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ZFP36 proteins and mRNA targets in B cell malignancies

Maria del Amor Alcaraz-Serrano

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Abstract

The ZFP36 proteins are a family of post-transcriptional regulator proteins that bind to adenine uridine rich elements (AREs) in 3' untranslated (3'UTR) regions of mRNAs. The members of the human family, ZFP36L1, ZFP36L2 and ZFP36 are able to degrade mRNAs of important cell regulators that include cytokines, cell signalling proteins and transcriptional factors. This project investigated two proposed targets for the protein family that have important roles in B cell biology, *BCL2* and *CD38* mRNAs. *BCL2* is an anti-apoptotic protein with key roles in cell survival and carcinogenesis; *CD38* is a membrane protein differentially expressed in B cells and with a prognostic value in B chronic lymphocytic leukaemia (B-CLL), patients positive for *CD38* are considered to have a poor prognosis.

This project provides evidence of a functional interaction between the three ZFP36 proteins and the 3'UTR AREs of *BCL2* and *CD38* mRNAs. 3'UTR dual luciferase reporter assay results showed that the three ZFP36 proteins bound the 3'UTR ARE of *BCL2* mRNA and *CD38* mRNA. Zinc finger mutant versions of ZFP36L1 failed to bind the 3' UTR AREs for each target, proving that intact zinc finger domains are the functional binding domains of the protein and are required for interaction with AREs. A complete ARE sequence is also needed and when mutated *BCL2* 3'UTR ARE was tested, lacking the adenine uridine rich core element, the *BCL2* transcript was not bound by ZFP36L1 protein. For *CD38* further experiments have demonstrated that down regulation of ZFP36L1 by siRNAs in HeLa cells resulted in an increase in *CD38* expression as measured by immunofluorescence and flow cytometry and by Western blot analysis. These results provide further evidence that ZFP36L1 negatively regulates *CD38* mRNA.

Analysis of *BCL2*, *CD38* and ZFP36L1 protein expression in primary B-CLL cells by Western blot analysis did not show an inverse relationship between the proposed targets and ZFP36L1. Protein expression analysis in B-CLL for the whole family of ZFP36 proteins showed that ZFP36L1 was heterogeneously expressed; ZFP36L2 was detected at very low levels or was undetectable and ZFP36 was low and homogeneously expressed. In cell lines representing different B cell stages, but mainly representing mature and plasma cell stages, ZFP36L2 was detected at relatively high levels but also heterogeneously and there was very low or undetectable expression of ZFP36L1 in all cells. Immunohistochemistry analysis of ZFP36L1, *BCL2* and *CD38* in normal lymphoid tissue and FL indicated that areas of normal lymphoid tissues associated with highest levels of *BCL2* and *CD38* were associated with low or undetectable levels of ZFP36L1. In FL (FL) ZFP36L1 was detected in follicular centre cells, where *BCL2* is also reported to be highly expressed due to a translocation that leads to over expression of *BCL2*. *CD38* expression was also detected within FL follicle centres with some cells showing a high level of expression within the neoplastic follicle and amongst scattered cells outside of it.

Overall, the results support the hypothesis that ZFP36L1 (and also ZFP36 and ZFP36L2) negatively regulates *BCL2* and *CD38* mRNAs. In a wider context, the results of this project support the view that ZFP36L1, and perhaps other ZFP36 family proteins, play important roles in controlling mature B cell survival and differentiation by targeting important regulatory mRNAs in these cells.

Dedication

Para tí, abuela. Por tí.

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Author's declaration

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed:

Date: 15th September 2015

Abbreviations

ADP: adenosine diphosphate
ADPRC: ADP ribosyl cyclase
AIDS: Acquired immune deficiency syndrome
ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks
AU: Adenine uridine
AUBPs: AU binding proteins
ARE/s: Adenine uridine rich element/s
B-CLL: B cell chronic lymphocytic leukaemia
BCR: B cell receptor
BLIMP1: B- lymphocyte-induced maturation protein-1
CBs: Centroblasts
CCs: Centrocytes
COSMIC: Catalogue of Somatic Mutations in Cancer
DLBCL: Diffuse Large B cell Lymphoma
ER: Endoplasmic reticulum .
FADD: Fas associated death domain
FL: Follicular lymphoma
FISH: Fluorescence *in situ* hybridisation
GC/c: Germinal centre/s
H: Heavy chain
HL/s: Hodgking Lymphoma/s
IgH: Immunoglobulin heavy chain
IHC: Immunohistochemistry
L: light chain
IL: Interleukin
IFN: Interferon
ICG DCC: International Cancer Genome Consortium Data Coordination Centre
LDLR: Low density lipoprotein receptor
LPS: lipopolysaccharide
LHR: luteinizing hormone receptor
MAPKAPK2: mitogen-activated protein kinase activated protein kinase 2
mESCs: embryonic stem cells
MGUS: Asymptomatic monoclonal gammopathy of undetermined significance
MM: Multiple myeloma
MHC (major histocompatibility complex)
MS: Multiple sclerosis
NAD: nicotinamide adenosine dinucleotide
NHL: Non Hodgkin lymphoma
PARN: Poly A specific ribonuclease
PB: Processing bodies
RBP: RNA binding protein
PKB/Akt: kinase B
RISC: RNA induced silencing complex
SG: Stress granules
SHM: Somatic hyper mutation
SNP: single nucleotide polymorphism
T-ALL: T-cell acute lymphoblastic leukaemia
TPA: 12-O-tetradecanoylphorbol-13-acetate
UTR: Untranslated region
ZFD: Zinc finger domain

Chapter 1

General Introduction

1.1 The Zinc Finger Protein 36 (ZFP36) family

The ZFP36/Tis11/Tristetraprolin is a protein family composed of 4 mammalian members, 3 of them are found in humans: ZFP36L1/Tis11b/Berg36/BRF1/ERF1/cMG1, ZFP36L2/Tis11d/BRF2 and ZFP36/Tis11/ Nup475/GOS24, also known as TTP, tris-tetra-proline, the prototype protein giving the name to the family for its three Pro-Pro-Pro-Pro repeats (Lai et al. 1990). ZFP36L3, the fourth member of the family was not found in humans but in the mouse placenta and yolk sac of rodents (Blackshear et al. 2005). A fifth member, ZFP36L4, xC3H-4 (Noiret et al. 2013, Tréguer et al. 2013) has been reported in frogs and fish and yeast (Blackshear 2002, Blackshear et al. 2005). Amongst species, the ZFP36 family have orthologs in all vertebrates, although not in birds which do not have a version of ZFP36 (Blackshear and Perera 2014). The relevance of the presence of the proteins amongst different species highlights their importance within the machinery for control of gene expression.

