Functional Role of ZAP70 in Chronic Lymphocytic Leukaemia

UNIVERSITY OF CAMBRIDGE

Vijitha Sathiaseelan

Department of Haematology

Cambridge Stem Cell Institute

This dissertation is submitted for the degree of

Doctor of Philosophy

Wolfson College

September 2019

Declaration of Originality

- This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.
- It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.
- It does not exceed the prescribed word limit for the relevant Degree Committee.

Functional Role of ZAP70 in Chronic Lymphocytic Leukaemia

Vijitha Sathiaseelan

1. Abstract

Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia. It is common in older people and rare in people under 40. CLL is characterised by the accumulation of monoclonal CD5+CD23+ B lymphocytes in the blood, bone marrow and secondary lymphatic tissues. There are 2 main subtypes of CLL based on the IgHV status: Un-mutated CLL (UM-CLL) and Mutated CLL (M-CLL). M-CLL leads to good prognosis whereas UM-CLL is the more aggressive form of the disease that could lead to Richter syndrome. Prognostics markers for CLL are the IgHV mutational status, chromosomal aberrations, CD38 expression and ZAP70 expression. Of these 4 prognostic markers, some are very labour intensive and not easy to perform on a routine basis, however ZAP70 expression has been a reliable predictor for the prognosis of CLL.

In this study, I investigated the role of ZAP70 in CLL. This was done using P505-15, a dual selective SYK and ZAP70 inhibitor, to reduce kinase activity of ZAP70. This was followed by siRNA knockdown experiments to disentangle effects of SYK and ZAP70 and reduce off target effects from other kinases. In addition, to better characterise protein interactors of ZAP70, B cell lines expressing both BirA ligase as well as ectopic expression of ZAP70 were generated. Then, immunoprecipitation of endogenous ZAP70 in patient CLL cells was carried out followed by mass spectrometry analysis.

BCR signalling is known to play a fundamental role in CLL cell survival and proliferation. Here, I have shown that P505-15 reduces calcium mobilization, cell survival and proliferation in ZAP70 positive CLL cells. Knockdown of ZAP70 using siRNA in CLL patient cells was achieved, however efficiency varied between patients. Knockdown of ZAP70 using siRNA showed no significant change in calcium mobilization compared to non specific siRNA. Using the ZAP70 knockdown cells, I further investigated changes downstream of the BCR signalling pathway. Comparing siZAP70 to siNSC, siZAP70 showed an increase

in pAKT levels in CLL cells post anti IgM activation, however no changes in NF-κB activity were observed.

Finally, mass spectrometry using BJAB cells and CLL cells positive for ZAP70, confirmed the role of ZAP70 in BCR signalling. In addition, it unexpectedly unveiled ZAP70's role in CLL cell migration and it's potential role in the DNA damage pathway in CLL cells.

Table of Contents

Acknowledgements

This PhD study has been a life changing experience and would not have been possible to do without the support and guidance from many people.

I would like to express my sincere gratitude to my supervisor, Dr. Ingo Ringshausen for giving me the opportunity to be part of his lab and providing great support, mentorship, motivation and immense knowledge throughout my PhD as well as providing a supportive environment for me to shape my scientific career. Many thanks to Dr. Hodson, my second supervisor for his technical advice throughout my PhD work.

Members of my lab as well as Hodson's lab have been incredibly supportive throughout my PhD, always happy to share tips / advice / support. They also gave me opportunities to try out activities during team building day, which I normally would not have dared to do. Special thanks to Andrew and Rebecca for taking time to proof read my thesis and Jade, Jane, Jingyu (3J's) for all their support and encouragement.

I thankfully acknowledge Cancer Research UK for their financial support to successfully complete my PhD work and all the patients donating samples without whom this work would not have been possible.

I am eternally grateful to my lovely parents for believing in me and encouraging me to follow my dreams. My heartfelt thanks to them as well as my mother in law, who were so supportive throughout my PhD. From skyping to help my children with homework to spending time with my children during their holiday visits.

Finally, my beautiful family, my husband Arjuna who has been my greatest support and my lovely children Nandha and Kumara. All their compromises and all the missed family dinner meals and holidays just to allow me to complete successfully will always be appreciated and remembered.

Abbreviations

2-ME	2-Mercaptoethanol
A	Alanine
ABC DLBCL	Activated B-cell like diffuse large B cell lymphoma
AKT	AMP activated protein kinase
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and RAD 3
BCL-2	B-cell lymphoma 2
BCR	B cell receptor
BIM	BCL-2-interacting mediator of cell death
BirA	Biotin ligase
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
Ca	Calcium
CD40L	CD40 ligand
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CLL	B-cell chronic lymphocytic leukaemia
cNLS	Classical nuclear localization signal
CpG	5'-C-phosphate-G-3'
DAPI	4'6-diamidino-2-phenylindole
DMEM	Dulbecco's Modifies Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxycycline
DSB	Double stranded break
EDTA	Ethylene diamine tetraacetic acid
ERK	Extracellular signal regulated kinase
EV	Empty vector
F	Phenylalanine
FACS	Fluorescence activated cell sorting

FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GC	Germinal centre
GCB DLBCL	Germinal centre B-cell like diffuse large B cell lymphoma
GFP	Green fluorescent protein
GSEA	Gene set enrichment analysis
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Homologous recombination
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgHV	Variable region of the immunoglobulin heavy chain
ITAM	Immunoreceptor tyrosine based activation motifs
K	Lysine
Lck	Lymphocyte specific protein tyrosine kinase
M-CLL	Mutated CLL
NaCl	Sodium chloride
NF-κB	Nuclear factor ‘kappa light chain enhancer’ of activated B-cells
NHEJ	Non homologous end joining
NSC	Non specific control
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PE	Phycoerythrin
Puro	Puromycin
RNAi	RNA interference
siRNA	Small interfering Ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TCR	T cell receptor
UM-CLL	Unmutated CLL
µl	Microliters
Y	Tyrosine
ZAP70	Zeta-chain associated protein

2. Introduction

2.1 B Cell Development

B Lymphocytes originate from haematopoietic precursors in the bone marrow (BM). The earliest cell type to differentiate from the haematopoietic progenitor cells (HSC) and commit to the B cell lineage is a Pro B cell (Patton, Plumb, & Abraham, 2014). Pro B cells in the bone marrow have to generate functional B cell receptors. The B cell receptor is composed of 2 parts: Membrane bound immunoglobulin molecules of one isotype (IgD, IgM, IgA, IgG or IgE) and a signal transduction moiety. The membrane immunoglobulin, also known as antigen binding subunit, is composed of two immunoglobulin light chains (IgLs) and two immunoglobulin heavy chains (IgHs). Specifically, the antigen binding site is composed of three distinct genetic regions: variable (V), diversity (D), and joining (J), that undergo a two step DNA recombination process. This is an error prone process involving the combinatorial rearrangement of the V,D,J gene segments in the heavy chain locus and V,J gene segments in the light chain loci (Nguyen, Pawlikowska, Firlej, Rosselli, & Aoufouchi, 2016). These variations result in the diverse ($>10^{10}$) combinations of functional VDJH and VJL rearrangements encoding the BCR (Ralph & Matsen, 2016), allowing for the recognition of antigens from all types of pathogens including bacteria, parasites, and viruses. During this two-step DNA recombination process in the bone marrow, Pro B cells undergo rearrangement of immunoglobulin (Ig) heavy chain genes, to become pre B cells. Mutations or alterations at the pre B cell stage could result in the development of cancer, immunodeficiency or autoimmunity. These cells further undergo light chain rearrangement to enable cells to express surface IgM, to become immature B lymphocytes. Upon exiting the BM, they enter the peripheral blood and start to express IgD to become naive B cells. Further activation by antigen presenting cells results in differentiation into plasma cells or memory B cells (Figure 1).

Throughout B cell development, the cells undergo several checkpoints to ensure the BCR is functional. Checkpoint 1 occurs after the rearrangement of the

functional heavy chain which pairs with the light chain to form pre BCR. Successful intracellular signalling events then prompt the B cells to rearrange and express the functional light chain. Failure to transduce the pre BCR signal results in arrest of further development of the B cell. The second checkpoint in B cell development is to test the interaction of BCR with bone marrow derived antigens (autoreactivity). Here, the diverse antibody repertoire is generated and checks that the BCRs recognise foreign and not self antigens. A vast majority of B cells react with self-antigens, resulting in cell death (negative selection), however non-reactive BCRs that survive are positively selected to exit the bone marrow and enter the peripheral blood.

In this thesis, I will be focusing on one particular B cell malignancy: Chronic Lymphocytic Leukaemia (CLL).

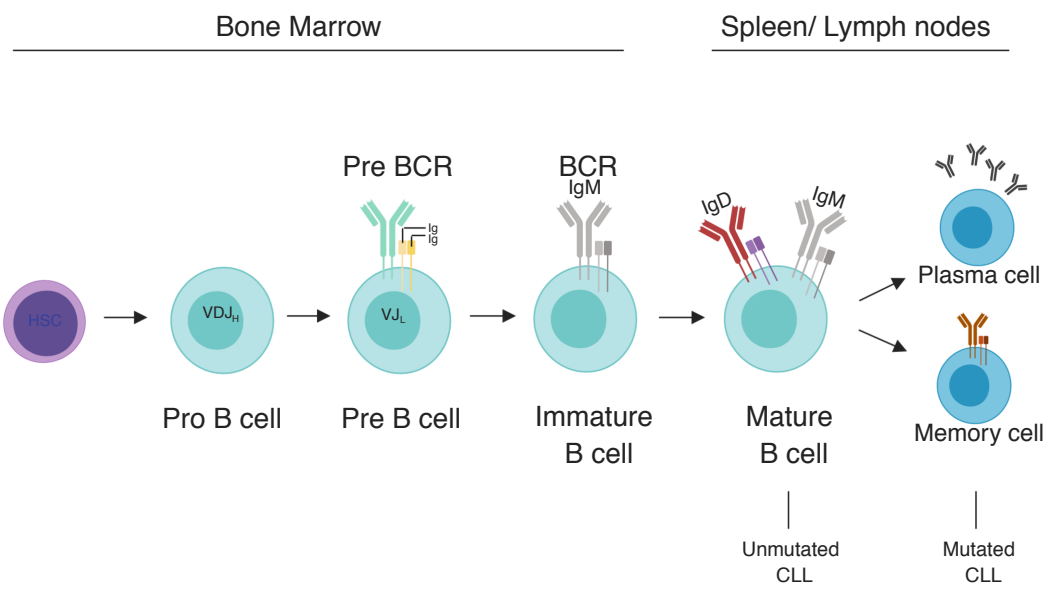


Figure 1: Key steps in B cell development: modified from (Ten Hacken, Gounari, Ghia, & Burger, 2019). B cell development starts in the bone marrow and progresses from haematopoietic stem cells through to pro B, pre B and immature pre B cell stages. Pre B cell receptor expression, with IgM heavy chain and surrogate light chains are achieved post VDJ rearrangement.

2.2 B Cell Chronic Lymphocytic Leukaemia (CLL)

2.2.1 Biology and diagnosis of CLL

According to cancer statistics, compiled by CRUK, in 2016 there were 1008 deaths due to CLL in the UK. Incidence rate is highest in people between the ages of 85-89 (2014-2016), with a higher frequency in males. There has been an 18% increase in incident rate over time.

CLL is a very prevalent type of leukaemia due to long survival of CLL patients. Leukemic cells build up very slowly over time with minimal symptoms, however the accumulation and spread of CLL lymphocytes to other parts including lymph nodes, liver and spleen can lead to a symptomatic disease (Rawstron et al., 2018; Rodrigues et al., 2016).

Early diagnosis can be achieved through blood tests showing the presence of at least 5,000 B-lymphocytes per microliter in the peripheral blood (Molica et al., 2014). CLL is known to be a very clinically heterogeneous disease, some patients show a prolonged survival while others experience an aggressive form of the disease requiring early treatment and suffering from frequent relapse (Rassenti et al 2004). Despite the many promising ongoing research studies, CLL has been incurable to date.

CLL cells arise from both pre and post-germinal centre B lymphocytes (Figure 1) and is characterized by the accumulation of monoclonal CD5+CD23+ B lymphocytes in the blood, bone marrow and secondary lymphatic tissues (Ponader et al., 2012). Specifically, CLL phenotype is distinctive with leukaemic B cells expressing B cell markers such as CD19, low level of CD20 and high level of CD23 (Hamblin, Davis, Gardiner, Oscier, & Stevenson, 1999). About 2-10% of CLL patients develop to Richters syndrome (Parikh, Kay, & Shanafelt, 2014), an aggressive form of lymphoma.

2.2.2 Current treatments for CLL/ B Cell immune response

Over the years, inhibitors such as ibrutinib, idelalisib and venetoclax have been used as a choice of treatment for CLL.

Ibrutinib is a targeted drug that blocks bruton tyrosine kinase protein (BTK). BTK plays a main role in B-cell receptor signalling and signals CLL cells to proliferate and survive. BTK plays a role in multiple downstream signalling pathways including signal transducer and activator of transcription 3 (STAT3) involved in pathogenesis of CLL and inducing/sustaining tumour immune tolerance (Kondo et al., 2018).

There are also several oral SYK inhibitors including fostamatinib (R788), entospeletinib (GS-9973), and cerdulatinib (PRT062607) being used in clinical trials. SYK is a cytosolic non-receptor protein tyrosine kinase (PTK) discovered in 1990 and expressed in haematopoietic cells. SYK is a critical element in the BCR signalling pathway and in cooperation with other BCR signalling molecules, plays a vital role in signal transduction independent from the BCR (Liu & Mamorska-Dyga, 2017). In particular, PRT062607 (also known as P505-15 referred to in this thesis) is an oral kinase inhibitor against SYK, and ZAP70. In vitro studies using this inhibitor in ABC and GCB lymphoma cell lines induced apoptosis and cell cycle arrest. In primary CLL cells, treatment with P505-15 inhibited BCR and IL4 induced downstream signalling in CLL cells and reduced CCL3/CCL4 productions (Blunt MD, Koehnrer S, Dobson RC, Larrayoz M, Wilmore S, Hayman A, Parnell J, Smith LD, Davies A, Johnson PWM, Conley PB, Pandey A, Strefford JC, Stevenson FK, Packham G, Forconi F, Coffey GP, Burer JA, 2017).

2.2.3 Prognostic Markers of CLL

Disease prognosis in CLL is extremely variable however abnormality in the immunoglobulin heavy chain variable (IgHV) has been used as a prognostic marker. However this technique is very laborious, expensive and time consuming for most medical centres to carry out as a routine procedure. Therefore, other predictors of prognosis and treatment response for CLL patients were sought which included expression of CD38 surface marker, level of zeta associated protein 70 (ZAP70) (Rassenti et al. 2004) and other chromosomal aberrations discussed in detail below.

IgHV mutational status

CLL patients are classified as: Mutated IgHV (M) or Unmutated IgHV (UM) based on the degree of somatic mutations. The criteria for mutated IgHV is greater than 2% deviation from germ line sequence (Fais et al., 1998). As mentioned above, after antigen engagement, V,D,J gene segments in heavy chain and V, J segments in light chain are modified by somatic mutations to higher affinity.

CLL cells that carry the UM-IgHV likely undergo malignant transformation prior to the entry into germinal centres and result in poor prognosis, on the other hand CLL cells with the M-IgHV that transit through the germinal centre and then undergo final development generally have good prognosis (Hamblin et al., 1999) (Figure 1). Both subtypes have similar gene expression patterns, hence it's been suggested that the difference in disease progression is due to BCR signalling which is the most important pathway activated in CLL cells in proliferative areas within lymphatic tissues (Burger & Chiorazzi, 2013) discussed in detail in section 2.5.

Chromosomal aberrations in CLL

CLL cells are known to carry a number of recurrent genomic aberrations that appear during the course of the disease and can be used as another prognostic marker. These aberrations are 13q14 (deletion on the long arm of chromosome 13, occurring in > 50% of patients), trisomy 12 (10-20% of patients) and 11q22-q23 deletion (10-20% cases). In regards to disease prognosis, patients with 17pDEL and 11qDEL experience more aggressive disease, whereas patients

with 13qDEL as the only abnormality have an indolent clinical outcome (Puiggros, Blanco, & Espinet, 2014; Stilgenbauer, Bullinger, Lichter, & Döhner, 2002).

In a few of these aberrations, a molecular target has been identified: in the case of 11q22-23 deletion, the ATM (Ataxia telangiectasia mutated) tumour suppressor gene is localized at this deleted site (Jiang et al., 2016). This is further discussed in section 2.8.3. The other deletions in chromosomal band 13q14.3 are targeting genes encoding microRNAs miR-15a/16-1. miRNAs are short RNAs, between 19-25 nucleotides, that regulate gene expression. Their deregulation can alter expression levels of genes involved in the development and progression of tumours. CLL patients with various IgHV and ZAP70 kinase status have shown a unique miRNA signature in miR-15/16, miR-29 and miR-155 and are composed of the most frequently deregulated miRNAs in the various malignancies. Particularly, miR-29 was found to be down regulated in CLL and a strong predictor of aggressive disease (Stamatopoulos et al., 2009). In addition, it was found that miR-21 expression stratifies survival of patients with 17pDEL (Rossi et al., 2010). Aberrations in chromosome 17 and the p53 mutations (17p 13.1) play vital roles in the pathogenesis of CLL. The gene p53 is a tumour suppressor, mutations in this gene result in an aggressive CLL disease with rapid progression and poor prognosis with low survival in the patients (Lens et al., 1997).

This feature of chromosomal aberrations is associated with the DNA damage response (DDR) pathway and dysregulation of the cell cycle. CLL progression is greatly affected by defects in DDR pathway and interacting cells in the microenvironment. Sequencing studies have identified somatic gene mutations in TP53, ATM, CHK1, CHK2, and BRCA1 in CLL patients, which are all involved in the DDR pathway explained in detail in section 2.8.

CD38 surface marker

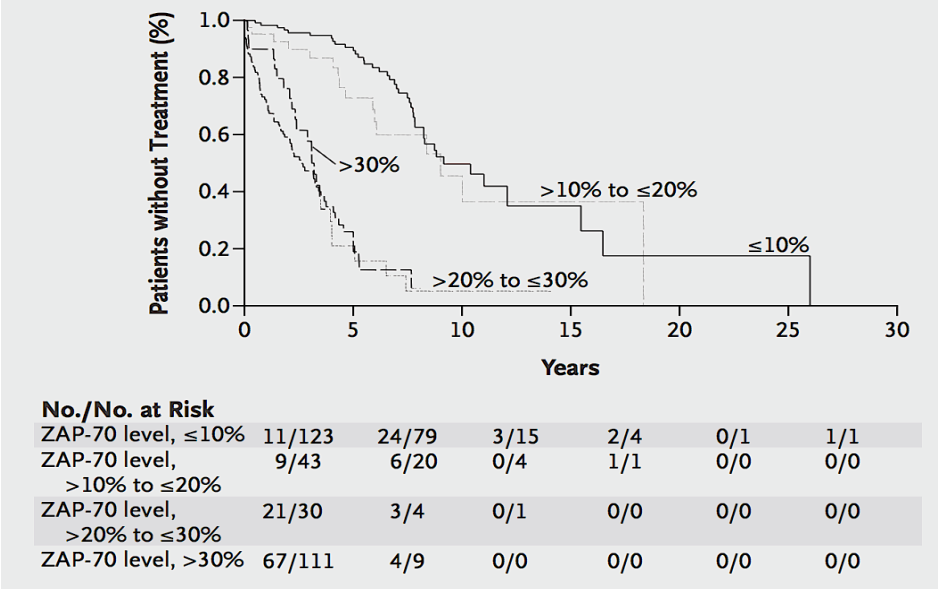
CD38 is considered to be a reliable marker of unfavourable prognosis in CLL. B lineage progenitors in the BM, B-lymphocytes in the germinal centre, activated tonsils and terminally differentiated plasma cells all express high levels of CD38. However, memory B cells express low levels of CD38. CD38 is an integral

surface marker and known to localize in membrane lipid microdomains in close proximity with the BCR complex (CD19/CD81) and with molecules regulating homing (CXCR4 and CD49d). The threshold of CD38 positivity in CLL cells is >= 30% to be considered positive. Increased CD38+ve clones are more responsive to BCR signalling *in vitro* (Malavasi et al., 2011). In addition, Vaisitti et al, showed an association between CD38 and migratory behaviour of CLL cells, and a direct functional cooperation between CD38 and CXCR4 signalling pathways (Vaisitti et al., 2010).

ZAP70

Another important prognostic marker in CLL is ZAP70 expression. Wiestner, A. *et al* have elegantly shown using a cohort of 107 patients that ZAP70 gene expression can be used to distinguish the UM-CLL and M-CLL subtypes. UM-CLL cells expressed 5.54 fold more ZAP70 compared to the M-CLL, which correctly predicted the IgHV status in 93% of the patients (Wiestner, 2003). Similarly, the majority of leukaemic cells without IgHV mutations expressed high levels of ZAP70, whereas leukaemic cells with IgHV mutations had very low levels of ZAP70 (Crespo et al., 2003). In addition it's been shown by flow cytometric analysis that ZAP70 expression alone can be used as a prognostic marker using the 20% cut off for ZAP70 positive B cells criteria and giving a simple and reliable surrogate marker for the identification of IgHV mutations. However, based on large cohort data, quantitative ZAP70 and CD38 expressions cannot replace IgVH analysis but in contrary addition of quantitative data can further specify prognoses of patients with CLL.

As shown in Figure 2, lower expression of ZAP-70 is associated with better prognosis (Rassenti et al. 2004), but exact mechanisms are not clearly understood.



No./No. at Risk						
ZAP-70 level, ≤10%	11/123	24/79	3/15	2/4	0/1	1/1
ZAP-70 level, >10% to ≤20%	9/43	6/20	0/4	1/1	0/0	0/0
ZAP-70 level, >20% to ≤30%	21/30	3/4	0/1	0/0	0/0	0/0
ZAP-70 level, >30%	67/111	4/9	0/0	0/0	0/0	0/0

Figure 2: Relationship between ZAP70 expression level and the time from diagnosis to initial therapy (Rassenti et al. 2004). CLL cells with ZAP70 expression ≤ 10% had median times from diagnosis to initial treatment as 9.2 years, which was not significantly different from CLL patients with ≥ 10% but ≤ 20%. Similarly two patient cohorts, ≥20 to ≤30% and >30%, had a median survival time of 3 years from diagnosis to initial therapy.

2.3 ZAP70 Zeta chain associated protein kinase 70

2.3.1 Discovery of ZAP70

ZAP70 is a SYK related tyrosine kinase first identified in T Cell Receptor (TCR) stimulated Jurkat cells (Chan et al. 1991). ZAP70 is expressed in T cells and Natural killer (NK) cells and is important for proximal TCR and NK cell signalling (Chan et al. 1992). Studies have shown that ZAP70 is expressed in CLL B cells (Chen et al. 2008) and expression of ZAP70 (> or =20% of B cells by flow cytometry) has been associated with an increased risk for an adverse outcome in B CLL cells and is considered an important risk factor in these patients. ZAP70 is highly homologous to SYK, hence defined as a member of the SYK/ZAP70 kinase family (Taniguchi et al., 1991). Specifically, amino acid sequence comparison between ZAP70 and porcine SYK revealed a 60 amino acid divergent region between the SH2(C) and the kinase domains (residues 248-308

in ZAP70 and residues 247-334 in porcine SYK) that includes a 26 amino acid insertion in SYK not present in ZAP70. Similarly an additional 17 amino acid C terminal extension is present in ZAP70 and absent in SYK. These changes were suggested to mediate specific interactions of ZAP70/SYK with effectors upstream or downstream (Andrew C. Chan, Iwashima, Turck, & Weiss, 1992).

The ZAP70 gene is located on chromosome 2q11.2 and is composed of 14 exons. ZAP70 is composed of two N terminal SH2 domains and a C terminal kinase domain. There are also 2 linker domains: Interdomain A which connects both SH2 domains, and interdomain B that connects the SH2 domain to the kinase domain (Figure 3). The SH2 domain plays an important role in double phosphorylation of the immunoreceptor tyrosine based activation motif (ITAM) and Interdomain B contains many tyrosine residues that aid in regulating the ZAP70 kinase activity. Specifically, Interdomain B contains three tyrosine residues: Y292, Y315 and Y319 which when phosphorylated by lymphocyte specific protein tyrosine kinase (Lck) upon TCR activation serve as docking sites for downstream signalling molecules (H. Wang et al., 2010). These tyrosine residues are explained in detail in section 2.4.1. The kinase domain of ZAP70 contains a conserved region similar to other protein tyrosine kinases. Specifically, there is 38% amino acid identity with abl and the src family PTKs (Andrew C. Chan et al., 1992). Major differences between ZAP70 and src family PTKs are that ZAP70 lacks a negative regulatory C terminal tyrosine residue and also lacks a myristylation site at amino acid 2 but contains a catalytic domain (Andrew C. Chan et al.,1992).

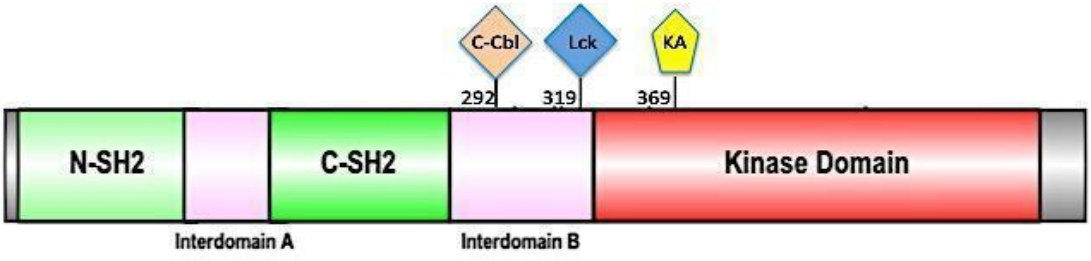


Figure 3: Schematic of ZAP70 domains and phosphorylated tyrosine residues modified from (Yan et al., 2013). ZAP70 contains two SH2 domains connected by two interdomains (A and B) and a kinase domain. SH2 domains are vital for ITAM phosphorylation and Interdomain B contains tyrosine residues important for phosphorylation.

2.3.2 ZAP70 Localization

Using a chimeric ZAP70 protein fused to variant GFP expressed in epithelial cells, ZAP70 has been shown to be expressed throughout the quiescent cells and rapidly recruited to the plasma membrane upon cellular activation of T cells (Sloan-Lancaster et al. 1997). Interestingly, the same group had published that ZAP70 resides in the nucleus of quiescent and activated Jurkat T cells. TCRε chain plays a major role in the redistribution of ZAP70 enhanced with co-expression of the active form of LCK (Sloan-Lancaster et al., 1998). Due to the large size of the ZAP70 protein, it cannot passively diffuse across the nuclear lamina. Therefore presence of nuclear localization signals (NLS) in the protein is needed for active transportation across nuclear lamina (unpublished: Ringshausen group). Here, using *in silico* analysis, it was shown clearly that ZAP70 carries three potential classical NLS which was confirmed using HELA cells transfected with ZAP70 (WT) GFP_ZAP70 wild type, ZAP70 (KA) GFP ZAP70 Mutant K369A and various other truncated forms: ZAP70(Trunc1) (excludes N terminal SH2), ZAP70(Trunc2) (excludes both SH2), ZAP70(Trunc3) excluded whole N terminal except kinase domain, ZAP70 (Trunc 4) (expresses truncated kinase domain only) (Figure 4) (Unpublished: Ringshausen group).

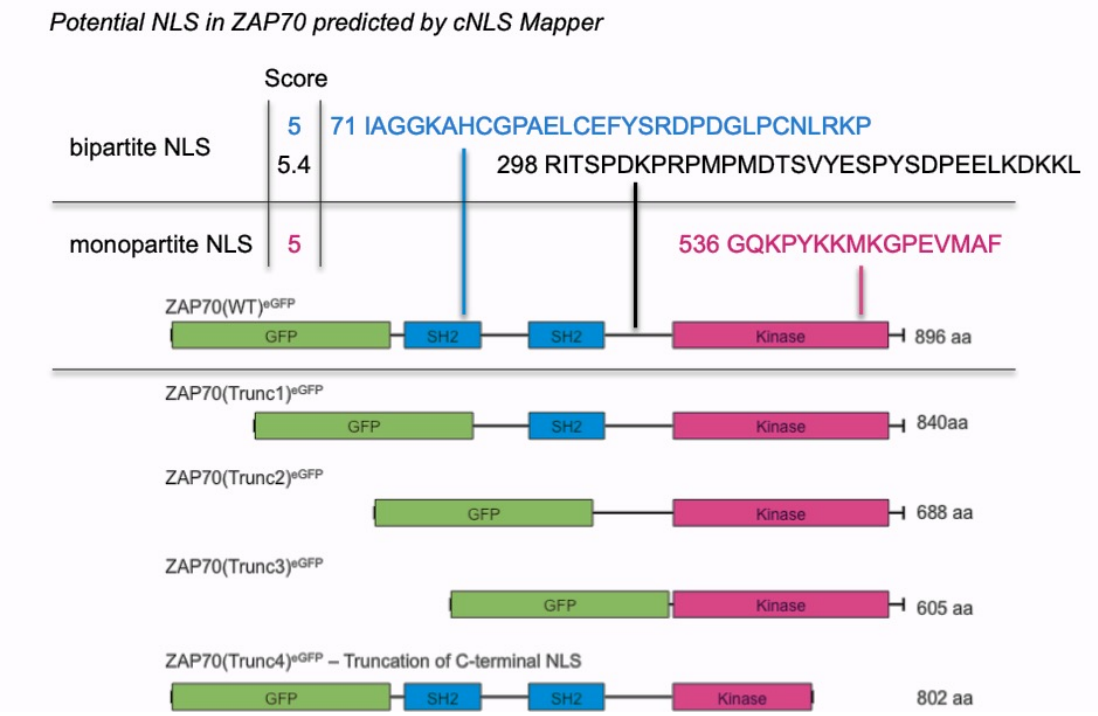


Figure 4: Potential NLS in ZAP70 predicted by cNLS Mapper (Unpublished: Ringshausen group). In silico analysis of potential nuclear localization sequence in the ZAP70 amino acid sequence. Established the three N-terminal truncation and one C-terminal truncation of the predicted monopartite NLS of ZAP70.

Here, HELA cells (non haematopoietic cell line) were used to study ZAP70 diffusion throughout the cell body (Unpublished: Ringshausen group) (Figure 5) and confirms presence of ZAP70 in the cytoplasm as well as nucleus.

In addition, it was also shown that nuclear ZAP70 upon stimulation via TCR can be phosphorylated (Sloan-Lancaster et al 1997).

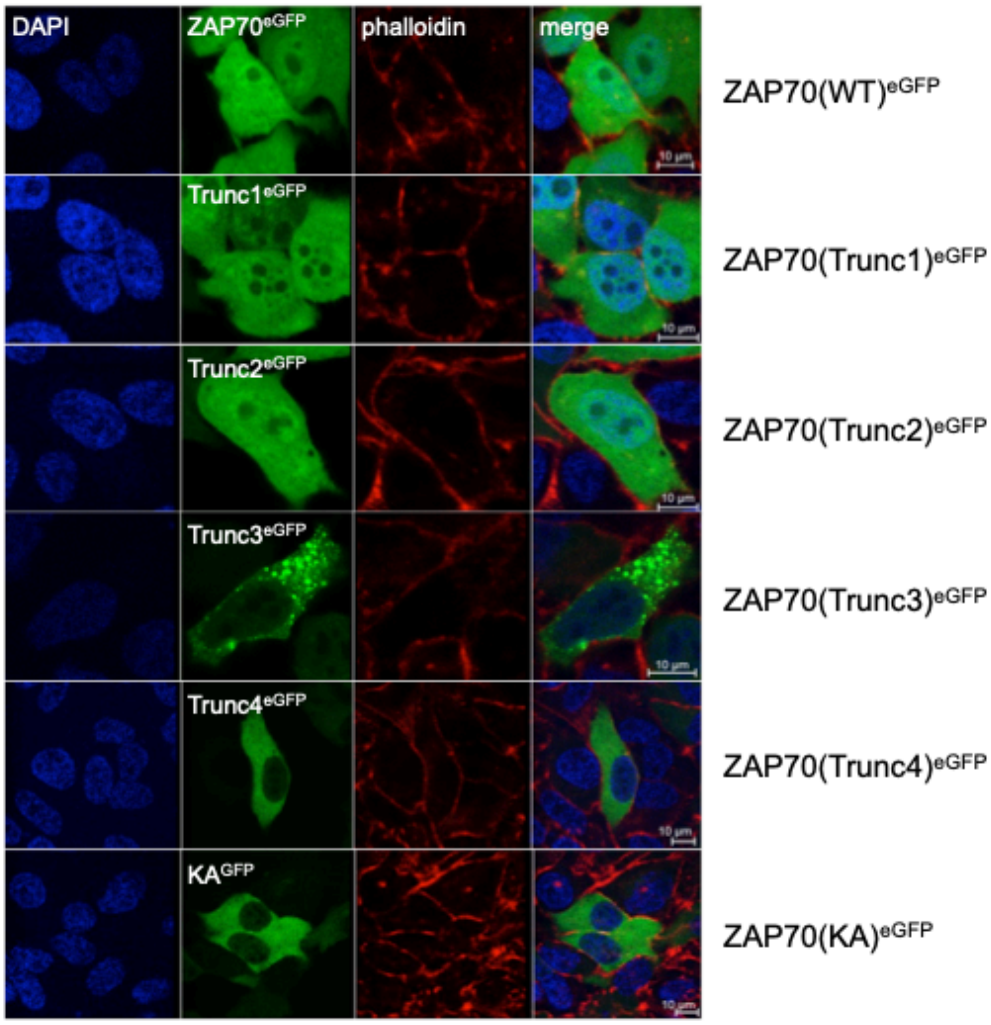


Figure 5: Nuclear translocation of ZAP70 (Unpublished: Ringshausen group). Immunofluorescence of HELA cells transfected with 6 different truncated versions of ZAP70 using lipofectamine. Trunc 1-3, N-terminal truncated products, one C-terminal truncated product and kinase deficient truncated product. Cells were counterstained with phalloidin for visualization of the cytoskeleton and with DAPI for nuclear depiction.

2.4 ZAP70 signalling in T cells

T lymphocytes (T cells) play a very important role in the cell mediated adaptive immune response by providing antigen recognition and cytotoxic reaction towards infected cells. T cells originate from haematopoietic stem cells in the bone marrow. T cells can be distinguished from other lymphocytes such as B cells and NK cells by the presence of the TCR on the cell surface.

