptionally active genes are enriched for these marks within these locations [317, 318]. Figure 5.2 shows that both H3Ac and H3K4me3 marks are associated with the *PRKCB* promoter in CLL and normal B cells, and that the extent of this association is significantly higher in the former compared to the latter. Figure 5.2 also shows that these marks are present on *PRKCB* chromatin 500bp up- and downstream of the transcriptional start site, and that the level of

association is greater in CLL compared to normal B cells. These results clearly show that the H3Ac and H3K4me3 marks are enriched on *PRKCB* chromatin in CLL cells. Because these marks are associated with chromatin relaxation consistent with active transcription, these results suggest that increased access of Sp1 to the *PRKCB* promoter in CLL cells may be the result of such chromatin relaxation.

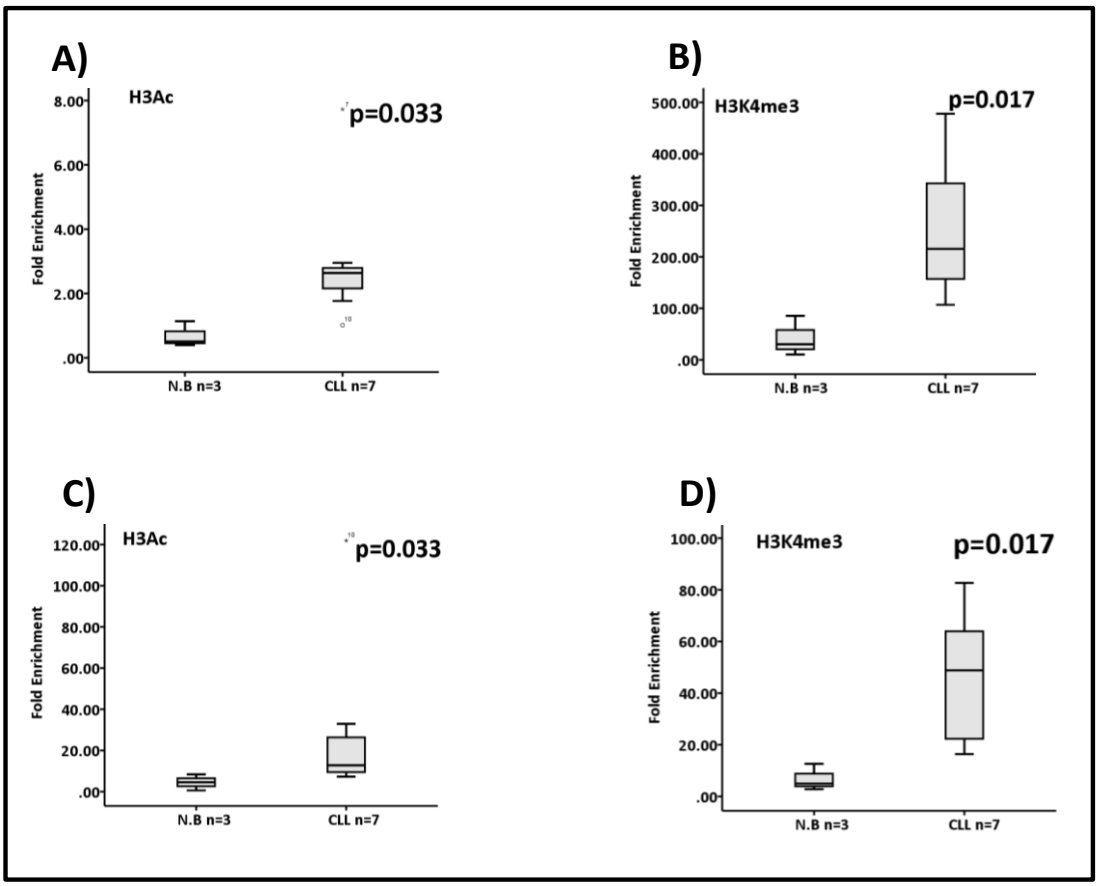


Figure 5.2: *PRKCB* chromatin is enriched with permissive histone marks as an indicator of active transcription status of its promoter in CLL cells. Chromatin was prepared from $1x10^7$ purified normal B and CLL cells, and the permissive Histone marks (H3Ac and H3k4me3) were immunoprecipitated using specific antibodies. The association of H3Ac and H3K4me3 were detected by qPCR and primers covering the region upstream and downstream of the TSS of *PRKCB* promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. **A)** and **C)** Show comparison of H3Ac association upstream and downstream of TSS within *PRKCB* promoter in normal B and CLL cells respectively. **B)** and **D)** Show comparison of H3K4me3 association upstream and downstream of TSS within *PRKCB* promoter in normal B and CLL cells respectively. Statistical analysis was performed using a Mann-Whitney U-test.

5.2.3 Expression of p300 in normal B and CLL cells

p300 is a histone acetyltransferase (HAT) that is important for acetylating lysine residues in histone3 [319]. I next examined the protein levels of p300 in CLL and normal B cells using Western blot. Figure 5.3 shows that p300 protein levels are generally higher in CLL compared to normal B cells. Some cases, such as #1873 and #1872 seem to overexpress p300 at very high levels. Considering that p300 is recruited to chromatin by co-association with transcription factors such as Sp1 [320, 321], it seemed logical to explore whether p300 played a role in regulation of *PRKCB* transcription.

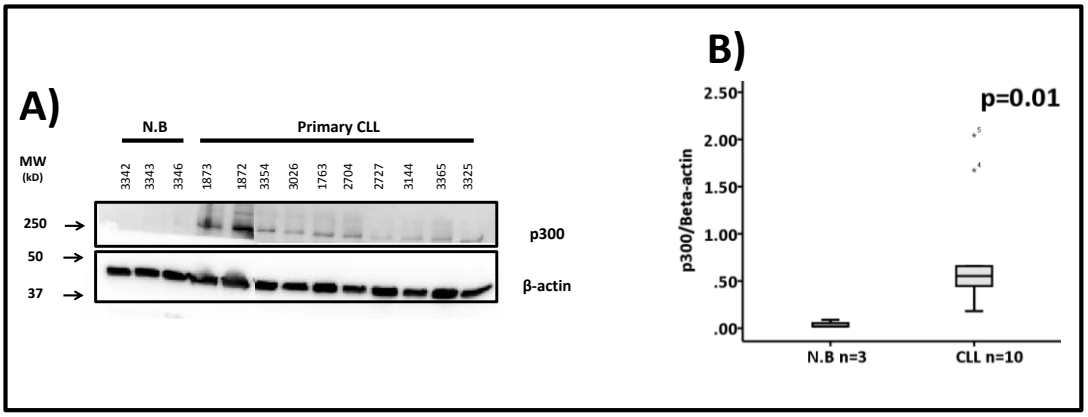


Figure 5.3: p300 protein expression in normal B and CLL cells. Western blot analysis of cell lysates prepared from purified CLL and normal B cells. 10 µg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to p300 and β-actin. **A)** Shows p300 protein expression in normal B (n=3) and CLL (n=10) cells. **B)** Quantitative analysis of p300 protein expression in CLL and normal B cells depicted in part A. Statistical analysis was performed using a Mann-Whitney U-test.

5.2.4 Inhibition of p300 decreases H3Ac and H3K4me3 marks associated with *PRKCB* chromatin in CLL cells, and suppresses PKCβ gene expression

C646 is a HAT inhibitor that specifically targets p300 [310]. I used this compound in combination with ChIP analysis to examine how treatment of CLL cells with C646 affected the H3Ac and H3K4me3 marks. Figure 5.4 shows that treatment of CLL cells with this inhibitor resulted in a decrease of H3Ac and H3K4me3 chromatin marks associated with *PRKCB* either upstream or downstream of the transcriptional start site. I next examined how this decrease of H3Ac and H3K4me3 chromatin marks associated with *PRKCB* affected transcription. Figure 5.5A shows that treatment of CLL cells with C646 resulted in a reduction of PKCβ mRNA levels. This reduction in PKCβ mRNA correlated with decreased association of Sp1 to the *PRKCB* promoter in treated CLL cells as analysed by ChIP (Figure 5.5B). Finally, Sp1 and PKCβII protein levels in CLL cells remained unaffected by treatment with C646 (Figure 5.5C). Taken together, these data show that inhibition of p300 with C646 suppresses PKCβ gene expression, and suggest that the mechanism of this suppression is through alteration of the chromatin marks that are permissive of transcription.

The next logical step would be to examine how knockdown of p300 affected *PRKCB* transcription. Figure 5.6 shows that treatment of MEC1 cells with siRNA targeting p300 resulted in a reduction of p300 mRNA and protein and PKCβ mRNA and protein. However, Sp1 protein and mRNA levels were also reduced by knockdown of p300, a result that indicates that the reduction in PKCβ gene expression observed in this experiment is as a result of suppressed Sp1 expression.

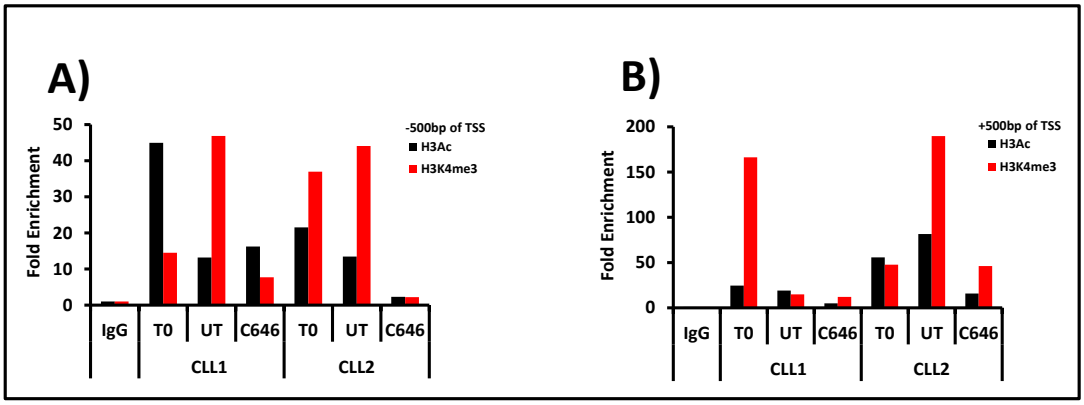


Figure 5.4: C646 decreases H3Ac and H3K4me3 associated with *PRKCB* promoter in CLL cells. Chromatin extracts from 1x10^7 CLL cells at T0 (time zero, just after thawing), or from cells cultured for 24 h in the presence of 10 µM C646 or DMSO vehicle control (UT) were prepared. Then, H3Ac and H3K4me3 were immunoprecipitated using specific antibodies. *PRKCB* promoter 500bp upstream and downstream of the TSS associated with H3Ac and H3K4me3 were detected using qPCR and specific primers covering these regions. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. Results show a summary of single experiments using two different CLL patient samples.