The human gene encoding the prototype protein, ZFP36, located in chromosome 19q13.1, was cloned and identified by different groups as an early response gene (DuBois et al. 1990, Lai et al. 1990, Taylor et al. 1991). Initially a partial sequence had been published after a study using fibroblasts stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Varnum et al. 1989, Varnum et al. 1991) and later the full length sequence was described as a result of cloning of the mouse cDNA from 3T3- L1 fibroblasts stimulated with insulin. Shortly after that, another study published the same sequence description as Nup475 also isolated from fibroblasts but stimulated with serum (DuBois et al. 1990, Lai et al. 1990). The final version of the protein sequence was corrected in another paper (Ma and Herschman 1991) and later on other studies described the DNA sequence in human cells (Taylor et al. 1991).

The second family member, ZFP36L1, had been identified in murine cells (Varnum et al. 1991); with chromosome location 14q22-24, it was identified from cDNAs representing early response genes from activated B cells and cloned from chronic lymphocytic cells after TPA treatment (Murphy and Norton 1990, Ning et al. 1996). The name ERF1, early response factor 1, was given after cloning and characterizing the ZFP36L1 gene isolated from cDNA libraries using a as a probe a rat homologue, rat cMG1 (Bustin et al. 1994), that had been reported earlier (Gomperts et al. 1990). The coding sequence of ZFP36L1 was published (Barnard et al. 1993). The sequence of *ZFP36L1* gene was reported to have 2 exons (Bustin et al. 1994) and mapped to its chromosome location using FISH (Maclean et al. 1995). Ning et al. (1996) cloned *ZFP36L1* identifying it as B cell early response gene of 36kD (BERG36), and reported that the protein contained 338 amino acids. The group also investigated its gene function in regulating Ramos B cell apoptosis. **They showed that the gene could be induced by calcium ionophore in Ramos B cells and that IL4 could block it but this was not the case for CD40 ligation.** Taken together, these observations indicated that ZFP36L1 induction promoted B cell apoptosis and IL4 signalling inhibited ZFP36L1 induction and promoted B cell survival (Ning et al. 1996).

ZFP36L2, 2p22.3-p21, the third member of the family in mammals was identified in mice (Varnum et al. 1991) and later was isolated from cDNA libraries probed with mouse *Zfp36l2* mRNA (Nie et al. 1995). It was suggested the protein was 52 KDa, and about 493 amino acids. Other group also cloned ZFP36L2, naming it TIS11D, TPA-inducible sequence 11, proposing the protein to have 492 amino acids and two binding motifs, detected at variable levels in all tissues and also in leukemic cells (Ino et al. 1995). The gene was reported to have 2 exons, being CG rich, apart from the 3' untranslated (3' UTR) region which was reported to have 5 repeats of ATTTA, the adenine uridine (AU) rich element (ARE) (Ino et al. 1995).

The gene sequences of the three *ZFP36* family members are very similar especially in their zinc finger structure (Varnum et al. 1991), which is the most conserved region as opposed to the C and N terminal domains which have less amino acids in common (Lai et al. 2000) (fig. 1). The genes differ mainly in the non-coding regions- introns and 3'UTR- (Blackshear et al. 2003b). A polymorphism in ZFP36 causing a C to T modification, is known to be associated with rheumatoid arthritis in African Americans (Carrick et al. 2006); ZFP36L1 has a single nucleotide polymorphism (SNP) in its intron (Blackshear et al. 2003b), but its effect or function is not yet known. A ZFP36L1 SNP is also linked to multiple sclerosis (MS) (Gourraud et al. 2012) and its uses as a molecular biomarker for the disease are being discussed (Paap et al. 2013). Recently an application for patent regarding MS prediction markers was published using ZFP36L1 polymorphisms as an indicator (within a number of other genes) to assess the course (benign, poor) of MS (Achiron and Gurevich 2010). *ZFP36L1* is increasingly being looked at in MS. Another 2010 study showed that there are changes in the genetic signature of MS during pregnancy, the study looked at a number of individuals and concluded that expression of inflammation related genes is changed in pregnant women, where *ZFP36L1* showed no pregnancy-related regulation (Gilli et al. 2010).

Table 1-1 The ZFP36 gene family.

Gene	Alternative names	Species	Chromosomal location	Size	
				Gene (kb)	Protein (kD)
<i>ZFP36</i>	TTP, TIS11, NUP475, GOS24	Human	19q13.1	2.5	34.0
		Mouse	7qA3	2.4	33.6
		Rat	1q21	2.5	33.7
<i>ZFP36L1</i>	BRF-1, TIS11b, ERF-1, cMGI, Berg36	Human	14q22-q24	5.4	36.3
		Mouse	12qC3	5.2	36.4
		Rat	6q24	5.0	36.4
<i>ZFP36L2</i>	BRF-2, TIS11d, ERF-2	Human	2p22.3-p21	4.2	51.0
		Mouse	17qE4	4.0	50.0
		Rat	6q12	4.0	50.0
<i>Zfp36l3</i>	AY661338 , RGD1559581	Mouse	XqA5	3.7	72.3
		Rat	Xq3.6	N.A.	72.2*

Table modified from Sanduja et al. (2011) showing basic details of the ZFP36 family members