ZAP70 is essential for normal development of T cells and in TCR signalling. This can be seen in ZAP70 deficient patients which have no functional T cells in their peripheral blood and suffer from severe combined immunodeficiency (SCID) (Elder et al., 1994). In 1994, Arpaia *et al* had reported that patients with mutations in the ZAP70 gene usually had severe T cell deficiencies, specifically these mutations were in the catalytic domain of ZAP70. Studies on ZAP70 deficient mice have shown that T cell development is arrested at the transition from double positive (CD4+CD8+) stage, where positive selection occurs (Kadlecek et al., 1998).

In 1991, Chan et al described a model describing TCR signalling mediated by ZAP70, in which upon the TCR encountering the peptide antigen bound to MHC complex on APC, the co-receptor associated LCK is brought near the CD3 complex and phosphorylates tyrosines in the ITAMS. When ITAM is doubly phosphorylated, ZAP70 is recruited with high affinity by binding to the tandem SH2 domains of ZAP70 (Figure 6). In addition, to achieving catalytic activity, the tyrosines in the activation loop of ZAP70 are then phosphorylated by LCK or ZAP70 itself. There are also other signalling proteins like LAT and SLP76 that are recruited in the process. All of these signalling events lead to T cell activation, proliferation and differentiation (A C Chan, Irving, Fraser, & Weiss, 1991).

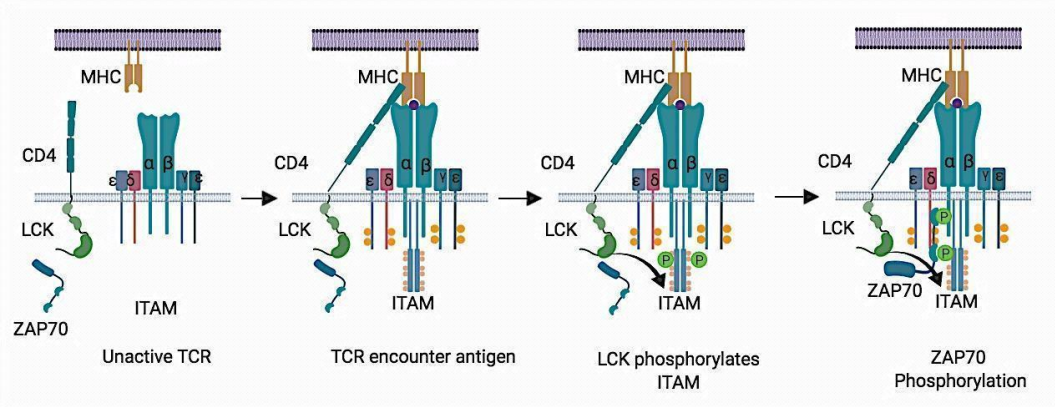


Figure 6: A sequential model for T cell activation modified from (Yan et al., 2013). TCR encounters the antigen, brings LCK nearer the CD3 complex and phosphorylates tyrosines in the ITAMS. Double phosphorylation of ITAM recruits ZAP70.

2.4.1 Roles of Y292, Y315 and Y319 during ZAP70 signalling

Tyrosine residue at Y292 has a negative regulatory function due to the recruitment of E3 ubiquitin ligase c-CBL. Zhao et al reported a negatively regulated role of Y292 in interdomain B. Tyrosine residues Y315 and Y319 have positive regulatory sites of phosphorylation. Both tyrosine resides become phosphorylated post TCR cross-linking by LCK and ZAP70 itself (Williams, 1999). Mutations of both Y315 and Y319 to phenylalanine (F) can render the kinase inactive (Wang et al. 2010). Another important role of interdomain B is stabilization of the active conformation and providing docking sites for other molecules that control ZAP70 function and downstream signalling (Wang et al. 2010). Tyrosine residue Y493, which is located in the activation loop of ZAP70, and its phosphorylation is important for full kinase activity of ZAP70 (Chan et al. 1995). Finally, ZAP70 mutation K369, that will be used in this study and referred to as KA mutant in this report, is a point mutation that changes lysine (K) to an alanine (A) at position 369. This mutation is located in the kinase domain of ZAP70 and it renders ZAP70 kinase inactive by preventing ATP binding (at position 345 to 352) due to alterations in the catalytic binding site (Johnson et al. 1996).

2.4.2 Mechanism of deregulation of ZAP70

In 2005, studies showed association of ZAP70 expression status in CLL with the methylation status of the intron1-exon2 boundary region of ZAP70 (Corcoran et al., 2005). Subsequently, in 2010, pyrosequening analysis of ZAP70 methylation in CLL uncovered several alternative sites in intron 1 and suggested a correlation of methylation with CD38 expression and IGHV mutation status (Chantepie et al., 2010). In addition, it was shown that ZAP70 promoter is associated with the active histone modifications and reduced DNA methylation led to transcriptional de-repression of ZAP70, therefore suggesting that expression of ZAP70 in CLL cells seems to be epigenetically regulated (Amin et al., 2012). Specifically, enrichment of di and tri-methylation of lysine 4 of histone H3 and higher levels of global acetylation are associated with the ZAP70 promoter regions in ZAP70 positive primary CLL cells.

2.5 BCR signalling

BCR signalling is very important in the pathogenesis of B cell lymphomas and has been extensively studied over many years (Herzog, Reth, & Jumaa, 2009). The BCR complex is composed of membrane immunoglobulin paired with the signal transduction component, Igα-Igβ heterodimers (CD79A and CD79B). Upon activation by an antigen, ITAMs in the cytoplasmic tail of CD79A and CD79B are phosphorylated by LYN, and other Src family kinases (Fyn, BLK). This results in recruitment of kinases, such as SYK and BTK, and subsequent activation of SYK (Rowley, Burkhardt, Chao, Matsueda, & Bolen, 1995). Further association of SYK with adaptor molecule B cell linker protein (BLNK) and its downstream signalling components BTK and PLC-γ2 results in B cell selection, proliferation, differentiation and antibody production (Moroni et al., 2004; Wossning et al., 2006) (Figure 7). As detailed in the review by Woyach et al, 2012, SYK causes the double phosphorylation of the CD79A/CD79B complex and LYN forms a complex with CD19 and provides continued amplification of the BCR. Initial recruitment of kinases/adaptor protein results in BCR activation via three main pathways: BTK, PLC_Y2 and PI3K. BTK self phosphorylates, however SYK and LYN leads to phosphorylation of PLC_Y2. In the phosphorylation of PLC_Y2, BLNK is recruited and is rapidly phosphorylated by SYK. Interaction of SYK, BTK, BLNK, and PLC_Y2 further dually phosphorylates PLC_Y2 to produce second messengers diacylglycerol (DAG: activator of protein kinase C) and inositol-1-4-5 triphophate (IP3) from the plasma membrane lipid phosphatidylinositol 4,5-biphosphate (PIP2). Presence of IP3 causes calcium influx from endoplasmic reticulum and extracellular compartment. This finally leads to translocation of NF-κB (nuclear factor “kappa light chain enhancer” of activated B cells) to the nucleus (Woyach, Johnson, & Byrd, 2012a).

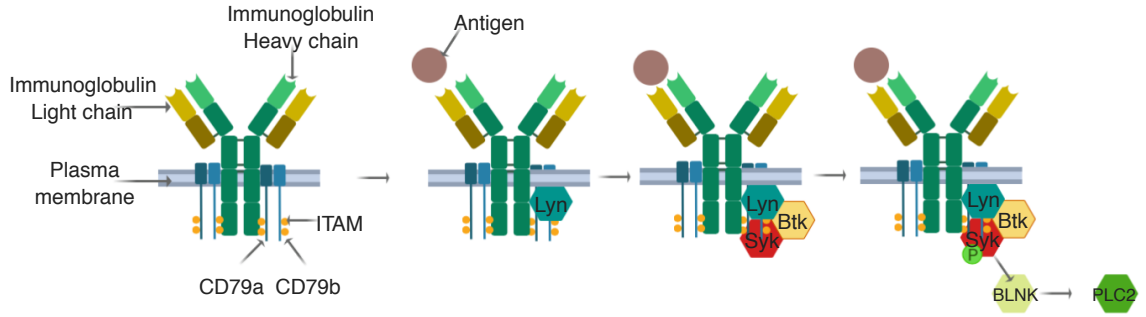


Figure 7: B cell receptor signalling pathways modified from (Woyach, Johnson, & Byrd, 2012b). Upon antigen activation, ITAM is phosphorylated by LYN resulting in the recruitment of BTK and SYK. Phosphorylation of SYK leads to recruitment of BLNK and activation of PLC_Y2 and further activation of downstream pathways.

2.5.1 BCR signalling in CLL

Despite the current view that the majority of immature B cells that express autoreactive B cell receptors are negatively selected, it has been shown that polyreactive BCRs recognize multiple self antigens, induced autonomous signalling and selective expansion of B cell precursors in a similar manner to pre BCR (Köhler et al., 2008). Autonomous signalling occurs whereby the surrogate light chain induces ligand independent pre BCR crosslinking and leads to expansion, which is needed for positive selection of B cells.

In B cell lymphomas, there are two different mechanisms of BCR activation: Antigen dependent or ligand independent ("tonic" BCR signalling) resulting in B cell selection, proliferation, differentiation and antibody production. Activating mutations in the BCR pathway are common in DLBCL, however in CLL, BCR pathway activation does not involve activating mutations, hence it is antigen dependent (auto antigen or microbial antigens present in the microenvironment), which results in the oncogenic transformation and disease progression (Binder et al.,2013; Minden et al., 2012). Similarly, functional snapshots of the CLL B cell phenotype suggest that CLL cells are aberrantly held in a hyperactive state similar to B cells exposed to antigen (Damle et al., 2002). "Tonic" activation was suggested due to the observation that phosphorylated LYN, SYK, and ERK were seen in un-activated primary CLL B cells (Contri et al., 2005; S. Gobessi et al.,

2009). This observation was supported by two key papers in 2012 and 2013 that showed an unexpected form of auto reactive BCR activation in CLL. In 2012, Duhren et al had shown that by transferring the heavy chain complementarity determining region (HCDR3) of a CLL derived BCR provides autonomous signalling capacity to a non autonomously active BCR, whereas mutations in the internal epitope abolishes this capability (Minden et al., 2012). This was followed by a report of an alternative epitope for CLL BCR self-recognition located in the FR3 of Igs playing a vital role in BCR signalling in CLL (Binder et al., 2013).

2.6 Effect of ZAP70 expression on BCR signalling in CLL

ZAP70 in the TCR was discovered many years ago, however the presence of ZAP70 in B cells is still unclear. ZAP70 plays a vital role in CLL and the presence of ZAP70 is strongly correlated with poor clinical outcome (Dürig et al., 2003). ZAP70 has been found to be associated with the B-cell receptor signalling in CLL (Chen et al. 2002). Furthermore, it has been shown that BCR signalling can be reconstituted by ZAP70 in SYK deficient B cells (Toyabe, Watanabe, Harada, Karasawa, & Uchiyama, 2001).

It is important to note that SYK is structurally homologous to ZAP70. BCR mediated activation of ZAP70 is very inefficient/ negligible compared to SYK in CLL, however the ability of ZAP70 to recruit downstream signalling molecules in response to activation is preserved (Stefania Gobessi et al., 2007). This was shown using avian lymphoma B cells, where even in the absence of SYK, ZAP70 was able to restore some BCR signalling events (Stefania Gobessi et al., 2007). Furthermore, in CLL B cells, introduction of ZAP70 (adenoviral gene transfer) in the presence of SYK, resulted in stronger activation of certain BCR signalling molecules including SYK, BLNK and PLC_Y (Pede et al. 2013). Furthermore, stronger calcium mobilization was observed in ZAP70 positive CLL cells compared to ZAP70 negative cells upon BCR ligation (Chen et al., 2005a). This suggests that the function of ZAP70 in CLL cells could be to enhance BCR signalling (Wang et al. 2010). Finally, it was reported that ZAP70 positive CLL cells showed increased proliferative response and enhanced survival upon TLR9 stimulation compared to ZAP70 negative cells, suggesting that ZAP70 may

operate as an amplifier for TLR9 mediated activation of the BCR signalling (Wagner et al., 2016).

Similar to the importance of BCR signalling in CLL, there are two other vital pathways that have an impact on CLL cells: the DNA damage pathway, (discussed briefly in the chromosomal aberrations section 2.2.3) and CLL cell migration. These 2 aspects and their relation to CLL will be discussed in detail in sections 2.8 and 2.9.

2.7 ZAP70 in other B cell neoplastic disorders

As mentioned in section 2.3.1, ZAP70 is expressed in CLL B cells (Chen et al. 2008) and > or =20% of B cells by flow cytometry have been associated with an increased risk for an adverse outcome in B CLL cells and is considered an important risk factor in these patients. In addition to CLL, studies using immunohistochemistry have characterised ZAP70 expression in other hematologic malignant neoplasms. Here, the vast majority of mature B-cell neoplasms do not express ZAP-70, however, they have shown that a small percentage of DLBCL (diffuse large B-cell lymphoma), MCL (mantle cell lymphoma) also express ZAP-70 (Sup et al., 2004). In 2005, another study confirmed the results and had assessed ZAP70 expression in a varied spectrum of B cell lymphoid neoplasms by immunohistochemistry. Here, the staining was compared to flow cytometry and relationship between ZAP70 and mutational status was assessed. ZAP70 positivity was obtained in 65% CLL, 31% Burkitts lymphoma, 29% lymphoblastic lymphomas, 8% MCL and 4% in marginal zone lymphomas and 2% diffuse large B cells lymphomas and absent in 14 samples of Hodgkin lymphomas and 19 follicular lymphomas (Carreras et al., 2005).

2.8 Relationship between CLL and DNA Damage pathway in CLL

2.8.1 DNA Damage Response Pathway

The DNA damage response (DDR) pathway functions to detect DNA damage, signal its presence, mediate DNA repair and maintain genomic stability (Kastan & Bartek, 2004). Both endogenous and exogenous DNA damage results in double

stranded breaks (DSB) and cells respond to DSB by activating DDR, which includes phosphorylation of the histone H2AX producing γH2AX (Kuo & Yang, 2008; Podhorecka, Skladanowski, & Bozko, 2010). H2AX is part of the H2A family, a component of the histone octomer in nucleosomes. H2AX is phosphorylated by two major kinases: ATM/CHK2 and ATR/CHK1 (Ray, Blevins, Wani, & Wani, 2016). This process of phosphorylation is the first step in recruiting and localizing DNA repair proteins resulting in formation of gamma H2AX foci (which is a biomarker for damage). A disturbed DDR plays a vital role in promoting CLL cell survival (Frenzel, Reinhardt, & Pallasch, 2016). Discussed in the next section are the two DSB repair mechanisms.

2.8.2 DNA double strand break repair

In mammalian cells, there are 2 major DSB repair mechanisms: error prone non-homologous end joining (NHEJ) pathway and homologous recombination (HR) mediated DSB repair (Arnoult et al., 2017). The NHEJ pathway takes place throughout all cell cycle phases and does not depend on an intact DNA replication product as a template for repair (Figure 8). NHEJ depends on catalytic activity of DNA-PK recruited by non catalytic subunits Ku70 and Ku80 (Wu et al., 2019a). KU is one part of the DNA-PK related to ATM checkpoint kinase. Specifically, NHEJ is a 3-step process: DNA end binding and bridging, terminal end processing and ligation. During the first step, NHEJ is initiated by recognition and binding of KU proteins to the damaged end. The KU heterodimer forms a DNA binding component for DNA-PK and acts to support and align the DNA ends to protect from degradation. This leads to recruitment of DNA-PK, serine threonine kinases in the PI3K family. DNA-PK phosphorylates DNA ligase IV. If ends are incompatible, artemis proteins trim off single stranded overhangs. Blunt ends are ligated accordingly (Davis & Chen, 2013).

On the other hand, HR requires the presence of an intact DNA replication product as a template and is an error free DSB repair mechanism (Figure 8). Due to its dependence on a template, this repair mechanism is only used in the late S and G2 cell cycle phase (Wu et al., 2019b). The HR repair pathway involves 3 steps: presynapsis, synapsis and post synapsis. During the presynaptic phase,

RAD51 is loaded onto single stranded DNA (ssDNA), the RAD51-ssDNA (presynaptic filament) comprises of six RAD51 molecules and 18 nucleotides per helical turn. During the next phase of synapsis, RAD51 supports the formation of a physical connection between invading DNA substrate and homologous duplex DNA template leading to formation of D loop. Here, RAD51-dsDNA filaments are formed with the aid of invading and donor ssDNA strands within the filament. Finally, post synapsis DNA is synthesized using the invading 3' end as a primer, RAD51 dissociates from dsDNA to expose 3"-OH required for DNA synthesis (Krejci, Altmannova, Spirek, & Zhao, 2012).

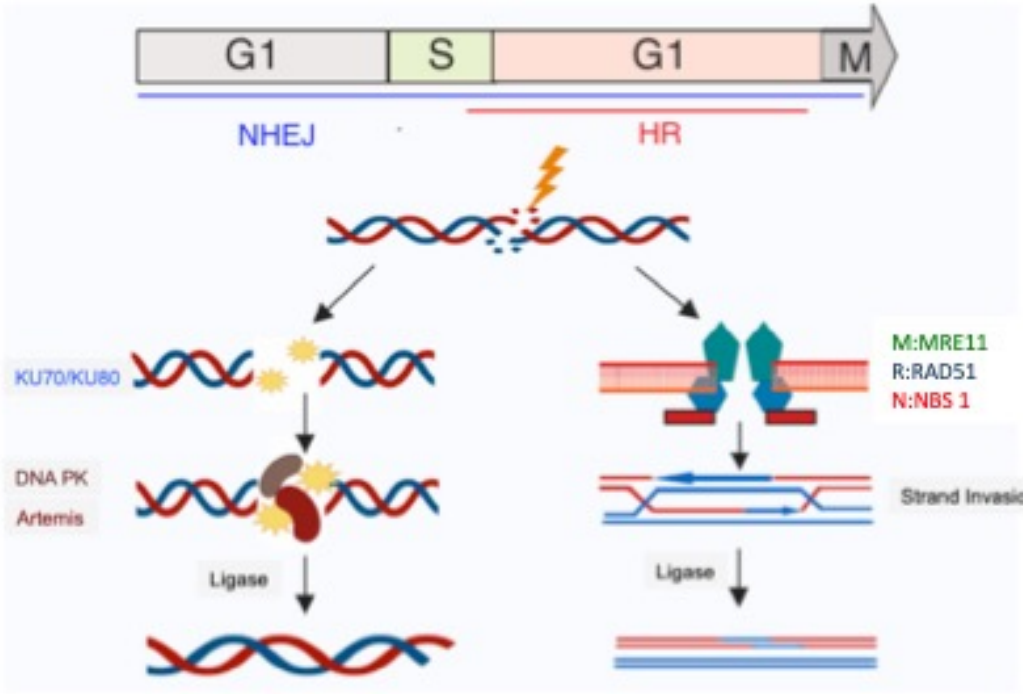


Figure 8: DNA damage repair mechanisms (modified from (Brandsma & Gent, 2012). NHEJ begins with recognition of DNA ends by KU70/80 that recruits DNA-PKs. Artemis trims the end if incompatible and ligase seals the break. In HR repair pathway, DSB ends are recognised and processed to 3'-OH ending single stranded tail. DNA strand invasion by RAD51 generates D loop, invading strand is disengaged after DNA synthesis and annealed with the second end (Li & Heyer, 2008).

2.8.3 ATM/ATR signalling

As mentioned earlier, initiation of the activities of the PI(3)K kinases, ATM and ATR are the first steps to inhibit cell cycle progression after DNA damage. The ATM gene maps to chromosome 11q22.q23, within the region that is deleted in CLL, resulting in ATM gene inactivation which is associated with defective apoptosis in response to chemotherapeutic agents (Guarini et al., 2012). The ATM is a nuclear serine/threonine kinase of 350kDa and a member of the PI3K family , which are activated in response to DNA double stranded breaks, induced by alkylating agents, topoisomerase inhibitors or ionizing radiation.

ATR is also a very large protein (301KDa) and belongs to the PI3K family, however it responds to a broader spectrum of DNA damage. Both ATM and ATR activate a second wave of phosphorylation through their activation of CHK1, CHK2 protein kinases upon induction due to single stranded breaks or DNA lesions (Matsuoka, Huang, & Elledge, 1998). Both ATM and ATR activate the cell cycle arresting target genes, DNA repair and apoptosis via p53 activation. Studies have shown that patients lacking ATM are unaffected, therefore suggesting that it is not essential for critical cellular functions such as normal cycle progression or cellular differentiation (Shiloh & Kastan, 2001). In the absence of DNA damage, ATM is present as a homodimer in which the kinase domain is blocked by its tight binding to an internal domain of the protein surrounding serine 1981, a major damage inducible phosphorylation site. Upon DSB, ATM undergoes conformational changes that stimulates phosphorylation of serine 1981 resulting in dissociation of the homodimer (Bakkenist & Kastan, 2003) and further phosphorylates p53.

In CLL, the pro-apoptotic ATM-CHK2-p53 signalling pathway is frequently mutationally inactivated through large deletions on chromosome 11q (ATM) or 17p (TP53) or through protein damaging mutations (Goy et al., 2017) as mentioned earlier. Most B-CLL cells undergo apoptotic death in response to DNA damage. However, the aggressive subset of B-CLL is completely resistant in vitro to irradiation-induced apoptosis (Vallat et al., 2003). Some of the therapies in CLL include alkylating agents and the combination of these DNA damaging drugs with purine nucleoside analogs. A current treatment option is combination

therapy of bendamustine and fludarabine. Results have confirmed that combination of both drugs together were synergistic (El-Mabhouh et al., 2014).

2.9 Importance of migration in CLL

2.9.1 Role of chemokines in CLL

Another aspect of CLL that is extensively studied is cell trafficking (Calpe et al., 2011; Quiroga et al., 2009). CLL cells in the peripheral blood (PB) are arrested in the G0/G1 cell cycle stage, and hence do not proliferate in the PB. CLL cell proliferation is thought to take place in proliferation centres in the LN and BM. Here CLL cells associate with CD40L+/CD4+ T helper cells to aid proliferation. Therefore CLL cells ought to migrate from PB to LN/BM (Richardson et al., 2006). Some of the factors involved are chemokines.

Chemokines are a family of small proteins (cytokines) secreted either constitutively or in response to stimulation by cells and play a major role in the immune system, specifically in regulating cell trafficking and homing of various immune cells. The presence of chemokine receptors are needed for a cell to respond to chemokines. Chemokine receptors belong to the seven transmembrane domain G protein coupled GPCRs family, that when activated initiate intracellular signalling, resulting in activation of small GTPases, and then downstream activation of integrins (molecules involved in cell adhesion) leading to cell movement. There are about 47 known chemokines and 19 chemokine receptors (Burger 2010). T and B lymphocytes both express receptors for various chemokines that are continuously expressed in the tissue microenvironment with their expression and function modulated during differentiation and lymphocyte activation. Chemokines and their receptors are important factors for organizing CLL cell trafficking and homing as well as their cellular interactions between CLL and accessory cells. Critical chemokines for CLL include CXCL12, CXCL13, CCL19/21 and their receptors CXCR4, CXCR5 and CCR7 respectively. Chemokines secreted by CLL cells are important in the recruitment of accessory cells. Other proteins that play a vital role in directing CLL cell migration within the

tissue microenvironment are integrins such as VLA-4 (CD49d) as well as selectins, MMP-9 and CD44 (Davids & Burger, 2013).

The CXCR4 chemokine receptor was one of the first to be discovered in CLL and has a high level of expression on the surface of peripheral blood CLL cells compared to B cells from healthy donors (Burger et al. 1999) (Richardson et al., 2006). CXCR4 expression is downregulated by its ligand CXCL12 (previously known as stromal cell derived factor-1 / SDF-1) via receptor endocytosis. CXCL12 is produced within primary and secondary lymphoid tissues, in non lymphoid tissues (homeostatic), and areas of increased encounter with antigens. Furthermore, CXCL12 induces phosphorylation of molecules involved in the formation of focal adhesions and reorganization of the cytoskeleton (Ticchioni et al., 2002). This chemokine has two major effects on CLL cells mediated through the CXCR4 receptor: (1) migration towards stromal cells and (2) provision of survival signals. CXCL12 not only affects CLL cell migration, but also provides a pro survival effect on CLL cells (Burger 2010). Quiroga et al. (2009) had shown that BCR signalling results in down regulation of CXCR4, along with enhanced chemotaxis towards CXCL12 and CXCL13.

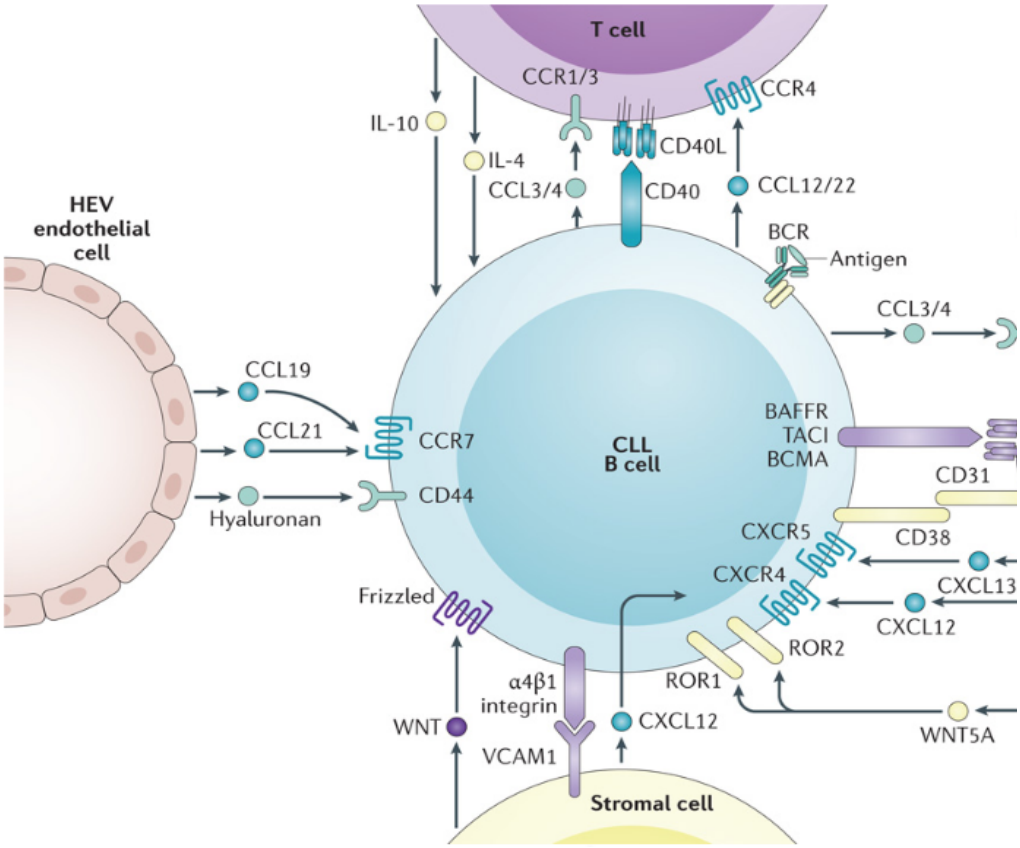


Figure 9: CLL cell migration (Devereux & Cuthill, 2017). Migration of CLL cells to proliferation centres is mediated by chemokine ligand 19 (CLL19) and 21(CCL21) via CC-chemokine receptor 7 (CCR7). These chemokines are produced by endothelial cells. In addition, migration can be mediated through CXCR4 in response to CXC-chemokine ligand 12 (CXCL12).

In addition to CXCR4, another receptor in CLL is the CCR7 receptor for chemokines CCL19 and CCL21, which are constitutively expressed by fibroblastic reticular cells, high endothelial venules (HEVs) and dendritic cells (DCS). In addition, CCR7 is expressed by CLL cells and migrates across vascular endothelium in response to CCL19 and CCL21 (Till et al. 2002). These chemokines are important in lymph node homing of naïve and regulatory T cells and DCS (Burger 2010). Finally, there is a correlation between migration index of B-CLL in response to CCR7 ligand and presence of clinical lymphadenopathy (López.Giral et al., 2004) (Figure 9).

Two other chemokines that have been found to be highly expressed and secreted by activated CLL cells are CCL3 and CCL4. These chemokines are

elevated in the plasma of CLL cells and are associated with poor prognosis. Data suggests that these chemokines function by recruiting CCR5+ regulatory T cells close to B cells, to provide highly efficient survival signals to B cells (Davids & Burger, 2013). CLL cells upregulate and secrete CCL3/4 in response to BCR stimulation in co culture with nurse like cells (NLCs) imitating the lymphatic tissue microenvironment.

2.9.2 Role of ZAP70 in Migration

ZAP70 expression is associated with higher responsiveness to BCR stimulation. ZAP70 positive CLL cells display increased chemotaxis and survival in response to CXCL12, CCL19 and CCL21 compared to ZAP70 negative CLL cells similar to CD38+ve CLL cells (Burger 2010). As previously mentioned, CXCR4 is more greatly expressed in B CLL cells compared to healthy donors, however comparing ZAP70 positive and ZAP70 negative patients, there is no significant difference in expression (Richardson et al., 2006). Ticchioni *et al*, 2002 showed that ZAP70 is required for CXCR4 signal transduction. Inhibition or absence of ZAP70 (Jurkat T cells/ primary CD4+ T cells ZAP70 negative patients) showed a lack of transendothelial migration rescued by transfection of ZAP70. Also, CXCL12 resulted in increased intracellular levels of F actin but no significant difference between ZAP70 positive and ZAP70 negative patients. However, CLL cell migration was significantly greater in ZAP70 positive CLL cells compared to ZAP70 negative cells towards CCL19 and CCL21 but not CXCL12 (Richardson et al., 2006).

Furthermore, CXCL12 induced phosphorylation of ZAP70, VAV1 and ERK, suggesting cross talk between ZAP70 and CXCR4. This crosstalk is needed for T cell migration (Ticchioni et al., 2002). Similarly, CXC12 treatment induced quick phosphorylation of ERK1 and ERK2 as early as two minutes with sustained signalling for up to 2 hours in ZAP70 positive cells (Richardson et al., 2006). Using an ectopic expressing Ramos cell line, it was shown that there was increased expression of CCR7 via ERK1/2 post IgM signalling which resulted in increased response and migration toward CCL21 (Calpe et al., 2011).

2.10 Aims of this Study

CLL is a malignancy of CD5+ B cells (Devereux & Cuthill, 2017). The differences between the two subtypes (M-CLL and UM-CLL) and their effects on disease prognosis has been well reported. In CLL, ZAP70 is a prognostic marker, however, the role of ZAP70 in B CLL remains elusive.

My goal in this study is to characterise involvement of ZAP70 in the pathogenesis of CLL. Based on published data (Chen et al., 2008; Gobessi et al., 2007; J. et al., 2010; Laufer, Lyck, & Legler, 2018), I set out to test my hypothesis that ZAP70 enhances BCR signalling with ramifications for cell proliferation, survival and migration. Specifically, reduction of ZAP70 kinase activity would decrease calcium mobilization, survival and proliferation of B CLL. Further downstream to BCR signalling, I hypothesise that reduced levels of ZAP70 would reduce phosphorylation levels of AKT and p65. In addition to the effect on BCR signalling, a further hypothesis is that ZAP70 in B CLL increases CLL cell migration. Lastly, based on data from the Ringshausen lab, where it was found that CLL cells had nuclear expression of ZAP70, and ZAP70 positive patients had poor prognosis. I hypothesise that nuclear ZAP70 contributes to DNA instability following genotoxic insults. All of my hypotheses will be investigated using the selective dual SYK/ZAP70 inhibitor P505-15 as well as using siRNA to knockdown ZAP70 in primary CLL cells. Finally, different mass spectrometry approaches will be explored to understand interacting partners of ZAP70 in BJAB and B CLL cells at both the steady state as well as upon anti IgM activation. This information would be extremely relevant not only to CLL but also to other B-cell malignancies.

3. Materials and Methods

3.1 Materials

3.1.1 Overview of siRNA primers

ZAP70 siRNA (siZAP70): Life Technologies: HSS187732, HSS187733, HSS187734.

Non-specific siRNA (siNSC): Thermofisher Scientific 12935300

SYK siRNA (siSYK): Life Technologies: SYKHSS110401, SYKHSS110402, SYKHSS186171

3.1.2 Retroviral vectors

pRetroCMV /T0/ Puro/GFP

pRetroCMV /T0/ Puro/

3.1.3 Overview of western blot antibodies used in this study

Table 1 – List of Antibodies used for western blotting

Antigen	Catalogue Number	Company
MOUSE ANTI ZAP-70 KINASE	610240	BD Bioscience
ZAP70 (L1E5) Mouse mAb	2709	Cell Signalling
pZAP70 Tyr 319/pSYK Tyr 352 (65E4) Rabbit mAb	2717	Cell Signalling
ZAP70 (LR)	SC-574	Santa Cruz

pBTK	87141	Cell Signalling
p-pERK	9101	Cell Signalling
β actin 13 E5 Rabbit mAb (HRP conjugate)	5125	Cell Signalling
RPl29	GTX101833	GeneTex
RPL37	GTX104688	GeneTex
Anti V5	R960-25	Invitrogen
FLAG	F3165	Sigma
APC Mouse Anti human CD19	555415	BD Pharmingen
Alexa Fluor 647 Mouse anti H2AX(pS139)	560447	BD Pharmingen
SYK (D115Q) Rabbit mAb	12358	Cell Signalling
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb	3033	Cell Signalling
NF-κB p65 (L8F6) Mouse mAb	6956	Cell Signalling
Phalloidin iFluor 488	AB176753	Abcam
Mouse monoclonal antibody to human/mouse ZAP-70 R-PE CLONE IE7.2	MHZAP7004	Invitrogen

Phospho Btk (Tyr223)	5082	Cell Signalling
Anti BTK	Ab137503	Abcam
Anti Mouse IgG RMG07	Ab197767	Abcam
Anti Rabbit IgG(H+L) F(ab')2 488 conjugate	4412	Cell Signalling
Phospho-Histone H2A-X(Ser139)	2577	Cell Signalling
Histone H2A	2578	Cell Signalling
Alexa 647 Mouse IgG1 K isotype control	557783	BD Bioscience
Rabbit IgG Polyclonal Isotype control	Ab27478	Abcam
Phospho-AKT(Ser 473)	4060	Cell Signalling
ZAP70 (IE7.2)	Sc-32760	Santa Cruz

3.1.4 Overview of cell lines

Table 2 - List of B cell lines used in this study.