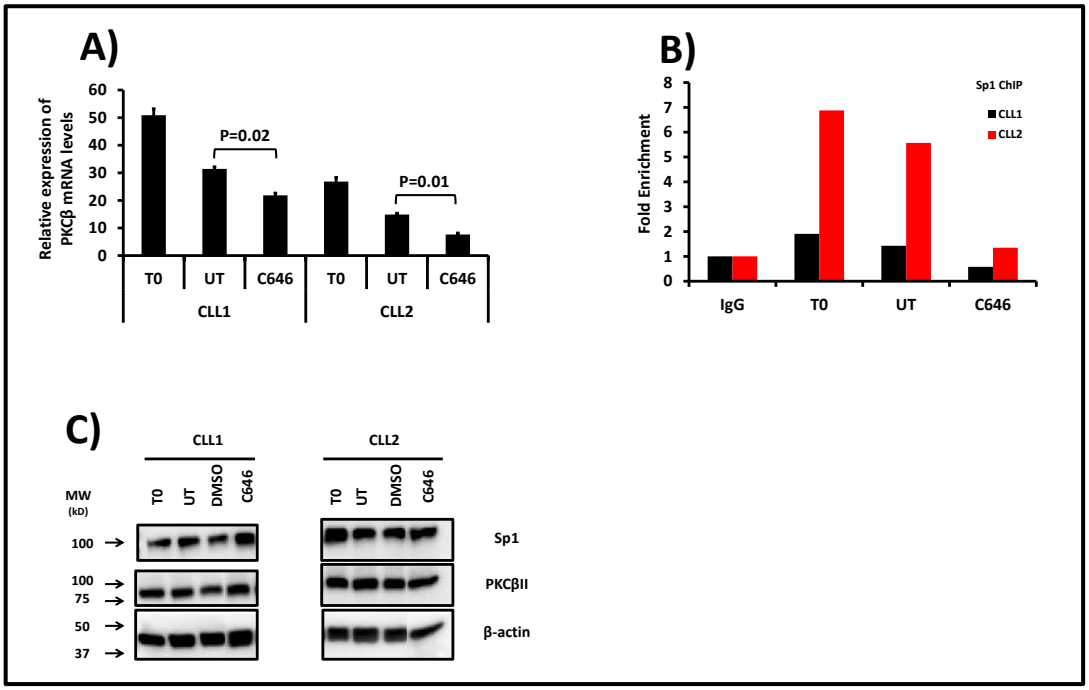


Figure 5.5: Effect of C646 on PKCβ mRNA levels, Sp1 association within *PRKCB* promoter and Sp1 protein levels in CLL cells. 1x10^7 CLL cells were cultured in absence (UT) or presence of DMSO vehicle control or 10 µM C646. Cell lysates, chromatin and mRNA extracts were prepared from harvested cells after 24 h culturing in addition to samples taken at T0 (time directly after thawing). PKCβ mRNA levels were measured using qRT-PCR relative to the reference gene RNA Polymerase II and presented as arbitrary units. 10µg of protein were separated by SDS-PAGE and were transferred to PVDF membranes and blot for Sp1, PKCβII, and β-actin as loading control. Sp1 was immunoprecipitated using specific antibodies. *PRKCB* promoter associated with Sp1 was detected using qPCR and specific primers covering Sp1 binding regions. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. **A)** Shows PKCβ mRNA levels. **B)** Shows Sp1 association with *PRKCB* promoter. **C)** Shows PKCβII and Sp1 protein levels. This experiment illustrates n=3 separate biological replicates and the results presented as (mean±SE) for parts A from two different CLL patients. Results show a summary of single experiments using two different CLL patient samples for part B.

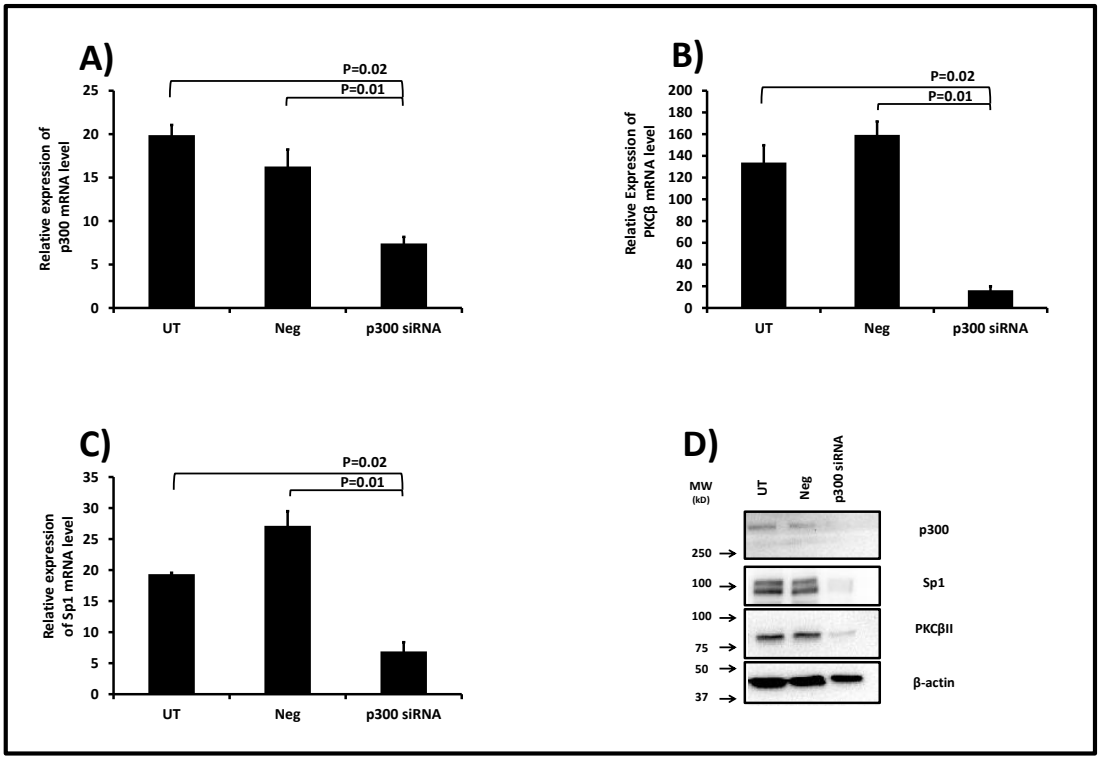


Figure 5.6: Effect of p300-specific siRNA on p300, PKCβII, and Sp1 mRNA and protein levels in MEC1 cells. $2x10^6$ MEC1 cells were nucleofected with 2 µM of p300-specific or control siRNA oligonucleotides and then cultured under the conditions described in the materials and methods. Harvested cells after 72h were analysed for protein expression by Western blot and mRNA levels by qRT-PCR. All the mRNA levels were measured relative to the reference gene RNA Polymerase II and expressed as arbitrary units. **A)** Shows p300 mRNA levels. **B)** Shows PKCβ mRNA levels. **C)** Shows Sp1 mRNA levels. **D)** Shows the protein levels for p300, PKCβII, Sp1, and β-actin. 10 µg of the prepared whole cell lysates were separated by SDS-PAGE and transferred into PVDF membranes, and then were immunobloted for the indicated antibodies. The mRNA levels in parts A, B, and C presented as the mean±SE of n=3 separate replicates. Statistical analysis for parts A, B, and C in this figure was performed using a student's t-test for paired data.

5.2.5 HDAC inhibitor increases H3Ac and H3K4me3 enrichment associated with *PRKCB* promoter in CLL cells

Chromatin remodelling is also catalysed by HDAC enzymes, which silence gene expression by altering the chromatin structure to a state which is less permissive of transcription by removing acetyl groups from lysine residues in histones [322]. Romidepsin is a class I HDAC inhibitor, and I used this compound to investigate its effect on CLL cells. ChIP analysis of H3Ac and H3K4me3 associated with the *PRKCB* promoter ±500bp from TSS showed that these marks increased in CLL cells treated with romidepsin (Figure 5.7). However, similar to C646, treatment of CLL cells with romidepsin also decreased PKCβ mRNA levels and Sp1 association with the *PRKCB* promoter (Figure 5.8A and B) without affecting Sp1 protein expression (Figure 5.8C). These results show that HDAC inhibition in CLL cells results in changes to chromatin that is potentially permissive of transcription, but does not lead to increased PKCβ gene expression.

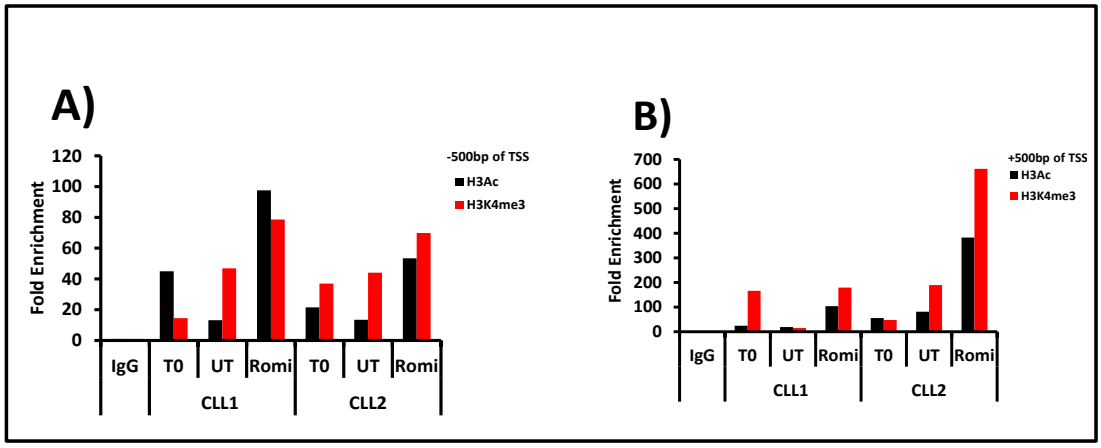


Figure 5.7: Romidepsin increases acetylation H3 and tri-methylation on lysine 4 of H3 associated with *PRKCB* promoter in CLL cells. Chromatin extracts from $1x10^7$ CLL cells at T0 (time zero, just after thawing), or from cells cultured for 24 h in absence of Romidepsin (UT) or in presence of Romidepsin (Romi) (6 nM) were prepared. Then, H3Ac and H3K4me3 were immunoprecipitated using specific antibodies. The association of H3Ac and H3k4me3 within the **A)** -500bp and **B)** +500pb of the TSS regions of *PRKCB* promoter were detected by using qPCR and primers covering the indicated regions within *PRKCB* promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. Results show a summary of single experiments using for two different CLL patient samples.