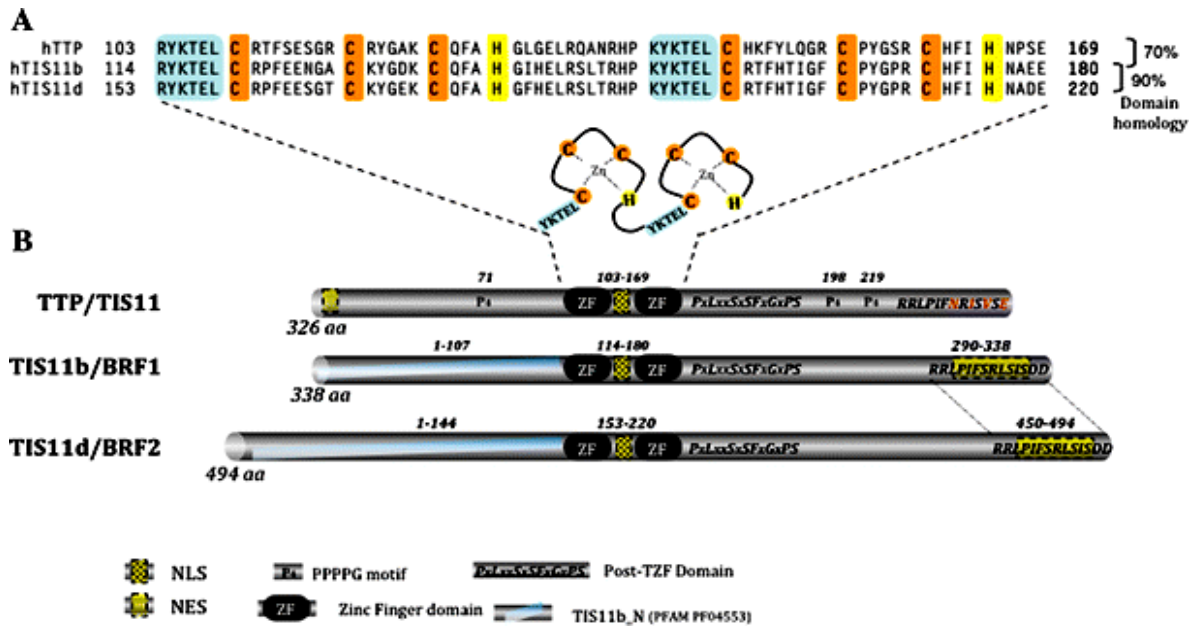


Figure 1-1 Similarities between the members of the ZFP36 family.

A. ZFP36L1 has a zinc finger domain (ZFD) protein sequence similarity of about 70% with ZFP36L2 and 90% with ZFP36. B. The 3 proteins have a nuclear localization sequence (NLS) within the ZFD; a nuclear export signal sequence (NES) for ZFP36L1 and ZFP36L2 is present in the C termini, for ZFP36 it is found in its N terminus. The N terminus of ZFP36 differs more from those of the other two proteins which are closely related with each other. Reproduced from Ciais et al. (2013).

The three *ZFP36* family mRNAs are expressed at different levels in a variety of tissues. The expression level of all three mRNA transcripts was quantitated in a variety of normal human tissues, and in the National Cancer Institute 60 cell lines, showing that each mRNA may be found in different tissues where another member of the family might not be expressed, or/and if more than one family member is found in a tissue their level of expression would often differ (Carrick and Blackshear 2007). The highest level for each mRNA was found as follows: *ZFP36* highest expression level in cervix, *ZFP36L1* highest expression level in lung and *ZFP36L2* highest expression level in the thymus. Figure 1- 2 summarises the expression profiles of the three mRNAs in a variety of tissues.

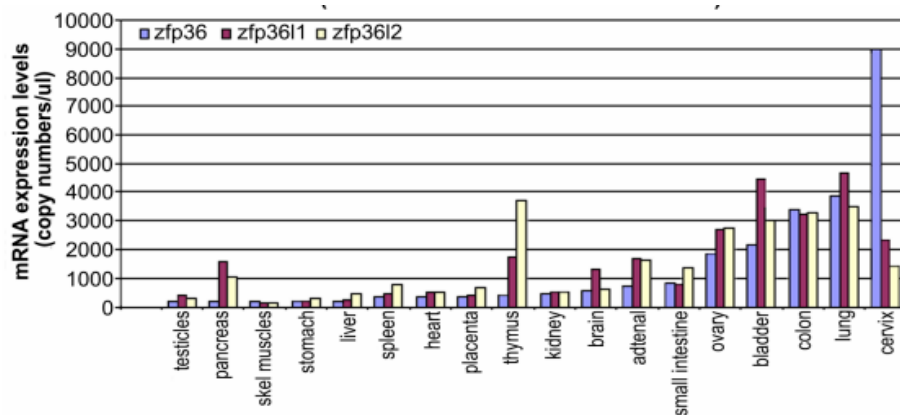


Figure 1-2 ZFP36 family members mRNA levels in normal human tissues.

mRNA levels were measured using qPCR, the levels of expression found for each mRNA in different normal tissues varied among tissues and between proteins. Levels shown in the figure are normalised for total cDNA content. Figure modified from Carrick and Blackshear (2007).

1.1.2 ZFP36 proteins in post-transcriptional gene regulation

1.1.2.1 ZFP36 proteins RNA binding motifs

The three human ZFP36 proteins have shared characteristics; they all have two tandem zinc finger motifs separated by 18 amino acids. There are different classes of ZFD, and they may be present in many DNA and RNA binding proteins; figure 1-3 shows a schematic representation of a ZFD showing zinc atoms that stabilise the motif and a beta sheet and an alpha helix as part of its structure. The zinc atoms are co-ordinated by two cysteine residues on the beta sheet, and two histidine residues on the alpha helix. A single zinc finger binds only to a few DNA or RNA bases hence these domains appear usually in tandem repeats (fig.1- 3). In the DNA binding ZFD the alpha helix binds to the bases in the target site through amino acid side chain interactions, the identity of these side chains determines the recognition of the target sequence (Krishna et al. 2003). CCCH zinc fingers can bind single and double stranded RNA. The CCCH motif of ZFP36L2 shed light in the binding mode of the proteins to single stranded RNA (Brown 2005). **CCCH zinc fingers are thought to use sequence-specific recognition of single-stranded RNA through an interaction between hydrogen bonds and Watson-Crick edges of the RNA bases (Steffl et al. 2005).**