Cell line	Type	Source
Mec 1	CLL	Dr Ringshausen
BJAB	DLBCL	Dr Hodson
HBL1	DLBCL	Dr Hodson
RAJI	DLBCL	Dr Hodson

3.1.5 Overview of qRT-PCR primers used in this study

Table 3 - List of qRT-PCR primers

Primer	Sequence
ZAP70 Forward	TATGGGAAGACGGTGTACCA
ZAP70 Reverse	AGAGCGTGTCAAACTTGGTG
GAPDH Forward	GTGAAGGTCGGAGTCAACG
GAPDH Reverse	TGAGGTCAATGAAGGGGTC

3.1.6 Cloning Primers

Table 4 - List of Primers used during cloning of ZAP70

Name	Sequence
Flagbio_Bamhi_ZAP70 F	AGGCGCGCCGAGCTCGAGGATCCTCCCAGACCCCGCGGCGCACCT
BAMHI ZAP70 F	GGATCCTCCCAGACCCCGCGGCGCACCTGCCCTTCTTCTACGGCAGCATCTCGCGTGCCGAGGCCGAG
BAMHI ZAP70 R	CCACCACACTGGACTAGTGGATCCTCAGGCACAGGCAGCCTCAG

3.1.7 ZAP70 Sequencing primers

Table 5 - List of ZAP70 sequencing primers

Name	Sequence
huZAP70_363_F	CATGGTGAGGGACTACGTGA
huZAP70_26_F	CCTTCTTCTACGGCAGCATC
huZAP70_1731_R	GCCTGTCCTCCCACTTGTAG
huZAP70_157_R	AAGTGGTGGAACCTCACGTC
huZAP70_1540_F	AGGAAGTTCAGCAGCAGGAG
huZAP70_1411_R	ATCTTGGCGTAGTGCCTGTT

huZAP70_1229_F	TGATGCTGGTGATGGAGATG
huZAP70_1136_R	CCTCATCATCTCCTCGGTGT
huZAP70_941_F	TGTACGAGAGCCCCTACAGC
huZAP70_847_R	CTGTTCAGGGTGTCGATCCT
huZAP70_702_F	GCTGGTGGAGTACCTGAAGC
huZAP70_544_R	GAACTTGCCGTCGGTCTG

3.1.8 Overview of Chemokines

Table 6: List of chemokines used in this study.

Chemokine	**Company**	**Catalogue number**
CCL21 Recombinant Human Exodus-2	Peprotech	300-35
CCL19 Recombinant Human MIP-3B	Peprotech	300-29B
CXCL12 Recombinant Human SDF-1A	Peprotech	300-28A

3.1.9 Overview of inhibitors

Table 7: List of Inhibitors used in this study.

Company	Catalogue Number	Inhibitor
Selleckchem	S8032	PRT062607 (P505-15, BIIB057) HCL
Cayman Chemical	16336	Ku-559332-(4-morpholinyl)-6-(1-thianthrenyl)-4H-Pyran-4-one
Selleckchem	S1212	Bendamustine HCL

3.1.10 Media and Buffers

CLL media: 500ml RPMI1640 + 5% Foetal Bovine Serum (FBS) + 50IU/ml Penicillin/streptomycin (Gibco) + 2mM L–Glutamine (Gibco) + 10mM HEPES (Gibco) + 0.7X Non-Essential Amino acids (Gibco) + 1mM Sodium Pyruvate (Gibco) + 0.05mM 2.mercaptoethanol

Cell lines: RPMI 1640 +10% FBS + penicillin/streptomycin/Glutamax

DMEM +10% FBS + penicillin/streptomycin

Staining buffer: 500ml of PBS + 1% FBS + 0.01% Sodium Azide

Flow cytometry (FACS buffer): 1X PBS + 0.5% BSA + 0.01% Sodium Azide

Nuclear extraction buffer A: 10mM HEPES pH7.9 +10mM KCL+300mM Sucrose+1.5mM MgCl2+ 0.5mM DTT+ 0.1% NP.40+ 0.5mM PMSF +1x cOmplete mini protease inhibitor

Nuclear extraction buffer B: 20mM HEPES pH7.9+ 100mM KCL+ 100mM NaCl+ 0.5mM DTT+ 20% glycerol + 0.5mM PMSF+ 1X cOmplete mini protease inhibitor (Roche)

Western Blot Buffers:

10X Running Buffer

Tris base (30.2g) + SDS (10g)+Glycine (144g) +water (up to 1000ml)

10X Transfer buffer

Tris base (30.2g)+ Glycine (144g) + water (up to 1000ml)

1X Transfer buffer

10X transfer buffer (100ml) + Methanol (200ml) + water (700ml)

10X TBS

Tris HCL (70.04g) + Tris base (6.66g) + NaCl (87.6g)

1X TBST

10X TBS (100ml) + Tween (1ml) + water (up to 1000ml)

Blocking buffer

3% BSA in TBST

Western blot polyacrylamide gel recipes:

Table 8 - Recipes for Running Gels (2 x (4ml) gels)

Reagent	Percentage Gel		
	8%	**10%**	**12%**
ddH2O	3.7ml	3.2ml	2.6ml
30% Acrylamide	2.13ml	2.67ml	3.2ml
1.5M Tris pH 8.8	2ml	2ml	2ml
10% SDS	80µL	80µL	80µL
10% APS	80µL	80µL	80µL
TEMED	8µL	8µL	8µL

Table 9 - Recipe for Stacking Gels (2 x (2ml) gels)

Reagent	Percentage
	6%
ddH2O	2.13ml
30% Acrylamide	0.8ml
0.5M Tris pH 6.8	1ml
10% SDS	40µL
10% APS	40µL
TEMED	4µL

3.2 Methods

3.2.1 Cell culture

BJAB BirA, HBL.1 BirA and Raji BirA cell lines were obtained from Dr Daniel Hodson and cultured in RPMI 1640 (Gibco) + 10% FBS + penicillin streptomycin and Glutamax (Gibco).

HEK 293 cells were cultured with DMEM (Sigma) + 10% FBS and penicillin/ streptomycin (Gibco)

For the inducible system, 100ng/ml of Doxycycline Hydrochloride (Sigma D3072) was used.

3.2.2 Isolation of PBMC from CLL patient blood samples.

Peripheral blood was collected from consented patients followed by PBMCs isolation using Ficoll-Paque plus as follows: blood was firstly diluted 1:1 in PBS and then carefully layered over 15ml of Ficoll dropwise. The blood Ficoll mixture was then centrifuged for 15 minutes at 880rcf at room temperature with no brakes. The cloudy interphase was then transferred into a new 50ml falcon tube and centrifuged for 10 minutes at 450rcf with brakes. The pellet was then resuspended in 10ml of CLL media and centrifuged again for 5 minutes at 880rcf. The pellet was then resuspended in 10ml of Pharmlyse (BD Biosciences) and incubated at room temperature for 10 minutes. After centrifugation, the cells were resuspended in 10ml of CLL media and cell purity experiments were carried out as described in section 3.2.5 and ZAP70 status determined as described in 3.2.6. Vials of white blood cells (WBC) were frozen down for all future experiments.

3.2.3 Flow cytometry

Antibodies and isotype controls used for flow cytometry measurements were purchased from Biolegend, ebioscience or BD Biosciences. All flow cytometry analyses were made using BD LSR II.

3.2.4 CLL Cell purity

Following isolation of PBMC, $1X10^6$ cells were washed once with staining buffer and centrifuged for 5 minutes at 500rcf. Cells were then suspended in 100µl of staining buffer. For CLL cell staining, 2µl of CD5 and 2ul of CD19 antibody were added to the tube and incubated for 20 minutes in the dark at room temperature. The cells were then washed once with 500µl of staining buffer and analysed using flow cytometry. Samples greater that 85% CLL purity (CD5+19+) were used for the experiments.

3.2.5 ZAP70 expression in CLL cells

For the ZAP70 staining, appropriate samples were stained with CD19 only for 20 minutes at room temperature in the dark and then fixed with 300µl of fixation buffer and incubated for 15 minutes at room temperature in the dark followed by 2 washes with 1x permeabilisation/wash buffer. The cells were then suspended in 100µl of 1x permeabilisation/wash buffer and 5µl of R.PE. conjugated monoclonal antibody against ZAP70 (clone 1E7.2), appropriate isotype controls were added to the cells in appropriate tubes, before incubating for 20 minutes at room temperature in the dark. Finally the cells were washed with staining buffer, resuspended in 300µl of staining buffer and analysed by flow cytometry using BD LSR II (BD Biosciences, San Jose, CA). Figure 10 shows the flow cytometry plot of a ZAP70 positive (31.4%) and ZAP70 negative (12.8%) patient sample. The cut off for ZAP70 positivity is greater than 20% of ZAP70 positive cells in CD19+ cells.

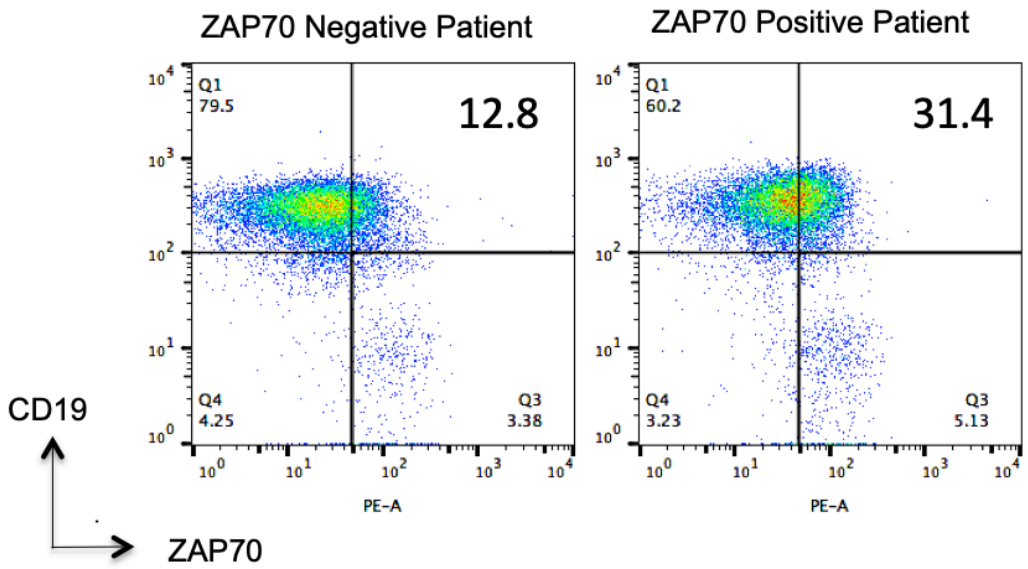


Figure 10: CD19/ZAP70 staining on CLL patient samples. Left panel shows a negative patient (CD19+ZAP70+= 12.8%) below 20% cut off . Right panel shows a positive patient (CD19+ZAP70+= 31.4%) above 20% cut off.

3.2.6 Cloning of pRCMV_FL_BIO_ZAP70 wild-type/mutant (+/- GFP).

ZAP70 was PCR amplified from a pcDNA4 ZAP70 WT or Mutant vector (Ringshausen group) respectively and inserted into the pRetroCMV /T0/ Puro/GFP and pRetroCMV /T0/ Puro vector (Dr. Daniel Hodson) by Gibson assembly (Figure 11).

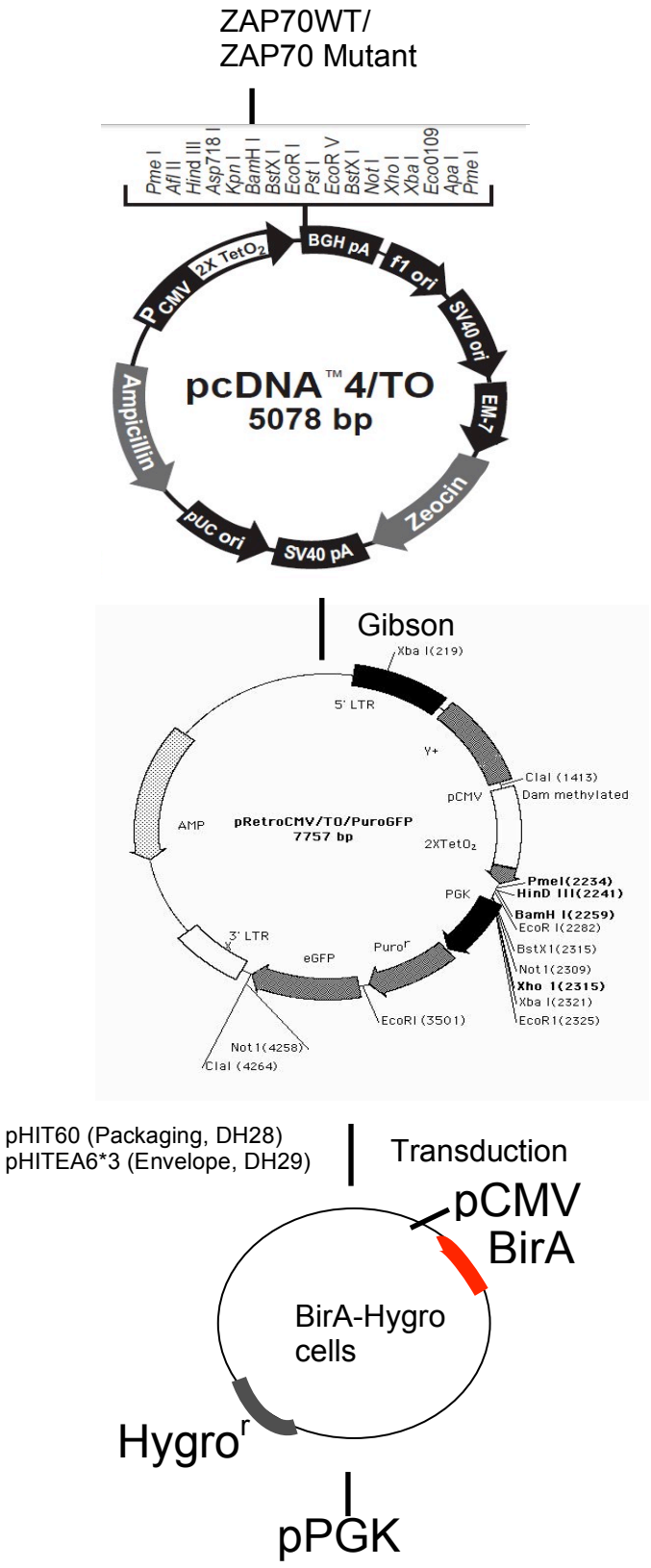


Figure 11: Schematic of cloning to generate cell lines utilized for in vivo biotinylation. ZAP70 was amplified from pCDNA4 and inserted int pRetroCMV using Gibson assembly following by transduction into cell lines expressing BirA.

3.2.7 Transfections and Retroviral Transductions

Single suspensions of HEK293T cells were plated on a 6 well dish to achieve about 40% confluency in preparation transfection. Transfection was performed 24 hours after seeding using room temperature TransIT-293 (Mirus) (vortexed well). In 200µl of 1x PBS (no FBS), 3.6µl of TransIT-293 was added, mixed well and incubated for 10 minutes at room temperature. After incubation, 200ng of packaging plasmid (DH28 and DH29) and 800ng of retroviral plasmid were vortexed and added. This mixture was further incubated for 45 minutes at room temperature and then added drop wise onto the cells, which was swirled around gently to mix with the cell media. The cells were then incubated overnight at 37^{o}C. Retroviral supernatant was harvested 48 hours post transfection and spun down at 2500rpm for 10minutes at 4^{o}C. The supernatant had 25mM HEPES and 10µg/ml Polybrene added, which was then dispensed over the cells in a 24 well plate, plated with BJAB/HBL1/Raji cells at 40% confluency, and mixed gently. The cells and supernatant were then spun at 2500g for 1 hour and 30 minutes at 30^{o}C. After centrifugation, most of the media was aspirated and replaced with cell line appropriate media. After protein expression was seen (48-72 hours), antibiotic selection was carried out by adding 2µg/ml of puromycin and allowed to grow for a few more days.

3.2.8 *In vivo* biotinylation

Cell lines (BJAB, HBL1 and RAJI) containing the BirA were obtained from Dr Daniel Hodson's lab. BirA is the Escherichia coli biotin ligase. This approach is based on the fusion of an *Escherichia coli* biotin protein ligase fused to a targeting protein. It recognizes and biotinylates a lysine side chain within a 15 amino acid acceptor peptide (*bio* sequence). These lines have the advantage of the BirA expression system which was used for pull down / selection etc.

3.2.9 Streptavidin pull down

10% of total lysate was saved as “input”. Remaining lysate was incubated for 4 hours with 10µl of MyOne streptavidin T1 (Dynabeads 65601) at 4^0C with rotation brought up to 500µl using lysis buffer, the flow through was saved and the beads were washed five times with PBS prior to elution. Western blot analysis was performed using 5% of each fraction.

3.2.10 Pull down of endogenous ZAP70 from primary cells (IgM activation)

Rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) protocol (Mohammed et al., 2016) was followed with a few modifications as described below.

Each condition utilised $20x10^6$ purified CLL cells. Post activation of cells were fixed DSG (disuccinimidyl glutarate) for 20 minutes at room temperature. 1% formaldehyde in serum free media was added to the DSG +cells for 10 minutes at room temperature. Equal volume of 1.25M Glycine was added to stop fixation. Cells were washed twice with ice cold PBS. Cells were then re-suspended in 1ml of Pierce IP lysis buffer (87787) and freeze/thawed three times on dry ice. Lysed cells were spun at 14,000rcf for 10 minutes. Supernatant (cytoplasmic fraction) was added to prewashed Protein G Dynabeads (Life Technologies 10003D) or Protein A Dynabeads (Life Technologies 10001D) and rotated for 4 hours at 4^oC. The supernatant was added to a magnetic rack whereby the beads bound to the magnet. Some of the supernatant was saved as input for western blots, the remaining supernatant was divided into two tubes containing: ZAP70 Antibody (20µg) and anti IgG (20µg) respectively and incubated overnight at 4^oC with rotation. The following day, prewashed beads were added and further incubated for 4 hours at 4^oC with rotation. After incubation, the beads were bound to a magnetic rack and the supernatant was saved as flow through. Beads were washed 5X with 1X cold PBS. On the last wash 10µl of beads were saved for western blots. Remaining beads were washed with cold ammonium hydrogen carbonate (AMBIC) solution twice. The beads were dried and submitted for mass spectrometry on dry ice.

3.2.11 Western blotting

Cells were collected in a conical tube and centrifuged for 5 minutes at 500rcf. The pellet was then resuspended in 10ml of PBS and centrifuged for 5 minutes at 500rcf. The pellet was resuspended in 1ml of PBS and transferred to a 1.5ml microcentrifuge tube before pelleting at 12,000rcf for 5 minutes. Supernatant was discarded and cell pellets were lysed using RIPA buffer (1M tris pH 7.4, 5M NaCl, Sodium Deoxycholate, IGEPAL, SDS and water) supplemented with HALT phosphatase inhibitor cocktail (Thermo Scientific: 78420) and HALT protease inhibitor cocktail (Thermo Scientific: 87786) which was incubated for 30 minutes on ice or RIPA 10X buffer (Cell Signalling: 9806). The tubes were then spun down at 16,000rcf for 5 minutes at 4^{o}C. Supernatant (protein lysate) was collected and quantified using the BCA assay. Equal amounts of samples were then loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) and further transferred on PVDF membranes. Primary antibodies were used with the HRP immunodetection system to detect human proteins. Western blot antibodies were purchased from either BD Biosciences, Santa Cruz Biotechnology, Cell Signalling or Abcam (Table 1).

3.2.12 Calcium flux assay

Media without any supplements was warmed in water at 37^{o}C. 5x10^{6} cells were spun down at 500rcf for 5 minutes. The cell pellet was resuspended in 500µl of serum free media before adding 2µl of Fluo-4 (Invitrogen) to each sample and incubating for 15 minutes at room temperature in the dark. Fluo-4 is a fluorescent dye developed for quantification of cellular Ca2+ concentration. Cells were then washed once with HBSS (Ca++ free) + 1% FBS. Cells were resuspended in 100µl of HBSS and incubated for 20 minutes on ice in the dark with 20µg of biotinylated IgM. Cells were then washed with HBSS and resuspended in 500µl of HBSS and incubated for 20 minutes at 37^{o}C. Before measurements were taken on the flow cytometer, DAPI was added to each sample and measurements were taken by recording background signalling, pausing after 30 seconds by removing the tube and quickly adding 20µg of streptavidin (N7021S)

and resuming measurements for 3 minutes. Change in calcium influx was calculated by subtracting fluorescence intensity peak value before stimulation from fluorescence intensity peak value after stimulation.

3.2.13 BCR Stimulation

BCR stimulation was performed by adding Biotin SP conjugated affinipure Fab fragment goat anti IgM $Fc_5\mu$ fragment specific (Jackson Immunoresearch Laboratories: 109-067-043) at a final concentration of 20µg/ml for 20 minutes on ice. Cells were washed with ice cold PBS and incubated for 20 minutes at 37^{o}C. 20µg of streptavidin was then added at varying timepoints according to experimental requirements.

BCR activation was also carried out with immobilized IgM with Dynabeads M280 Streptavidin, (Invitrogen 11205D). For this IgM and prewashed streptavidin beads were rotated for 1 hour at room temperature, this mixture was then added to cells according to the BCR stimulation protocol above.

3.2.14 Subcellular fractionization

Cell pellets were washed with ice cold PBS and lysed with nuclear extraction buffer A for 5 minutes on ice. The nuclear pellets were centrifuged for 5 seconds at 13,200rpm. The supernatant was saved as cytosolic fraction. The pellet was then further lysed with nuclear extraction buffer B and sonicated for 30 seconds. The lysate was then centrifuged for 5 seconds at 13,200rpm to pellet the debris, and the supernatant (nuclear fraction) was saved. Protein lysate was run on SDS PAGE gel.

3.2.15 Quantitative expression of ZAP70 by real time PCR (qPCR)

Cultured cells were spun down at 500rcf for 5 minutes. Cell pellet was washed once with PBS and RNA extraction using the QIAGEN RNA isolation kit following the manufacturers protocol. The RNA was quantified using the Nanodrop and 500-1,000ng of RNA was used to make cDNA using the high capacity cDNA reverse transcriptase kit (Life Technologies). The cDNA was then used alongside primers specific to genes of interest and the SYBR green qPCR mix (Life

Technologies) for real time PCR assay. Normalization was carried out using GAPDH and fold changes were calculated using ΔΔct method.

3.2.16 Knockdown of ZAP70 using siRNA

Patient samples were thawed, counted and $10x10^6$ cells were used for nucleofection. The CLL cells were resuspended in 100µl of Nucleofection solution (Lonza) and 0.67µg of each ZAP70 siRNA (set of 3) or 2µg of NSC siRNA and transferred to cuvette for nucleofection using Amaxa nucleofector programme X-001. Post nucleofection, cells were transferred to eppendorf tubes and spun at 500rcf for 5mins. The cell pellet was resuspended using CLL media and plated on stromal cells (plated the day before) in CLL media. Cells were incubated for 1 week at 37^oC 5% CO_2.

3.2.17 DNA Damage (irradiation/chemotherapy and treatment)

Patient samples were thawed, counted and plated on stromal cells to recover for 24 hours. Cells were pre treated with KU55933 (Selleckchem), ZAP70 inhibitor or siRNA knockdown nucleofection was carried out prior to DNA damage. Irradiation dose rate used was 4Gy. Cells were transferred to a 37^oC 5%CO2 incubator and cells were harvested for western blot analysis 1 hour post radiation. Bendamustine (Selleckchem) was used at a concentration of 30µM for 24 hours to induce DNA damage.

Apoptosis detection: Annexin V FACS staining was carried out 48 hours post radiation or chemotherapy drug treatment.

3.2.18 Comet assay

Cells were prepared and treated with inhibitor prior to radiation to induce double stranded breaks (DSB), carried out as described previously. The comet agarose bottle was heated at 95^oC in a water bath for 20 minutes to liquefy. The agarose bottle was then transferred to 37^oC until required. Reagents were prepared according to the manufacturers protocol (OxiSelect Comet assay Kit: STA-350). Briefly, cells were prepared and combined with comet agarose at 1:10 ratio by pipetting and 75µl/well was immediately transferred onto the top of the comet

agarose base layer. The slide was then transferred horizontally to 4^{o}C in the dark for 15 minutes. The slide was then transferred to a small basin containing pre chilled lysis buffer carefully before immersing in the buffer for 60 minutes at 4^{o}C in the dark. The lysis buffer was then gently aspirated and replaced with pre chilled alkaline solution for another 30 minutes at 4^{o}C in the dark. The alkaline buffer was then aspirated and replaced with pre chilled TBE electrophoresis solution for 5 minutes and repeated once more. The slide was gently placed in the electrophoresis chamber and a voltage of 1volt/cm was applied for 10-15 minutes. The slide was moved to a container containing pre chilled DI H_2O. The slide was then placed in cold 70% Ethanol for 5 minutes and allowed to dry. 100µl/well of diluted vista green DNA dye was added to the slide and incubated at room temperature for 15 minutes. The slides were viewed under a fluorescent microscope using a FITC filter.

3.2.19 Migration assay

CLL cells post treatment were resuspended in RPMI1640 with 5% FBS. From this 5x10^{5} cells were added to the top chamber of a 6.5mm diameter transwell culture insert with a pore size of 5µM. Filters with cells were transferred into wells containing medium with the chemokines CXCL12 (100ng/ml), CCL19 (1µg/ml), and CCL21 (1µg/ml) or media alone. The chambers were incubated for 3 hours at 37^{o}C in 5% CO_2. Post incubation cells in the lower chamber were thoroughly resuspended, stained with CD5/CD19 and counted using CountBright Absolute counting beads according to the manufacturers protocol. The migration index was calculated as the number of cells transmigrating with chemokine divided by the number of transmigrating cells in the absence of chemokine.

3.2.20 Proliferation assay

CLL cells were co-cultured with EL08 cells expressing CD40/IL21. EL08 is a murine cell line characterised as a stromal cell line to aid in maintaining CLL cells *in vitro*. After inhibitor treatment, 2x10^{6} CLL cells were harvested and washed once with PBS. The cell pellet was then incubated with 2.5µM CFSE dye, resuspended in PBS (37^{o}C), and incubated for 20 minutes at 37^{o}C in an incubator whilst being protected from light. Culture media was added to the cells

and incubated for 5 minutes to remove any free dye. Cells were then centrifuged and resuspended in fresh media and incubated for 6 days at 37^{o}C prior to FACS staining and analysis.

3.2.21 Apoptosis assay

The apoptosis assay was carried out using the Annexin V kit (Biolegend) and DAPI (4'6-diamidino-2-phenylindole) according to manufacturers instructions. Double negative (DAPI negative and Annexin V negative) cells were referred to as live cells in this thesis.

3.2.22 OPP Protein Synthesis assay

Click-iT OPP (O-propargyl-puromycin) kits (Thermofisher) was used to detect newly synthesized proteins. Experiments were conducted according to the manufacturer's protocol.

3.2.23 Statistical analysis

Significant differences between groups were assessed using paired T test using Graphpad Prism 6 software. Significance were denoted as follows: ns ($P>0.05$), * ($P\leq0.05$), **($P\leq0.01$) and ***($P\leq0.001$). Error bars used throughout this thesis is mean with standard deviation.

4. Results

4.1 Function of ZAP70 in CLL cells

BCR signalling plays an important role in chronic lymphocytic leukaemia. *In vivo*, mutated CLL (good prognosis) is driven towards anergy whereas unmutated CLL show less anergy. Continuous anergy has a decreasing effect on adhesion and migration, thereby having an overall effect on cell signalling, cell survival and proliferation (Packham et al., 2014). It has been suggested that ZAP70 enhances BCR signalling which causes increased CLL cell survival via Bim regulation, and proliferation (Paterson et al., 2012) .

4.1.1 P505-15 decreases calcium mobilization in CLL cells

UM-CLL cells are more responsive to surface IgM activation (increased calcium mobilization) resulting in tumour progression. CLL cells transduced with ZAP70 wildtype, a kinase defective ZAP70 (ZAPP-70 KA 369) or ZAP70 unable to bind to c-CBL have shown increased intracellular calcium flux compared to mock transduced CLL cells lacking ZAP-70 (Chen et al., 2008). It has been shown that cells from ZAP70 positive patients have greater calcium flux compared to ZAP70 negative CLL patient cells (Chen et al., 2005b) and activation of PLC_Y induces mobilization of intracellular calcium that can be measured via flow cytometry.

Here, P505-15 was used to inhibit ZAP70 kinase activity and ibrutinib to inhibit BTK activity (positive control). ZAP70 positive patient CLL cells were cultured in CLL media and treated with two concentrations of P505-15, 5μM and 0.25μM. Post treatment, calcium mobilization was measured using the calcium flux assay (Figure 12). After acquiring calcium flux measurements post addition of IgM, Ionomycin was subsequently added to release all calcium. Calculations on how peak fluorescent changes were measured is described in the methods sections calcium flux assay 3.2.12.

Figure 12A shows quantification results of peak fluorescent intensity using 9 ZAP70 positive CLL cells and representative FACS plots (Figure 12B) of the changes seen post treatment. Specifically, significant reduction in peak fluorescence intensity was seen comparing untreated to high concentration of

P505-15 inhibitor. A similar level of reduction is seen with ibrutinib at both 5μM and 0.25μM concentrations.

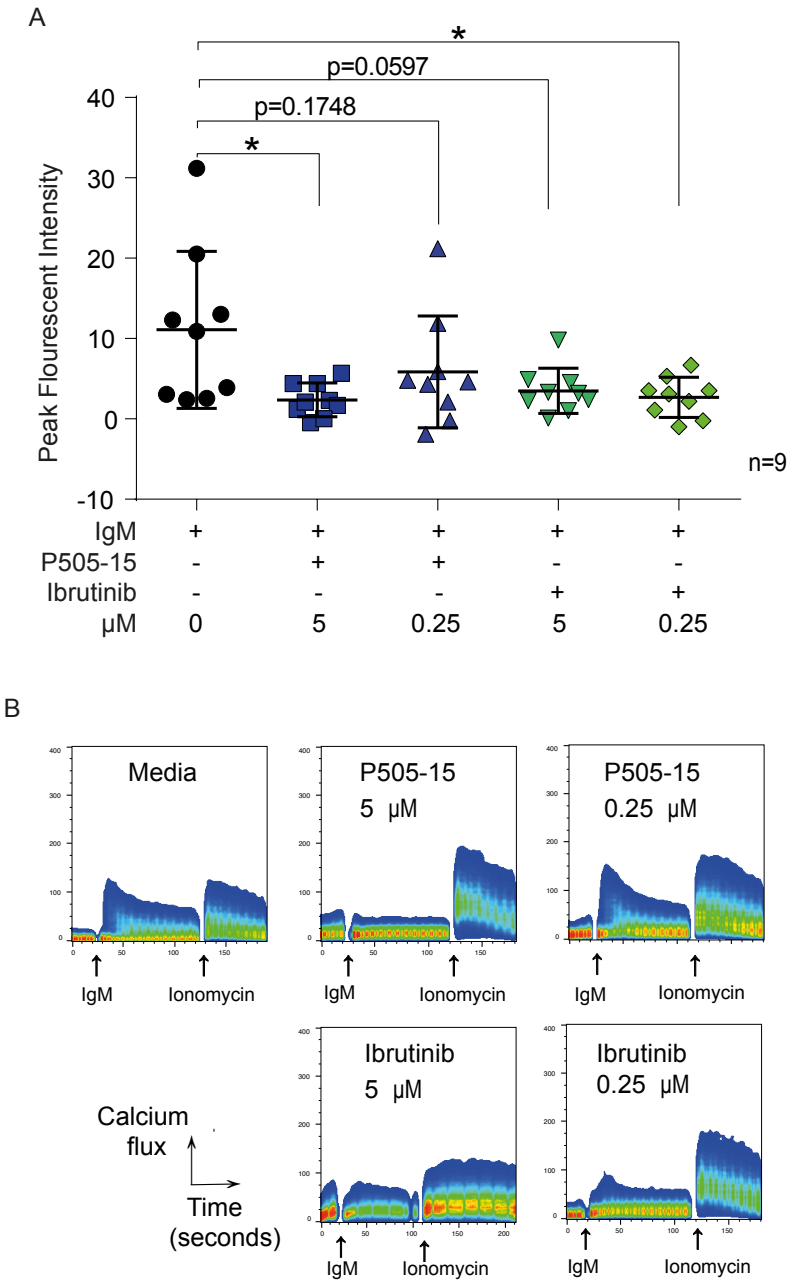


Figure 12: P505-15 treatment reduces BCR signalling in CLL cells. Quantification of calcium flux using cells from ZAP70 positive patients, n=9 (A) and representative plots (B). Inhibitor treatment was carried out for 1hour.

4.1.2 P505-15 reduces CLL cell viability

In non transformed cells, anergy increases susceptibility to apoptosis however in leukemic cells this is countered by overexpression of the B-cell lymphoma-2 survival protein (BCL2) (Packham et al., 2014). In normal cells, anti-IgM induced phosphorylation of BIM_{EL}, however in CLL cells surface IgM stimulation increased phosphorylation of 2 BIM isoforms: BIM_{EL} and BIM_{L} resulting in BIM degradation. This IgM induced phosphorylation in CLL correlated with unmutated IgHV gene status and disease progression (Paterson et al., 2012).

To investigate whether inhibition of ZAP70 kinase activity affects CLL cell survival, Annexin V staining was performed on CLL patient cells treated with P505-15 and ibrutinib, in the presence or absence of IgM activation for 24 hours (Figure 13A) and 48 hours (Figure 13B). The assay showed significant reductions in viability with 5μM but not at 0.5μM of P505-15 at 24 hours compared to the vehicle control. However, at 48 hours significant reduction was observed at both 5μM and 0.5μM of P505-15. Similar observations were made irrespective of IgM addition as well as with ibrutinib treatment used as positive control (Figure 13). For both 24 and 48 hour timepoints, all conditions were normalised to individual patient Media no IgM.