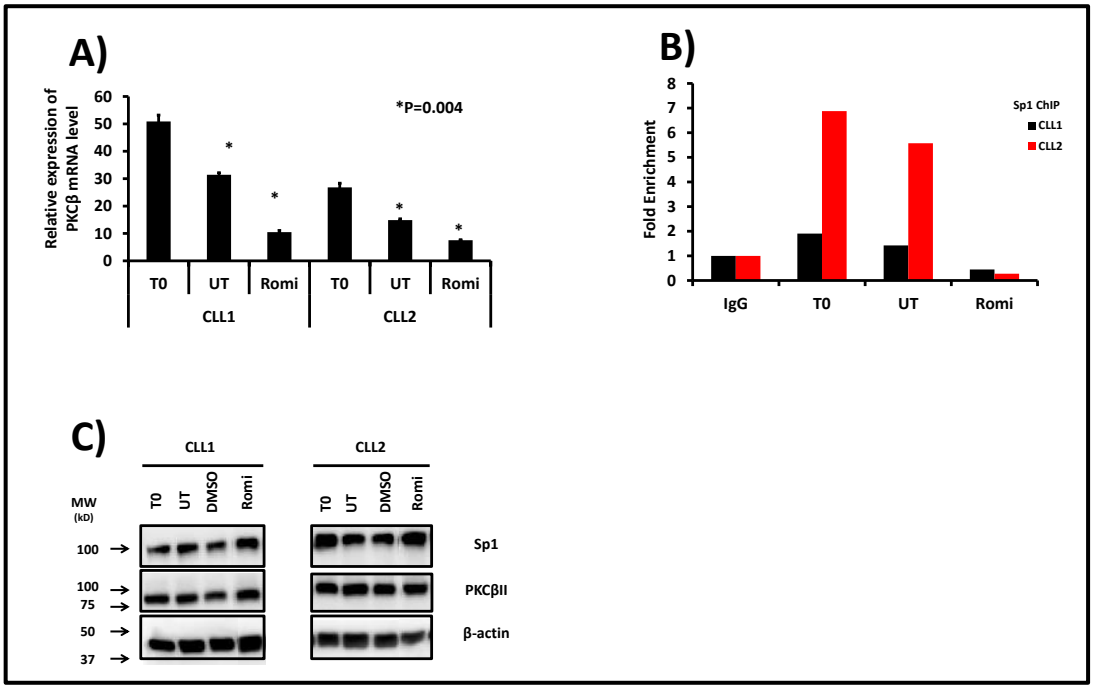


Figure 5.8: Effect of Romidepsin on PKCβ mRNA levels, Sp1 association within *PRKCB* promoter and Sp1 protein levels in CLL cells. 1x10^7 CLL cells were cultured in the absence (UT) or presence of DMSO vehicle control or 6 nM Romidepsin (Romi). Cell lysates, chromatin and mRNA extracts were prepared from harvested cells after 24 h culturing in addition to samples taken at T0 (time directly after thawing). PKCβ mRNA levels were measured using qRT-PCR relative to the reference gene RNA Polymerase II and presented as arbitrary units. 10 µg of protein were separated by SDS-PAGE and were transferred to PVDF membranes and then blotted for Sp1, PKCβII, and β-actin as loading control. Sp1 was immunoprecipitated using specific antibodies. *PRKCB* promoter associated with Sp1 was detected using qPCR and specific primers covering Sp1 binding regions. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. **A)** Shows PKCβ mRNA levels. **B)** Shows Sp1 association to *PRKCB* promoter. **C)** Shows Sp1 and PKCβII protein levels. This experiment illustrates n=3 separate biological replicates and the results presented as (mean±SE) for parts A from two different CLL patients. Results show a summary of single experiments using two different CLL patient samples for part B.

5.3 Discussion

Epigenetic control of gene expression is important for maintaining cell phenotype, allowing heritable expression of particular genes without changing nucleotide sequences in DNA [113]. In cancer this process can be deregulated to allow over/under expression of particular genes that are important for the neoplastic behaviour of the malignant cell [323, 324]. Therefore, studying this phenomenon and its regulatory mechanisms is key to gaining insight into cancer cell pathophysiology. In this Chapter I present data which shows that chromatin containing the PKCβ gene in CLL cells is more highly associated with transcriptionally permissive histone marks than that in normal B cells. Furthermore, my data also suggest that the presence of these histone marks allows greater accessibility of Sp1 because treatment of CLL cells with HAT inhibitors reduces the level of transcriptionally permissive histone marks and Sp1 association with the *PRKCB* promoter. These data, for the first time, implicate a role of chromatin structure in the regulation of PKCβ gene expression.

Epigenetic control of gene expression is principally mediated by three different mechanisms [314]; changes in the status of gene promoter methylation [17], histone modifications controlling chromatin condensation [18] and by miRNA disruption of translation or destabilisation of mRNA [20]. In the first instance, we investigated *PRKCB* promoter methylation in CLL and normal B cells and we showed that it is similar and unmethylated in these two cell types. The CLL cell epigenome has been investigated and shown to be generally hypomethylated, specifically for genes that play key roles in the pathogenesis of these malignant cells [162, 325]. This implies that overexpression of these genes is the result of hyopmethylation of their promoter regions as is suggested as a mechanism for how DNA methylation affects gene expression [326, 327]. For the purposes of the current study it was important to study methylation of the *PRKCB* promoter because it is known to contain CpG islands, which are the target of the DNA methyltransferases and demethylases that are responsible for DNA methylation status [326]. Also, promoter methylation status affects the ability of Sp1 to access gene promoters [186, 328], and this is important regarding my results which indicate that Sp1 drives

PKCβ gene expression. And finally, a recent study by Hagiwara *et al*. showed that the *PRKCB* promoter is a target of *PROX1*, a DNA methyltransferase, in a HeLa cell model system where it limits access of Sp1 to its consensus binding sequence in the *PRKCB* promoter due to hypermethylation [5]. That the methylation status of the *PRKCB* promoter in CLL and normal B cells is unmethylated indicates that *PROX1* likely does not play a role in regulating PKCβ gene expression in CLL cells, and that the increased access of Sp1 to this promoter in CLL cells is not the result of changes to gene methylation. In this regard, control of *PRKCB* transcription in CLL is similar to that controlling *PRKCE* (the gene coding for PKCε) in breast cancer cells; expression of this gene is also driven by Sp1, but although its promoter can be methylated it remains unmethylated between normal and breast cancer cells [329].

With regard to histone modifications, I studied H3Ac and H3K4me3 which are permissive histone marks of euchromatin and are generally associated with gene promoters where active transcription is taking place [42, 316]. In particular, the H3K4me3 mark promotes the recruitment of enzymes which facilitate the formation of an "open" structure of chromatin that is conducive to increased access of transcription factors and induction of transcription [316]. The data I present in this Chapter clearly show that the *PRKCB* promoter in CLL cells has these permissive histone marks to a greater extent than that in normal B cells. Interestingly, recent reports have indicated that expression of the class of enzymes responsible for regulating histone acetylation, known as HDACs and HATs [330, 331], are deregulated in CLL cells, and may relate to disease prognosis and pathogenesis [332-334]. p300 is an example of a HAT protein that transfers an acetyl group to histone proteins, and, in particular to histone H3 at lysine residues. I found the p300 was more highly expressed in CLL compared to normal B cells, suggesting that this enzyme might catalyse the more permissive transcriptional environment observed in the former cell type. C646 is a drug that was developed to specifically inhibit the activity of p300 [245]. In this Chapter I used this drug to explore how p300-mediated histone modification affected PKCβ gene expression in CLL cells. I found that inhibition of p300 in CLL cells with C646 lead to reduced levels of H3Ac and H3K4me3, as well as Sp1 associated with the *PRKCB* promoter. Thus, the

presence of C646 inhibited the expression of PKCβ in CLL cells, and because this compound had no effect on Sp1 protein levels the proposed mechanism of downregulated PKCβ gene expression is due to chromatin condensation caused by inhibition of p300. I attempted to confirm this conclusion by knocking down p300 expression in MEC1 cells with siRNA. Such knockdown resulted in a profound reduction in PKCβ mRNA levels, but this could be caused by reduced Sp1 expression because knockdown of p300 in these cells also resulted in attenuation of mRNA and protein levels of this transcription factor. The reason for this discrepancy could be because I used CLL cells to test the effects of C646 and MEC1 cells to test the effects of p300 knockdown. Nevertheless, although I cannot directly demonstrate a role for p300 in regulating PKCβ gene expression, the data presented do suggest that chromatin structure is important.

HDAC enzymes are antagonistic to HATs, and remove acetyl groups from histone and other proteins. HDACs are a family of enzymes that are divided into four different classes [322], and inhibiting these enzymes using pan or specific inhibitors leads to increased acetylation of lysine residues within proteins. When this happens within histones this result in relaxation of chromatin and allows more accessibility for transcription factors [308, 335]. I have examined histone marks associated with *PRKCB* chromatin in romidepsin-treated and untreated CLL cells. I found that culture of CLL cells with romidepsin results in an increase in the H3Ac and H3K4me3 marks associated with the *PRKCB* promoter. However, the increased association of these marks with the *PRKCB* promoter did not result in increased PKCβ gene transcription, nor increased Sp1 association with the *PRKCB* promoter. The failure of romidepsin-treated CLL cells to upregulate PKCβ gene expression in the presence of relaxed chromatin was not due to changes in Sp1 protein levels as was suggested by others using MS-275, another class I HDAC inhibitor [336]. Alternatively, because HDACs also catalyse removal of acetyl from other proteins such as transcription factors [337], it could be that Sp1 in CLL cells became acetylated and this inhibited its function. This notion is supported by studies showing that such modification of Sp1 inhibits the ability of this transcription factor to bind its target genes [191, 338, 339].

Taken together, the findings of this Chapter show that *PRKCB* in CLL cells is more permissive for transcription than that in normal B cells. Future work will need to determine the nature of the enzymes catalysing the permissive environment, and, in particular, focus on the potential role of p300.

❖ Chapter Six: Role of vascular endothelial growth factor (VEGF) and Bryostatin in regulating transcription of *PRKCB* expression in CLL cells

6.1 Introduction and aim

So far in this thesis I have focussed on the role of two cell-intrinsic factors, transcription factors and epigenetic modifications, in regulating PKCβ gene expression in CLL cells. An additional level of regulation can occur through stimulation of gene expression by extrinsic factors such as growth factors. This is important with respect to PKCβ because a previous report from this Department has shown that *PRKCB* transcription in CLL cells is stimulated by VEGF [143], whilst another has demonstrated that expression of this gene can be inhibited by the PKC agonist Bryostatin [137]. However, these studies have not addressed the mechanism by which *PRKCB* transcription is stimulated/inhibited by these factors. The previous work of this thesis identified Sp1 as the major driver of *PRKCB* transcription, and the work of Farren *et al.,* [8] shows that STAT3 can suppress expression of this gene. Therefore, the aim of this chapter is to investigate how VEGF and Bryostatin regulate Sp1/STAT3 interaction with the *PRKCB* promoter in CLL cells.