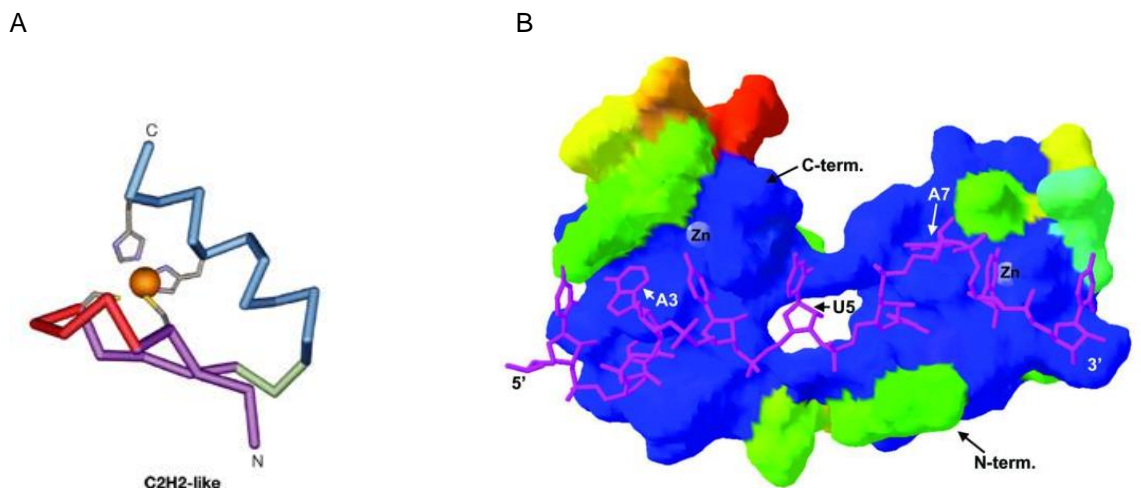


Figure 1-3 Representation of zinc finger motifs.

(A) A prototype DNA binding zinc finger motif, C2H2, it has an alpha helix (shown in blue), a short antiparallel beta sheet (purple) and coordinating zinc atoms. Histidine and cysteine residues are shown bonded to a zinc ion. C2H2 zinc fingers are arguably the best studied. The image is from Kadmas and Beckerle (2004). (B) The ZFP36 proteins have a ZFD that is made up of Proposed model of tandem ZFD bound to the 9-mer sequence UUAUUUUUU. The amino-terminal of zinc finger is on the right, the C-terminal zinc finger is on the left. The RNA oligonucleotide runs from left to right in the 5' to 3' orientation. The zinc residues are highlighted, as are certain bases in the RNA. The model is based on the ZFP36L2 ZFD structure and the colours show level of identity between the model shown here for ZFP36 and ZFP36L2, being cool colours, blue and green, significant of a greater chemical similarity between ZFP36L2 and ZFP36. The image is from Brooks and Blackshear (2013) where it was reproduced from Carrick et al. (2004).

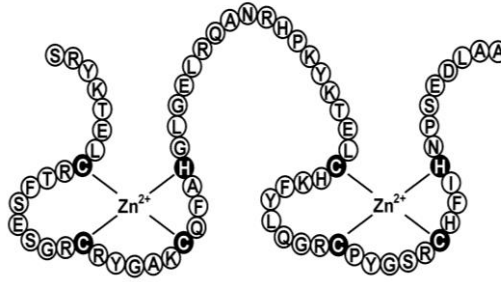


Figure 1-4 Two zinc finger domains of ZFP36

ZFP36/Tis11/TTP, ZFP36L1/Tis11b/Berg36 and ZFP36L2/Tis11d/BRF2 share structural characteristics including two zinc-binding domains of the form Cys-X8-Cys-X5-Cys-X3-His. The image is from Brewer et al. (2004b) .

1.1.2.2 Mechanism of mRNA degradation by ZFP36 proteins via adenine-uridine-rich elements

AREs regulate mRNA decay by interacting with ARE-binding factors. Many mRNAs including many encoding oncoproteins and inflammatory mediators contain AREs in their 3' UTR. Although, more recently, evidence has been found of the role of ZFP36 in regulating gene expression via an ARE independent pathway (Liang et al. 2009, Schichl et al. 2009) their main reported role in studies to date is their function as post-transcriptional regulators by promoting mRNA degradation through recognizing and binding to AREs in 3' UTR regions of mRNAs via their ZFD.

Following binding of AREs ZFP36 proteins induce deadenylation and decapping leading to recruitment of the mRNA by the exosome for 3'-5' degradation or 5'-3' degradation via exonuclease activity, XRN1. These processes result in the destruction of the RNA transcript (Blackshear 2002) hence performing a central role in post-transcriptional gene expression.

The proposed model by which ZFP36 proteins promote degradation of the mRNA transcripts follows deadenylation, shortening of the poly A tails of their targets. Removal of the poly A tail is necessary for mRNA degradation with engagement of Poly A specific ribonuclease activity (Lai et al. 2003). Initial reports of ZFP36 shortening the Poly A tails of mRNA transcripts derived from TNF α by (Lai et al. 1999) and GMCSF by (Carballo et al. 2000). Poly A RNase (PARN) deadenylation seems to be enhanced by the ZFD of ZFP36, and it did not occur in presence of mutated ZFP36 which was unable to bind to the ARE (Lai et al. 2003). Although deadenylation is thought to be promoted by the action of PARN, immunoprecipitation studies showed that this action is not performed by direct contact with ZFP36 or ZFP36L1 (Lai et al. 2003, Lykke-Andersen and Wagner 2005). Other deadenylation enzymes reported to interact with ZFP36 are CAF1a and CAF1b, an interaction that is prevented when ZFP36 is phosphorylated resulting in lack of deadenylation activity (Marchese et al. 2010). NOT1 association via ZFP36's C terminal seems to be required for CAF1s recruitment by the protein (Sandler et al. 2011). The recruitment of deadenylation complex (CCR4-NOT) has also

been confirmed for ZFP36L1 and ZFP36L2 on binding to the 3'UTR mRNA of low density lipoprotein receptor (LDLR) (Adachi et al. 2014).

Decapping by decapping enzymes has also been reported in ZFP36 led mRNA degradation. A number of enzymes (hDcp1, hDcp2-decapping enzymes); hXrn1, (5'-3' exonuclease), hCcr4 (a deadenylase) and components of the exosome such as hRrp4, were seen to co-immunoprecipitate with ZFP36 (Fenger-Grøn et al. 2005, Lykke-Andersen and Wagner 2005); a number of groups further reported ZFP36 interacting with XRN and DCP1 and CDP2 (Hau et al. 2007, Kim et al. 2010b); and involvement of the exosome had been further supported by (Chen et al. 2001). Co-immunoprecipitation of ZFP36 with the enzymes mentioned happened via N terminal proposing that localisation of ZFP36 proteins within processing bodies (PBs) happens via C terminal (Lykke-Andersen and Wagner 2005). The three ZFP36 proteins have been reported to localise within PBs, which are sites of mRNA degradation, containing decapping enzymes (Stoecklin and Anderson 2007). Degradation of the mRNA transcript is also reported to occur via exosome and Xrn1 exonuclease (Wilusz et al. 2001). The suggested mechanism of action of ZFP36 proteins is mRNA degradation is shown in figure 1-5.