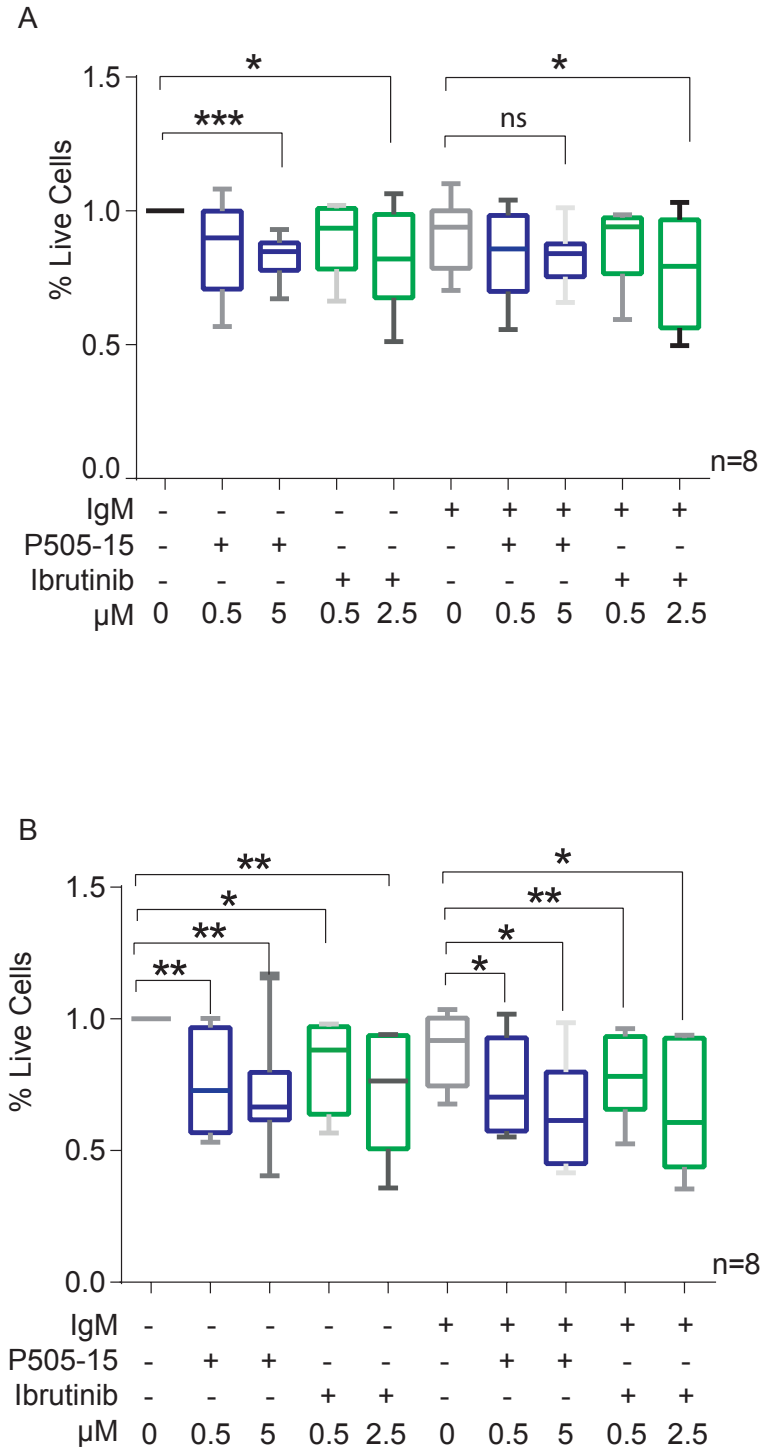


Figure 13: P505-15 decreases cell viability. Reduction in cell viability after P505-15 and ibrutinib treatment of ZAP70 positive CLL cells at 24 hours (A) and 48 hours (B) irrespective of IgM activation. Cells treated with P505-15 and ibrutinib were harvested at respective timepoints for CLL cell and Annexin V staining. % live cells: DAPI and Annexin V double negative cells.

4.1.3 P505-15 significantly reduces CLL cell proliferation

CLL cells do not proliferate in the peripheral blood, however they are found associated with CD40L+/CD4+ T helper (Th) cells and dendritic cells in the proliferation centres (PC). Higher proliferative capacity was observed in the UM-CLL compared to M-CLL correlated with higher phospho-BTK and greater sensitivity to Ibrutinib. BTK activity is responsible for increased cell proliferation confirmed by siRNA knockdown studies (Guo et al., 2016).

To investigate whether the inhibition of ZAP70 kinase affects CLL cell proliferation, CLL cells were co-cultured with EL08 cells expressing CD40L/IL21. Cells were then treated with P505-15 and changes in proliferation were assessed. As seen in Figure 14, there is a dose dependent significant decrease in percent proliferation (CFSE) upon treatment of CLL patient cells with P505-15. Ibrutinib was used as a positive control to reduce proliferation.

Based on these results, it is clear that P505-15 causes a significant decrease in calcium mobilization, viability and proliferation in ZAP70 positive CLL cells. Since, P505-15 is not specific for ZAP70, it is vital to specifically unravel the role of ZAP70 expression with minimal effect on other tyrosine kinases such as SYK which is done using ZAP70 siRNA (section 4.2.1)

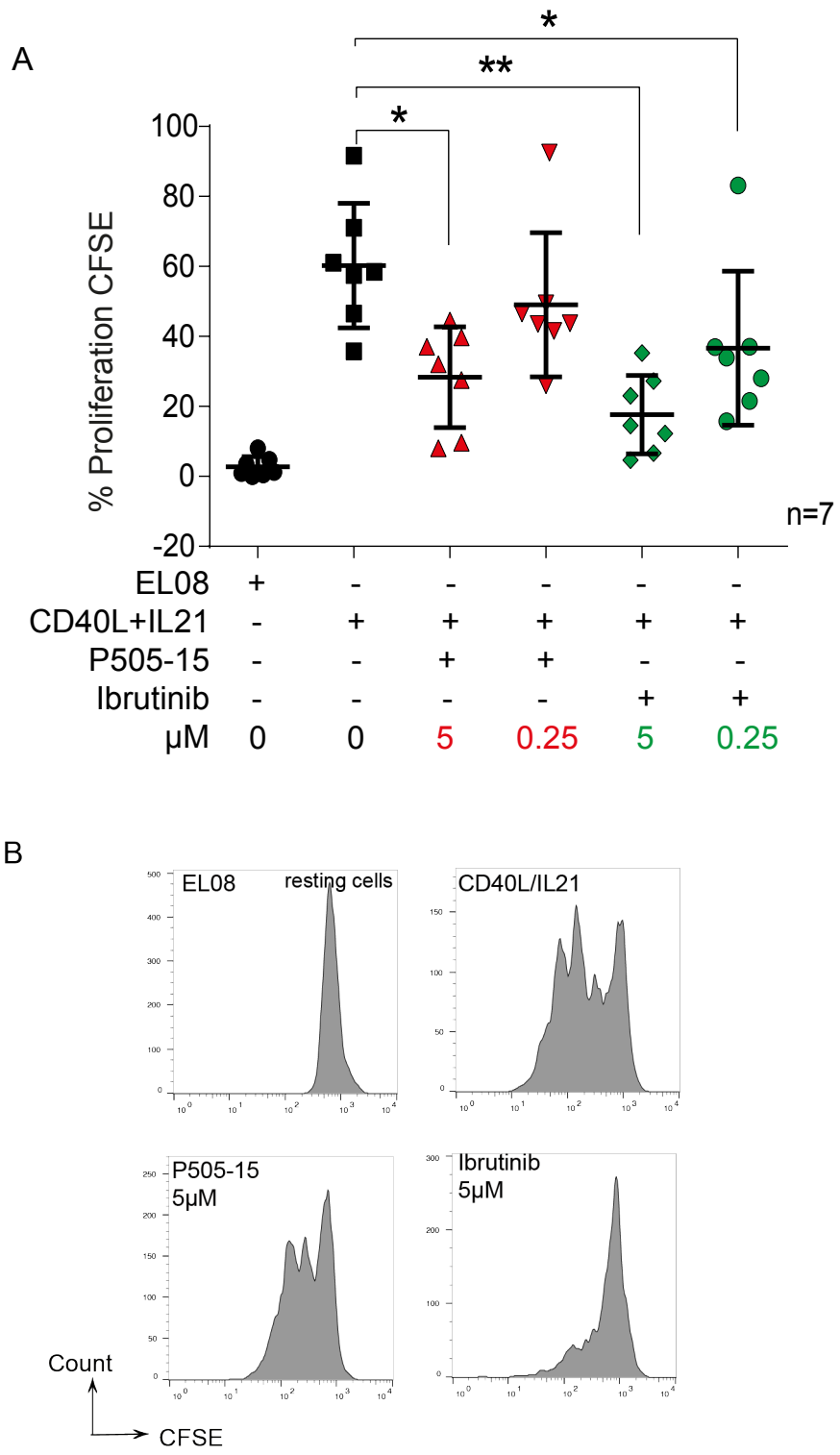


Figure 14: P505-15 decreases CLL cell proliferation. Reduction in proliferation post P505-15 inhibition, quantification of proliferating cells (A) and representative FACS plots showing division of cells with CFSE dye (B). Proliferation was determined by dilution of CFSE dye as cells divided, showing approximately 3-4 cell divisions over 6 days.

4.2 siZAP70 lowers ZAP70 levels with no effect on SYK expression.

Specific knockdown of ZAP70 expression was achieved using siRNA nucleofection with minimal effect on other proteins. Notably, due to a long half-life of ZAP70, knock down required the prolonged culture of nucleofected cells on stroma for a minimum of 6 days. Figure 15A shows ZAP70 siRNA knockdown in two ZAP70 positive patient cells with varying amount of endogenous ZAP70 as seen in media (un-nucleofected cells). ZAP70 positive cells were nucleofected with 2µg siRNA and co-cultured with EL08 cells for 6 days. Comparing siZAP70 to siNSC, there is a decrease in ZAP70 expression at day 6 post-nucleofection with minimal change in SYK expression. Figure 15B shows quantification of ZAP70 and SYK expression levels using results from 7 patients. Image J was used for quantification of ZAP70 and SYK expression normalized to β actin.

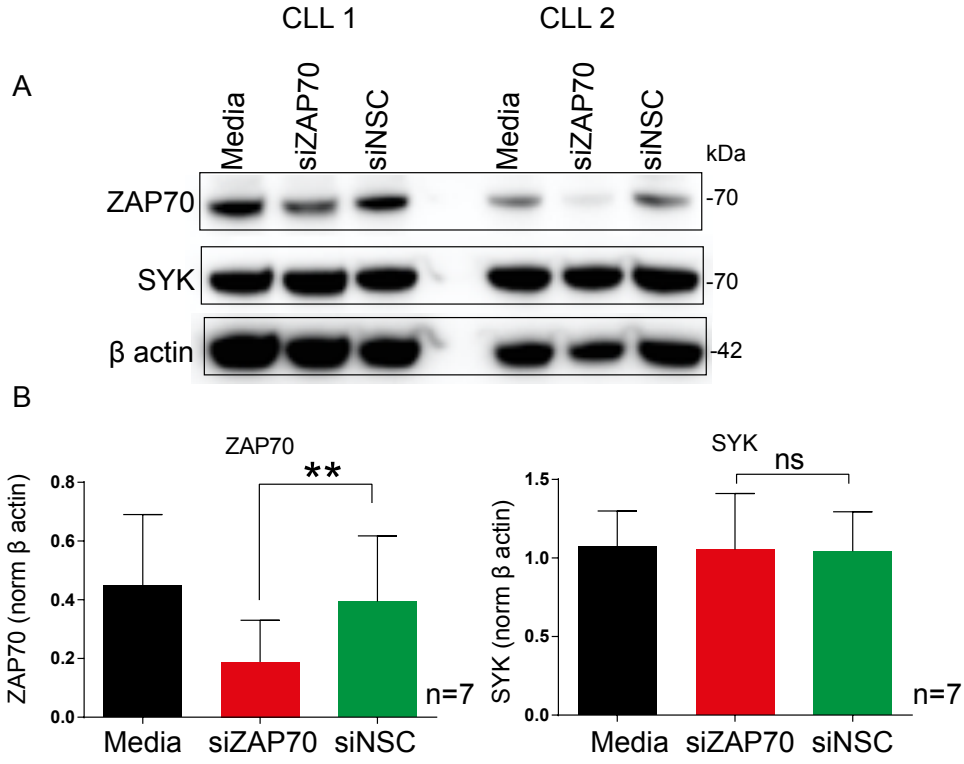


Figure 15: Reduction of ZAP70 expression post ZAP70 siRNA nucleofection with minimal effect on SYK expression using ZAP70 positive CLL patient cells. (A) representative western blot of ZAP70, SYK and β actin and (B) ImageJ quantification of ZAP70 and SYK expression normalized to β actin.

4.2.1 siZAP70 has no effect on calcium mobilization on CLL cells.

To test if ZAP70 siRNA knockdown affects calcium mobilization, calcium flux assay was carried out on ZAP70 and NSC siRNA treated cells. As seen in Figure 16, there was no significant difference in calcium mobilization between siZAP70 and siNSC cells. Non significant reduction in cell viability post knockdown was observed as shown in figure 37.

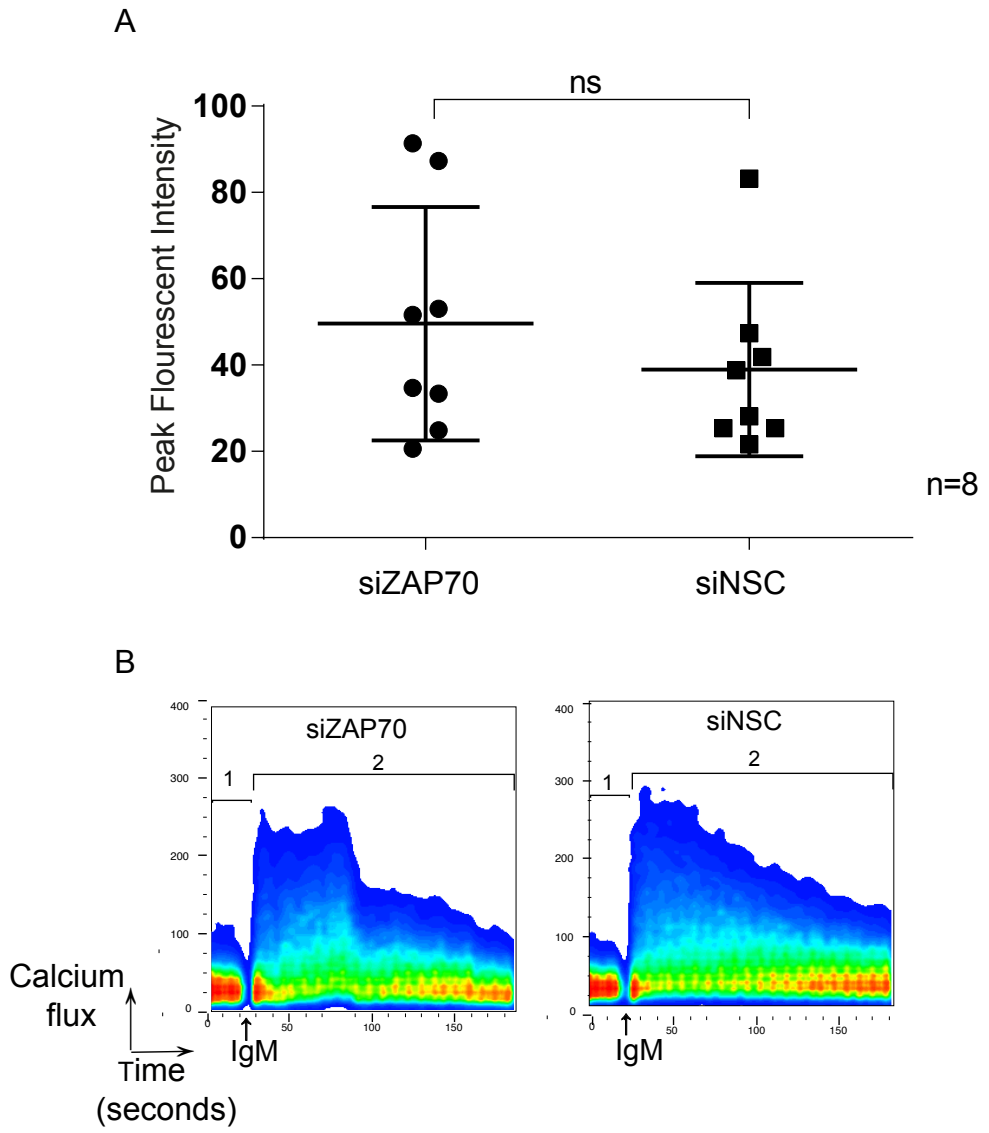


Figure 16: Unchanged BCR signalling post ZAP70 knockdown.
ZAP70 positive CLL cells were nucleofected with siZAP70 and siNSC and co cultured with EL08 for 6 days. On day 6, cells were harvested for calcium flux assay. Quantification is based on 8 CLL ZAP70 positive patient cells. Quantification achieved by subtracting peak fluorescent intensity in region 1 (baseline) from peak fluorescence intensity of region 2 (A). Representative FACS plots showing calcium flux kinetics (B).

4.3 Regulation of AKT in CLL cells

AKT is downstream of the signalling cascade following BCR ligation. B cells co cultured with marrow stromal cells highlighted the importance of PI3K/AKT signalling to support survival of CLL B cells (Ding et al., 2010)

In unstimulated CLL cells, Akt is fully activated with phosphorylation at both serine 473 and threonine 308 and inhibition of AKT results in apoptosis of CLL cells associated with rapid loss of MCL1, mediated by GSK3 and increased expression of p53. This shows that AKT is important in the survival of unstimulated CLL cells (Zhuang et al., 2010). Furthermore, single cell profiling of phosphorylated protein levels in CLL showed that (pS473) was more induced in IgHV UM- CLL and significantly higher than in M-CLL (Myhrvold et al., 2018). As mentioned briefly in the introduction, BCR signalling is propagated via PI3K. Here, PI3K phosphorylates PIP2 to create PIP3, which recruits BTK and AKT and activates the signalling effector mTOR (Bellacosa et al., 1998). It has been shown that the mTOR signalling cascade is differentially regulated in distinct prognostic CLL cohorts. In addition dual mTORC1/2 inhibitors preferentially induce apoptosis of poor prognostic CLL subsets (Cosimo et al., 2019)

4.3.1 siRNA knockdown of ZAP70 increased pAKT in CLL cells.

Firstly, I wanted to identify the most suitable method and timepoint for BCR activation using IgM (soluble/ immobolised) to result in phosphorylation of AKT, ERK and BTK. Immobolised IgM resulted in higher and sustained activation compared to soluble IgM (not shown). Figure 17A shows pAKT, pERK and pBTK levels in the cells activated with immobolised IgM and harvested at 15 minutes, 60 minutes and 180 minutes (Figure 17A). Maximum phosphorylation of AKT and ERK was seen at 1 hour and phosphorylation of BTK observed at 15 minutes.

To specifically understand the role of ZAP70, I investigated if depletion of ZAP70 by knockdown would affect AKT phosphorylation. Here, I nucleofected ZAP70 positive CLL cells with ZAP70 and non-specific siRNA for 6 days co-cultured with

EL08 cells. On day 6, cells were activated with immobilised IgM for 1 hour and then harvested for western blot assay. ZAP70 siRNA treated cells show increased pAKT compared to non specific siRNA treated cells (Figure 17B) and Figure 17C shows pAKT levels normalized to total AKT relative to siNSC.

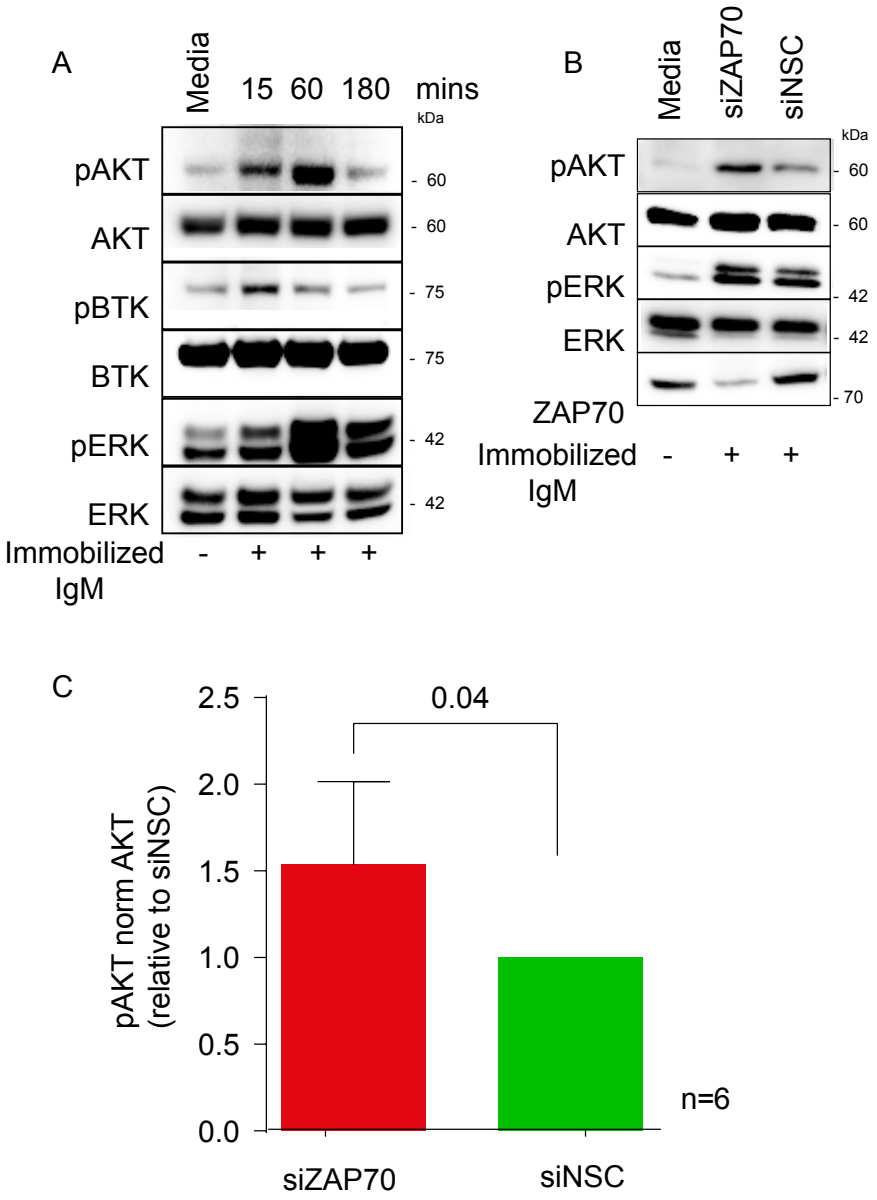


Figure 17: ZAP70 knockdown results in increased AKT phosphorylation. Representative western blot showing pAKT 473, pBTK, and pERK at various timepoints post immobilised IgM activation (A), Changes in phosphorylation of AKT, and ERK on ZAP70 and NSC knockdown cells post IgM activation (B) and ImageJ quantification using ZAP70 and NSC siRNA treated cells (n=6) (C).

4.3.2 NF-κB activity is unchanged following ZAP70 knockdown

CLL cells have been reported to exhibit high constitutive NF-kB activation compared to normal B cells due to AKT activation, BCR signalling, and CD40 ligation (Hewamana et al., 2008). NF-κB is regulated via 2 pathways, in the canonical pathway, the inhibitor IκBα binds cytosolic p50 and p65. Once activated, IKKβ phosphorylates IκBα thereby promoting its degradation and release of p50 and p65, which translocates to the nucleus and activates transcription. These transcription factors are key regulators of differentiation and survival in B cells. These factors include: c-Rel, RelB, p50, p52, and p65 (RELA) (Shih, Tsui, Caldwell, & Hoffmann, 2011) Interestingly, gene expression profiling of CLL cells in the LN showed upregulation of BCR gene signature, indicating B cell receptor and NF-κB activation is consistent with phosphorylation of SYK and IkBα respectively. The expression of BCR target genes was stronger in UM-CLL than in M-CLL (Herishanu et al., 2011). Specifically, introduction of functional ZAP70 protein in CLL cells with minimal endogenous ZAP70 expression, resulted in an increase in IL1B, IL6 and IL8 upon BCR stimulation through an IKK dependent mechanism (Pede et al., 2013). In addition, comparing CLL samples to normal donors, overexpression of AKT/mTOR related signalling proteins resulted in higher phosphoprotein levels of PDK1, 4EBP1, p70S6K, BAD and PRAS40 (Shull et al., 2015). Also, CLL cells co-cultured with EL08-1D2 indicate that BAD, PRAS40 and glycogen synthase kinase 3-β (GSK3-β) are phosphorylated, suggesting a pro-survival signal via PI3K and AKT in CLL cells (Mangolini et al., 2018).

Here, I am investigating if ZAP70 plays a role in NF-κB activation by knocking down ZAP70 expression in ZAP70 positive CLL cells. Firstly, I assessed changes in phospho p65 post IgM activation. Figure 18A shows a lack of change in phospho p65 in 3 CLL patients post IgM activation following ZAP70 positive cells being nucleofected with 2ug of siRNA and cocultured with EL08 for 6 days. On day 6, cells were activated with immobolised IgM for a further 24 hours and on day 7, cells were harvested for western blot. Figure 18B shows no changes in

phospho-p65 comparing ZAP70 and non-specific siRNA. These western blots are representative of 6 patient samples.

Figure 18C and 18D shows the minimal changes in phospho PRAS40 in the ZAP70 siRNA knockdown cells compared to NSC knockdown. Quantification of PRAS40 normalised to total PRAS40 was done using ImageJ from 6 patients. Comparing ZAP70 and NSC knockdown, despite being non significant (p=0.1650) showed an increase in siZAP70 compared to siNSC.

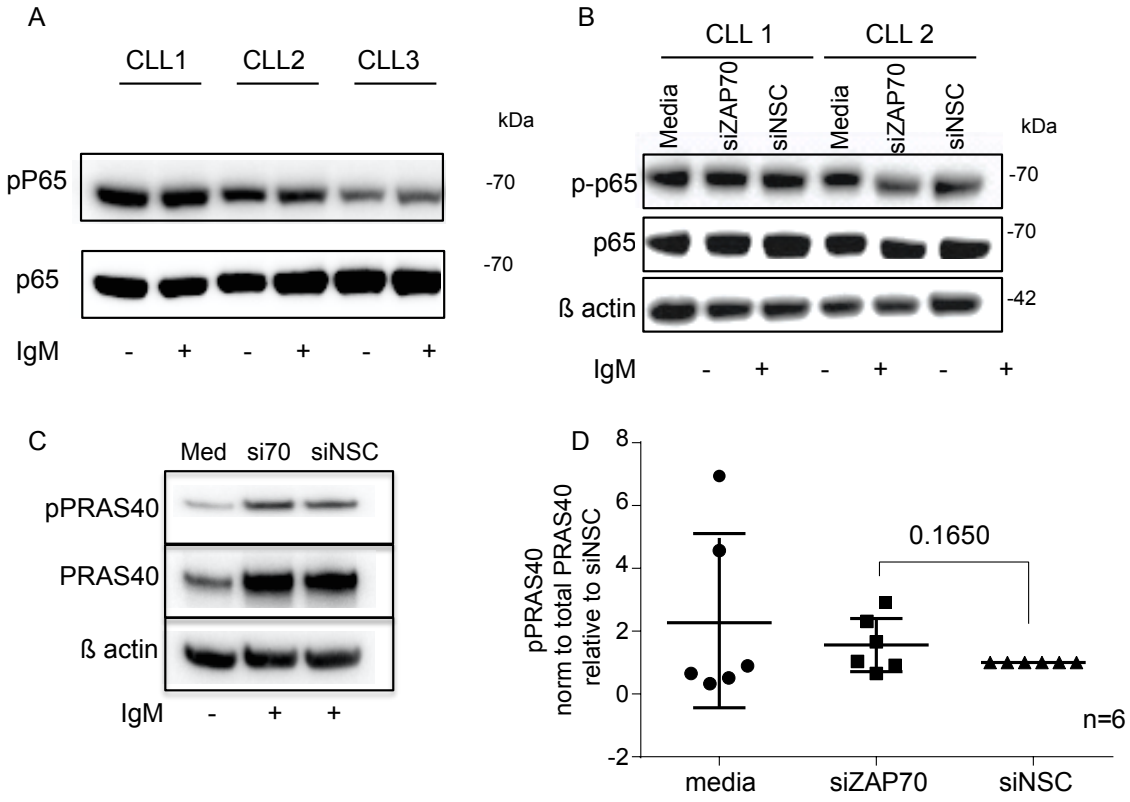


Figure 18: NF-κB activity post ZAP70 knockdown. (A) Representative western blot image of phospho p65 after immobilised IgM activation. (B) Representative Western blot of 2 CLL patients that were treated with ZAP70 and NSC siRNA followed by 24 hours of immobilised IgM activation at day 6 post nucleofection. (C) Changes in phosphor PRAS 40 in the ZAP70 and NSC nucleofected cells (D) Quantification of phospho PRAS40 in the ZAP70 and NSC knockdown cells post immobilised IgM activation.

5. Results: Mass spectrometry unveils ZAP70 protein interactors

5.1 RIME/BirA mass spectrometry approach using BJAB cells.

Unravelling protein-protein interactions is a major barrier to understanding complex biological processes. Functions of these proteins can only be fully understood in the context of networks of interaction. Many techniques like affinity capture complex purification and yeast two hybrid strategies have been used in the past, but these have limitations such as loss of insoluble or weak/transient proteins that are part of the protein interactome and difficulties in extraction, digestion and separations, whereby proteins are underrepresented in mass spectrometry analysis.

To enhance identification of these interacting proteins the BirA system was utilised. This method was used to identify candidate proteins that are near to or interact with human Lamin A, a well characterised component of the nuclear envelope as proof of concept (Roux, Kim, Raida, & Burke, 2012). In CLL, isobaric labelling and mass spectrometry proteomics was carried out on 14 CLL samples and compared to B cells from healthy individuals. A clear signature independent of subtype was identified consisting of established proteins of CLL such as CD5, BCL2, ROR1 and CD23 overexpression as well as PIGR, CKAP4, CLEC17A some of which are involved in the BCR signalling (Johnston et al., 2018). In addition, proteomics analysis was used to analyse both M-CLL and UM-CLL. PCA analysis indicated significant differences in these two CLL subtypes. Specifically, increased levels of F actin capping protein B subunit, 14-3-3 B protein and laminin binding precursor were observed in the M-CLL compared to UM-CLL. Also nucleophosmin, a cellular protein implicated in mRNA transport, chromatin remodelling, apoptosis and genome stability was present in M-CLL and absent in UM-CLL (Cochran et al., 2003).

5.1.1 Generation of ZAP70 expressing B cell lines

To investigate specific interacting partners of ZAP70, B cell lines BJAB (GCB DLBCL), HBL1 (ABC DLBCL) and RAJI (Burkitt's lymphoma) that contains the BirA ligase described earlier with ectopic expression of ZAP70 were generated. These cell lines had minimal endogenous ZAP70. Namalwa cells/inducible MEC1 Tet on/off ZAP70 cells were used as a positive control for the ZAP70 western blot assay as seen in Figure 19A. In addition, RNAseq data from Dr Hodson's lab (not shown) confirmed low endogenous level of ZAP70 in BJAB, HBL1 and RAJI cells. To generate the expression system in the cell lines, ZAP70 was fused to flag tag and expressed using the retroviral transduction system as mentioned in the materials and methods section 3.2.6. Since one of the goals of this project is to study the role of ZAP70 before and after IgM activation, the calcium flux assay was used to confirm that the BCR could be activated in the selected cell lines (Figure 19B). The results confirmed that the B cell receptor could be stimulated upon IgM activation, marked by the significant difference in calcium flux in the cells after anti IgM stimulation. This was performed alongside ZAP70 positive patient samples as a positive control.

Following confirmation of minimal endogenous ZAP70 and activatable BCR in the parental cell lines, ZAP70 WT and ZAP70 mutant expressing cells were generated in BJAB, HBL1 and RAJI cell lines (Figure 19C). Comparing the ZAP70 WT and ZAP70 mutant expressing cells, western blots showed an increase in ZAP70 protein expression in ZAP70 WT cells compared to ZAP70 mutant expressing cells (K369A). Consistently, presence of FLAG tag was seen in the wild-type and mutant constructs and not present in the empty vector construct (Figure 19C). Figure 19D shows BCR signalling of the BJAB empty and BJAB ZAP70 Wild-type cells. In the BJAB cells, there is minimal significant difference in terms of magnitude of activation between the ZAP70 wild-type and empty vector. In addition, Real time PCR was carried out to verify the over expression of ZAP70, the cell lines were normalized to GAPDH and fold change was relative to the ZAP70 expression level of Namalwa (positive control). It was noted that the expression levels of BirA varied significantly between the cell lines, with BJAB cells expressing the highest amount and lower expressions seen in HBL1 and RAJI cells.