6.2 Results

6.2.1 VEGF stimulates the expression of PKCβII gene in CLL cells

To begin this investigation I repeated the findings reported by Abrams *et al.,* [143]. Figure 6.1 shows confirmation of these findings. PKCβ mRNA levels decrease in CLL cells that have been cultured overnight, whilst inclusion of VEGF in these cultures results in PKCβ mRNA levels being maintained or further increased. Considering the role of Sp1 in driving PKCβ gene expression, I next examined the effect of VEGF on association of this transcription factor with the *PRKCB* promoter.

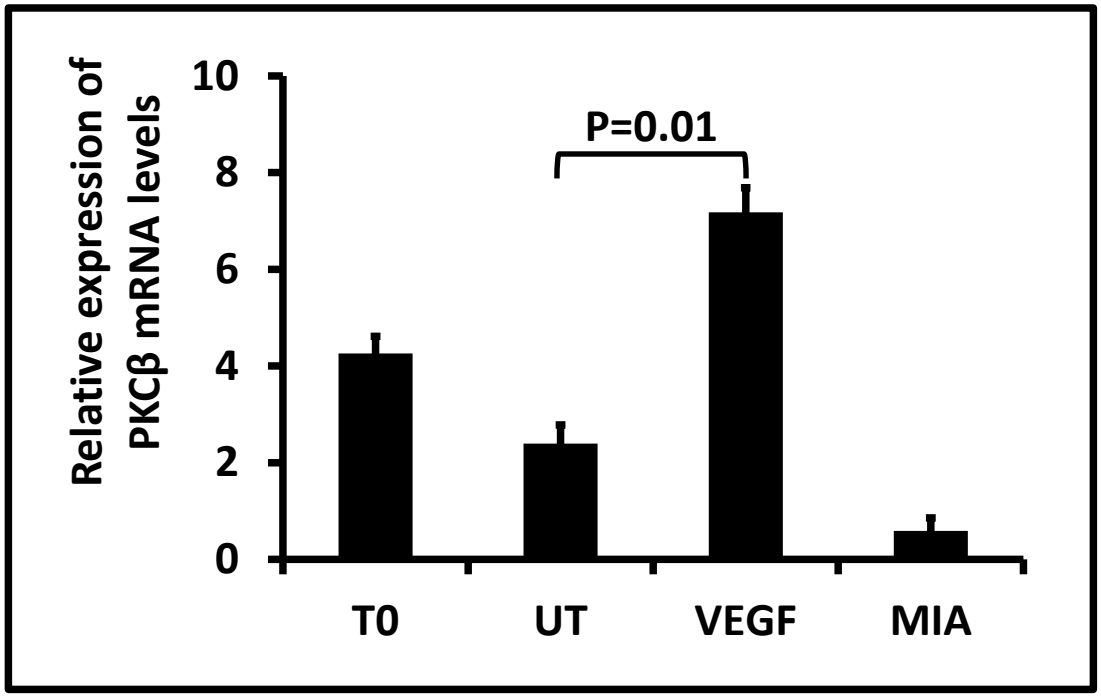


Figure 6.1: VEGF stimulates PKCβ gene expression in CLL cells. $1x10^7$ CLL cells were cultured for 24 h in the absence (UT) or presence of 100 ng/mL VEGF, or with 200nM mithramycin, and then mRNA was harvested. T0 indicates cells from which mRNA was extracted just after thawing (time zero). PKCβ mRNA level was measured by qRT-PCR and presented relative to the reference gene RNA Polymerase II in arbitrary units. The data are presented as mean ± SE of n=3 experiments using CLL cells from a single patient. These data are representative of the results generated using cells from three different CLL patient samples. Statistical analysis for this figure was performed using a student's t-test for paired data.

2.6.2 VEGF stimulates Sp1 association with the *PRKCB* promoter in CLL cells

To investigate the notion that VEGF may modulate the association of Sp1 to the *PRKCB* promoter I performed ChIP analysis. Figure 6.2 shows that CLL cells stimulated with VEGF have significantly increased levels of Sp1 in association with the *PRKCB* promoter than do either unstimulated CLL cells, or CLL cells that have had chromatin extracted from them directly after thawing. In these experiments mithramycin was included as a control to displace Sp1 from the *PRKCB* promoter. Taken together with the data showing that VEGF stimulates PKCβ gene expression,

these results show clearly that the stimulation of *PRKCB* transcription in CLL cells by VEGF is due to induction of Sp1 binding to the promoter of this gene.

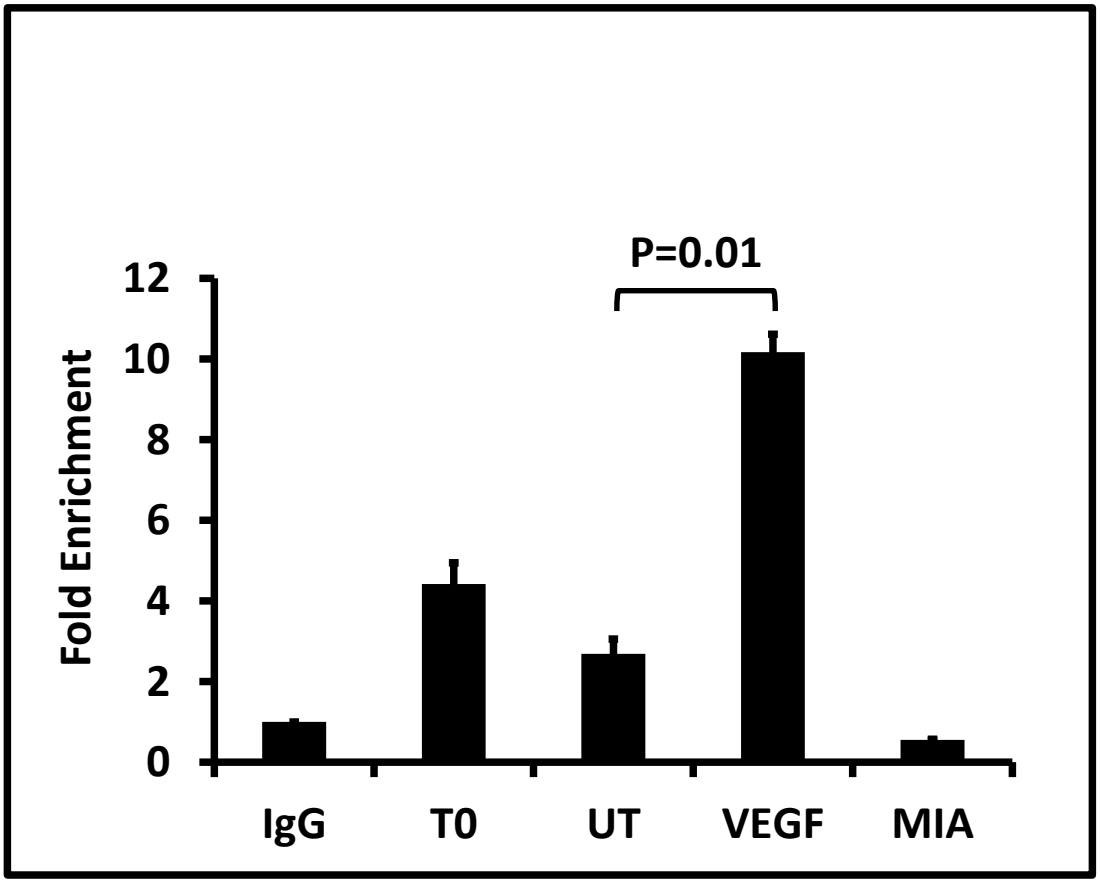


Figure 6.2: VEGF stimulates Sp1 association with the *PRKCB* promoter in CLL cells. $1x10^7$ CLL cells were cultured for 24 h in the absence (UT) or presence of VEGF (100ng/ml), or with MIA (200 nM). Following harvest of these cells chromatin extracts were prepared, and Sp1 was immunoprecipitated using specific antibody. T0 indicates cells from which chromatin was extracted just after thawing (time zero). The association of Sp1 to the *PRKCB* promoter was detected using qPCR and primers which covered the Sp1 binding site within this promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control. Results are presented as mean ± SE of n=3 independent experiments using cells from a single CLL patient sample. These results are representative of similar experiments performed using cells from three different CLL patient samples. Statistical analysis for this figure was performed using a student's t-test for paired data.

2.6.3 VEGF decreases association of STAT3 to the *PRKCB* promoter after 24h of culturing with CLL cells

In Chapter 4 I showed that STAT3 association with the *PRKCB* promoter was induced after overnight culture of CLL cells. Considering the role of STAT3 in suppression of PKCβ gene expression [8], it seemed necessary to investigate whether there is a relationship between STAT3 and Sp1 in relation to VEGF. Figure 6.3A shows, as in Figure 4.21, that overnight culture of CLL cells resulted in induction of STAT3 association with the *PRKCB* promoter in CLL cells. Figure 6.3A also shows that when VEGF is present, this induction is blocked and the level of STAT3 association with the *PRKCB* promoter remains similar to that observed using fresh cells.

To explore whether STAT3 suppresses *PRKCB* promoter activity I next used MEC1 cells where I transfected pGL3-pkcβ-1.2kb and assayed for luciferase. Figure 6.3B shows that VEGF stimulates *PRKCB* promoter activity in line with its ability to stimulate PKCβ gene expression in CLL cells (Figure 6.1). Similarly, treatment of MEC1 cells with an inhibitor of STAT3 which blocks its ability to interact with DNA also results in increased *PRKCB* promoter activity. In contrast, stimulation of MEC1 cells with IL-6 or IL-21 (which both activate STAT3), or with LY333531 (an inhibitor of PKCβ and of the ability of VEGF to stimulate PKCβ gene expression [143]) suppressed *PRKCB* promoter activity (Figure 6.3B). Taken together, these results suggest that STAT3 also functions as a suppressor of PKCβ gene expression in CLL cells. Furthermore, when considered in combination with the ChIP data showing regulated association of STAT3 and Sp1 with the *PRKCB* promoter in CLL cells, these results further suggest that STAT3 works to influence the ability of Sp1 to associate with the *PRKCB* promoter through a mechanism involving VEGF-stimulated activation of PKCβ.