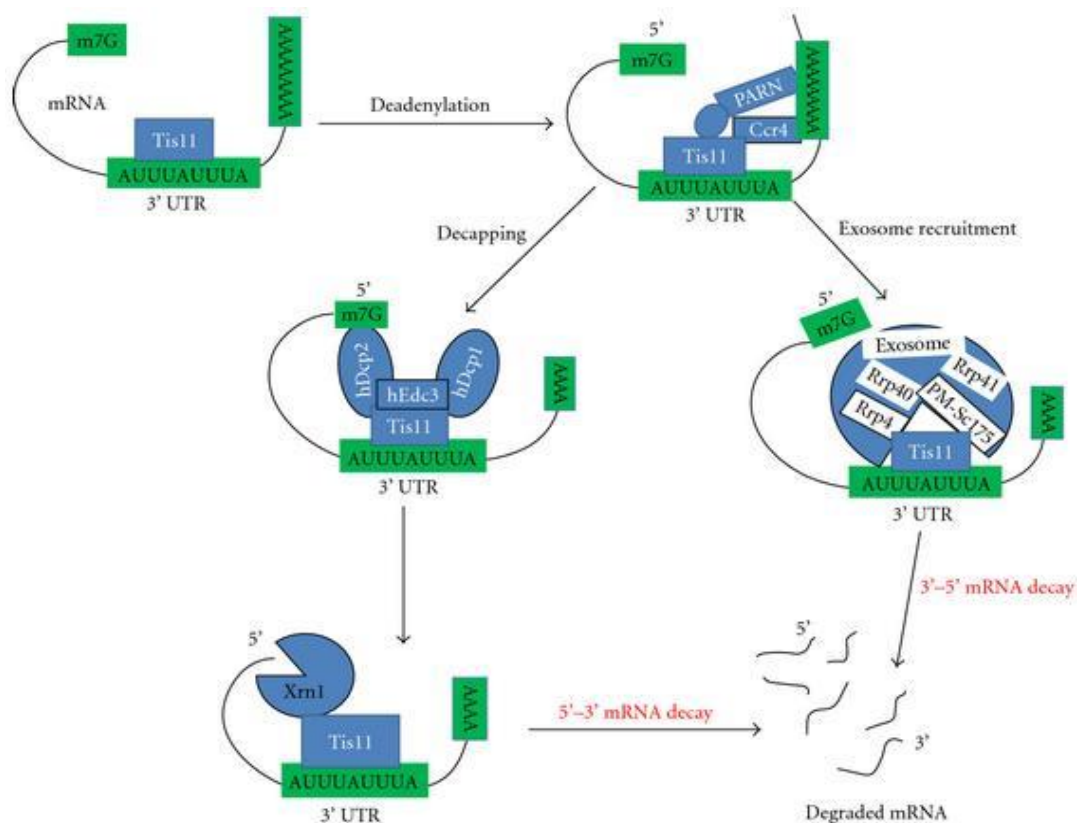


Figure 1-5 Pathways and major components of ARE-mediated mRNA decay by TIS11/ZFP36 family of proteins.

Reproduced with permission from Baou et al. 2009. Proteins bound to 3' UTR, deadenylases (hCcr, 4 PARN) are recruited; deadenylated mRNA to exosome containing Rrp4, Rrp40, Rrp41, PM-Scf75 (3' to 5' exoribonuclease complex). Decapping after deadenylation driven by decapping enzymes (Dcp1, Dcp 2) and degradation of mRNA by Xrn1 exonuclease.

There is still some debate as to what the optimal ARE recognition sequence is for the ZFP36 protein family. Some authors have proposed a nonamer as the optimal target sequence, UUAUUUAUU (Worthington et al. 2002, Blackshear et al. 2003a, Cao 2004, Hudson et al. 2004, Lai et al. 2005, Lin et al. 2008, Hodson et al. 2010), many reports indicated that the minimal sequence required for mediated mRNA degradation was a nonamer rather than a pentamer (Lagnado et al. 1994, Zubiaga et al. 1995). but other studies reported that only about 44% of ZFP36's mRNAs targets showed the nonamer as their binding sequence (Stoecklin et al. 2008). Other groups have reported that the pentamer AUUUA is the target sequence, and other sequences such as UUUUUUUU or UUUUUUAUU (Michel et al. 2003, Brewer et al. 2004a, Kim et al. 2010a) have also been proposed. Further studies challenged the idea of the nonamer being the basic minimal sequence or minimal consensus sequence in all AREs; more so since many early response genes with known functional AREs do not have it (Chen and Shyu 1994). ZFP36L1 is reported able to bind to class II AREs, as already reported for TNF α (Lai et al. 2000) and VEGF (Bell et al. 2006).

AREs are the most common determinants of RNA stabilisation (Chen and Shyu 1995), they serve as binding sites for micro RNAs (miRNAs) and RNA binding proteins (RBPs). RBPs exert their regulatory effect, for instance, stabilisation (HuA, HuB, HuC, HuD, HuR) or degradation (AUF1, ZFP36, ZFP36L1, TIA-1, TIAR, and KSRP) of transcripts, by recognising and binding to these AREs which are present in about 8% of human mRNAs (Bakheet et al. 2001). The AU segment can cover from 50 and up to 150 nucleotides and it is common the presence of repeats of the pentameric AUUUA sequence (Caput et al. 1986, Shaw and Kamen 1986).

AREs tend to be composed of varying numbers of the AUUUA or UUAUUUAUU sequences, an initial classification of AREs was done looking at the presence or lack of AUUUA (Chen and Shyu 1994). After this type of classification a second attempt was done looking at categorising the diverse AREs depending on whether they contained a few copies of the pentamers scattered along the ARE with U rich sequences, or whether AREs in which various copies of AUUUA would be present clustered together (Chen and Shyu 1995). Another group classified AREs into 3 different groups experimentally (Xu et al. 1997) and more recently, researchers produced the ARE containing mRNA database (ARED); the database compilation based on repeats of AUUUA, comprising of five subsets of AREs under class II relating to the number of nonamers (Bakheet et al. 2006), class II in the experimental based classification by Xu et al. (1997). A final effort in classifying the AREs and trying to join both prior groupings together was attempted leading to the classification depicted in table 1-2 (Wilusz et al. 2001):

Table 1-2 Classification of AREs.