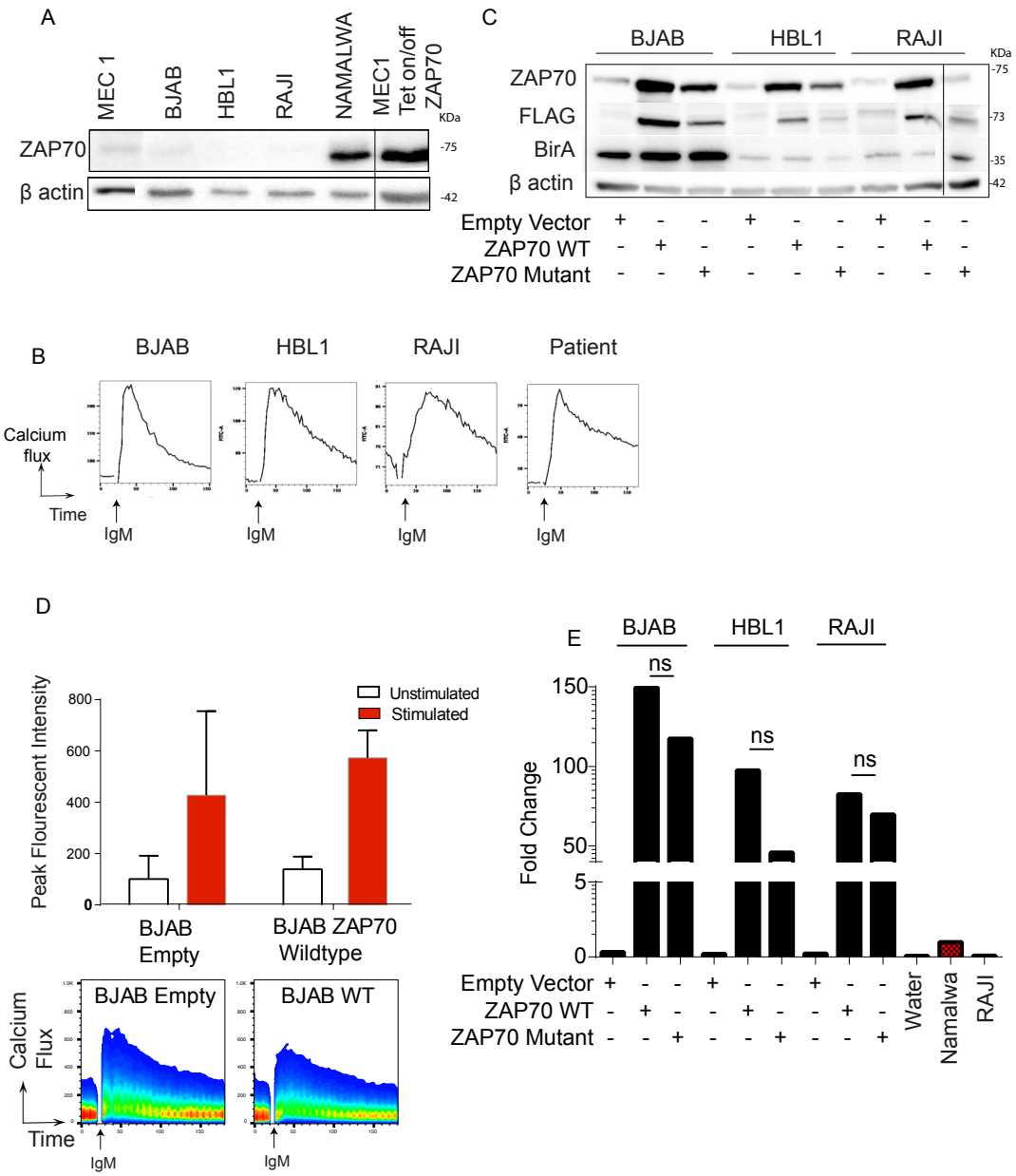


Figure 19: Generation of EV, ZAP70 WT and ZAP70 mutant expression systems. (A) Endogenous ZAP70 levels in cell lines were verified by western blot analysis. Thawed vials of cells were cultured and passaged 3 times prior to harvesting. Namalwa was used as positive control and β actin used as loading control. (B) BCR activation of parental cell lines confirmed by calcium flux assay. (C, E) ZAP70, FLAG tag, BirA expression levels in the EV, WT and mutant expressing cells confirmed by western blot (C) and real time PCR (E). (D) Calcium flux assay to confirm BCR activation using anti IgM in the EV and WT BJAB cells.

5.1.2 Mass spectrometry of BJAB cells with ectopic expression of ZAP70

Due to its high throughput technology coupled with specificity and sensitivity, mass spectrometry (MS) has become the choice for protein identification and quantification. Here, mass spectrometry coupled with the BirA approach was used to efficiently pull down ZAP70. Figure 20A shows the workflow of the mass spectrometry pull down experiment on the BJAB cells utilizing the BirA system. First, cells with and without IgM activation were cross-linked using 1% formaldehyde and further lysed with COIP lysis buffer. From the cell lysis, 50 µl from the cytoplasmic fraction was saved as “input”, remaining supernatant was incubated with streptavidin dynabeads. Beads bound to ZAP70 were captured using magnetic columns and the supernatant was saved as flow through. ZAP70 bound beads were washed 5 times with ice cold PBS and two final washes with ambic solution was carried out. The beads were then submitted for mass spectrometry analysis. Figure 20B shows the principal component analysis on the BJAB empty and BJAB wild type in both the inactivated and activated state. Samples were done in duplicate and segregated into 4 distinct groups.

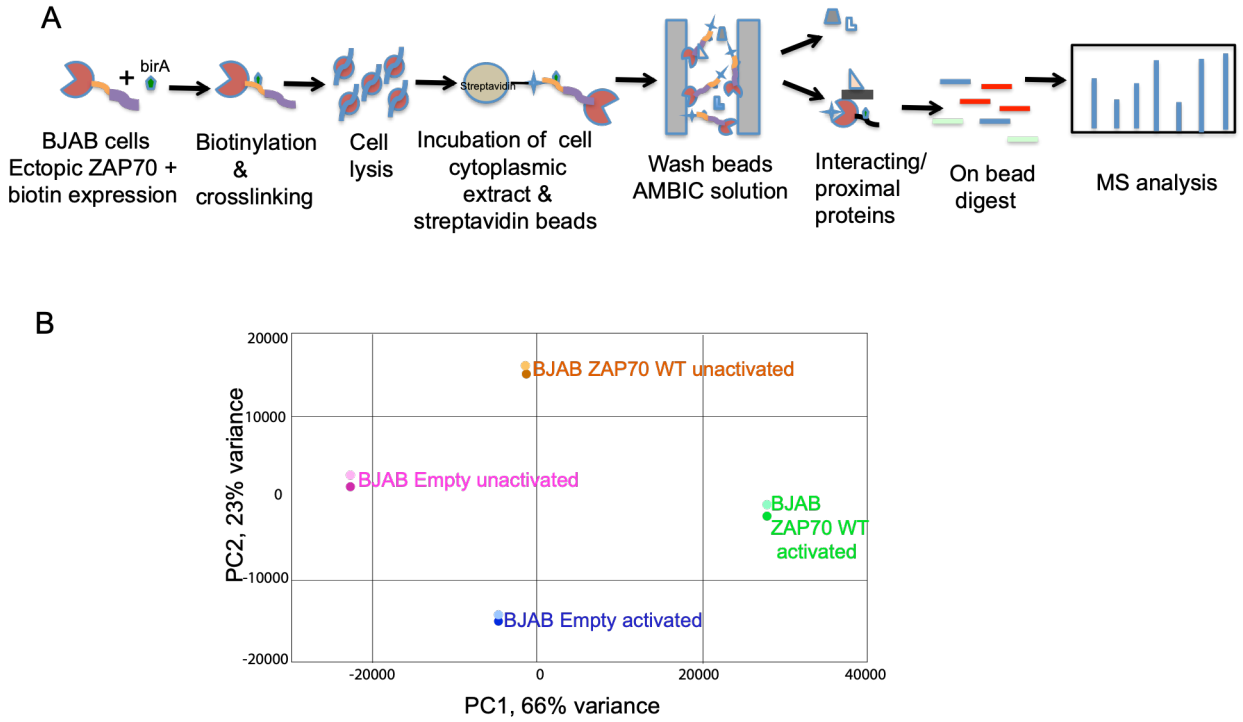


Figure 20: Mass spectrometry workflow on B cell line. (A) Schematic of pull down utilizing the BirA and overexpressed ZAP70 WT, with and without IgM activation. Cells were cross-linked using 1% formaldehyde (Mohammed et al., 2016). Cells were then lysed and ZAP70 pull down was achieved using streptavidin beads and used for mass spectrometry. (B) Principal component analysis showing segregated clusters. BJAB cells incorporated with the BirA ligase and clustering of BJAB empty unactivated (pink), empty activated (purple), WT unactivated (orange) and WT activated (green). This experiment was performed in duplicate.

5.1.3 Efficient pull down of tagged ZAP70 in transfected cells

Figure 21 shows that ZAP70 was successfully pulled down with minimal contamination represented by the absence of β actin in the elute (Figure 21). There is minimal ZAP70 present in the flow through in HBL1 and RAJI cells but this is absent in BJAB. This protocol was scaled up to purify ZAP70 for mass spectrometry to identify partners that interact with ZAP70.

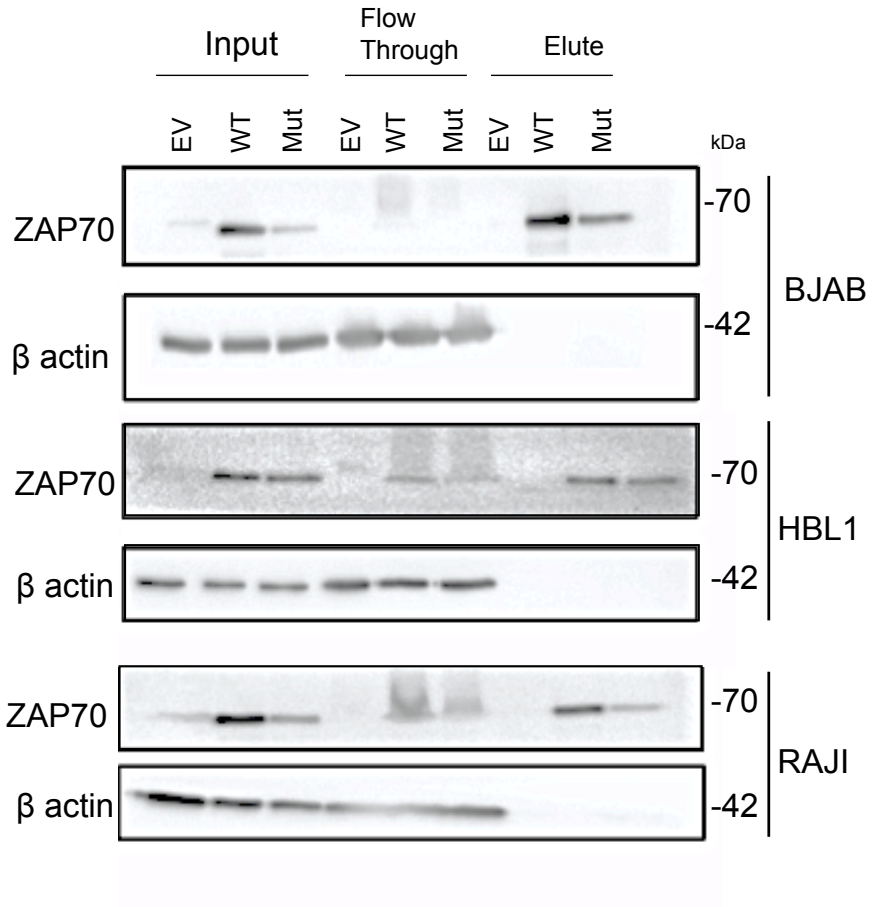


Figure 21: Streptavidin pull down of ZAP70 using BJAB, HBL and RAJI cells. Each cell line consists of Empty Vector (EV), ZAP70 WT (WT) and ZAP70 Mutant (Mut) expressing cells. Western blot shows input, flow through and elute for each cell category. β actin used to show purity of elute.

5.1.4 Protein expression changes post ectopic expression of ZAP70

ZAP70 plays a vital role in BCR signalling and is activated in response to IgM treatment. Here, to identify interacting partners of ZAP70 at both steady state and activated state, ZAP70 was pulled down from ectopic expressing BJAB cells with BirA expression using streptavidin beads. Stringent filtering was applied and volcano plots show the differentially up and down regulated proteins in BJAB cells under 3 conditions: inactivated (Figure 22A), activated (Figure 22B) and comparison between activated and steady state specifically for ZAP70 wild type cells (Figure 22C). Positive confirmation of the successful pull down was verified by presence of kinases belonging to BCR signalling such as BTK, LYN, CD79a, and SYK, with amplified expression levels post IgM activation (Figure 22). The results showed 702 proteins upregulated and 761 proteins downregulated in the unactivated state. Upon activation, there were 1163 proteins up-regulated and 354 proteins down-regulated. Specific to ZAP70 WT at the activated state, 1253 proteins were up-regulated and 1019 proteins were down-regulated (Figure 22)

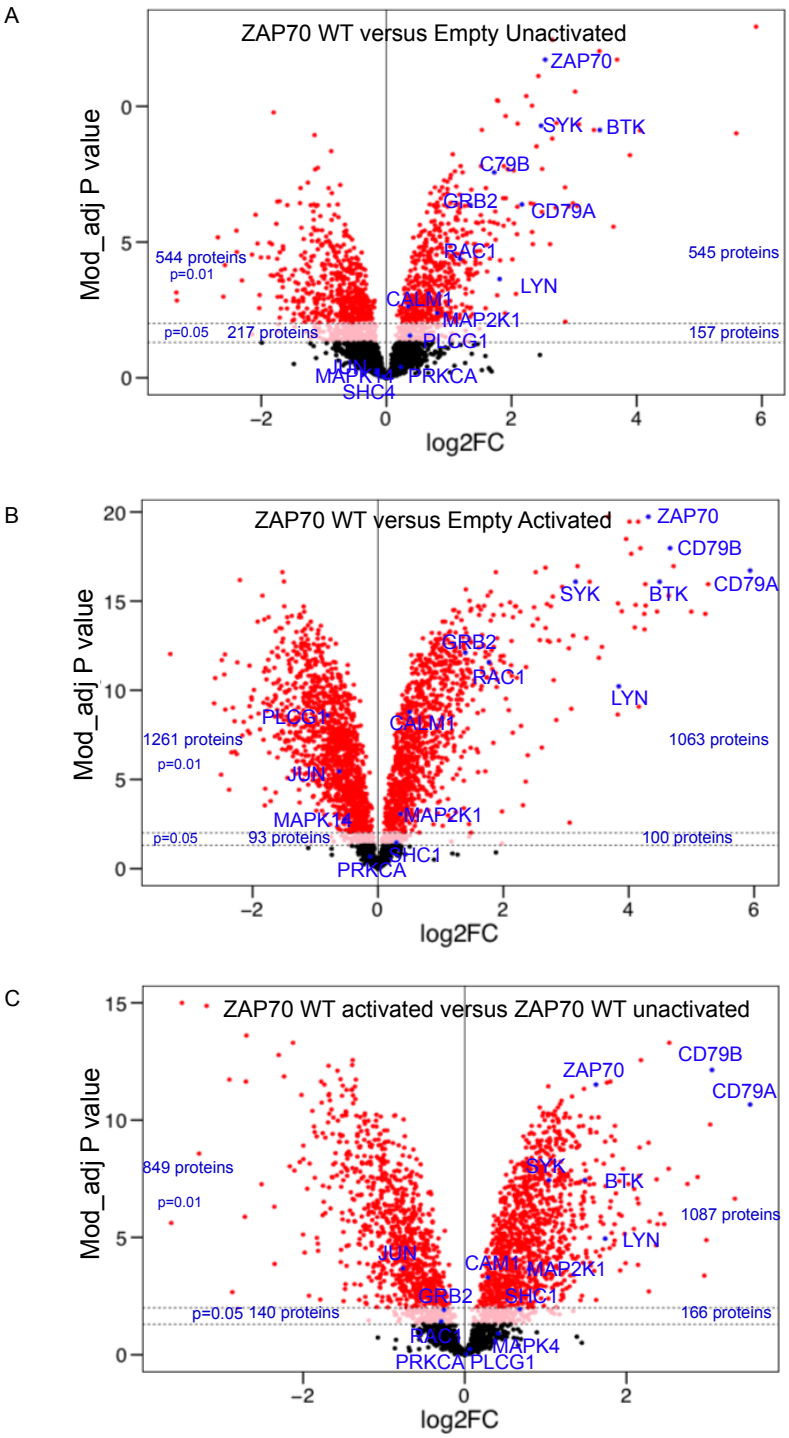


Figure 22: Volcano plots showing differentially up and down regulated proteins in BJAB ZAP70 WT cells. Unactivated (A), Activated (B) and specific to ZAP70 WT in the activated state (C). FDR of 0.05 was applied. Red data points indicate significant regulations with up regulation on the right and down regulation on the left side of the volcano plot and black data points are not significant. Proteins associated in BCR signalling are highlighted in blue.

Quantitative 2 dimensional fluorescence difference gel electrophoresis was used to compare the 2 CLL subtypes before and after IgM activation. Differences between unstimulated UM-CLL and M-CLL cells were observed (Perrot et al., 2011). The study had shown that anti IgM stimulation induces significantly more proteomic response in the UM-CLL compared to M-CLL cells. This is consistent with the knowledge that UM-CLL cells are more sensitive to anti IgM activation compared to M-CLL. Few proteins upregulated after IgM activations are HNRPK (role in pre- mRNA processing) and LSP1 (F actin binding cytoskeletal protein) (Perrot et al., 2011).

In the ZAP70 pull down mass spectrometry experiment, data was subjected to gene set enrichment analysis (GSEA) that identified transcriptional changes in gene sets involved in BCR signalling, migration and ribosomal pathway (Figure 23A and 23B). Figure 24A, 24B and 24C also shows the heatmap of proteins involved in some of the relevant pathways such as KEGG BCR signalling, KEGG actin cytoskeleton and KEGG ribosome respectively. As mentioned earlier, kinases such as BTK, SYK, and LYN that are known to be relevant in BCR signalling in CLL cells were up-regulated in the ZAP70 WT cells. In addition, there were proteins related to migration and focal adhesion such as Rac1, Rac2, PTPN6, SWAP70, CDC42 present in the ZAP70 wild-type cells specifically post IgM activation. In regards to the ribosome pathway, the majority of the ribosomal proteins were upregulated in the ZAP70 WT cells specifically post IgM activation. Up-regulation of ribosomal proteins was confirmed as specific by carrying out full proteome mass spectrometry followed by GSEA analysis on BJAB ZAP70 WT cells with and without IgM activation, where the ribosomal signature was present.

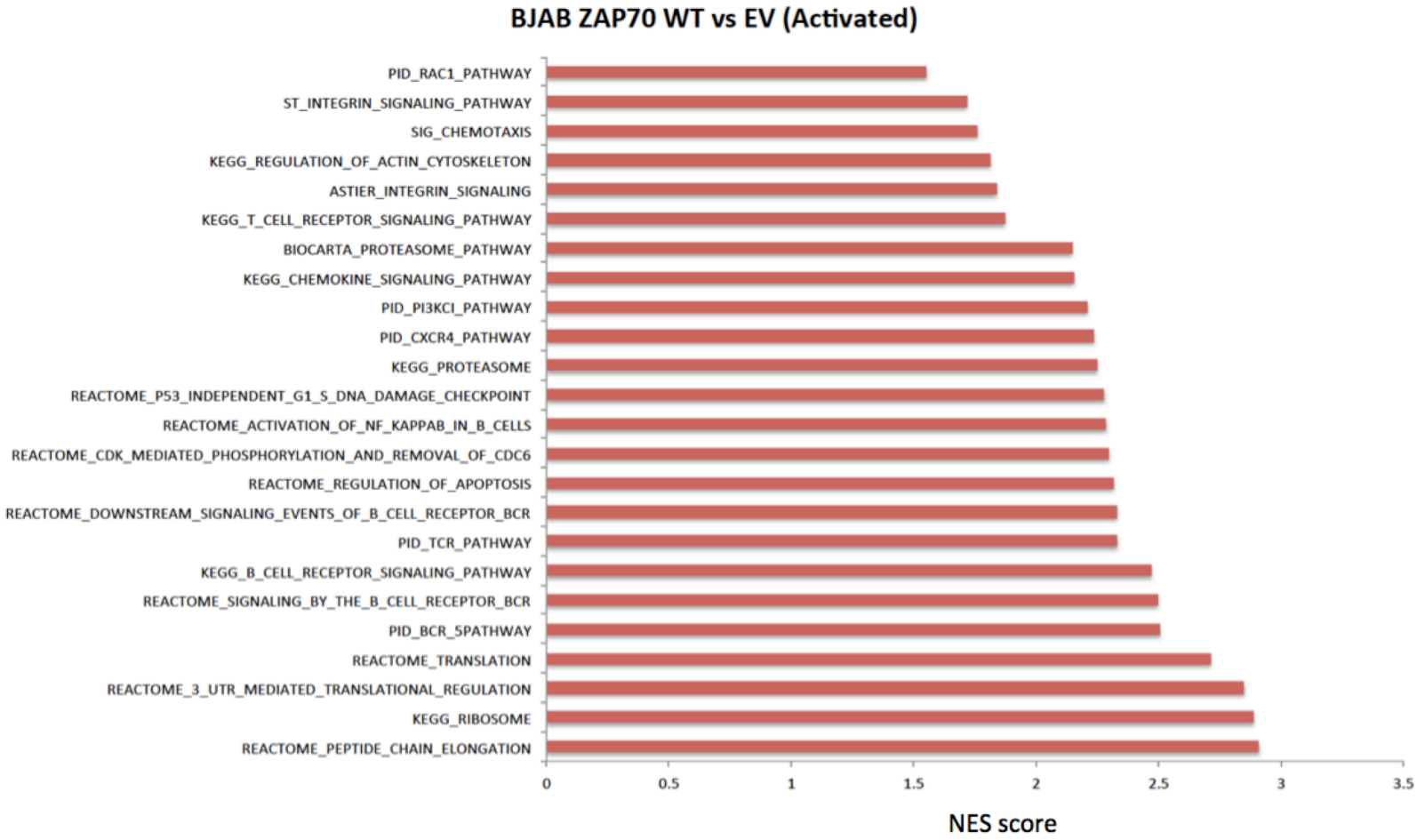


Figure 23A: **Gene enrichment analyses (GSEA)**. Relevant pathways in both EV and WT BJAB cells in IgM Activated state. Gene sets are listed in order of Normalised enrichment scores. FDR q values for all are below 0.25.

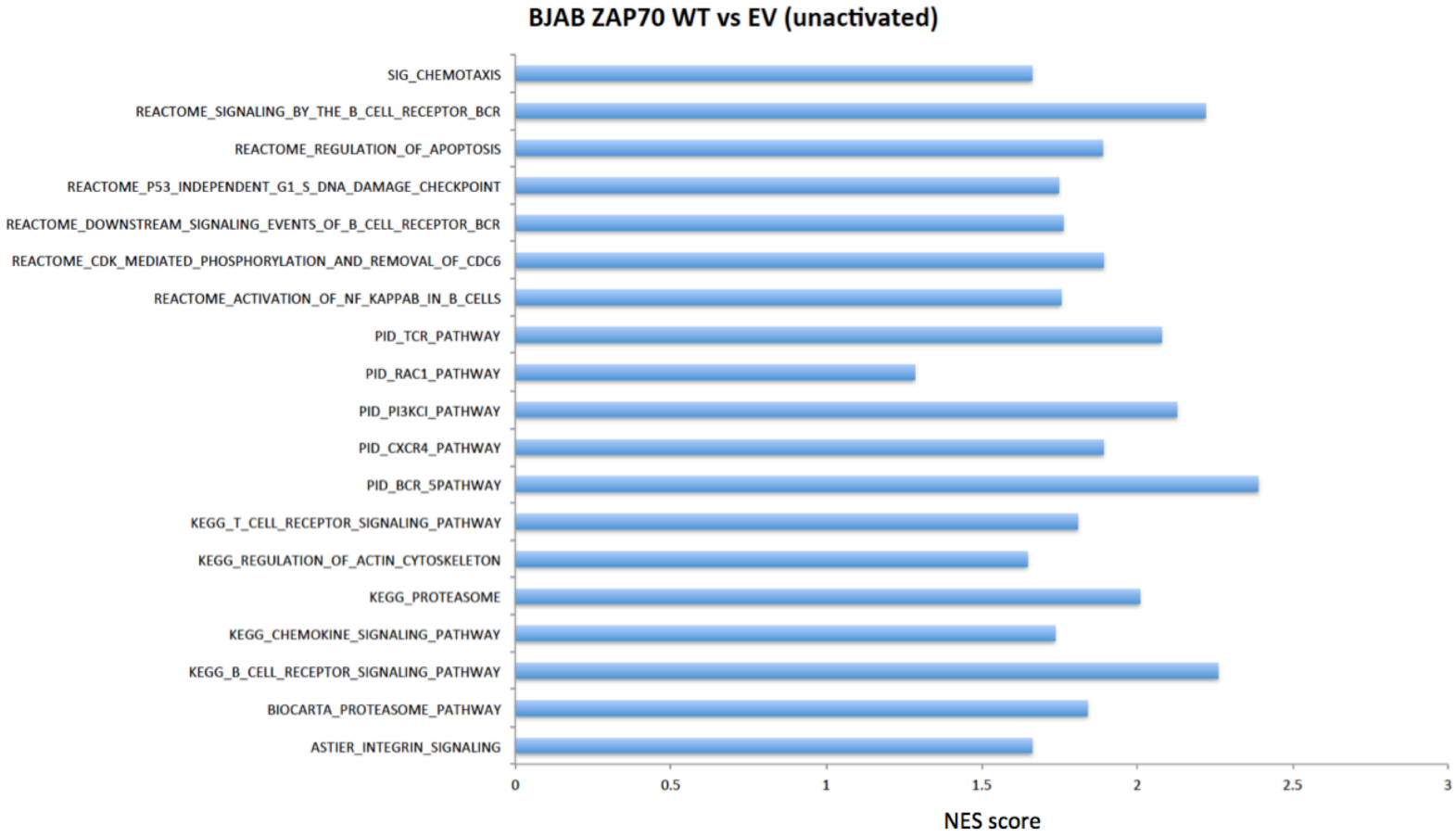


Figure 23B: Gene enrichment analyses (GSEA). Relevant pathways in both EV and WT BJAB cells in unactivated state. Gene sets are listed in order of Normalised enrichment scores. FDR q values for all are below 0.25.

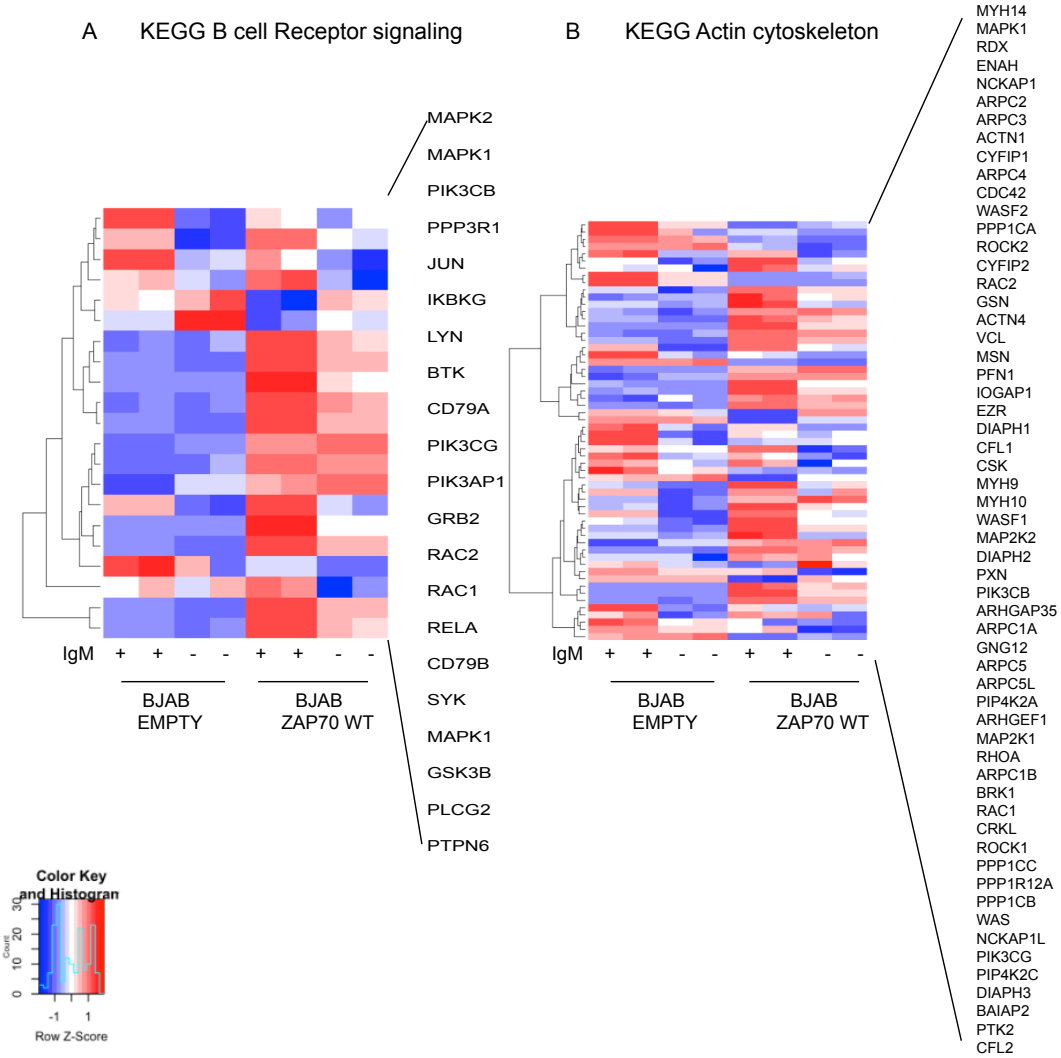


Figure 24: KEGG pathway analysis. Changes in key proteins involved in BCR signalling (A) and Actin Cytoskeleton (B) in BJAB cells at both unactivated and activated state.

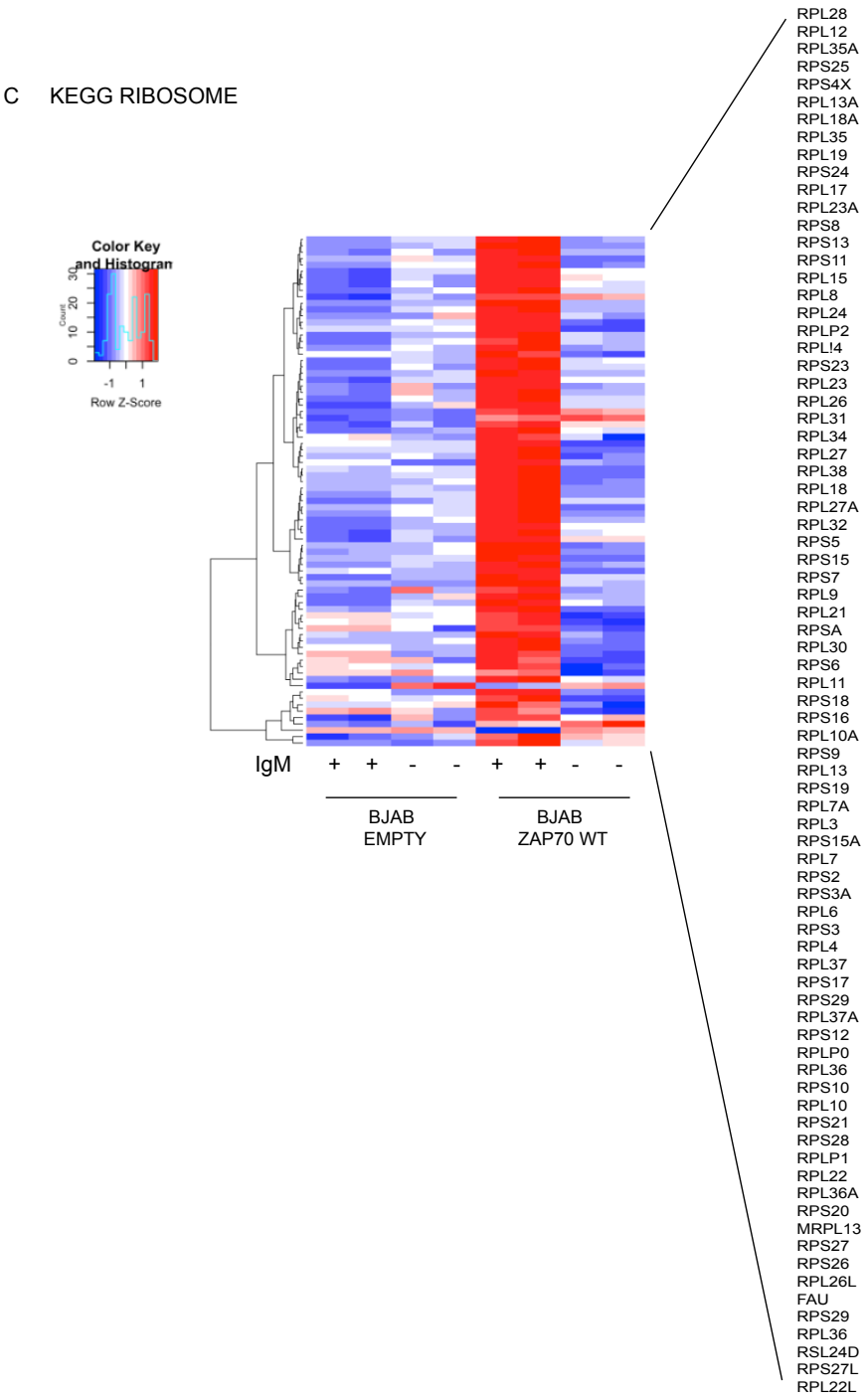


Figure 24C: KEGG pathway analysis. Changes in key proteins involved in ribosomal pathway in BJAB cells at both unactivated and activated state.

5.2 RIME/multiplexed Mass spectrometry of CLL patient cells

Mass spectrometry using BJAB cells has confirmed involvement of ZAP70 in BCR signalling, as well as its potential role in migration. However, it was still vital to explore interacting ZAP70 in CLL patient cells. In order to understand the physical interaction between neighbouring endogenous proteins that define regulation and their function, sensitive protein purification and identification of protein complexes was necessary. Here, a quantitative multiplexed method was used that integrates rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) method with isobaric labelling and tribrid mass spectrometry (Papachristou et al., 2018), which can unveil protein interactors of ZAP70 at steady state and upon IgM activation with increased sensitivity. Briefly, ZAP70 positive patient cells, with or without IgM activation were double cross-linked (formaldehyde and DSG crosslinking as described in the methods section). The cells were then harvested, lysed and incubated with ZAP70 antibody overnight. ZAP70 and interacting proteins were captured by incubating cells and relevant antibodies with washed Protein G beads. Protein G beads were bound to a magnet and used for mass spectrometry analysis. Figure 25A shows the schematic of endogenous ZAP70 pull down using quantitative multiplexed RIME method. Figure 25B shows the principal component analysis showing the segregated clustering of ZAP70 proteins (steady state (red) / IgM activated (blue)) and IgG (steady and activated pooled: green).