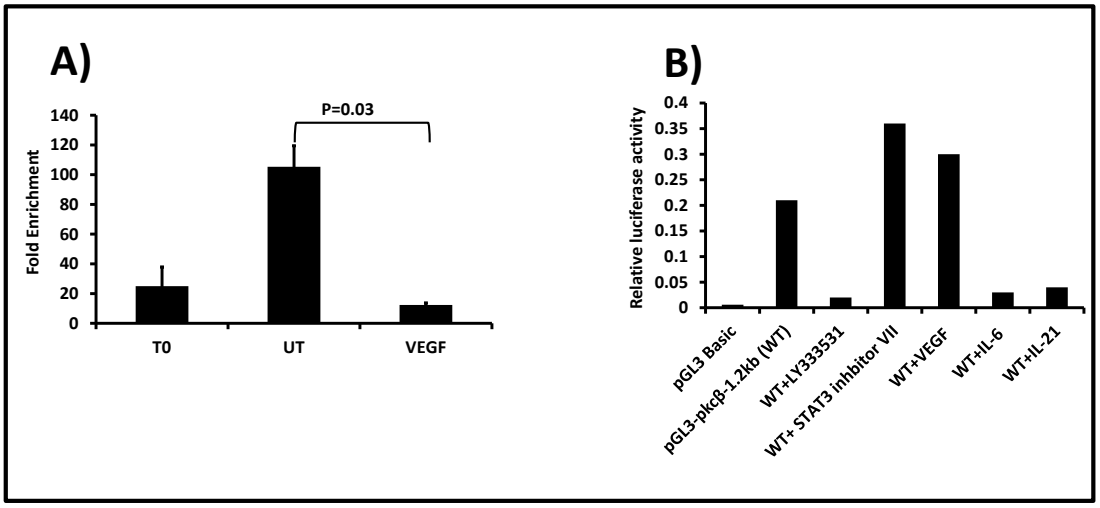


Figure 6.3: Effect of VEGF on STAT3 association with the *PRKCB* promoter in CLL cells. A) STAT3 association with the *PRKCB* promoter. $1x10^7$ CLL cells were cultured for 24 h in the absence (UT) or presence of 100 ng/ml VEGF. T0 refers to cells used just after thawing. Chromatin extracts were prepared from harvested cells and STAT3 was immunoprecipitated using specific antibodies. The association of STAT3 within the *PRKCB* promoter were detected using qPCR and primers covering the STAT3 binding site region. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control, and as a mean±SE summary of three experiments using different CLL patient samples. Statistical analysis for this figure was performed using a student's t-test for paired data. **B)** Effect of different stimulators and inhibitors on *PRKCB* promoter activity in MEC1 cells. $2x10^6$ MEC1 cells were co-transfected with 0.1 µg pRL and 2 µg wt pGL3-pkcβ-1.2kb (WT), and were either left untreated, or were treated with 100 nM LY333531, 0.5 µM STAT3 inhibitor VII, 100 ng/ml VEGF , 150 ng/ml IL-6, or with 50 ng/ml IL-21. Luciferase assays were performed following 24 h culture of the cells under serum-rich conditions, and are reported relative to renilla expression. This is a single experiment.

2.6.4 Bryostatin inhibits PKCβII mRNA and protein levels in CLL cells

Abrams *et al*. [143] and the experiments with LY333531 (Figure 6.3B) show that PKCβII activity is important for the induction of *PRKCB* transcription. Bryostatin is a PKC agonist, and as such, treatment of CLL cells with this agent should stimulate PKCβ gene expression. However, work from our department and others shows that treatment of CLL cells with Bryostatin inhibits transcription of *PRKCB* and expression of PKCβII protein [137, 340]. Figure 6.4 shows confirmation of these

results; PKCβII transcript and protein levels in CLL cells were reduced by treatment with Bryostatin relative to untreated or control (DMSO vehicle) samples.

Previous work in this thesis has shown that a reduction of Sp1 protein levels in CLL cells leads to a reduction in *PRKCB* transcription. However, Figure 6.5A shows that Sp1 protein levels remain similar between control and Bryostatin-treated CLL cells. Since Bryostatin stimulates CLL differentiation [341] it seemed necessary to investigate how this agent affected the presence of permissive transcription marks associated with the *PRKCB* promoter. Treatment of CLL cells with Bryostatin induced an increase in permissive H3Ac histone marks associated with the *PRKCB* promoter (Figure 6.5B), indicating that epigenetic changes are not responsible for the suppression of PKCβ gene expression. Thus, the suppressive effects of bryostatin must be mediated by regulation of transcription factor access to the *PRKCB* promoter. I investigate this notion in the next section.

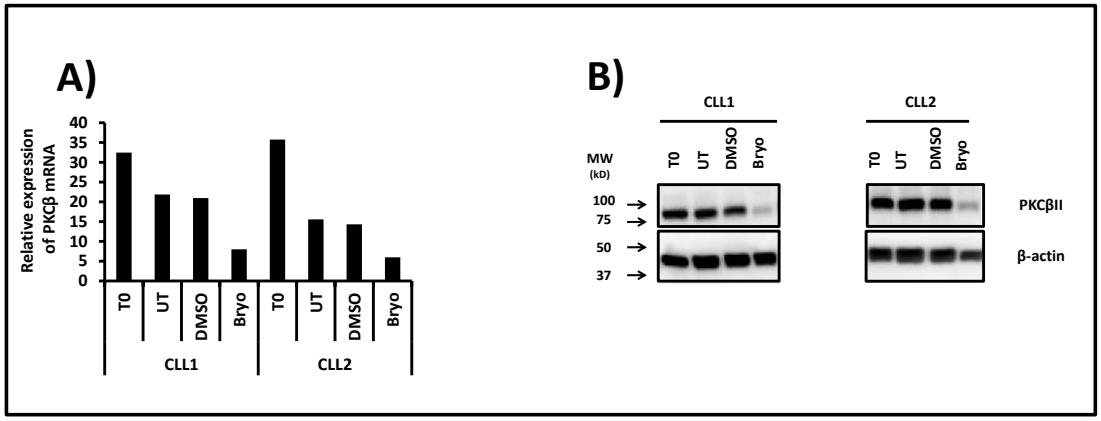


Figure 6.4: Bryostatin inhibits PKCβII mRNA and protein levels in CLL cells. 1x10^7 of CLL cells were cultured in absence (UT) or presence of Bryostatin (Bryo) (50 nM). Cell lysate for Western blot analysis and PKCβ mRNA was prepared from CLL cells harvested after 24 h culturing or at T0 (time zero, just after thawing). PKCβ levels were measured by qRT-PCR relative to the reference gene RNA Polymerase II and presented as arbitrary units. For Western blot analysis 10 µg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to PKCβII and β-actin. **A)** Shows PKCβ mRNA levels. **B)** Shows protein levels. The results in this figure presents n=1 experiment in two different CLL patients samples.

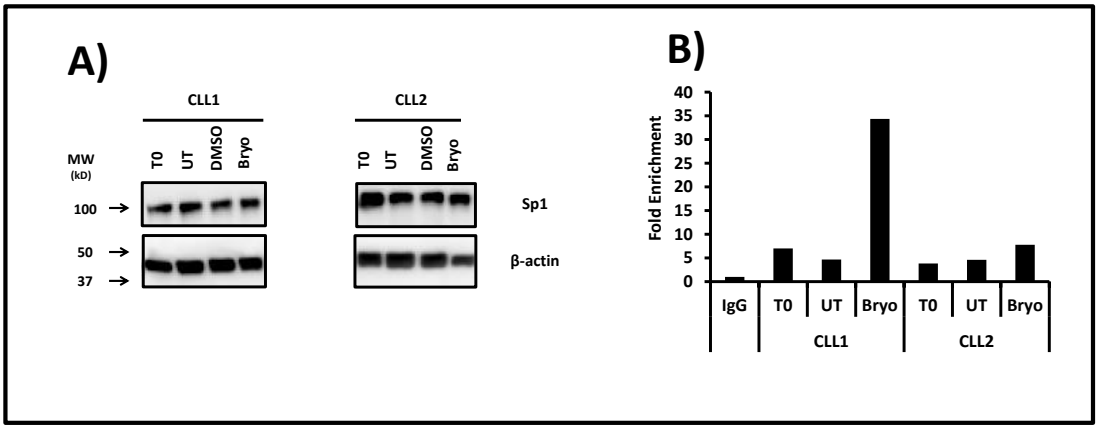


Figure 6.5: Effect of Bryostatin on Sp1 protein levels and H3Ac associated with the *PRKCB* promoter in CLL cells. $1x10^7$ of CLL cells were cultured in absence (UT) or presence of Bryostatin (Bryo) (50 nM) for 24 h. T0 refers to cells used just after thawing. **A)** Sp1 protein levels Sp1. Cell lysates were prepared from harvested cells, and 10µg of protein was separated on 10% acrylamide SDS-PAGE gels, and transferred to PVDF membranes, which were then probed with antibodies to Sp1 and β-actin**. B)** H3Ac levels increases in the treated CLL cells with bryostatin. Chromatin was prepared and H3Ac was immunoprecipitated using specific antibodies. The association of H3Ac with the *PRKCB* promoter were detected by using qPCR and primers covered -500bp region from TSS of *PRKCB* promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control in two different CLL patient samples. The results for part B in this figure presents single experiment in two different CLL patients samples.

2.6.5 Bryostatin decreases Sp1 and increases STAT3 association with the *PRKCB* promoter in CLL cells

To explore whether Bryostatin changes transcription factor accessibility to the *PRKCB* promoter in CLL cells I used ChIP and analysed for Sp1 and STAT3. Treatment of CLL cells with Bryostatin results in decreased Sp1 association with the *PRKCB* promoter, and increased STAT3 association (Figure 6.6). Considering the role of STAT3 in suppressing PKCβ gene expression, these results suggest that the mechanism through which Bryostatin acts to inhibit PKCβ gene expression is through activation of STAT3 binding to the promoter and consequent block of Sp1 access.