Group	Motif	Examples
I	WUJUUAW and a U-rich region	c-fos, c-myc
IIA	AUUUAUUUAUUUAUUUAUUUA	GM-CSF, TNF- α
IIB	AUUUAUUUAUUUAUUUA	Interferon- α
IIC	WUJUUUUUUUUUUAW	cox-2, IL-2, VEGF
IID	WUJUUUUUUUUUUWW	FGF2
IIE	WUJUUUUUUUUUUWW	u-PA receptor
III	U-rich, non-AUUUA	c-jun

Proposed classification of AREs based on two previous groups, modified from Wilusz et al. 2001.

1.1.3 Phosphorylation of ZFP36 proteins

The proteins in this family have a high proportion of serines and threonines, which are amino acids susceptible of phosphorylation (Cao 2004, Benjamin et al. 2006). The phosphorylation of ZFP36 proteins impacts their localization, stability and mRNA decay function (Brook et al. 2006, Hitti et al. 2006, Maitra et al. 2008). ZFP36 is phosphorylated by mitogen-activated protein kinase activated protein kinase 2 (MAPKAPK2) which promotes binding of 14-3-3 proteins and this hinders deadenylation by preventing the binding of deadenylation proteins to ZFP36 (Johnson et al. 2002, Chrestensen et al. 2004, Stoecklin et al. 2004, Marchese et al. 2010, Clement et al. 2011, Sandler et al. 2011). In the case of ZFP36L1 and ZFP36 phosphorylation of the proteins in two serines leads to the binding of 14-3-3 proteins protecting against dephosphorylation and impeding mRNA decay activity (Schmidlin et al. 2004, Benjamin et al. 2006, Sun et al. 2007). Previous reports had already highlighted that ZFP36L1 mRNA degradation function was regulated by kinases, specifically, kinase B (PKB/Akt) (Schmidlin et al. 2004, Benjamin et al. 2006) which stabilises ARE transcripts by phosphorylating ZFP36L1. They observed *in vitro* mRNA decay by a recombinant ZFP36L1 but if the protein was phosphorylated by PKB/Akt the transcript was stabilised, the stabilisation was not down to inhibition of binding of ZFP36L1 to the 3' UTR but the phosphorylation induced a complex formation with the scaffold protein 14-3-3. *In vivo* and *in vitro* data support a model (fig. 1-6) where PKB/Akt causes ARE-mRNA stabilization by inactivating ZFP36L1 through binding to 14-3-3 (Schmidlin et al. 2004, Benjamin et al. 2006).

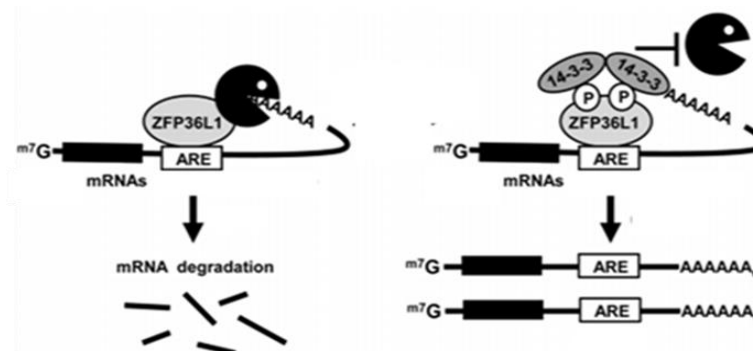


Figure 1-6 Phosphorylation of ZFP36 proteins impacts their function.

Schematic representation of ZFP36 proteins function regulated by phosphorylation. Phosphorylated ZFP36L1 bind 14-3-3 proteins inhibiting the binding of the deadenylation complex. The image has been adapted from Lin et al. (2012).

Stress granules (SGs) form in the cell cytosol when the cell undergoes stress conditions, such as, hypoxia or heat shock. The SGs contain untranslated mRNA or silenced mRNA. It is thought that the function of SGs could be the protection of these mRNAs under unfavourable circumstances for the cell. It has been reported that in cells under stress ZFP36 might be recruited to the SGs (Kedersha and Anderson 2002), however phosphorylation and subsequent formation of the complex with the 14-3-3 proteins halted ZFP36 entering the SGs (Stoecklin et al. 2004, Rigby et al. 2005). Furthermore a mutated ZFD in ZFP36 protein also prevented the protein entering SGs (Murata et al. 2005). SGs can physically associate with PBs, places of RNA degradation, and this association may facilitate the degradation of selected mRNAs within SGs which are passed to the PBs (Kedersha and Anderson 2002, Kedersha et al. 2005). The three ZFP36 proteins are reported to localise within PBs, alongside decapping enzymes (Stoecklin and Anderson 2007), and it has been proposed that transporter protein Transportin has a role in delivering ZFP36 between SG and PBs (Gallouzi and Di Marco 2009).

1.1.4 ZFP36 family proteins physiological roles

Besides the shared characteristics of the three human ZFP36 family members: highly similar gene sequences, conserved ZFDs and the ability to recognise similar AREs within the 3' UTR of mRNA transcripts, these three proteins have different expression patterns in different tissues and also might differ in the ARE type they preferentially bind to. The biological roles of the ZFP36 family have been highlighted through a variety of studies showing involvement in processes such as cell proliferation (Shimada et al. 2000), cell differentiation (Wegmüller et al. 2007, Busse et al. 2008), or cell death (Ning et al. 1996, Johnson et al. 2000).