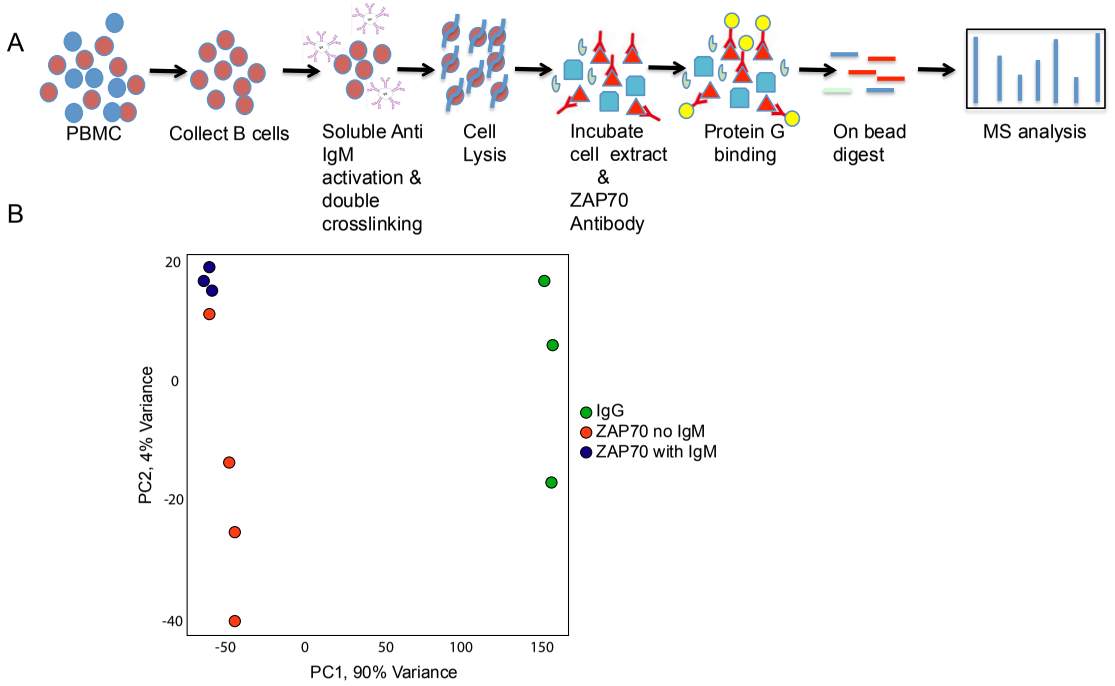


Figure 25: Endogenous ZAP70 pull down from CLL patient cells. Schematic of workflow of the endogenous ZAP70 immunoprecipitation using the integrated approach of RIME and isobaric labelling and tribrid mass spectrometry. Cells with or without activation were double cross-linked, and harvested for cell lysing. Cytoplasmic fraction was then incubated with ZAP70 antibody overnight. ZAP70 and interacting proteins were eluted using Protein G dynabeads and submitted for mass spectrometry analysis (A). Principal component analysis showing well segregated clustering of IgG (pooled unactivated and activated: green), ZAP70 unactivated (red) and ZAP70 activated (blue) (B).

5.2.1 Mass spectrometry of CLL patient cells

Similar to immunoprecipatation performed with BJAB cells, stringent filtering conditions were used to identify proteins expressed. Figure 26A, 26B, 26C shows volcano plots of upregulated proteins bound to ZAP70 at steady state (1420 genes), upon activation (1352 genes) and specific to ZAP70 in the activated state (298 genes) respectively.

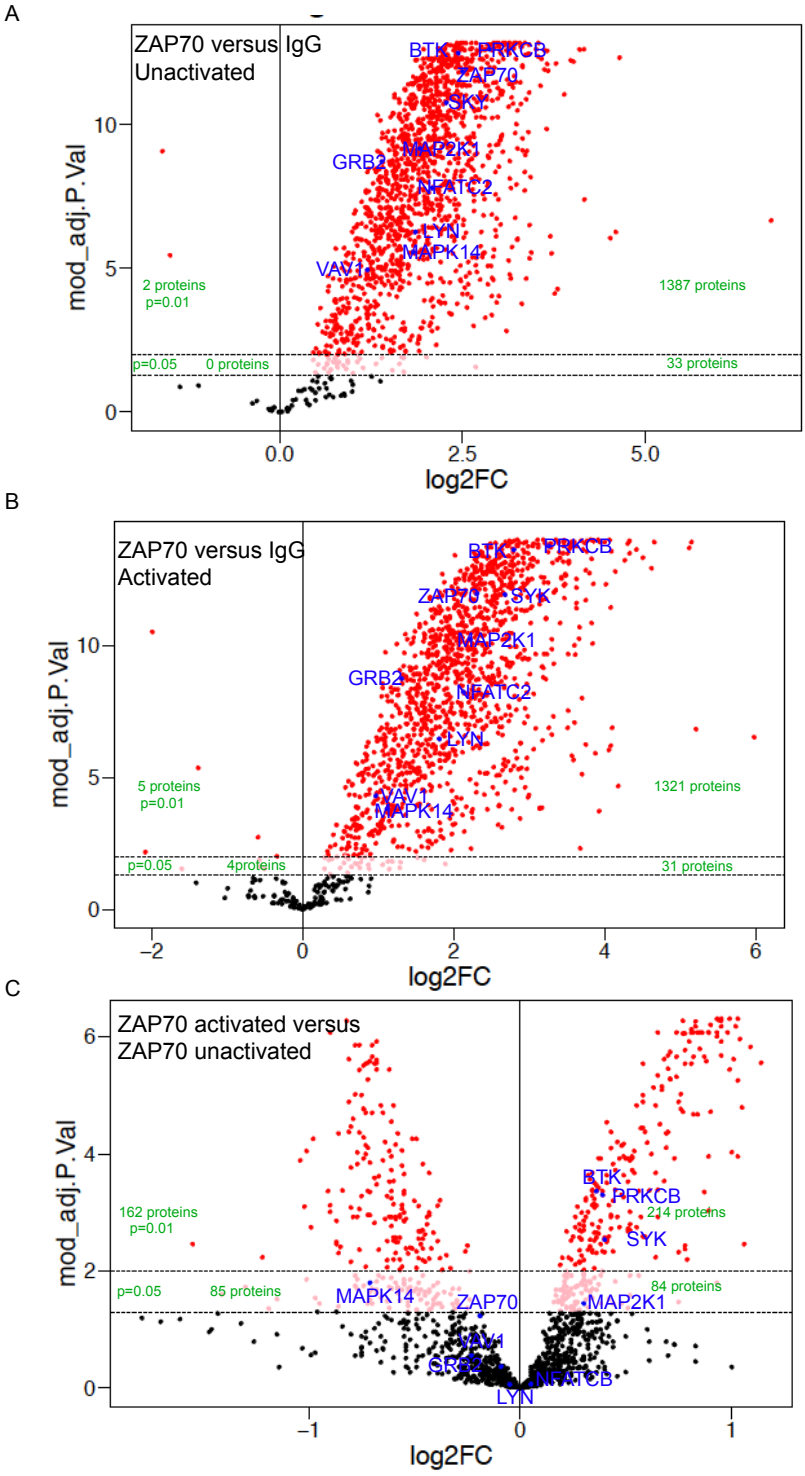


Figure 26: Patient cells mass spectrometry analysis. Volcano plots showing differentially regulated proteins (red), upregulated proteins on the right and down regulated proteins on the left in patient cells specific to ZAP70 versus IgG in the unactivated(A), activated (B) and specific to ZAP70 in the activated state versus steady state (C). Proteins highlighted in blue below to the BCR signalling complex.

Figure 27A highlights the results from GSEA analysis, where KEGG B Cell receptor signalling showed enrichment of proteins such as SYK, BTK, CARD11, PLCγ2, upon activation. Figure 27B shows changes in proteins involved in cell motility and Figure 28A once again shows majority of the ribosomal proteins were enriched upon IgM activation in ZAP70 positive patient cells as well. To better understand the role of ZAP70 in protein synthesis the puromycin analog (OPP) assay was used. OPP was fed to ZAP70 positive cultured CLL cells and incorporated into proteins during active protein synthesis. My hypothesis is that ZAP70 positive cells will have increases protein synthesis compared to ZAP70 negative CLL cells with a further increase upon IgM activation. Figure 28B shows results from the OPP assay. IgM activation does not seem to cause an increase in OPP labelling which is contrary to CpG treatment in CLL cells. CpG-ODN is a relatively strong stimulating agent for CLL cells and has shown greater OPP labelling compared to IgM (Yeomans et al., 2016). A condition including the addition of CpG-ODN2006 was added to ensure positive OPP labelling (Figure 28B).

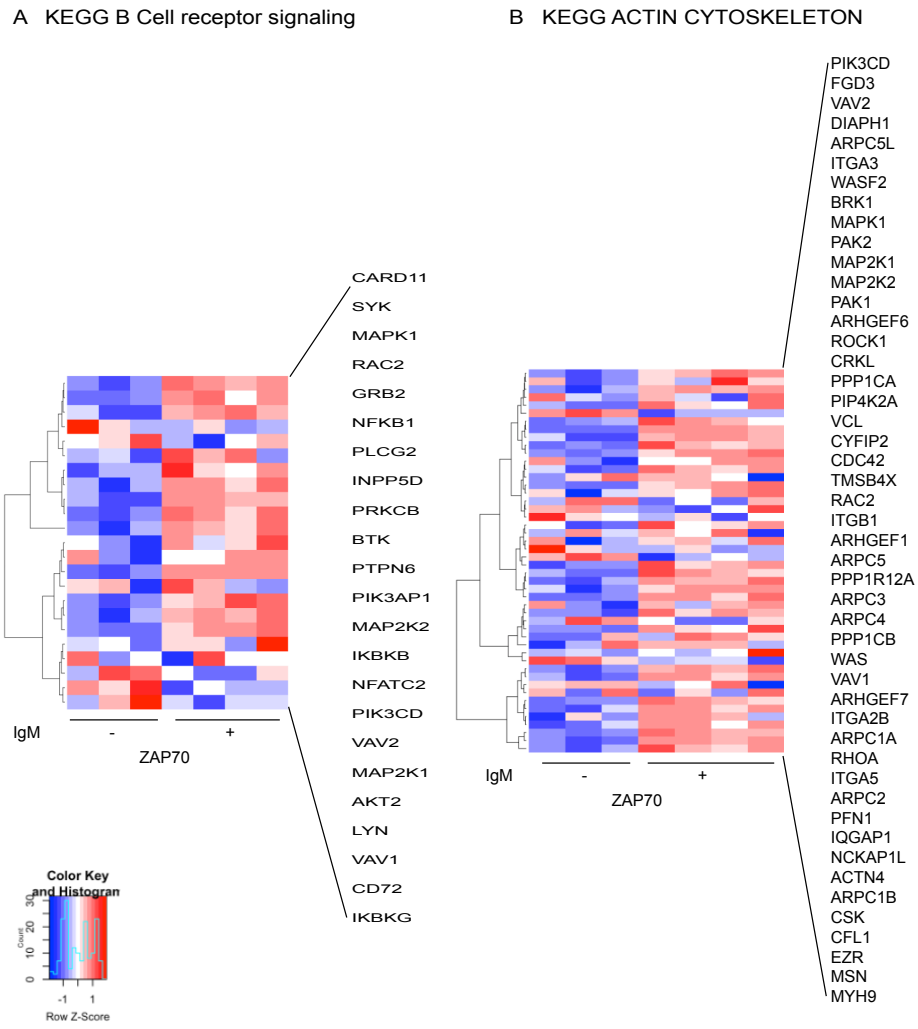


Figure 27: KEGG B cell receptor signalling. Changes in key proteins involved in BCR signalling (A) and Actin cytoskeleton (B) in Patient cells with greater than 20% endogenous ZAP70 in B cells at both unactivated and activated state. KEGG ribosome pathway analysis on patient cells showing striking upregulation of ribosomal protein in the ZAP70 immunoprecipitated post IgM activated cells.

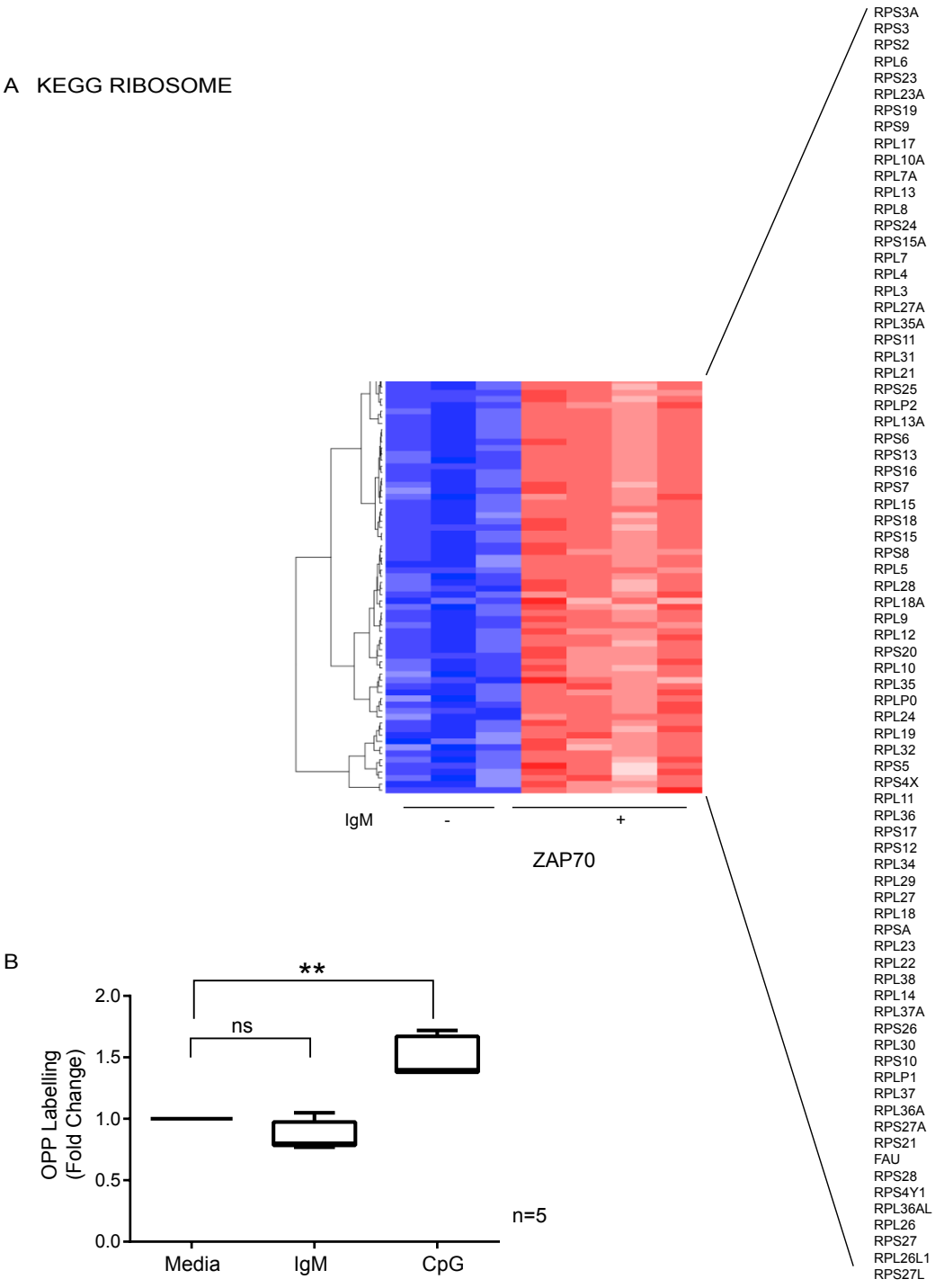


Figure 28: KEGG ribosomal pathway. Changes in key proteins involved in Ribosomal pathway in Patient cells with greater than 20% endogenous ZAP70 in B cells at both unactivated and activated state. KEGG ribosome pathway analysis on patient cells showing striking upregulation of ribosomal protein in the ZAP70 immunoprecipitated post IgM activated cells (A) CLL cells treated with immobilized IgM or CpG-ODN2006 or left untreated for 24hours before OPP labelling. Graph shows results from 5 representative ZAP70 positive CLL patient cells (B)

5.3 Common ZAP70 interactors in BJAB and CLL Patient cells

In order to focus on proteins interacting with ZAP70, results from both ectopically expressing ZAP70 BJAB cells and ZAP70 positive patient cells were compared. Using the stringent conditions for both BJAB and patient CLL mass spectrometry data, comparisons were performed to investigate proteins in common. Figure 29 shows a Venn diagram of proteins that are expressed preferentially in the BJAB ZAP70 WT and CLL patient groups. The criteria used is log2FC>2 and p ≤0.05. This highlights 27 proteins found in common consisting of many BCR related proteins such as ZAP70, SYK, SWAP70, PLCγ2, CSK, some ribosomal proteins: RPS15A, RPS17, RPS20, and RPS19 and proteins related to migratory pathways: SWAP70, PTPN6, LCP1 as well. Figure 30 shows the correlation plot of interacting proteins between BJAB and Patient mass spectrometry. Here, data from one patient was used to compare to BJAB data. Proteins that fall exactly or closer to the straight line shows the strongest correlation and correlation becomes weaker for the proteins that become more scattered.

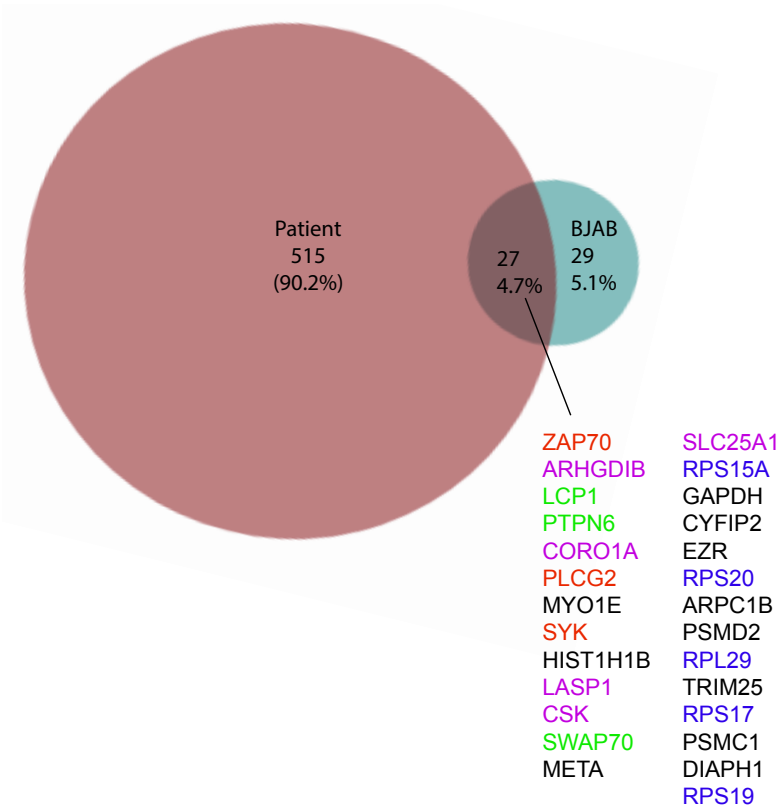
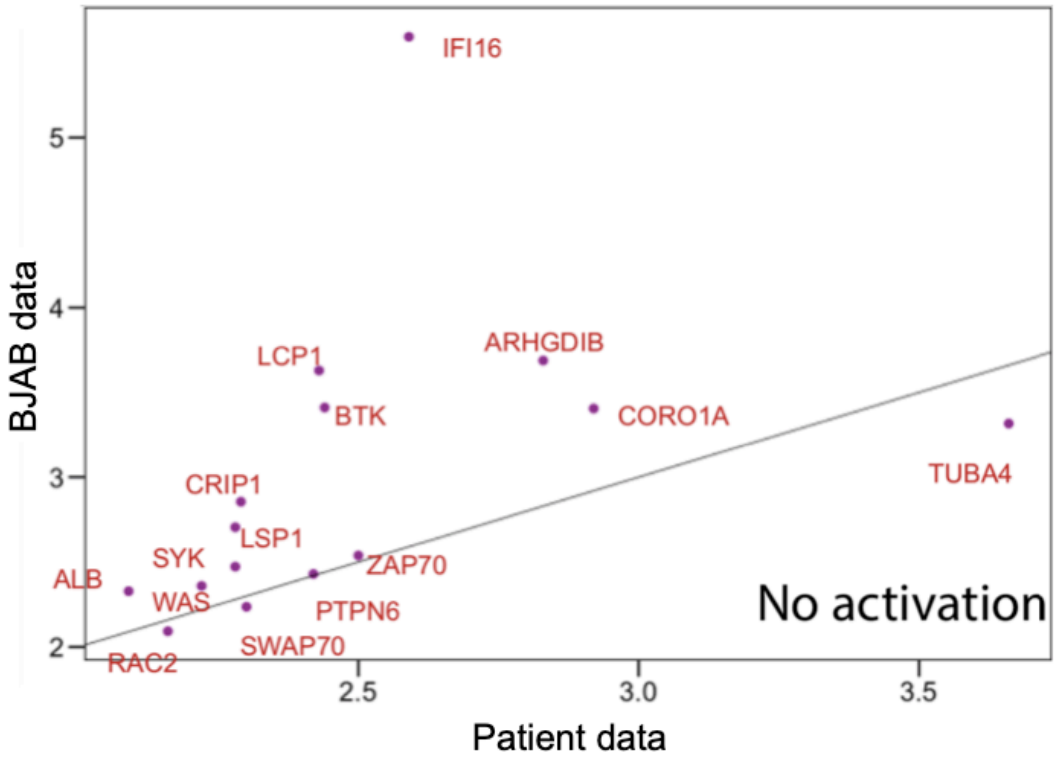


Figure 29: Venn diagram illustrating the overlap in ZAP70 interacting proteins between BJAB cells and CLL patient cells. Stringent filtering criteria of log2FC >2 and p ≤ 0.05 was used. Proteins involved in selected pathways are highlighted: BCR (red), ribosomal (blue), migration (green) and associated with CLL (purple).



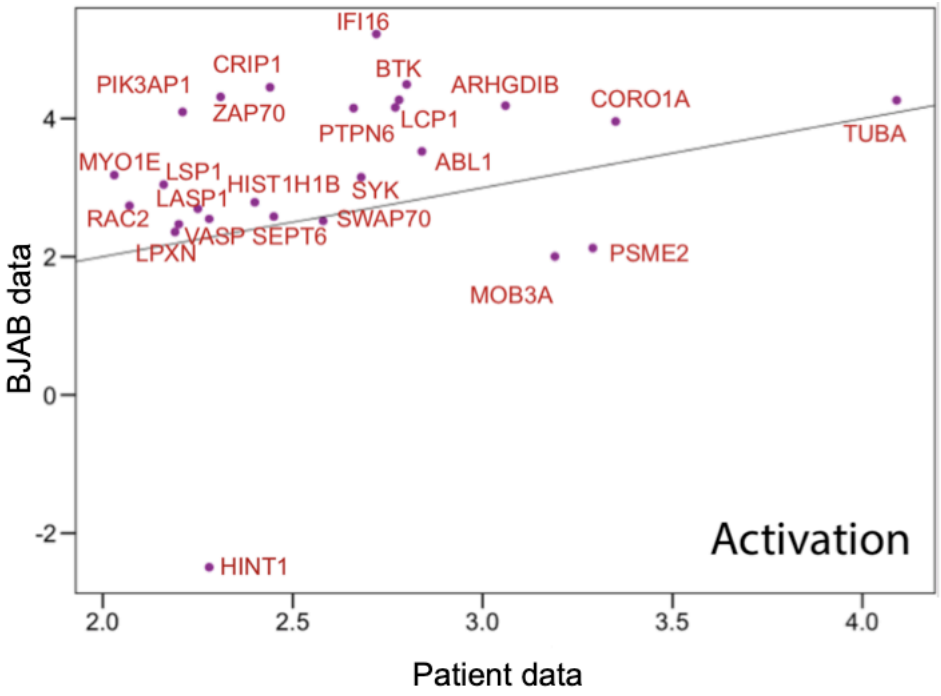


Figure 30: Correlation plot of common proteins between BJAB and Patient CLL cells. Proteins in common from mass spectrometry analysis between BJAB ZAP70 and endogenous ZAP70 in CLL patient cells

5.4 CLL Cell Migration

One of the important signatures relating to ZAP70 identified in the BJAB and CLL patient mass spectrometry was involving migratory proteins. In CLL cell trafficking, homing and interactions between CLL and accessory cells involves various chemokines and their receptors secreted by CLL cells or stromal cells. This interaction results in CLL chemotaxis into the tissue microenvironment where the cells respond to survival and proliferation signals (Davids & Burger, 2013). To investigate the role of ZAP70 in migration, I treated ZAP70 positive patient cells with 5μM P505-15 and ibrutinib (positive control) and performed a migration assay. Both inhibitors resulted in a significant decrease in migration index in response to CXCL12, CCL19 and CCL21 (Figure 31).

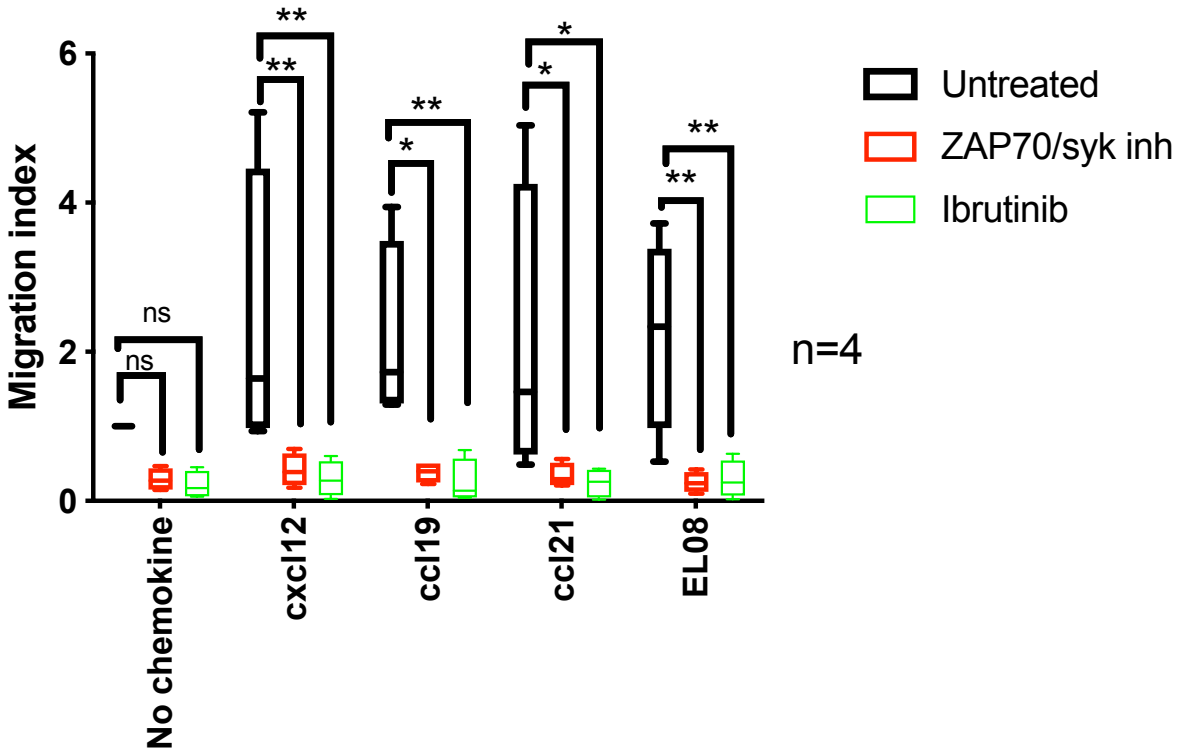


Figure 31: Effect of ZAP70 on CLL cell migration.
A significant decrease in migration observed after inhibitor treatment in the presence of chemokines CXCL12, CCL19 and CCL21 and when cultured with EL08 stromal cells.

To specifically determine ZAP70 effect, ZAP70 positive patient cells were nucleofected and on day 6 used for the migration assay. Comparing siZAP70 when normalised to siNSC showed a reduction (p=0.0552) in the presence of CCL19, and a non significant change in the presence of CCL21 (Figure 32A).

Similarly to varying endogenous ZAP70 expression levels among patient cells, knockdown efficiency differs as well. Figure 32B shows the correlation between ZAP70 knockdown efficiency and CLL cell migration index. Increase in ZAP70 levels showed increased CLL cell migration.

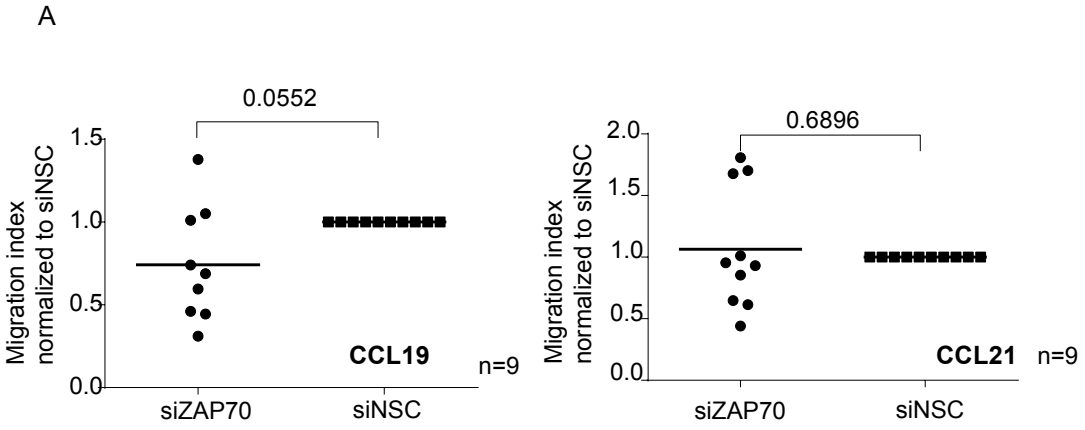


Figure 32a: siZAP70 reduces migration index. Changes in migration index comparing siZAP70 and siNSC cells in the presence of CCL19 and CCL21. Migration index was calculated as the number of cells transmigrating with chemokine divided by the number of transmigrating cells in the absence of chemokine (A).

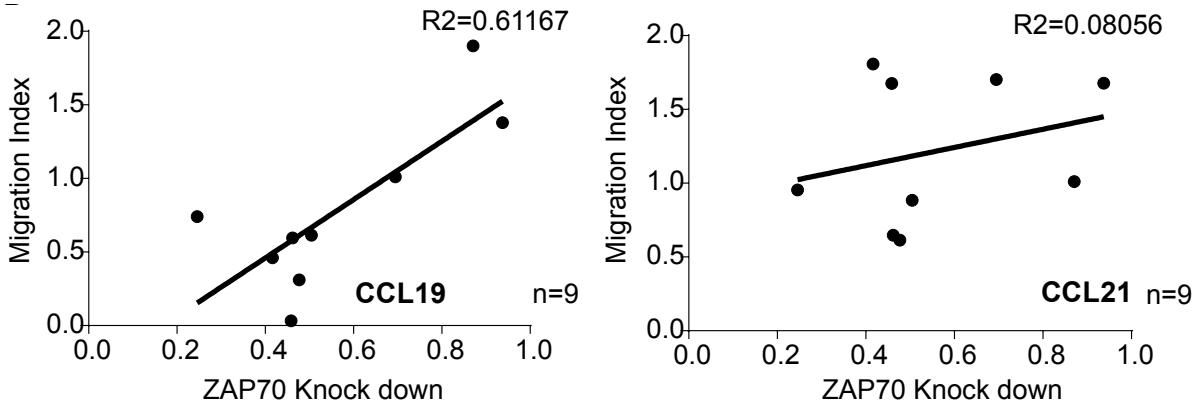


Figure 32b: Correlation between ZAP70 expression and migration in siRNA treated CLL cells.

6. Results: Role of ZAP70 in DNA Damage in CLL Cells

6.1 Formation of γH2AX foci

6.1.1 P505-15 increases formation of γH2AX foci

As mentioned earlier, CLL is a malignancy with chromosomal aberrations. DNA damage and alterations of the DDR are features of genetic instability common in CLL (Popp et al., 2017). Furthermore, DNA damage driver mutations were found in higher proportions in the UM-CLL compared to only 3 driver mutations 9 MYD88, CHD2, and del13q found in the M-CLL (Landau et al., 2015). In 2006, it was shown that in CpG stimulated CLL cells, there was a correlation (p=0.034) between UM-CLL and unbalanced translocations (Dicker, Schnittger, Haferlach, Kern, & Schoch, 2006; Haferlach, Dicker, Schnittger, Kern, & Haferlach, 2007). Comprehensive genetic characterization of CLL confirmed that del11q is overrepresented in UM-CLL. In addition 6qdel, 17pdel and trisomy 12 as the sole abnormality were significantly more frequent in UM-CLL patients with p=0.0001,0.003 and 0.015) respectively (Haferlach et al., 2007). Specifically, unpublished data from the Ringshausen lab suggest that ZAP70 is present and very mobile in the nucleus of CLL cells. My hypothesis is that in ZAP70 positive patients cells, higher levels of phospho gamma H2AX foci are expected compared to ZAP70 negative patient cells.

To investigate the role of ZAP70 in DNA damage, CLL patient cells were irradiated with 4Gy and immunofluorescence staining was carried out to assess γH2AX foci formation. This is representative of greater than 3 patients used for the study. Upon 4Gy irradiation, formation of γH2AX foci can be seen which is absent in the IgG and non-radiated controls (Figure 33).

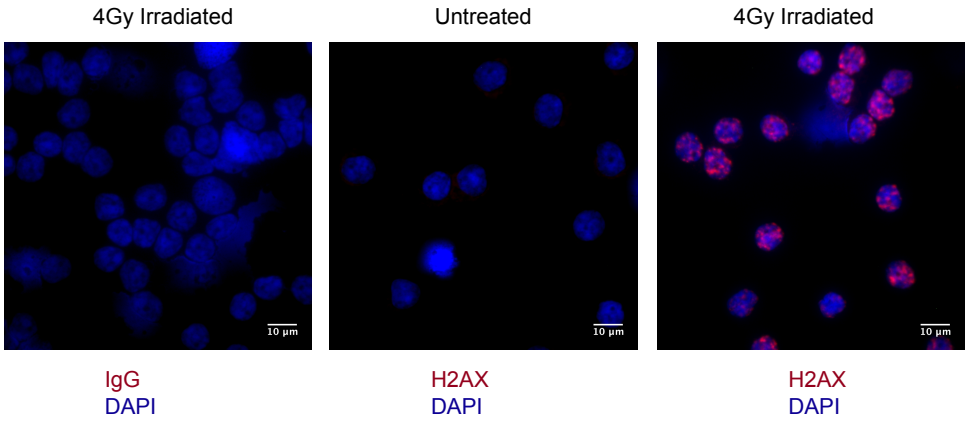


Figure 33: Immunostaining of γH2AX in CLL patient cells after 4Gy irradiation.