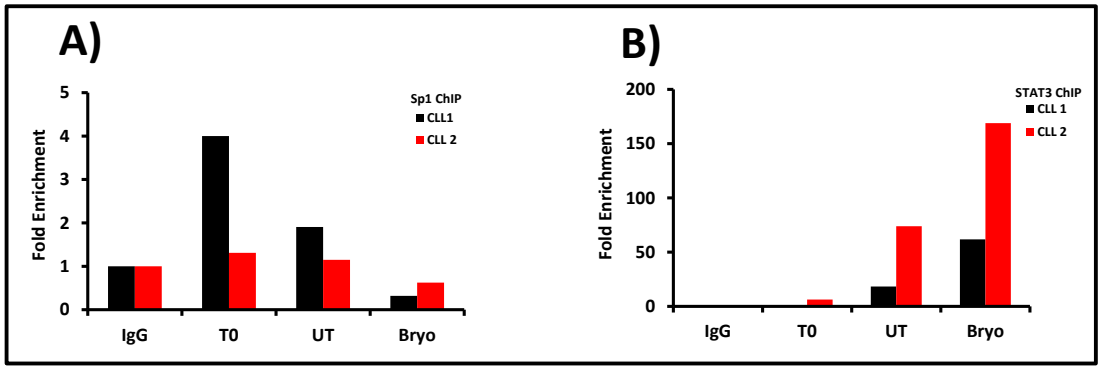


Figure 6.6: Bryostatin increase Sp1 and decrease STAT3 association within *PRKCB* promoter in CLL cells. $1x10^7$ CLL cells were cultured in absence of (UT) or the presence of Bryostatin (50 nM) (Bryo).Chromatin extracts were prepared from harvested cells after 24 h culturing or at T0 (time zero, just after thawing) then, Sp1 or STAT3 were immunoprecipitated using specific antibodies. The association of Sp1 or STAT3 within *PRKCB* promoter were detected by using qPCR and primers covered Sp1 or STAT3 binding site region within *PRKCB* promoter. The results are presented as fold enrichment compared to *PRKCB* promoter sequences associated with the non-specific IgG immunoprecipitation control in two different CLL patient samples.

6.3 Discussion

Earlier studies showed that VEGF stimulates [143] while Bryostatin inhibits [137, 340] PKCβ gene expression in CLL cells. These findings deserved investigation because of the apparent contradiction of result despite a common mechanism of activation; both VEGF and bryostatin activate PKCβ, and PKCβ activity is known to play a role in stimulating *PRKCB* transcription [143, 164, 262]. In this Chapter I

investigated how these two cell extrinsic factors stimulated *PRKCB* expression at the transcriptional level. I found that these factors principally affected the ability of Sp1 to drive and STAT3 to inhibit PKCβ gene expression. These findings are important because they provide further insight into the mechanisms that are responsible for the overexpression of PKCβII in CLL cells.

It is well established that VEGF plays a key role in survival and development of cancer cells, and this also includes CLL cells [342, 343]. A key player in the signal transduction pathway stimulated by VEGF is PKCβ [143], and it is established that active PKCβII can auto-stimulate expression of its gene [143, 164, 262]. Previous work from this Department showed that the activity of PKCβII in CLL cells decreased with overnight culture, and that this activity could be maintained by including VEGF within these cultures [143]. The decreased activity of PKCβII corresponded with a drop in PKCβ mRNA levels that were maintained in cells stimulated with VEGF. Here I show that these changes in PKCβ mRNA levels correlate with decreased or increased association of Sp1 to the *PRKCB* promoter. These results therefore suggest that PKCβII activity must regulate the ability of Sp1 to associate with the promoter region of *PRKCB* and induce transcription. However, my results also show that the changes in PKCβ mRNA levels I observe in CLL cells cultured in the presence or absence of VEGF also correlate with decreased or increased association of STAT3 to the *PRKCB* promoter. A previous report by another group showed that STAT3 operated as a repressor of *PRKCB* transcription [8]. Thus, there appears to be a relationship between STAT3 and Sp1 whereby Sp1 access to the *PRKCB* promoter is governed by the presence of STAT3. This would then mean that PKCβII activity must influence STAT3 association with the *PRKCB* promoter, and that this is involved in the mechanism governing the ability of Sp1 to stimulate PKCβ gene expression.

The ability of active PKCβII to influence STAT3 and Sp1 regulation of PKCβ gene expression deserved further modelling. Here, I used MEC1 cells and transfected them with a *PRKCB* promoter construct that included the STAT3 and Sp1 binding sites. Stimulation of these transfected cells with either IL-6 or IL-21, which both stimulates STAT3 activity; result in inhibition of promoter activity. In contrast,

treatment of CLL cells with STAT3 inhibitor VII (which blocks the ability of STAT3 to interact with DNA) results in induction of promoter activity. These results, when taken together with those presented in Figure 4.19B showing that mutations of the STAT3 binding sites within the promoter construct that I used lead to increased promoter activity, strongly suggest a predominant role of STAT3 as a repressor of PKCβ gene expression. A role for active PKCβII within this system is implied when transfected MEC1 cells are treated with LY333531, which when used at 100 nM is highly specific for the inhibition of PKCα and β [344]. Such inhibition of PKCβ leads to suppressed *PRKCB* promoter activity within my system. Finally, stimulation of transfected MEC1 cells with VEGF generated increased promoter activity of a similar magnitude observed when cells were treated with STAT3 inhibitor VII. This implies that VEGF-stimulated PKCβ activity removes the suppressive effects of STAT3 on the promoter.

The above findings are consistent with those reported by Farren *et al*., who showed that there is a relationship between PKCβII activity and the block of suppressor function of STAT3 on the *PRKCB* promoter [8]. The mechanism proposed to explain this effect is that active PKCβII causes a reduction in the expression of receptors for VEGF on the surface of undifferentiated myeloid cells. This explanation, however, does not apply to CLL cells because PKCβII is active in these cells, and VEGF receptors are both expressed and functional [143].

Bryostatin is a PKC agonist which can activate classical PKC isoforms[345]. I used this compound to stimulate CLL cells, and found that bryostatin inhibited PKCβII expression both at the protein and at the mRNA levels. While it could be expected that bryostatin treatment leads to increase degradation of PKCβII protein as it was described before [111, 159, 346] the effect on PKCβ gene expression required further investigation. Thus, I used ChIP analysis to show that bryostatin treatment of CLL cells leads to reduced levels of Sp1 and increased levels of STAT3 association with the *PRKCB* promoter. Bryostatin is a potent inducer of differentiation in CLL [165, 347], and I demonstrate that such treatment relaxes the chromatin structure surrounding the promoter of *PRKCB* to potentially make it more permissive of Sp1 association and transcription. Therefore, decreased association of Sp1 with the

PRKCB promoter in bryostatin-treated CLL cells is not because of chromatin landscape changes. Importantly, it is likely the increased association of STAT3 to the *PRKCB* promoter results in suppression of gene expression. STAT3 is likely to have become activated by CLL cell stimulation with bryostatin due to autocrine stimulation by secreted factors as is described by Battle and Frank [165]. Interestingly, Obeid *et al*. [4] showed that stimulation of cells transfected with the basal region of the *PRKCB* promoter with phorbol ester, another PKC agonist, leads to hyperactivation of promoter activity. Assuming that PKCβ protein expression within the cell system used by Obeid *et al.* [4] would be decreased by phorbol ester treatment in a similar way as is observed in CLL cells, it is therefore likely that PKCβ activity is required for modulating STAT3 association with the promoter rather than with simulating Sp1 association. Future work will need to address this proposition.

Taken together, these findings are of importance because they show a new relationship between STAT3 and Sp1 in the regulation of PKCβ gene expression, and provide further insight into the role of VEGF in the pathophysiology of CLL cells. Within proliferation centres it is reasonable to assume that CLL cells would be exposed to factors such as IL-6 [348-350]and IL-21 [351, 352] which would stimulate and activate STAT3, to possibly suppress PKCβ expression. VEGF is also present within proliferation centres [353], and its function is to modulate the suppressive effects of STAT3 activation by IL-6/IL-21 and promote the high PKCβII expression that is observed. Potentially, VEGF-stimulation of PKCβII activity inhibits STAT3 suppression of *PRKCB* transcription. The mechanism how is mediated now needs further investigation.

❖ Chapter Seven: General Discussion and conclusions

PKCβII overexpression is a hallmark feature of CLL cells. At the beginning of this work there were few studies investigating the regulation of *PRKCB*, the gene encoding PKCβII. The earliest of these studies described the basal promoter of the *PRKCB* gene, and gave hints regarding the factors which drove activity of this promoter [4, 161]. Subsequent studies identified roles for RUNX1 [6], MITF [7] and STAT3 [8], but the binding sites for these transcription factors are outside of the basal promoter region of *PRKCB* and do not majorly contribute to driving transcription of this gene. Additionally, epigenetic factors were also taken into consideration because the *PRKCB* promoter region is rich in CpG islands making it a candidate for methylation [4], as were cell extrinsic factors such as VEGF and Bryostatin which were shown to respectively stimulate or inhibit PKCβ gene expression [143, 340]. None of these studies identified the central transcription factor regulating *PRKCB* transcription. Thus, a clear and full understanding of the mechanisms regulating this gene was missing.

The main aim of this thesis was to provide insight into the regulatory mechanisms of *PRKCB* expression in CLL cells. I found that the overexpression of PKCβII in CLL cells is derived mainly by Sp1-mediated transcription of *PRKCB*. Importantly, the result I present in Figure 3.7 shows that Sp1 is overexpressed in CLL cells and correlates with PKCβII mRNA levels, suggesting that these factors are linked and potentially contribute to the pathophysiology of these malignant cells. PKCβII is known to be overexpressed in other cancer cell types such as lung and colorectal cells and this is correlated with disease progression [304, 354]. Interestingly, these malignant cells also show overexpression of Sp1 [355-357]. Thus, the overexpression of PKCβII in these cells may be due to deregulated expression of Sp1 in a similar way to my findings in CLL cells. Importantly, in CLL cells there are a number of genes such as Bcl2 [191], TCL-1 [269], and LEF1 [358] that are overexpressed, and which have Sp1 binding sites within their promoters. It is

therefore possible that overexpression of Sp1 in CLL cells may lead to deregulated expression of these genes as well.

Sp1 is a ubiquitous transcription factor regulating the expression of many genes. It can act to stimulate transcription of genes with TATA or TATA-less promoters [59, 143], and Sp1-driven expression of some genes has been found to play an important role in the pathogenesis of epithelial cancers [359, 360]. With respect to *PRKCB*, the promoter which governs expression of this gene is TATA-less [4]. This is important for the reliance of *PRKCB* on Sp1 to mediate transcription. The TATA box of gene promoters is responsible for coordinating assembly of the machinery responsible for gene transcription [361-363]. In the case of Sp1, the protein structure of this transcription factor is able to recruit this machinery to effect transcription from genes which lack the TATA box [364-370]. The studies describing the basal promoter of *PRKCB* suggested two potential binding sites for Sp1 based on recognition of consensus binding sites [4]. In Chapter 3 I show clearly that Sp1 is the major driver of *PRKCB* expression in CLL cells. Importantly, I show that both Sp1 binding sites are required for transcription to occur, mutation of either Sp1 binding site results in complete suppression of promoter activity. This finding is consistent with a model of how Sp1 mediates transcription from TATA-less gene promoters, such promoters tend to have multiple Sp1 binding sites where each plays a role in driving transcription of the gene [369, 371].