Recently the diversity of the targets these proteins reportedly bind to has increased with confirmed targets in lipid metabolism where ZFP36L1 and ZFP36L2 have been found to have a role in controlling the mRNA of the unstable low density lipoprotein receptor (LDLR), by binding to its 3'UTR, recruiting a deadenylase complex, CCR4-nOT, and destabilising this mRNA transcript (Adachi et al. 2014). The homology between the proteins is obvious, but there is evidence that each of the three ZFP36 proteins may also target distinct but over-lapping mRNAs. Results from gene knockout studies in mice indicate that some of their individual functions might be non-redundant where the deletion of each ZFP36 protein has had different effects. Although normal at birth mice with knockout *Zfp36* developed a variety of symptoms: cachexia, patchy alopecia, arthritis, conjunctivitis, autoimmune syndrome, myeloid hyperplasia, as a result of *TNF α* mRNA extended half-life; the symptoms were alleviated if anti-*TNF α* antibody was administered (Taylor et al. 1996). **In the case of ZFP36, inflammatory signals lead to expression of the protein which can negatively regulate the pro-inflammatory mediators resulting in an anti-inflammatory function in macrophages** (Carrick et al. 2004). *Zfp36l1* knockout mice died *in utero* due to failure in chorioallantoic fusion (Stumpo et al. 2004). *Zfp36l2* knockout mice showed defects in haematopoiesis (Stumpo et al. 2009). Mice with a truncated version of ZFP36L2, due to deletion of the first exon (but leaving the

RNA binding domain and nuclear export signal intact) seemed normal at birth and presented a normal phenotype but the females were infertile. A mutation in *Zfp36l2* leading to decreased expression of a form of *zfp36l2* (with the 29 N-terminal amino acid residues deleted), ΔN -*zfp36l2*, resulted in fertilised eggs being arrested in early development. Female mice homozygous for that truncated version ovulated normally and the eggs were fertilized, but embryos did not develop and were seen to arrest at the 2 cell stage, females also released 40% fewer eggs than the WT littermates (Ramos et al. 2004). The wild type version of the *zfp36l2* protein and mRNA was down-regulated faster than its mutated version (ΔN -*zfp36l2*) in response to lipopolysaccharide (LPS) exposure in bone marrow macrophages, it suggested that the embryonic arrest connected to the truncated *zfp36l2* might be linked to its resistance to stimulus induced down regulation, and so the truncation (lacking of a leucine repeat) stabilises the protein (Ramos 2012).

The role of ZFP36L2 influencing ovulation and oocyte maturation has been further researched and it has been proposed that it has a role in the molecular basis for unexplained human female infertility (Ball et al. 2014). Ovulation and oocyte maturation are both triggered by luteinizing hormone receptor (LHR) signalling, its activation is necessary for the proper hormonal functioning during reproduction. ZFP36L2 interacts with the 3'UTR of LHR mRNA and lowers levels of *Zfp36l2* expressed correlating with increased levels of LHR mRNA (Ball et al. 2014), whilst over expressing *zfp36l2* in the MLTC-1 cell line lowered *LHR* mRNA levels. The group proposed that lack of the physiological down regulation of *LHR* mRNA levels by ZFP36L2 in the ovaries is associated with anovulation and oocyte meiotic arrest and the low levels of ZFP36L2 (70% decrease in the ovary) in ΔN -*Zfp36l2* females might be insufficient to properly down-regulate the *LHR* mRNA induced by the LHR surge, thus resulting in anovulation (Ball et al. 2014).

Mice lacking ZFP36L1 and ZFP36L2 during thymotopoiesis developed T- Acute lymphoblastic leukaemia (T-ALL) and showed a higher expression of Notch1 (Hodson et al. 2010). Cells accumulated after having passed the β selection checkpoint and before the development of T-ALL. The 3' UTR ARE of *Notch1* mRNA was targeted by ZFP36L1 and ZFP36L2 leading to suppression of Notch1 expression, the opposite was seen in untransformed thymocytes where higher Notch1 levels was observed (Hodson et al. 2010).

ZFP36L1 is expressed in all human hematopoietic cells but not in erythroid cells (Vignudelli et al. 2010). ZFP36L1 overexpression in human cord blood CD34+ progenitor cells arrested red blood cell differentiation (Vignudelli et al. 2010). The inhibition in erythroid differentiation was linked to a down regulation of the signal transducer and activator of transcription 5b (*Stat5b*) protein through targeting and degradation of its mRNA (Vignudelli et al. 2010). Over expression of ZFP36 also inhibited erythroid differentiation; and the over expression of ZFP36 and ZFP36L1 seemed to have a cumulative effect in stopping red blood cell differentiation (Vignudelli et al. 2010). Also, both ZFP36 and ZFP36L1, were seen to bind to the ARE in the 3'UTR of *Stat5b* mRNA. IRF8, a transcription factor of the IFN family is a pro-apoptotic factor was also seen to increase when

ZFP36L1 was over expressed (Vignudelli et al. 2010). ZFP36L1 has been shown have a role in keeping the undifferentiated state of myeloid cells during differentiation (Shimada et al. 2000) and similarly in pluripotent stem cells (Wegmüller et al. 2007). The protein also negatively regulates plasmacytoid differentiation by targeting B lymphocyte-induced maturation protein-1 (BLIMP1) (Nasir et al. 2012).

Studies have shown a role for ZFP36L1 in promoting B cell apoptosis. In Ramos Burkitt's lymphoma B cells the presence of this protein seems to be needed for apoptosis induced by calcium signalling (Ning et al. 1996); ZFP36L1 expression was also reported to be required for Rituximab-induced apoptosis in B-Chronic Lymphocytic Leukaemia cells (B-CLL) (Baou et al. 2009b).

ZFP36L2 is found down regulated in red blood cell differentiation; knockdown of ZFP36L2 in transplanted erythroid progenitor cells prevented expansion of erythroid (Zhang et al. 2013). The protein binds to mRNAs highly expressed in terminal immature red blood cell differentiation and negatively regulates the expression level of those mRNAs (Zhang et al. 2013).

The prototype, ZFP36, has been well studied in inflammation, especially its relationship with $TNF\alpha$ and its role in destabilising the $TNF\alpha$ mRNA transcript (Taylor et al. 1996, Johnson and Blackwell 2002). The involvement of ZFP36L1 and ZFP36L2 negatively regulating inflammatory processes is less clear. A recent study reported that ZFP36L1 is greatly induced by infection but that it does not seem to be the main player in repressing inflammatory mediators (Hyatt et al. 2014). This group created myeloid deficient ZFP36L1 mice and concluded that in these mice the lung cytokine production, bacterial clearance or increased lung inflammation was not altered compared to wild-type mice. It was concluded that the prototype ZFP36 protein is the main player as a negative regulator of cytokines in macrophages and that it may compensate the loss of ZFP36L1 presence (Hyatt et al. 2014).