Figure 34 shows the effect of KU55933 (ATM inhibitor: used as positive control) and P505-15. Foci formation is reduced in the presence of 10μM KU55933 and γH2AX foci observed when 1μM of KU55933. Furthermore, in the presence of 5μM of P505-15, there is increased intensity of γH2AX foci compared to cells treated with 0.5μM P505-15 (Figure 34).

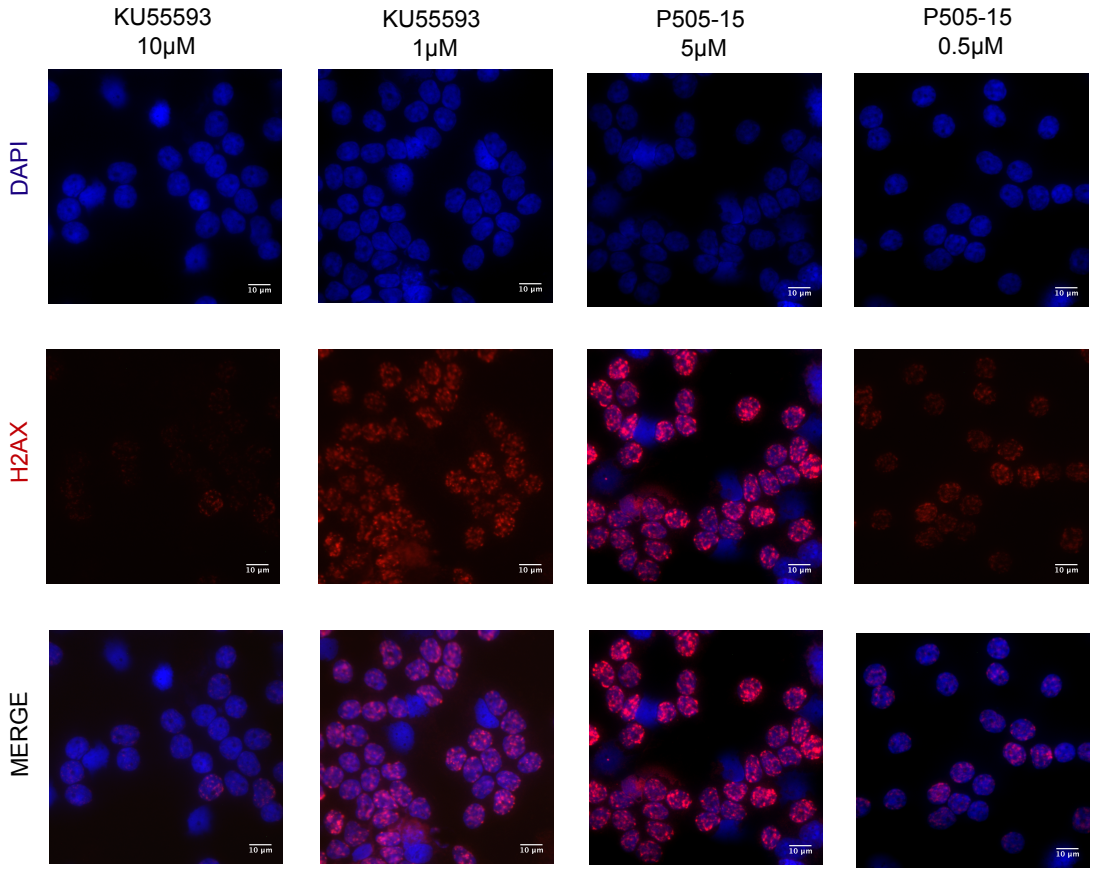


Figure 34: Effect of P505-15 on formation of γH2AX foci. Immunostaining of γH2AX in patient cells treated with KU55593 (ATM inhibitor) and P505-15 at two concentrations followed by 4Gy irradiation.

6.2 P505-15 increases double stranded DNA breaks (Comet assay)

To further quantify the extent of DNA damage upon P505-15 inhibition, the COMET assay was carried out to measure DNA strand breaks in cells. Inhibitor treatment was carried out for 1 hour followed by irradiation (4Gy). Immunofluorescence results showed increased tail length (DNA migrating) at 5μM compared to 0.5μM of P505-15 and quantified results using imageJ (Figure 35).

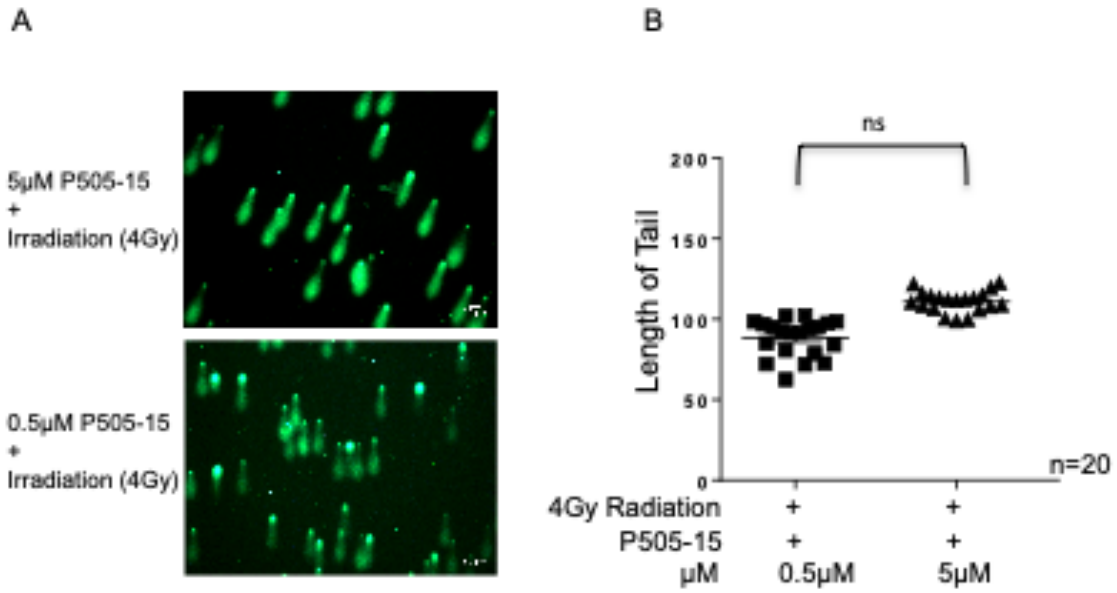


Figure 35: Effect of reduced ZAP70 kinase activity on double stranded DNA breaks upon irradiation. The comet assay of patient CLL cells treated with either 5uM (top image) or 0.5uM (bottom image) P505-15 after 4Gy irradiation, representative images are shown (A). The quantification of the comet assay (n=20) (B).

6.3 P505-15 causes further decrease in cell viability post DNA damage

In order to investigate the effect of P505-15 inhibition post chemotherapy or irradiation, CLL cells from ZAP70 positive samples were treated with P505-15 inhibitor at 5μM and 0.5μM concentrations followed by either bendamustine at 20μM/10μM (Figure 36A) or 4Gy irradiation (Figure 36B). Upon comparing untreated cells to bendamustine treatment, there is a significant decrease in live cells at both concentrations of bendamustine. In the presence of high concentration of P505-15 inhibitor, there is a significant decrease in cell viability and non-significant change at the lower concentration of P505-15 inhibitor. Inhibitors alone (coloured in green) show the baseline effect of inhibitors on CLL cells (Figure 36A). Figure 36 B on the other hand shows effect of P505-15 post 4Gy of radiation. Here, there is again a significant dose dependent change in cell viability.

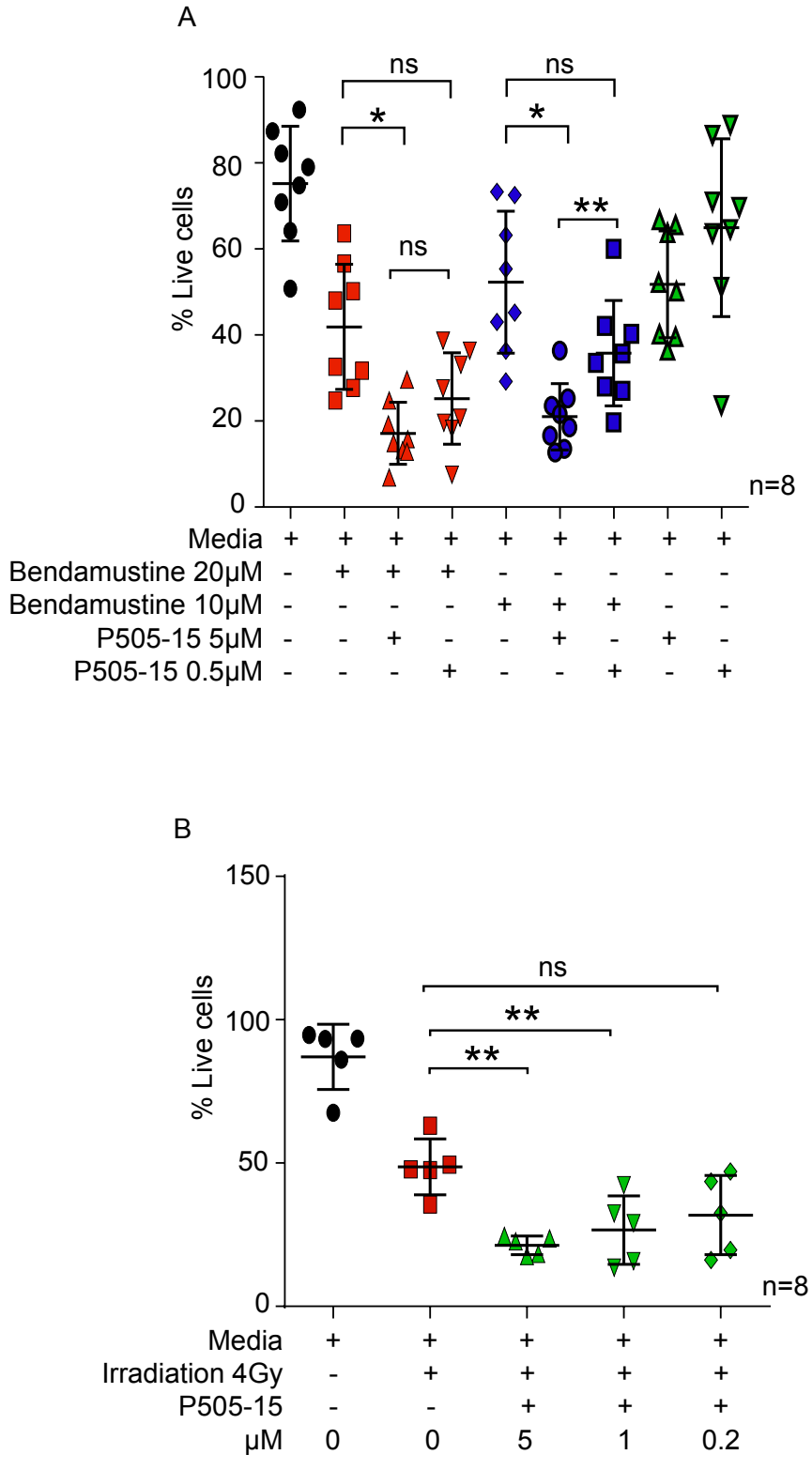


Figure 36: Reduction in cell viability in CLL cells treated with P505-15 prior to irradiation/chemotherapy. Cell viability of CLL cells treated with P505-15 followed by Bendamustine treatment (A) or 4Gy irradiation (B).

6.4 Cell viability is unaffected post ZAP70 siRNA knockdown

To investigate if the results seen above with inhibitor can be replicated using ZAP70 knockdown, ZAP70 siRNA and NSC nucleofected cells were used. On day 6 post nucleofection, cells were treated with Bendamustine (10μM) or irradiated (4Gy), decrease in viability was observed comparing control (non transfected) cells. However, no significant changes were observed when comparing the siZAP70 to siNSC, similar results were observed with irradiation experiments as well (Figure 37A and B).

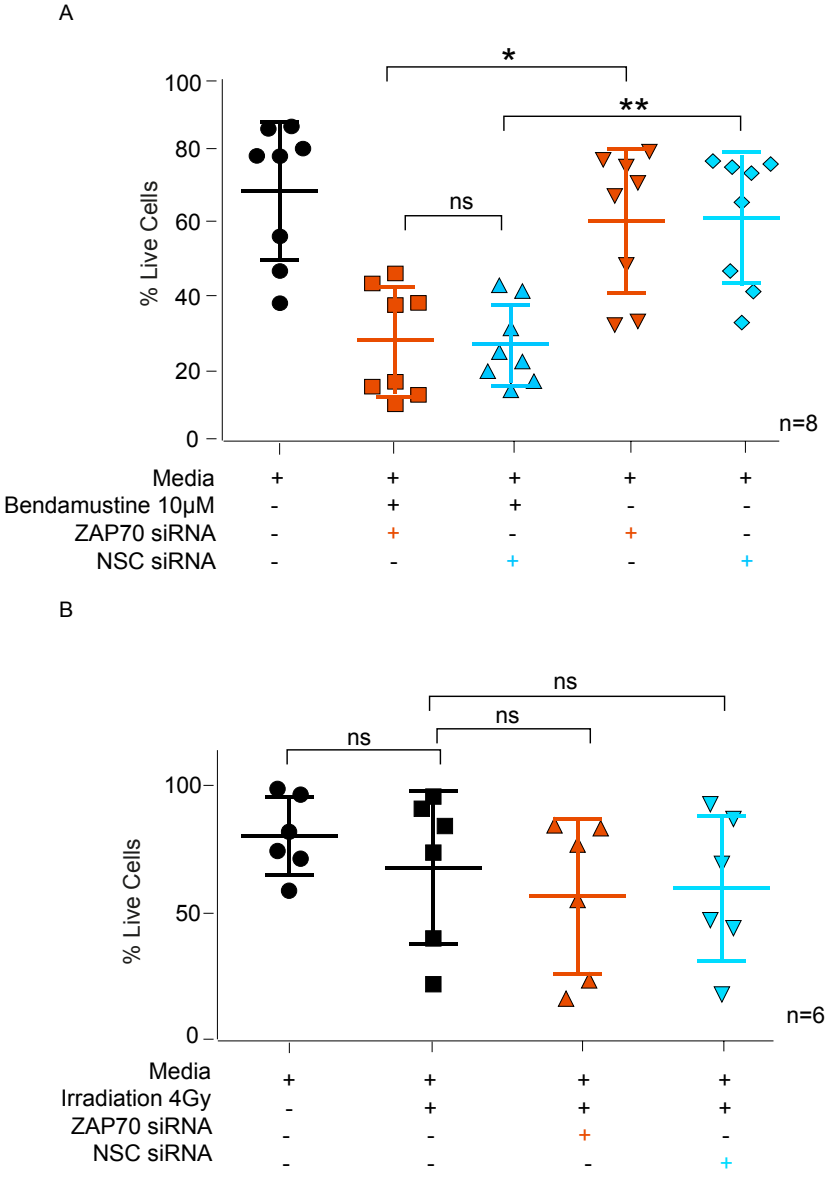


Figure 37: ZAP70 knockdown does not affect CLL cell viability. Cell viability of siRNA (ZAP70 and NSC) treated CLL cells followed by Bendamustine (A) or 4Gy irradiation (B).

7. Discussion

7.1 Role of ZAP70 in B CLL

Of the many prognostic markers of CLL, specifically, ZAP70 expression is a very reliable surrogate marker for the distinction between the Ig-mutated and Ig-unmutated CLL subtypes and ZAP70 status can be easily determined using flow cytometry. In 2004, it was shown that UM IgHV is strongly associated with the expression of ZAP70, ZAP70 is a stronger predictor of the need for treatment in B-cell CLL with low levels of ZAP70 being associated with better prognosis of CLL (Rassenti et al., 2004). Possible explanations for the effect on prognosis can be inferred from research showing that introducing ZAP70 ectopically into ZAP70 negative CLL cells, enhanced expression of NF-κB target genes such as IL1B, IL6, and IL8 upon BCR stimulation possibly by an IKK dependent mechanism, and NF-κB pathway inhibitors have been explored as novel therapeutic approaches for the treatment of CLL (Lopez-Guerra & Colomer, 2010; Pede et al., 2013). Therefore, suggesting that ZAP70 acts directly as an amplifier of BCR signalling in CLL, resulting in poorer prognosis. Another explanation for the difference in prognosis involves the BCL-2 family of apoptosis regulating proteins in CLL, hence why CLL is referred to as a disorder with failed apoptosis (decreased death). My aim in this project was to investigate the role of ZAP70 in CLL with particular focus on BCR signalling, apoptosis, migration and proliferation whilst adding on to the existing knowledge.

Here, I assessed the functional role of ZAP70 by using P505-15. P505-15 is a small molecule dual inhibitor of SYK/ZAP70, which has low nanomolar potency (IC_{50}=50%). ZAP70 positive CLL cells were treated with two concentrations of P505-15 (5μM and 0.5μM) and BTK inhibitor: Ibrutinib (used as positive control) and BCR signalling was assessed by calcium flux assay. Significant decrease in peak fluorescent intensity using both 5μm and 0.5μM of P505-15 was observed (Figure 12). In addition, a decrease in peak fluorescent intensity was seen with Ibrutinib, which abrogates signalling downstream of BTK. Alongside P505-15 inhibitor studies, ZAP70 siRNA knockdown assays were carried out. This was done to disentangle the effect of SYK from ZAP70 and minimize off target effects on other kinases. The advantage of this approach is individual patients with normal

versus decreased ZAP70 expression can be used to determine changes in signalling or cell survival keeping other factors constant within the same patient. Here I have successfully shown that ZAP70 siRNA significantly lowers ZAP70 expression with minimal changes in SYK expression (Figure 15). Using this knockdown system, calcium flux assays were carried out to assess BCR signalling, and apoptosis assays to understand cell survival in ZAP70 siRNA knockdown cells were done. Results obtained here do not show a significant decrease in calcium mobilization when comparing ZAP70 siRNA and non specific control siRNA (Figure 16). The absence of significant change between siZAP70 and siNSC could be due to the lack of complete loss of ZAP70, as siRNA is a transient knockdown that does not have full efficiency. I observed that siRNA efficiency varied across patient cells with knockdown ranging between 50%-80% of ZAP70 expression. The stability of remaining ZAP70 proteins post knockdown could potentially be masking the effect of knockdown in the BCR signalling and proliferation assays. Lastly, papers have suggested that ZAP70 acts to enhance the signalling capacity of the BCR complex in B cell CLL and is not essential for BCR signalling (Chen et al., 2002), so therefore a lack of a difference could suggest increased activity of SYK to reduce effect of lowered ZAP70 expression. This data contradicts results shown by Chen et al (2005), whereby post IgM ligations, ZAP70+ CLL cells had higher calcium flux than did ZAP70 negative CLL cells. The discrepancy between data presented in this thesis and published data in my opinion is due to the different experimental systems used which factors in varying levels of ZAP70 in patient and arbitrary cutoff of 20% that defines ZAP70 positive and negative B cells.

The knock down approach was also used to assess the effect on AKT phosphorylation. In my data, upon ZAP70 knockdown there was an increase in pAKT post IgM activation (Figure 17b), which was contrary to my hypothesis. This is also contrary to the findings from studies that used BJAB cells transfected with ZAP70. It was previously shown that BJAB cells with ZAP70 expression had stronger and prolonged activation of SYK, ERK, and AKT after BCR stimulation (Stefania Gobessi et al., 2007). This discrepancy in increase of phospho AKT observed in ZAP70 knockdown cells could be due to use of immobilised IgM activation rather than soluble IgM. Studies have found that ligation of the BCR with sol-IgM induced incomplete responses in CLL cells resulting in transient phosphorylation of ERK, and AKT. On the other hand stimulation with immobolised

IgM elicited a more complete BCR signal characterised by prolonged phosphorylation of ERK and AKT, indicating persistent signalling (Petlickovski et al., 2005).

I further assessed CLL cell survival of ZAP70 positive CLL in the presence/absence of soluble anti IgM ligation cells using an Annexin V apoptosis assay. At 24 hours, I observed a significant decrease in cell viability using 5μM of P505-15 in the absence of IgM and a non-significant decrease upon IgM activation (Figure 13). The differences seen with/without IgM supports the data from Hacken et al, in 2015 that reported that surface IgM is higher in UM–CLL and resulted in increased CLL viabililty compared to IgD activation (Hacken et al., 2015). This survival is characterised by caspase inhibition, induction of NF-kB and expression of antiapoptotic molecules (Bcl-2, Mcl-1) (Bernal et al., 2001) However, overall there is a significant increase in apoptosis in the UM-CLL compared to M-CLL cells (Coscia et al., 2011). Annexin V data at 48hrs using P505-15 shows a decrease in viable cell numbers at both concentrations of P505-15, with a greater significance in the absence of IgM ligation compared to IgM activation (Figure 13), again supporting earlier findings of IgM preventing apoptosis of B CLL cells. Therefore, these results suggest that P505-15 treatment for 48 hours reduces B CLL cell viability, however extent of apoptosis can be lowered with IgM ligation.

Lastly, in the context of proliferation, similar to calcium flux and cell survival, P505-15 shows a similar effect on CLL cell proliferation measured by carboxyfluorescein succinimidyl ester (CFSE) incorporation. ZAP70 positive CLL cells treated with varying concentration of P505-15 show a dose dependent decrease in proliferation when co-cultured with EL08 expressing CD40L+IL21 with similar levels of decrease in proliferation seen with Ibrutinib treatment (Figure 14). Studies have shown that CD40 ligation in CLL B cells is a strong stimulus to induce NF-κB signalling and survival (Hömig-Hölzel et al., 2008). In addition, my data supports the findings made by Schleiss et al, where they showed that ZAP70 positive CLL cells showed a proliferative advantage associated with increased phosphorylation of ZAP70/SYK and STAT 6 with a further increase upon TLR9 activation (Schleiss et al., 2019).

All of the above results suggest that ZAP70/SYK inhibition using P505-15 plays a direct role in CLL cell BCR signalling, proliferation and cell survival. Therefore to conclude, I have shown that P505-15 treatment, in the presence of IgM ligation,

results in a significant decrease in BCR signalling, cell survival and proliferation of CLL cells suggesting a vital role of ZAP70/SYK in these processes.

7.2 ZAP70 proteomics

Signalling via BCR plays an important pathogenic role in CLL. Within the CLL context, ZAP70, a negative prognostic factor, structurally homologous to SYK plays an analogous role in BCR signalling. A mass spectrometry based phosphoproteomic study comparing quantitative differences in the temporal dynamics of phosphorylation in simulated and unstimulated T cells, in both activated and unactivated T cells, with/without ZAP70 catalytic activity has been carried out. The results showed that kinase activity of ZAP70 stimulates a negative feedback pathway that targets LCK and controls the phosphorylation of ITAMS as well as downstream targets of CD3 and ζ chain components (Sjölin-Goodfellow et al., 2015). In my project, my aim was to understand protein interactors of ZAP70 in BJAB cells (B cell line) as well as in patient CLL cells. Mass spectrometry was used as it offers ways to dissect information about the vastly complicated protein interactions happening in cells.

CLL cells derived from patients can be extremely difficult to culture/propagate *in vitro* due to rapid apoptosis (maybe suggesting an advantageous *in vivo* microenvironment requirement for survival) thereby hindering long term studies including knockdown or over expression experiments to be carried out. I explored the use of B-cell lines with minimal endogenous ZAP70 to study ZAP70 interactions with other proteins. Here, I have ectopically introduced ZAP70 to try to answer the questions listed on the aims of my project section. So far 3 B-cell lines (BJAB, HBL1 and Raji) that have minimal endogenous ZAP70 expression (RNA seq by Dr. Daniel Hodson) have been identified. I have also shown that BCR in these cells can be activated with IgM at varying levels, this was confirmed by calcium flux assays (Figure 19B). The cell lines showed varying levels of activation, which could be due to the B cell subtype (activated B cell versus germinal centre B cell). In my experiments, comparing intracellular calcium in the empty vector control to the ZAP70 wild-type showed no significant differences (Figure 19D). However, previous studies undertaken by Chen et al, 2008 had shown increased intracellular

calcium in the adenovirus ZAP70 wild-type or ZAP70 KA369 expression system transduced into patient CLL cells compared to mock. This disparity could be due to differences in signalling efficiency between patient cells with endogenous level of protein versus cell lines with ectopic expression of protein.

For mass spectrometry, I utilized the *in vivo* biotinylation method. This method exploits the use of Escherichia coli biotin holoenzyme synthetase (BirA) that is known to perform highly selective, specific and rapid biotinylation of the fused protein (Fairhead & Howarth, 2015). Biotin-streptavidin interaction is one of the most strongest non covalent bonds known in biology, making it extremely advantageous for purification of the protein of interest (Kim, Cantor, Orkin, & Wang, 2009). In this study, there were varying levels of BirA in each of the cell lines, with BJAB cells showing the highest BirA expression (Figure 19C). A possible reason for this could be varying efficiency of transducing the BirA into the cell lines. Furthermore, since BirA is an active biotin ligase, low, medium and high BirA expressing cells all signify efficient biotinylation of the flag biotin tagged gene product (J. Wang, Cantor, & Orkin, 2009).

Here, I have shown that ZAP70 can be successfully pulled down with minimal contamination (Figure 21). It was noted that BJAB BirA cells resulted in higher levels of purified ZAP70 compared to the other two cell lines, this could be due to the varying BirA expression in each of the cell lines as mentioned earlier. For this reason, mass spectrometry was carried out on BJAB cells in the first instance. This mass spectrometry approach successfully unveiled ZAP70 interacting protein partners and allowed the study of the interaction of ZAP70 with the BCR complex as a whole. Some of the main highlights from the mass spectrometry results were the presence of known proteins involved in the BCR signalling, giving us positive confirmation that our mass spectrometry approach worked in pulling down ZAP70 and its interacting partners. GSEA analysis on the ZAP70 versus EV at the unactivated, activated and specific to ZAP70 (activated) state resulted in novel signatures relating to migration and ribosome biosynthesis (Figures 23-24).

After the successful pull down of ZAP70 from BJAB ZAP70WT cells, I explored ways to pull down endogenous ZAP70 from CLL ZAP70 positive cells. After trying various methods, the most specific and efficient pull down of endogenous ZAP70 was achieved using the qPLEX-RIME (quantitative multiplexed method in combination with rapid immunoprecipitation mass spectrometry of endogenous

method) (Papachristou et al., 2018). This method employs a two-step fixation procedure using disuccinimidyl glutarate (DSG) and formaldehyde (FA) to capture transient interactions efficiently.

When comparing ZAP70 pull down results from BJAB and patient cells, similar signatures were observed. In both BJAB and primary cells, there was a striking collection of ribosomal proteins interacting with ZAP70 upon activation. Previous studies reported reduced ribosomal activity in CLL patients, which correlated with a reduction in rRNA maturation in CLL. However, a study using polysome profiling coupled to microarray analysis examined the translatome of a panel of peripheral blood B cells isolated from 34 CLL patients identified a ribosome related signature in CLL patients with mRNAs encoding for ribosomal proteins and factors that modify ribosomal RNA (Sbarrato et al., 2016). My data suggest that upon IgM activation, ZAP70 has a direct role in the ribosomal complex. It could be that tyrosine phosphorylation of ribosomal proteins is a way of regulating translation. To investigate changes in protein synthesis, the OPP assay was carried out. It was seen that ZAP70 positive CLL patient cells showed no significant change upon IgM activation, in contrast a significant increase was observed in the presence of CpG.

Another trend observed in both the BJAB and patient mass spec was the collection of proteins involved in cell motility. Specifically, there were proteins involved in migration and focal adhesion: LCP1, SWAP70, PTPN6, RAC1, SHP1. Some of these proteins showed upregulation upon activation in the BJAB cells. In CLL, cells circulate freely in the peripheral blood but transmigrate to sites such as bone marrow and lymph nodes that provide anti-apoptosis and pro survival signals due to chemokine gradients supplied by stromal cells. Significant decrease in the migration index of CLL cells treated with P505-15 and Ibrutinib was observed (Figure 31). This data is consistent with findings that R406 abrogated both CLL cell survival and migration (Quiroga et al., 2009). In order to determine the specific role of ZAP70 in migration, I carried out ZAP70 siRNA knockdown followed by migration assays in the presence of CCL19 and CCL21. My results showed a decreasing trend in migration towards CCL19 but not CCL21 in the siZAP70 cells compared to siNSC. In addition, there was positive correlation achieved between ZAP70 expression (based on efficiency of siRNA) and migration index. This data in combination with results published by Calpe et al (2013) suggest that ZAP70 could play a role in chemokine receptor CCR7 upregulation (ERK1/2 dependent) leading

to increased migration in the presence of CCL19. The “trend” in decrease in migration index instead of significant decrease could be due to the extensive length of days of cells in culture to achieve knockdown hence affecting cell viability and responsiveness of cells to chemokines.

7.3 Effect of ZAP70 in DNA Damage in CLL

CLL pathogenesis is greatly influenced by alterations in DNA damage response (DDR). CD19+ cells in CLL patients have increased numbers of γH2AX foci, a marker of DNA double strand breaks, compared to CD19+ cells in MBL (monoclonal B-cell Lymphocytosis) patients and healthy individuals (Popp et al., 2019). MBL is a premalignant stage of CLL and mechanisms of genetic instability are involved in the transformation of MBL to CLL. Popp et al also stated that within the CLL subtypes, UM-CLL cells have slightly higher γH2AX foci levels compared to M-CLL but this difference was not significant. Here, I show that P505-15 treatment results in increased formation of γH2AX foci (Figure 34), which correlated with my results from the COMET assay, which showed increased DNA damage tail length using higher concentrations of P505-15 (Figure 35). Therefore suggesting that presence of ZAP70 in CLL is protecting cells from DNA damage. This was contrary to findings from Popp et al, where upon comparing CD38-, CD38+, ZAP70- and ZAP70 + CLL samples, all groups had similar γH2AX levels (minor differences between these groups were not significant, $p=0.6456$). A possible explanation for the difference in results could be the variability in patient samples.

Here, since ZAP70 is a protein kinase, it can be suggested that ZAP70 could operate as an epigenetic regulator similar to role of nuclear JAK2 (Dawson et al., 2009).

In addition, P505-15 treatment prior to irradiation or bendamustine treatment resulted in dose dependent significant decrease in cell viability (Figure 36). This decrease was not due to the inhibitor and inhibitor alone resulted in greater viability. However, comparing siZAP70 to siNSC, this change was not observed (Figure 37), probably due to inefficiency of knockdown.

8. Conclusion & Future work

My research has shown that inhibition of kinase activity of ZAP70/SYK using P505-15 decreases BCR signalling, survival as well as proliferation. However, this was not observed in cells treated with ZAP70 siRNA. Secondly, mass spectrometry results in both BJAB cells with ectopic expression of ZAP70 and endogenous ZAP70 in ZAP70 positive CLL cells confirm that ZAP70 plays a vital role in BCR signalling but interestingly, my data also suggest that ZAP70 plays an important role in CLL cell migration and protects CLL cells from DNA damage as well as show a striking role of ZAP70 in ribosomal biosynthesis. These findings are relevant not only to CLL but also to other B cell malignancies.

Overall, it is clear that results from my inhibitor studies are contradicting with the knockdown approach due to reasons already mentioned above. Specifically, the inhibitor targets both SYK and ZAP70 . A specific inhibitor that targets only ZAP70 would be recommended. Future work should be aimed at addressing some of the shortcomings of the current approaches. As an alternative approach to siRNA, CRISPR knockout of ZAP70 in CLL cells should be explored. This method should be able to eliminate ZAP70 at the DNA level and provide more consistent absence of ZAP70 in CLL cells. However, as stated previously, CLL cells are different to grow and proliferate *in vitro*, which would be a further challenge for CRISPR knockout.

Another factor contributing to the discrepancy in the results between the inhibitor and knockdown studies is due to the masking effect of SYK described earlier. One suggestion would be to use siRNA against both ZAP70 as well as SYK and investigate changes in BCR signalling, survival and proliferation. Alongside, it will be interesting to unravel knockdown effects of SYK only and a comparison performed with ZAP70 knockdown. This should provide insight to whether ZAP70 efficiency increases in the absence of SYK in B CLL. A siRNA or CRISPR approach would be advisable rather than assessing in overexpression system or comparison between ZAP70 positive and negative patient cells.

Also, as an alternative to the migration assay used, another system that provides a chemokine gradient to support migration should be researched. In addition, a role of F actin and adhesion proteins should be explored in B CLL cells lacking ZAP70

expression as migration in CLL cells seems to be dependent on ZAP70. Finally, to address the ribosomal signature observed, it would be worth carrying out polysome profiling to investigate changes in CLL cells due to levels of ZAP70 as well as upon IgM activation. Finally, RNA seq experiments on knockdown / CRISPR cells would provide insight into gene expression patterns at both steady state as well as upon IgM activation.

9. References

Allsup, D. J., Kamiguti, A. S., Lin, K., Sherrington, P. D., Matrai, Z., Slupsky, J. R., Zuzel, M. (2005). B-cell receptor translocation to lipid rafts and associated signaling differ between prognostically important subgroups of chronic lymphocytic leukaemia. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-03-1563

Aman, M. J., & Ravichandran, K. S. (2000). A requirement for lipid rafts in B cell receptor induced Ca2+ flux. *Current Biology*, *10*(7), 393–396. https://doi.org/10.1016/S0960-9822(00)00415-2

Arnoult, N., Correia, A., #1, M., Merlo, A., Garcia-Gomez, S., Maric, M., … Karlseder, J. (2017). Regulation of DNA Repair pathway choice in S/G2 by the NHEJ inhibitor CYREN Europe PMC Funders Group. *Nature*, *549*(7673), 548–552. https://doi.org/10.1038/nature24023

Bakkenist, C. J., & Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*. https://doi.org/10.1038/nature01368

Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., … Tsichlis, P. (1998). Akt activation by growth factors is a multiple-step process: The role of the PH domain. *Oncogene*. https://doi.org/10.1038/sj.onc.1201947

Bernal, A., Pastore, R. D., Asgary, Z., Keller, S. A., Cesarman, E., Liou, H. C., & Schattner, E. J. (2001). Survival of leukemic B cells promoted by engagement of the antigen receptor. *Blood*. https://doi.org/10.1182/blood.V98.10.3050

Binder, M., Müller, F., Frick, M., Wehr, C., Simon, F., Leistler, B., … Trepel, M. (2013). CLL B-cell receptors can recognize themselves: Alternative epitopes and structural clues for autostimulatory mechanisms in CLL. *Blood*. https://doi.org/10.1182/blood-2012-09-454439

Blunt MD, Koehnrer S, Dobson RC, Larrayoz M, Wilmore S, Hayman A, Parnell J, Smith LD, Davies A, Johnson PWM, Conley PB, Pandey A, Strefford JC, Stevenson FK, Packham G, Forconi F, Coffey GP, Burer JA, S. A. (2017). The dual Syk/JAK inhibitor Cerdulatinib Antogonizes B-cell receptor and microenvironmental signaling in Chronic Lymphocytic leukaemia. *Clinical Cancer Research*, *23(9)*, 2313–2324.