The reliance of *PRKCB* in CLL cells on Sp1 to mediate transcription is further supported by my studies demonstrating a relationship between Sp1 binding to the *PRKCB* promoter and PKCβ mRNA. This relationship partially explains the mechanism of how VEGF mediates PKCβ gene expression in CLL cells. Thus, work presented in this thesis and by others in this Department [143] show that PKCβ mRNA levels decrease in CLL cells that are cultured overnight. Stimulation of cultured CLL cells with VEGF results in maintenance or increased PKCβ mRNA levels. The work I present in this thesis shows that Sp1 binding to the *PRKCB* promoter in CLL cells decreases with overnight culture of unstimulated CLL cells, and increases

in cells that have been stimulated with VEGF. This data suggests that Sp1 binding to the *PRKCB* promoter for induction of transcription can be regulated by cell-extrinsic factors. VEGF may accomplish this by inducing post-translational modification of Sp1 to change its ability to associate with DNA. Indeed, Sp1 can be modified by phosphorylation, acetylation, ubiquitination and sumoylation [49, 176, 372]. However, the role of each of these modifications on Sp1 function seems specific to particular promoters and cells lines, and has not been clearly delineated owing to the ubiquitous role of Sp1 in transcribing many genes and a lack of appropriate molecular tools.

In Chapter 4 I addressed the role of other potential transcription factors in regulating the expression of *PRKCB* in CLL cells. I investigated RUNX1, Sp3, MITF, E2F1 and c-Myc as well as STAT3 because of the reported role of these transcription factors in regulating the expression of this gene [158, 162-164]. In the first instance I found that RUNX1 does not associate with the *PRKCB* promoter in primary CLL cells. My cell line model, MEC1, showed expression of RUNX1, but ChIP analysis showed no association with the *PRKCB* promoter in these cells although association could be demonstrated with *EVI1*, used as a positive control. Therefore, it is highly unlikely that RUNX1 contributes to PKCβ overexpression in CLL cells.

Investigation of MITF and E2F1 showed that these transcription factors were static with respect to transcription of *PRKCB*. Thus, MITF and E2F1 bound the promoter of *PRKCB* at higher levels in CLL than in normal B cells, but they did not appear to have a direct role in gene transcription. Knockdown of MITF resulted in a slight increase in PKCβ gene expression, whereas knockdown of E2F1 resulted in decreased PKCβ gene expression due to a reduction in Sp1 expression. These results suggest a passive role for MITF and E2F1, and it is possible that the increased binding observed in CLL compared to normal B cells could be due to the increased euchromatin surrounding *PRKCB* observed in the former cells. Alterations in chromatin structure play a role in regulating MITF access to and activation of gene promoters [373]. With respect to *PRKCB* the relaxation of chromatin structure allows MITF access to the promoter, but transcription does not occur for reasons unknown. A potential role for c-Myc is also included because it is expressed in CLL

cells from patients with progressive disease [374], and because these cells are likely to contain high levels of PKCβII [1]. However, c-Myc was not observed in association with the E-Box sites of the *PRKCB* promoter in either primary CLL cells or MEC-1 cells. Nevertheless, knockdown of c-Myc induced downregulation of PKCβ gene expression, and further analysis revealed a potential mechanism explaining this effect involving c-Myc regulation of E2F1, which, in turn, regulates Sp1 expression. Support for this mechanism is provided by studies showing that c-Myc regulates the expression of E2F1 [285], and that E2F1 can regulated the expression of Sp1 [257].

The consensus sequence that Sp1 and Sp3 bind to in DNA is very similar, and many genes show binding of Sp1 and Sp3 to their promoter regions [375-378]. Because of this similarity in consensus binding sequence, Sp3 is suggested to be a suppressor of Sp1 function particularly in the promoters which have more than one Sp1 binding sites [176, 276, 293]. Therefore, it was important to examine the potential role of Sp3 in *PRKCB* transcription. Importantly, Sp3 seems overexpressed in CLL compared to normal B cells. This implies that increased association of Sp1 with the *PRKCB* promoter is not the result of decreased Sp3 expression resulting in reduced competition for the binding sites. Indeed, ChIP analysis of Sp3 showed that it does not associate with the *PRKCB* promoter. Nevertheless, knockdown of Sp3 resulted in decreased levels of PKCβ mRNA indicating an indirect role for Sp3 in expression of *PRKCB*. This role was defined in experiments showing that knockdown of Sp3 reduced Sp1 expression in CLL and MEC1 cells. This notion is supported by studies showing that Sp3 regulates Sp1 gene expression [278], and is consistent with the observation that Sp3 shows higher levels of expression in CLL cells. Thus, high expression of Sp3 contributes to high expression of Sp1 and, effectively, PKCβII. High expression of Sp3 has been observed in other cancers where overexpression of Sp1 has also been observed [291, 379, 380]. Potentially, deregulation of Sp3 expression could contribute to the behaviour of the malignant cells in these diseases.

Analysis of the role STAT3 plays in regulating *PRKCB* transcription in CLL cells was important for two reasons; firstly, STAT3 was shown to suppress *PRKCB* promoter activity in a myeloid cell system [8], and, secondly, STAT3 is known to be

constitutively active in CLL cells [238]. Therefore, it is highly likely that STAT3 plays a role in regulating PKCβ gene expression in CLL cells. Indeed, examination of *PRKCB* promoter activity in MEC1 cells shows that STAT3 binding is important because mutation of the binding site results in increased activity. Moreover, incubation of MEC1 cells bearing the wt promoter construct with VEGF or with IL-6/IL-21 respectively shows increased and suppressed promoter activity. This role of STAT3 is echoed in experiments with primary CLL cells which showed increased association of STAT3 with the *PRKCB* promoter in cells cultured overnight in unstimulated conditions known to result in increased levels of pY-STAT3 [243], and decreased association of STAT3 in cells cultured in the presence of VEGF. Previous work from this Department showed that VEGF induced PKCβ gene expression through stimulation of PKCβII activity. In my experiments, inhibition of PKCβII activity blocked *PRKCB* promoter activity in transfected MEC1 cells. This suggests a potential role of PKCβII in regulating the suppressive effects of STAT3 on the *PRKCB* promoter. Further work is now necessary to clarify how PKCβII mediates its effect on STAT3, and whether this occurs when STAT3 is maximally activated by cytokines such as IL-6 and IL-21. The proliferation centres where CLL cells survive and expand contain high levels of VEGF, IL-6 and IL-21 as well as other stimuli [348-353]. PKCβII protein levels in CLL cells within this environment is shown to be high [143], suggesting that VEGF may override the suppressive effects of STAT3.

This work could potentially clarify the relationship between STAT3 and Sp1 on the *PRKCB* promoter. My experiments show that VEGF induces the association of Sp1 whilst decreasing the accessibility for STAT3 to *PRKCB*. Conversely, in unstimulated cells, the increased binding of STAT3 sees reduction of Sp1 association with the promoter. It seems logical from these experiments that STAT3 binding to the *PRKCB* promoter may limit the accessibility of Sp1, and this would be the mechanism by which STAT3 mediates its suppression function. How this is achieved could be due to cooperation with MITF, which I have also shown interacts with the *PRKCB* promoter. This cooperation between STAT3 and MITF could involve the participation of a third protein known as Protein inhibitor of activated STAT3 (PIAS3). This protein is known to interact with both MITF and STAT3 to limit their

transcriptional activity [381, 382]. In particular, IL-6 is demonstrated to stimulate co-association of STAT3, PIAS3 and MITF [383]. PIAS3 is potentially a target of PKC, and VEGF stimulation of CLL cells may influence the ability of IL-6 to stimulate STAT3-PIAS3-MITF complex formation. The ensuing result is STAT3 is unable to bind the *PRKCB* promoter and Sp1 is free to bind and upregulate expression of the gene. Support for this notion is suggested by my experiment where I knockdown expression of MITF and see a slight upregulation in PKCβ gene expression.

Access of Sp1 to the *PRKCB* promoter in CLL cells is not only regulated by STAT3, but also by epigenetic factors. During the course of this thesis a paper by Higawara *et al.* showed that Sp1 binding to *PRKCB* could be blocked by hypermethylation of its binding site by the gene product of *PROX1* [5]. However, my studies show that the *PRKCB* promoter in CLL cells is similar to that in normal B cells and is unmethylated. This indicates that gene methylation does not play a role in overexpression of PKCβII in CLL. Instead, my results suggest that the greater access of Sp1 to the *PRKCB* promoter in CLL cells is likely due to enrichment of H3Ac and H3K4me3 which are histone marks both connected with euchromatin [42]. This appears to be an active process in CLL because treatment of CLL cells with an inhibitor of HATs reduces the H3Ac and H3K4me3 histone marks and Sp1 association with the *PRKCB* promoter. The role of chromatin structure in regulating CLL cell phenotype and malignant cell behaviour is largely unexplored.

In conclusion, I demonstrate a clear role for Sp1 as a major driver of PKCβII overexpression in CLL cells. This role appears to be the result of changes in chromatin structure leading to increased access of Sp1 to the *PRKCB* promoter, increased expression of Sp1 itself (potentially driven by high expression of Sp3) and decreased ability of STAT3 to suppress *PRKCB* transcription caused by cell extrinsic factors which affect CLL survival and growth in proliferation centres (Figure 7.1). Sp1 regulates the expression of many genes within cells, and potentially the findings I present in this thesis could provide insight into the mechanism controlling the overexpression of other genes that define the malignant cell phenotype of CLL. Moreover, there is a well-established bidirectional relationship between CLL cells and their microenvironmental support cells where the latter provides survival

signals. Recently, Lutzny et al., have shown that CLL cells induce expression of PKCβII in microenvironmental stromal cells leading to NFκB activation and ultimate support of the malignant cells in this system [131]. My findings may explain the mechanism how CLL cells and other neoplastic B cells stimulate the expression of PKCβII in supportive stromal cells.

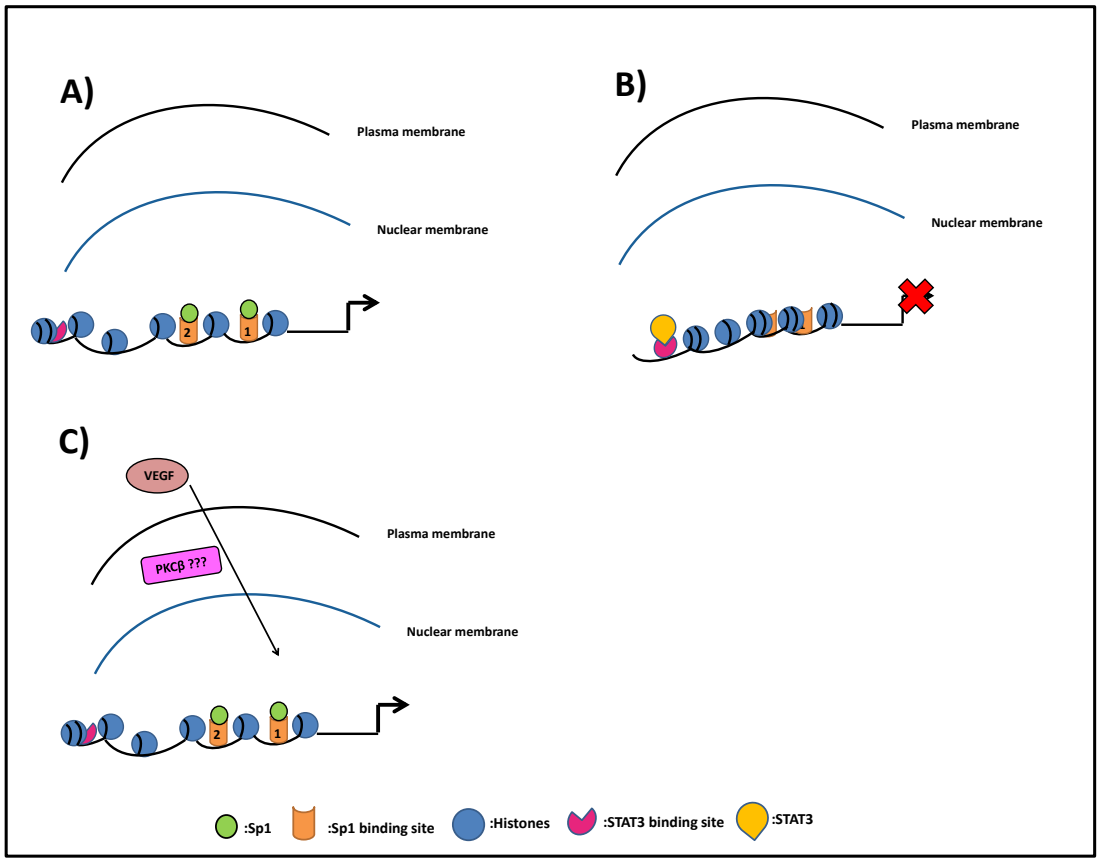


Figure 7.1: Model summary for the transcription of *PRKCB* in CLL cells. This Model represents the transcription of *PRKCB* in CLL cells. **A)** Represents basal promoter transcription driven mainly by Sp1. **B)** Represents inactive promoter status where binding of STAT3 suppresses transcription. **C)** Represents stimulation of *PRKCB* transcription by VEGF whereby STAT3 binding is reduced and Sp1 binding is increased.

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-Appendix A:

Clinical data of CLL patients used in this study

Sample ID	Gender	IgVH mutation	P53 status	CD38 (%)	Disease stage (Binet/Rai)	Cytogenetic abnormalities
3340	Male	3.44%	NK	NK	IV/ C	NK
3336	Female	NK	NK	NK	II/C	NK
2329	Male	NK	WT	NK	NK	Normal
3052	Male	3.44%	NK	NK	NK	NK
2172	Female	NK	NK	NK	NK	NK
3041	Male	NK	NK	NK	A	NK
2823	Male	NK	NA	NK	NK	NK
2155	Female	0	C	NK	NK	NK
2294	Female	NK	NK	NK	NK	NK
2354	Female	NK	NK	NK	NK	NK
2264	Female	NK	Normal	NK	NK	Normal
2532	Male	NK	NK	NK	NK	NK
3026	Female	NK	NK	NK	NK	NK
2027	Male	2.05%	WT	NK	NK	NK
2437	Male	NK	B	NK	B	NK
3105	Male	NK	NK	95.8%	NK	NK
1874	Male	NK	NK	5%	A/0	Normal
3144	Female	NK	NK	NK	C	NK
1786	Female	NK	NK	NK	B/IV	NK
2724	Female	NK	NK	NK	B	NK
3354	Female	NK	NK	NK	B/I	NK
3365	Female	NK	NK	NK	C/0	NK

3325	Female	5.21%	NK	NK	C/IV	Del17p13 (77%)
3244	Male	0.00%	NK	NK	B	NK
3028	Female	NK	NK	NK	A	NK
2727	Male	NK	WT	NK	A	NK
1767	Female	NK	NK	NK	B	NK
2686	Female	NK	NK	NK	NK	NK
3347	Female	NK	NK	NK	III	NK
2683	Male	NK	NK	NK	NK	NK
3221	Male	NK	NK	NK	NK	NK

NK: Not known

-Appendix B:

-Mycoplasma Test protocol:

The mycoplasma infection test was applied for the recent passaged cultured cell lines. The cells were suspended with 100µl of sterile PBS after washing them twice. Subsequently the cell suspension was vortexed for 5-10sec after heating for 10min at 95^{o}C. Then, the supernatant was collected in sterile eppendorff after spin down the cells at 13,000 RPM for 2min and kept in ice until it was used for the test. The next PCR step in the mycoplasma test protocol was done by the lab technicians. Briefly, 10µl of the cell supernatant was added as a PCR template to each e-Myco^{TM} Mycoplasma PCR detection kit (v.2.0) (Intro Biotechnologies, Korea) tube and were suspended with another 10µl of sterile water to give a total volume of 20µl of PCR reaction. Simultaneously, another 20µl of a negative control, sterile water only, and a positive control were running alongside the tested samples in the PCR reaction. The following thermal profile was used for the PCR reaction an initial denaturation step at 94^{o}C for 1min. followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 60^{o}C for 20sec, and extension at 72^{o}C for 1min. The reaction was further extended for one cycle at 72^{o}C for 5min.Finally, the PCR products beside the DNA leader were run in 2% agarose gel. The samples consider infected if they showed a similar band size like the positive control.

-Appendix C:

Sequence and the optimized conditions of qRT-PCR and ChIP primers

Sequence of primers and techniques that used for	Temperature of	
A) primers were used in qPCR for mRNA expression	**Annealing**	**Collecting data**
PKCβ For: (5'- TGGGGTGACACCCAAGACATTC-3') Rev: (5'- GCTGGATCTCTTTGCGTTCAAG-3')	58^{o}C	81^{o}C
Sp1 For: (5'-GACGTTGATGCCACTGTTG-GCAAG-3') Rev: (5' -TCAAGACCCACCAGAATAAGAAGGGAG-3')	64^{o}C	80^{o}C
Sp3 For:5'-CAGGAGGAACAACGCTTATC-3' Rev:5'-TCTCATTTCCAGAAACTGTGAC-3'	57^{o}C	80^{o}C
MITF For: (5'-CTCACAGCGTGTATTTTTCCCACAG-3') Rev: (5' -TGGTTCCCTTGTTCCAGCGCATG-3')	64^{o}C	80^{o}C
p300 For: (5'-CGCTTTGTCTACACCTGCAA-3') Rev: (5'-TGCTGGTTGTTGCTCTCATC-3')	57^{o}C	80^{o}C
BCL10 For: (5'-CCCGCTCCGCCTCCTCTCCTT-3') Rev: (5'-GGCGCTTCTTCCGGGTCCGG-3')	65^{o}C	80^{o}C
B) Primers were used in qPCR ChIP analysis for the *PRKCB* promote and the Positive controls primers		
PRKCB promoter proximal region For: (5'-GCACGCTTAGCCGCGAGG-3') Rev: (5'-AGCTGCTGCCGCTCGTCC-3')	56^{o}C	85^{o}C
PRKCB promoter distal region For: (5'-TATTGATCTACTGAAATCCTTCCTC-3') Rev: (5'-ATCCCATTGGTCATTCTGCA-3')	58^{o}C	85^{o}C
+500bp region of PRKCB Promoter TSS For: (5'-ACTTCATCTGGTGAGCGCGC-3') Rev: (5'-AGGACTGTCCATCCGGGAGT-3')	72^{o}C	85^{o}C
-500bp region of PRKCB Promoter TSS For: (5'-ACTTCATCTGGTGAGCGCGC-3') Rev: (5'-AGGACTGTCCATCCGGGAGT-3')	64^{o}C	85^{o}C
DHFR promoter Sp1/Sp3/E2F1 positive primer For: (5'- TCGCCTGCACAAATAGGGAC-3') Rev: (5'-AGAACGCGCGGGTCAAGTTT-3')	60^{o}C	85^{o}C
Nucleolin promoter c-Myc positive primer For: (5'- TTGCGACGCGTACGAGCTGG-3') Rev: (5'-ACTCCGACTAGGGCCGATAC-3')	65^{o}C	85^{o}C
EVI1 promoter RUNX1 positive primer For: (5'- TCACTTCGGACAGTTTCCTG-3') Rev: (5'-CCCGGCTTAGCAACGTAGA-3')	58^{o}C	81^{o}C
c-Fos promoter STAT3 positive primer (Cell Signalling, Technology, USA)	60^{o}C	85^{o}C

-Appendix D:

List of antibodies using in Western blot and ChIP analysis

Antibody	Source	dilution	Application
Anti-PKCβII rabbit polyclonal	Santa Cruz Biotechnology, Inc, USA	1:20000	W.B
ChIPAb+^{TM}Sp1 rabbit polyclonal	MerckMillipore, Watford, UK	1:10000 5µg	W.B ChIP
ChIPAb+ Acetyl-Histone H3 rabbit polyclonal	MerckMillipore, Watford, UK	5µg	ChIP
ChIPAb+ Trimethyl- Histone H3 (Lys4) rabbit monoclonal	MerckMillipore, Watford, UK	3µL	ChIP
Anti-Sp3 rabbit polyclonal	MerckMillipore, Watford, UK	1:5000 5µg	W.B ChIP
Anti-c-Myc (N-262)rabbit polyclonal	Santa Cruz Biotechnology, Inc, USA	5µg	ChIP
Anti-c-Myc monoclonal rabbit	Cell signalling Technology, USA	1:1000	W.B
Anti-E2F1 rabbit polyclonal (C-20)	Santa Cruz Biotechnology, Inc, USA	1:1000 2µg	W.B ChIP
Anti-MITF ChIP grade (ab122982) rabbit polyclonal	Abcam, Cambridge, UK	1:1000 1:200	W.B ChIP
Anti-P300 (N-15) rabbit polyclonal	Santa Cruz Biotechnology, Inc, USA	1:1000 7µl	W.B ChIP
BCL2 rabbit polyclonal	Cell signalling Technology, USA	1:1000	W.B
ERK1/2 mouse monoclonal	Santa Cruz Biotechnology, Inc, USA	1:2000	W.B
Anti-RUNX1 rabbit polyclonal	Calbiochem, UK	5µg	ChIP