Brandsma, I., & Gent, D. C. (2012). Pathway choice in DNA double strand break repair: Observations of a balancing act. *Genome Integrity*. https://doi.org/10.1186/2041-9414-3-9

Burger, J. A., & Chiorazzi, N. (2013). B cell receptor signaling in chronic lymphocytic leukaemia. *Trends in Immunology*. https://doi.org/10.1016/j.it.2013.07.002

Calpe, E., Codony, C., Baptista, M. J., Abrisqueta, P., Carpio, C., Purroy, N., … Crespo, M. (2011). ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation. *Blood*, *118*(16), 4401–4410. https://doi.org/10.1182/blood-2011-01-333682

Chan, A. C., Irving, B. A., Fraser, J. D., & Weiss, A. (1991). The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(20), 9166–70. https://doi.org/10.1073/pnas.88.20.9166

Chan, A. C., Iwashima, M., Turck, C. W., & Weiss, A. (1992). ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell*. https://doi.org/10.1016/0092-8674(92)90598-7

Chen, L., Apgar, J., Huynh, L., Dicker, F., Giago-McGahan, T., Rassenti, L., … Kipps, T. J. (2005a). ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukaemia. *Blood*, *105*(5), 2036–41. https://doi.org/10.1182/blood-2004-05-1715

Chen, L., Apgar, J., Huynh, L., Dicker, F., Giago-McGahan, T., Rassenti, L., … Kipps, T. J. (2005b). ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukaemia. *Blood*, *105*(5), 2036–2041. https://doi.org/10.1182/blood-2004-05-1715

Chen, L., Huynh, L., Apgar, J., Tang, L., Rassenti, L., Weiss, A., & Kipps, T. J. (2008). ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukaemia. *Blood*, *111*(5), 2685–92. https://doi.org/10.1182/blood-2006-12-062265

Chen, L., Widhopf, G., Huynh, L., Rassenti, L., Rai, K. R., Weiss, A., & Kipps, T. J. (2002). Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukaemia. *Blood*, *100*(13), 4609–4614. https://doi.org/10.1182/blood-2002-06-1683

Cheng, P. C., Dykstra, M. L., Mitchell, R. N., & Pierce, S. K. (1999). A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *The Journal of Experimental Medicine*, *190*(11), 1549–60. https://doi.org/10.1084/jem.190.11.1549

Cochran, D. A. E., Evans, C. A., Blinco, D., Burthem, J., Stevenson, F. K., Gaskell, S. J., & Whetton, A. D. (2003). Proteomic analysis of chronic lymphocytic leukaemia subtypes with mutated or unmutated Ig V(H) genes. *Molecular & Cellular Proteomics : MCP*. https://doi.org/10.1074/mcp.M300055-MCP200

Contri, A., Brunati, A. M., Trentin, L., Cabrelle, A., Miorin, M., Cesaro, L., … Donella-Deana, A. (2005). Chronic lymphocytic leukaemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI200522094

Coscia, M., Pantaleoni, F., Riganti, C., Vitale, C., Rigoni, M., Peola, S., … Massaia, M. (2011). IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. *leukaemia : Official Journal of the leukaemia Society of America, leukaemia Research Fund, U.K*, *25*(5), 828–837. https://doi.org/10.1038/leu.2011.12

Cosimo, E., Tarafdar, A., Moles, M. W., Holroyd, A. K., Malik, N., Catherwood, M. A., … Michie, A. M. (2019). Akt/mTORC2 inhibition activates FOXO1 function in CLL cells reducing B-cell receptor-mediated survival. *Clinical Cancer Research*, *25*(5), 1574–1587. https://doi.org/10.1158/1078-0432.CCR-18-2036

Crespo, M., Bosch, F., Villamor, N., Bellosillo, B., Colomer, D., Rozman, M., … Montserrat, E. (2003). ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukaemia. *The New England Journal of Medicine*, *348*(18), 1764–1775. https://doi.org/10.1056/NEJMoa023143

Damle, R. N., Ghiotto, F., Valetto, A., Albesiano, E., Fais, F., Yan, X. J., … Chiorazzi, N. (2002). B-cell chronic lymphocytic leukaemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. *Blood*. https://doi.org/10.1182/blood.V99.11.4087

Davids, M. S., & Burger, J. A. (2013). Cell Trafficking in Chronic Lymphocytic leukaemia. *Open Journal of Hematology*. https://doi.org/10.13055/ojhmt_3_s1_03.120221

Davis, A. J., & Chen, D. J. (2013). DNA double strand break repair via non-homologous end-joining. *Translational Cancer Research*. https://doi.org/10.3978/j.issn.2218-676X.2013.04.02

Del Principe, M. I., Dal Bo, M., Bittolo, T., Buccisano, F., Rossi, F. M., Zucchetto, A., … Del Poeta, G. (2016). Clinical significance of bax/bcl-2 ratio in chronic lymphocytic leukaemia. *Haematologica*, *101*(1), 77–85. https://doi.org/10.3324/haematol.2015.131854

Devereux, S., & Cuthill, K. (2017). Chronic lymphocytic leukaemia. *Medicine (United Kingdom)*, *45*(5), 292–296. https://doi.org/10.1016/j.mpmed.2017.02.001

Dicker, F., Schnittger, S., Haferlach, T., Kern, W., & Schoch, C. (2006). Immunostimulatory oligonucleotide-induced metaphase cytogenetics detect chromosomal aberrations in 80% of CLL patients: A study of 132 CLL cases with correlation to FISH, IgVH status, and CD38 expression. *Blood*. https://doi.org/10.1182/blood-2006-02-005322

Ding, W., Knox, T. R., Tschumper, R. C., Wu, W., Schwager, S. M., Boysen, J. C., … Kay, N. E. (2010). Platelet-derived growth factor (PDGF)-PDGF receptor interaction activates bone marrow-derived mesenchymal stromal cells derived from chronic lymphocytic leukaemia: Implications for an angiogenic switch. *Blood*, *116*(16), 2984–2993. https://doi.org/10.1182/blood-2010-02-269894

Dürig, J., Nückel, H., Cremer, M., Führer, A., Halfmeyer, K., Fandrey, J., … Dührsen, U. (2003). ZAP-70 expression is a prognostic factor in chronic lymphocytic leukaemia. *leukaemia*. https://doi.org/10.1038/sj.leu.2403147

El-Mabhouh, A. A., Ayres, M. L., Shpall, E. J., Baladandayuthapani, V., Keating, M. J., Wierda, W. G., & Gandhi, V. (2014). Evaluation of bendamustine in combination with fludarabine in primary chronic lymphocytic leukaemia cells. *Blood*. https://doi.org/10.1182/blood-2013-12-541433

Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A., & Parslow, T. G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science*. https://doi.org/10.1126/science.8202712

Fairhead, M., & Howarth, M. (2015). Site-Specific biotinylation of purified proteins using BirA. In *Site-Specific Protein Labeling: Methods and Protocols* (pp. 171–184). https://doi.org/10.1007/978-1-4939-2272-7_12

Fais, F., Ghiotto, F., Hashimoto, S., Sellars, B., Valetto, A., Allen, S. L., …

Chiorazzi, N. (1998). Chronic lymphocytic leukaemia B cells express restricted sets of mutated and unmutated antigen receptors. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI3009

Frenzel, L. P., Reinhardt, H. C., & Pallasch, C. P. (2016). Concepts of chronic lymphocytic leukaemia pathogenesis: DNA damage response and tumor microenvironment. *Oncology Research and Treatment*. https://doi.org/10.1159/000443820

Gobessi, S., Laurenti, L., Longo, P. G., Carsetti, L., Berno, V., Sica, S., … Efremov, D. G. (2009). Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukaemia B cells. *leukaemia*. https://doi.org/10.1038/leu.2008.346

Gobessi, S., Laurenti, L., Longo, P. G., Sica, S., Leone, G., & Efremov, D. G. (2007). ZAP-70 enhances B-cell-receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukaemia and lymphoma B cells. *Blood*, *109*(5), 2032–2039. https://doi.org/10.1182/blood-2006-03-011759

Goy, J., Gillan, T. L., Bruyere, H., Huang, S. J. T., Hrynchak, M., Karsan, A., … Gerrie, A. S. (2017). Chronic Lymphocytic leukaemia Patients With Deletion 11q Have a Short Time to Requirement of First-Line Therapy, But Long Overall Survival: Results of a Population-Based Cohort in British Columbia, Canada. *Clinical Lymphoma, Myeloma and leukaemia*, *17*(6), 382–389. https://doi.org/10.1016/j.clml.2017.04.001

Guarini, A., Marinelli, M., Tavolaro, S., Bellacchio, E., Magliozzi, M., Chiaretti, S., … Foà, R. (2012). Atm gene alterations in chronic lymphocytic leukaemia patients induce a distinct gene expression profile and predict disease progression. *Haematologica*. https://doi.org/10.3324/haematol.2011.049270

Guo, A., Lu, P., Galanina, N., Nabhan, C., Smith, S. M., Coleman, M., & Wang, Y. L. (2016). Heightened BTK-dependent cell proliferation in unmutated chronic lymphocytic leukaemia confers increased sensitivity to ibrutinib. *Oncotarget*. https://doi.org/10.18632/oncotarget.6727

Gupta, N., & DeFranco, A. L. (2003). Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. *Molecular Biology of the Cell*, *14*(2), 432–44. https://doi.org/10.1091/mbc.02-05-0078

Hacken, E. ten, Sivina, M., Ping, L., O'Brien, S., Wierda, W. G., Ferrajoli, A., … Burger, J. A. (2015). IgM and IgD Receptors Differentially Contribute to CLL Survival and Chemokine Secretion: Implications for CLL Biology and Treatment. *Blood*.

Haferlach, C., Dicker, F., Schnittger, S., Kern, W., & Haferlach, T. (2007). Comprehensive genetic characterization of CLL: A study on 506 cases analysed with chromosome banding analysis, interphase FISH, IgVH status and immunophenotyping. *leukaemia*. https://doi.org/10.1038/sj.leu.2404935

Hamblin, T. J., Davis, Z., Gardiner, A., Oscier, D. G., & Stevenson, F. K. (1999). Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukaemia. *Blood*.

Herishanu, Y., Pérez-Galán, P., Liu, D., Biancotto, A., Pittaluga, S., Vire, B., … Wiestner, A. (2011). The lymph node microenvironment promotes B-cell receptor signaling, NF-κB activation, and tumor proliferation in chronic lymphocytic leukaemia. *Blood*. https://doi.org/10.1182/blood-2010-05-284984

Hewamana, S., Alghazal, S., Lin, T., Clement, M., Jenkins, C., Guzman, M. L., … Pepper, C. (2008). The NF-κB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukaemia and represents a promising therapeutic target. *Blood*. https://doi.org/10.1182/blood-2007-11-125278

Hivroz, C., Grillot-Courvalin, C., Labaume, S., And, R. M., & Brouet, J. -C. (1988). Cross-linking of membrane IgM on B CLL cells: dissociation between intracellular free Ca2+ mobilization and cell proliferation. *European Journal of Immunology*. https://doi.org/10.1002/eji.1830181124

Hömig-Hölzel, C., Hojer, C., Rastelli, J., Casola, S., Strobl, L. J., Müller, W., … Zimber-Strobl, U. (2008). Constitutive CD40 signaling in B cells selectively activates the noncanonical NF-κB pathway and promotes lymphomagenesis. *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20080238

Jiang, Y., Chen, H. C., Su, X., Thompson, P. A., Liu, X., Do, K. A., … Plunkett, W. (2016). ATM function and its relationship with ATM gene mutations in chronic lymphocytic leukaemia with the recurrent deletion (11q22.3-23.2). *Blood Cancer Journal*. https://doi.org/10.1038/bcj.2016.69

Johnston, H. E., Carter, M. J., Larrayoz, M., Clarke, J., Garbis, S. D., Oscier, D., …

Cragg, M. S. (2018). Proteomics profiling of CLL versus healthy B-cells identifies putative therapeutic targets and a subtype-independent signature of spliceosome dysregulation. *Molecular and Cellular Proteomics*. https://doi.org/10.1074/mcp.RA117.000539

Kabouridis, P. (2006). Lipid rafts in T cell receptor signalling. *Molecular Membrane Biology*, *23*(1), 49–57. https://doi.org/10.1080/09687860500453673.Lipid

Kadlecek, T. A., van Oers, N. S., Lefrancois, L., Olson, S., Finlay, D., Chu, D. H., … Weiss, A. (1998). Differential requirements for ZAP-70 in TCR signaling and T cell development. *Journal of Immunology (Baltimore, Md. : 1950)*.

Kastan, M. B., & Bartek, J. (2004). Cell-cycle checkpoints and cancer. *Nature*. https://doi.org/10.1038/nature03097

Kim, J., Cantor, A. B., Orkin, S. H., & Wang, J. (2009). Use of in vivo biotinylation to study protein-protein and protein-DNA interactions in mouse embryonic stem cells. *Nat Protoc*, *4*(4), 506–517. https://doi.org/nprot.2009.23 [pii]\r10.1038/nprot.2009.23

Köhler, F., Hug, E., Eschbach, C., Meixlsperger, S., Hobeika, E., Kofer, J., … Jumaa, H. (2008). Autoreactive B Cell Receptors Mimic Autonomous Pre-B Cell Receptor Signaling and Induce Proliferation of Early B Cells. *Immunity*. https://doi.org/10.1016/j.immuni.2008.10.013

Kondo, K., Shaim, H., Thompson, P. A., Burger, J. A., Keating, M., Estrov, Z., … Rezvani, K. (2018). Ibrutinib modulates the immunosuppressive CLL microenvironment through STAT3-mediated suppression of regulatory B-cell function and inhibition of the PD-1/PD-L1 pathway. *leukaemia*. https://doi.org/10.1038/leu.2017.304

Krejci, L., Altmannova, V., Spirek, M., & Zhao, X. (2012). Homologous recombination and its regulation. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gks270

Kuo, L. J., & Yang, L. X. (2008). ??-H2AX- A novel biomaker for DNA double-strand breaks. *In Vivo*.

Landau, D. A., Tausch, E., Taylor-Weiner, A. N., Stewart, C., Reiter, J. G., Bahlo, J., … Wu, C. J. (2015). Mutations driving CLL and their evolution in progression and relapse. *Nature*. https://doi.org/10.1038/nature15395

Lens, D., Dyer, M. J. S., Garcia-Marco, J. M., De Schouwer, P. J. J. C., Hamoudi, R. A., Jones, D., … Catovsky, D. (1997). p53 abnormalities in CLL are

associated with excess of prolymphocytes and poor prognosis. *British Journal of Haematology*. https://doi.org/10.1046/j.1365-2141.1997.4723278.x

Li, X., & Heyer, W. D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Research*. https://doi.org/10.1038/cr.2008.1

Liu, D., & Mamorska-Dyga, A. (2017). Syk inhibitors in clinical development for hematological malignancies. *Journal of Hematology and Oncology*. https://doi.org/10.1186/s13045-017-0512-1

López-Giral, S., Quintana, N. E., Cabrerizo, M., Alfonso-Pérez, M., Sala-Valdés, M., de Soria, V. G. G., … Muñoz, C. (2004). Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukaemia and non-Hodgkin lymphomas with widespread nodular dissemination. *Journal of Leukocyte Biology*. https://doi.org/10.1189/jlb.1203652

Lopez-Guerra, M., & Colomer, D. (2010). NF-kappaB as a therapeutic target in chronic lymphocytic leukaemia. *Expert Opin Ther Targets*, *14*(3), 275–288. Retrieved from http://dx.doi.org/10.1517/14728221003598930

Malavasi, F., Deaglio, S., Damle, R., Cutrona, G., Ferrarini, M., & Chiorazzi, N. (2011). CD38 and chronic lymphocytic leukaemia: A decade later. *Blood*. https://doi.org/10.1182/blood-2011-06-275610

Mangolini, M., Götte, F., Moore, A., Ammon, T., Oelsner, M., Lutzny-Geier, G., … Ringshausen, I. (2018). Notch2 controls non-autonomous Wnt-signalling in chronic lymphocytic leukaemia. *Nature Communications*. https://doi.org/10.1038/s41467-018-06069-5

Matsuoka, S., Huang, M., & Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science*. https://doi.org/10.1126/science.282.5395.1893

Minden, M. D. Von, Übelhart, R., Schneider, D., Wossning, T., Bach, M. P., Buchner, M., … Jumaa, H. (2012). Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature*. https://doi.org/10.1038/nature11309

Mohammed, H., Taylor, C., Brown, G. D., Papachristou, E. K., Carroll, J. S., & D'Santos, C. S. (2016). Rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) for analysis of chromatin complexes. *Nature Protocols*. https://doi.org/10.1038/nprot.2016.020

Molica, S., Giannarelli, D., Mirabelli, R., Levato, L., Gentile, M., Lentini, M., & Morabito, F. (2014). Changes in the incidence, pattern of presentation and clinical outcome of early chronic lymphocytic leukaemia patients using the 2008 International Workshop on CLL guidelines. *Expert Review of Hematology*. https://doi.org/10.1586/17474086.2014.954542

Moroni, M., Soldatenkov, V., Zhang, L., Zhang, Y., Stoica, G., Gehan, E., … Mueller, S. C. (2004). Progressive loss of Syk and abnormal proliferation in breast cancer cells. *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-03-3520

Myhrvold, I. K., Cremaschi, A., Hermansen, J. U., Tjønnfjord, G. E., Munthe, L. A., Taskén, K., & Skånland, S. S. (2018). Single cell profiling of phospho-protein levels in chronic lymphocytic leukaemia. *Oncotarget*, *9*(10), 9273–9284. https://doi.org/10.18632/oncotarget.23949

Nguyen, T. V., Pawlikowska, P., Firlej, V., Rosselli, F., & Aoufouchi, S. (2016). V(D)J recombination process and the Pre-B to immature B-cells transition are altered in Fanca -/- mice. *Scientific Reports*. https://doi.org/10.1038/srep36906

Nicolas Blanchard1, Vincenzo Di Bartolo2, C. H. (2002). Nicolas Blanchard1, Vincenzo Di In the Immune Synapse, ZAP-70 Controls T Cell Polarization and Recruitment of Signaling Proteins but Not Formation of the Synaptic Pattern. *Immunity*, *17*(4), 389–399. https://doi.org/doi:10.1016/S1074-7613(02)00421-1

Packham, G., Krysov, S., Allen, A., Savelyeva, N., Steele, A. J., Forconi, F., & Stevenson, F. K. (2014). The outcome of B-cell receptor signaling in chronic lymphocytic leukaemia: Proliferation or anergy. *Haematologica*. https://doi.org/10.3324/haematol.2013.098384

Papachristou, E. K., Kishore, K., Holding, A. N., Harvey, K., Roumeliotis, T. I., Chilamakuri, C. S. R., … Carroll, J. S. (2018). A quantitative mass spectrometry-based approach to monitor the dynamics of endogenous chromatin-associated protein complexes. *Nature Communications*. https://doi.org/10.1038/s41467-018-04619-5

Parikh, S. A., Kay, N. E., & Shanafelt, T. D. (2014). How we treat Richter syndrome. *Blood*. https://doi.org/10.1182/blood-2013-11-516229

Patton, D. T., Plumb, A. W., & Abraham, N. (2014). The Survival and Differentiation of Pro-B and Pre-B Cells in the Bone Marrow Is Dependent on IL-7Rα Tyr 449 . *The Journal of Immunology*.

https://doi.org/10.4049/jimmunol.1302925

Pede, V., Rombout, A., Vermeire, J., Naessens, E., Vanderstraeten, H., Philippé, J., & Verhasselt, B. (2013). Expression of ZAP70 in chronic lymphocytic leukaemia activates NF-κB signalling. *British Journal of Haematology*, *163*(5), 621–630. https://doi.org/10.1111/bjh.12588

Pepper, C., Lin, T. T., Pratt, G., Hewamana, S., Brennan, P., Hiller, L., … Fegan, C. (2008). Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukaemia and is associated with other poor prognostic markers. *Blood*, *112*(9), 3807–3817. https://doi.org/10.1182/blood-2008-05-157131

Perrot, A., Pionneau, C., Nadaud, S., Davi, F., Leblond, V., Jacob, F., … Vallat, L. (2011). A unique proteomic profile on surface IgM ligation in unmutated chronic lymphocytic leukaemia. *Blood*. https://doi.org/10.1182/blood-2011-02-335125

Petlickovski, A., Laurenti, L., Li, X., Marietti, S., Chiusolo, P., Sica, S., … Efremov, D. G. (2005). Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukaemia B cells. *Blood*. https://doi.org/10.1182/blood-2004-07-2669

Pierce, S. K. (2002). Lipid rafts and B-cell activation. *Nature Reviews. Immunology*, *2*(2), 96–105. https://doi.org/10.1038/nri726

Podhorecka, M., Skladanowski, A., & Bozko, P. (2010). H2AX phosphorylation: Its role in DNA damage response and cancer therapy. *Journal of Nucleic Acids*. https://doi.org/10.4061/2010/920161

Ponader, S., Chen, S. S., Buggy, J. J., Balakrishnan, K., Gandhi, V., Wierda, W. G., … Burger, J. A. (2012). The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukaemia cell survival and tissue homing in vitro and in vivo. *Blood*. https://doi.org/10.1182/blood-2011-10-386417

Popp, H. D., Flach, J., Brendel, S., Ruppenthal, S., Kleiner, H., Seifarth, W., … Fabarius, A. (2019). Accumulation of DNA damage and alteration of the DNA damage response in monoclonal B-cell lymphocytosis and chronic lymphocytic leukaemia. *leukaemia and Lymphoma*. https://doi.org/10.1080/10428194.2018.1498494

Popp, H. D., Naumann, N., Brendel, S., Henzler, T., Weiss, C., Hofmann, W. K., & Fabarius, A. (2017). Increase of DNA damage and alteration of the DNA damage response in myelodysplastic syndromes and acute myeloid

leukaemias. *leukaemia Research*. https://doi.org/10.1016/j.leukres.2017.03.011

Puiggros, A., Blanco, G., & Espinet, B. (2014). Genetic Abnormalities in Chronic Lymphocytic leukaemia: Where We Are and Where We Go. *BioMed Research International*. https://doi.org/10.1155/2014/435983

Quiroga, M. P., Balakrishnan, K., Kurtova, A. V., Sivina, M., Keating, M. J., Wierda, W. G., … Burger, J. A. (2009). B-cell antigen receptor signaling enhances chronic lymphocytic leukaemia cell migration and survival: Specific targeting with a novel spleen tyrosine kinase inhibitor, R406. *Blood*. https://doi.org/10.1182/blood-2009-03-212837

Radeva, G., & Sharom, F. J. (2004). Isolation and characterization of lipid rafts with different properties from RBL-2H3 (rat basophilic leukaemia) cells. *The Biochemical Journal*, *380*(Pt 1), 219–230. https://doi.org/10.1042/BJ20031348

Ralph, D. K., & Matsen, F. A. (2016). Consistency of VDJ Rearrangement and Substitution Parameters Enables Accurate B Cell Receptor Sequence Annotation. *PLoS Computational Biology*. https://doi.org/10.1371/journal.pcbi.1004409

Rassenti, L. Z., Huynh, L., Toy, T. L., Chen, L., Keating, M. J., Gribben, J. G., … Kipps, T. J. (2004). ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukaemia. *New England Journal of Medicine*. https://doi.org/10.1056/NEJMoa040857

Rawstron, A. C., Kreuzer, K. A., Soosapilla, A., Spacek, M., Stehlikova, O., Gambell, P., … Montserrat, E. (2018). Reproducible diagnosis of chronic lymphocytic leukaemia by flow cytometry: An European Research Initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) Harmonisation project. *Cytometry Part B - Clinical Cytometry*. https://doi.org/10.1002/cyto.b.21595

Ray, A., Blevins, C., Wani, G., & Wani, A. A. (2016). ATR- and ATM-Mediated DNA damage response is dependent on excision repair assembly during G1 but not in S phase of cell cycle. *PLoS ONE*, *11*(7), 1–20. https://doi.org/10.1371/journal.pone.0159344

Richardson, S. J., Matthews, C., Catherwood, M. A., Alexander, H. D., Carey, B. S., Farrugia, J., … Prentice, A. G. (2006). ZAP-70 expression is associated

with enhanced ability to respond to migratory and survival signals in B-cell chronic lymphocytic leukaemia (B-CLL). *Blood*. https://doi.org/10.1182/blood-2005-04-1718

Rodrigues, C. A., Gonçalves, M. V., Ikoma, M. R. V., Lorand-Metze, I., Pereira, A. D., Farias, D. L. C. de, … Yamamoto, M. (2016). Diagnosis and treatment of chronic lymphocytic leukaemia: recommendations from the Brazilian Group of Chronic Lymphocytic leukaemia. *Revista Brasileira de Hematologia e Hemoterapia*. https://doi.org/10.1016/j.bjhh.2016.07.004

Rossi, S., Shimizu, M., Barbarotto, E., Nicoloso, M. S., Dimitri, F., Sampath, D., … Calin, G. A. (2010). MicroRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood*. https://doi.org/10.1182/blood-2010-01-263889

Roux, K. J., Kim, D. I., Raida, M., & Burke, B. (2012). A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.201112098

Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R., & Bolen, J. B. (1995). Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.270.19.11590

Salzer, U., & Prohaska, R. (2001). Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. *Blood*, *97*(4), 1141–1143. https://doi.org/10.1182/blood.V97.4.1141

Sbarrato, T., Horvilleur, E., Poÿry, T., Hill, K., Chaplin, L. C., Spriggs, R. V., … Willis, A. E. (2016). A ribosome-related signature in peripheral blood CLL B cells is linked to reduced survival following treatment. *Cell Death and Disease*. https://doi.org/10.1038/cddis.2016.148

Schleiss, C., Ilias, W., Tahar, O., Güler, Y., Miguet, L., Mayeur-Rousse, C., … Vallat, L. (2019). BCR-associated factors driving chronic lymphocytic leukaemia cells proliferation ex vivo. *Scientific Reports*. https://doi.org/10.1038/s41598-018-36853-8

Shih, V. F. S., Tsui, R., Caldwell, A., & Hoffmann, A. (2011). A single NFκB system for both canonical and non-canonical signaling. *Cell Research*. https://doi.org/10.1038/cr.2010.161

Shiloh, Y., & Kastan, M. B. (2001). ATM: Genome stability, neuronal development, and cancer cross paths. *Advances in Cancer Research*. https://doi.org/10.1016/S0065-230X(01)83007-4

Shull, A. Y., Noonepalle, S. K., Awan, F. T., Liu, J., Pei, L., Bollag, R. J., … Shi, H. (2015). RPPA-based protein profiling reveals eIF4G overexpression and 4E-BP1 serine 65 phosphorylation as molecular events that correspond with a pro-survival phenotype in chronic lymphocytic leukaemia. *Oncotarget*. https://doi.org/10.18632/oncotarget.4104

Sjölin-Goodfellow, H., Frushicheva, M. P., Ji, Q., Cheng, D. A., Kadlecek, T. A., Cantor, A. J., … Weiss, A. (2015). The catalytic activity of the kinase ZAP-70 mediates basal signaling and negative feedback of the T cell receptor pathway. *Science Signaling*. https://doi.org/10.1126/scisignal.2005596

Sloan-Lancaster, J., Presley, J., Ellenberg, J., Yamazaki, T., Lippincott-Schwartz, J., & Samelson, L. E. (1998). ZAP-70 association with T cell receptor ζ (TCRζ): Fluorescence imaging of dynamic changes upon cellular stimulation. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.143.3.613

Stamatopoulos, B., Meuleman, N., Haibe-Kains, B., Saussoy, P., Van Den Neste, E., Michaux, L., … Lagneaux, L. (2009). microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukaemia and improves disease risk stratification. *Blood*. https://doi.org/10.1182/blood-2008-11-189407

Stilgenbauer, S., Bullinger, L., Lichter, P., & Döhner, H. (2002). Genetics of chronic lymphocytic leukaemia: Genomic aberrations and VH gene mutation status in pathogenesis and clinical course. *leukaemia*. https://doi.org/10.1038/sj.leu.2402537

Taniguchi, T., Kobayashi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., … Yamamura, H. (1991). Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. *Journal of Biological Chemistry*.

ten Hacken, E., Gounari, M., Ghia, P., & Burger, J. A. (2019). The importance of B cell receptor isotypes and stereotypes in chronic lymphocytic leukaemia. *leukaemia*. https://doi.org/10.1038/s41375-018-0303-x

Ticchioni, M., Charvet, C., Noraz, N., Lamy, L., Steinberg, M., Bernard, A., & Deckert, M. (2002). Signaling through ZAP-70 is required for CXCL12-

mediated T-cell transendothelial migration. *Blood*. https://doi.org/10.1182/blood.V99.9.3111

Toyabe, S. I., Watanabe, A., Harada, W., Karasawa, T., & Uchiyama, M. (2001). Specific immunoglobulin E responses in ZAP-70-deficient patients are mediated by Syk-dependent T-cell receptor signalling. *Immunology*. https://doi.org/10.1046/j.1365-2567.2001.01246.x

Vaisitti, T., Aydin, S., Rossi, D., Cottino, F., Bergui, L., D'Arena, G., … Deaglio, S. (2010). CD38 increases CXCL12-mediated signals and homing of chronic lymphocytic leukaemia cells. *leukaemia*. https://doi.org/10.1038/leu.2010.36

Vallat, L., Magdelénat, H., Merle-Béral, H., Masdehors, P., De Montalk, G. P., Davi, F., … Delic, J. (2003). The resistance of B-CLL cells to DNA damage-induced apoptosis defined by DNA microarrays. *Blood*. https://doi.org/10.1182/blood-2002-06-1743

Wagner, M., Oelsner, M., Moore, A., Götte, F., Kuhn, P.-H., Haferlach, T., … Ringshausen, I. (2016). Integration of innate into adaptive immune responses in ZAP-70–positive chronic lymphocytic leukaemia. *Blood*, *127*(4), 436–448. Retrieved from http://www.bloodjournal.org/content/127/4/436.abstract

Wang, H., Kadlecek, T. A., Au-Yeung, B. B., Goodfellow, H. E. S., Hsu, L.-Y., Freedman, T. S., & Weiss, A. (2010). ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harbor Perspectives in Biology*, *2*(5), a002279. https://doi.org/10.1101/cshperspect.a002279

Wang, J., Cantor, A., & Orkin, S. (2009). Tandem affinity purification of protein complexes in mouse embryonic stem cells using in vivo biotinylation. *Current Protocols in Stem Cell Biology*, *March*, Chapter1: Unit1B.5. https://doi.org/10.1002/9780470151808.sc01b05s8.Tandem

Weintraub, B. C., Jun, J. E., Bishop, A. C., Shokat, K. M., Thomas, M. L., & Goodnow, C. C. (2000). Entry of B cell receptor into signaling domains is inhibited in tolerant B cells. *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.191.8.1443

Wiestner, A. (2003). ZAP-70 expression identifies a chronic lymphocytic leukaemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*. https://doi.org/10.1182/blood-2002-10-3306

Williams, B. L. (1999). Phosphorylation of Tyr319 in ZAP-70 is required for T-cell

antigen receptor-dependent phospholipase C-gamma 1 and Ras activation. *The EMBO Journal*. https://doi.org/10.1093/emboj/18.7.1832

Wossning, T., Herzog, S., Köhler, F., Meixlsperger, S., Kulathu, Y., Mittler, G., … Jumaa, H. (2006). Deregulated Syk inhibits differentiation and induces growth factor–independent proliferation of pre–B cells. *The Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20060967

Woyach, J. A., Johnson, A. J., & Byrd, J. C. (2012a). The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood*. https://doi.org/10.1182/blood-2012-02-362624

Woyach, J. A., Johnson, A. J., & Byrd, J. C. (2012b). The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood*. https://doi.org/10.1182/blood-2012-02-362624

Wu, Q., Liang, S., Ochi, T., Chirgadze, D. Y., Huiskonen, J. T., & Blundell, T. L. (2019a). Understanding the structure and role of DNA-PK in NHEJ: How X-ray diffraction and cryo-EM contribute in complementary ways. *Progress in Biophysics and Molecular Biology*, (xxxx). https://doi.org/10.1016/j.pbiomolbio.2019.03.007

Wu, Q., Liang, S., Ochi, T., Chirgadze, D. Y., Huiskonen, J. T., & Blundell, T. L. (2019b). Understanding the structure and role of DNA-PK in NHEJ: How X-ray diffraction and cryo-EM contribute in complementary ways. *Progress in Biophysics and Molecular Biology*, (xxxx). https://doi.org/10.1016/j.pbiomolbio.2019.03.007

Yan, Q., Barros, T., Visperas, P. R., Deindl, S., Kadlecek, T. A., Weiss, A., & Kuriyan, J. (2013). Structural Basis for Activation of ZAP-70 by Phosphorylation of the SH2-Kinase Linker. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.01637-12

Zhuang, J., Hawkins, S. F., Glenn, M. A., Lin, K., Johnson, G. G., Carter, A., … Pettitt, A. R. (2010). Akt is activated in chronic lymphocytic leukaemia cells and delivers a pro-survival signal: The therapeutic potential of Akt inhibition. *Haematologica*. https://doi.org/10.3324/haematol.2009.010272

zum Büschenfelde, C. M., Wagner, M., Lutzny, G., Oelsner, M., Feuerstacke, Y., Decker, T., … Ringshausen, I. (2010). Recruitment of PKC-betaII to lipid rafts mediates apoptosis-resistance in chronic lymphocytic leukaemia expressing ZAP-70. *leukaemia : Official Journal of the leukaemia Society of America,*

leukaemia Research Fund, U.K, *24*(1), 141–152.
https://doi.org/10.1038/leu.2009.216