Recently ZFP36L1 has been found to destabilise pluripotency transcripts hence maintaining the pluripotency of mouse embryonic stem cells (mESCs) (Tan and Elowitz 2014). The authors of this study determined that ZFP36L1 is able to post-transcriptionally regulate both, pluripotency and differentiation, responses downstream of Erk MAP kinase. Activation of the FGF-Erk signalling pathway stimulates mESC differentiation and it also leads to up regulation of ZFP36L1, this overexpression impaired proliferation under pluripotency conditions, but under differentiation conditions it promoted mesodermal determination (Tan and Elowitz 2014). The pluripotency associated mRNAs that ZFP36L1 regulates in mESCs after stimulation included Nanog, ZFP36L1 regulates Nanog's abundance and localization which leads to perturbing the network of transcription factors without inducing differentiation but arresting self-renewal. The same paper announced a number of pluripotency mRNAs targets for ZFP36L1 pulled out using RNA immunoprecipitation and sequencing (RIP-seq): ZFP36L1 bound to (AREs) in: Klf2, Kdm4c, and Zfp143 (Tan and Elowitz 2014).

1.1.5 mRNA targets for the ZFP36 protein family

The mRNA ARE that each family member targets may be distinct for each ZFP36 protein or overlapping targets where the proteins may recognise a subset of identical mRNA targets. A review of the roles of this protein family in mRNA regulation can be found in several papers showing the role of these proteins in a large variety of functions (Baou et al. 2009a, Sanduja et al. 2011, Ciais et al. 2013) .

A number of mRNA targets for ZFP36 family members have been identified in several studies. Most studies have focused on identifying candidate mRNAs for the prototype family member, ZFP36. Some mRNA targets have been identified in knockout mice models and/or in studies focused on different cell types where genes have been silenced (Emmons et al. 2008, Stumpo et al. 2009). Information on proposed targets published for ZFP36 family members can be seen in the appendix due to its length. Literature listing putative targets for the 3 proteins or a single family member, ZFP36 in this case, include research groups proposing 137 mRNAs (Stoecklin et al. 2008) and identifying 250 potential targets (Lai et al. 2006). Recently, Brooks and Blackshear, 2013 reviewed current mRNA binding targets for the prototype ZFP36 protein and the methods used to identify mRNA targets, this review highlights that since the first studied targets for ZFP36 in 2000 up to 293 papers have been published identifying mRNA targets for the protein or proteins, although the paper shows a table only for ZFP36.

Recently, data was published for pro-survival mRNA targets identified by *in silico* and *in vitro* analysis and identified *BCL2*, one of the putative targets researched in this thesis, as a candidate target for ZFP36L1 (Zekavati et al. 2014). Microarray expression profiling and ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks) (Margolin et al. 2006) *in silico* analysis of ZFP36L1 in B cells resulted in data showing possible mRNA targets with a role in B cell differentiation (Nasir et al. 2012). Some targets identified by this analysis like MCSF, VEGFA and IL-3 were already validated targets. Importantly this data identified a key factor in B cell maturation, *BLIMP1*, and led the way to the confirmation that ZFP36L1 regulates *BLIMP1* mRNA via the ARE (Nasir et al. 2012). This analysis also proposed *CD38* mRNA, the other candidate studied in this thesis, as a mRNA that is regulated by ZFP36L1. ZFP36L1 targets inferred from ARACNe analysis can be seen in Nasir et al. 2012, and a table is shown in the appendix.

TNF α is possibly the most studied mRNA target for this family of proteins; initially *TNF α* mRNA was reported a target for ZFP36 by Carballo et al. (1998), confirmed later by other groups (Maclean et al. 1998, Lai et al. 1999, Johnson and Blackwell 2002, Chen 2006, Jalonen et al. 2006b, Suzuki et al. 2006, Hau et al. 2007, Patil et al. 2008) establishing the degradation of *TNF α* mRNA via binding of ZFP36 to its ARE. For ZFP36 even its own mRNA has been reported as a target, suggesting a

potential for auto-regulation via negative feedback (Brooks et al. 2004; Lin, N.Y. et al. 2007; Tchen et al. 2004). *TNF α* mRNA ARE is also a target for ZFP36L1 and ZFP36L2, as are *GMCSF* and *VEGF* mRNAs (Lai et al. 2000, Stoecklin et al. 2002, Lai et al. 2003, Ciais et al. 2004, Fukae et al. 2005). The angiogenic gene, *Dll4*, was reported a target for ZFP36L1 only: silencing of the protein in endothelial cells led to up-regulation of *Dll4* (Desroches-Castan et al. 2011) **Other reported mRNA targets for ZFP36L1 include; steroidogenic acute regulator protein (STAR)** (Duan et al. 2009) **and Stat5b** (Vignudelli et al. 2010). For a recent review of the post-transcriptional regulation of angiogenesis via AREs and its potential for cancer therapeutics please see Planel et al. (2014). In this review, the role of ZFP36L1 is mentioned as one of the proteins with potential use in therapy, they based this on their previous work where they researched the potential role of ZFP36L1 in angiogenic and anti-tumor therapy. In their 2010 paper the group looked at developing a therapy using ZFP36L1's role as mRNA destabiliser. They further researched the role of ZFP36L1 as a negative post-transcriptional regulator of *VEGF* mRNA and obtained evidence of decreased *VEGF* mRNA half life and protein levels by a cell penetrating ZFP36L1 internalised into living cells of mice adrenal gland. In the same study they injected ZFP36L1 in Lewis Lung carcinoma cells implanted in mice showing a decreased in various inflammatory and angiogenic cytokines and reduction of vascularisation of the tumor (Planel et al. 2010), This work followed the earlier findings identifying *VEGF* mRNA decay by ZFP36L1 (Ciais et al. 2004, Bell et al. 2006).

1.1.6 ZFP36 proteins and miRNAs

miRNAs are short strands of RNA present in plants and animals and first described by Lee et al. (1993) although the term microRNA was not used until 2001 (Ruvkun 2001). They are initially coded as longer strands with a hairpin structure and processed by enzymes (Drosher) (Wu et al. 2000, Han et al. 2004) and Dicer (Bernstein et al. 2001) into shorter lengths. The result is a double stranded RNA of about 22 nucleotides, one strand is incorporated into the RNA induced silencing complex (RISC) by argonaute proteins. Argonaute proteins can function as endonucleases directed to the mRNA complementary to the miRNA they are bound to (Meister 2013). The strand bound to the argonaute protein is called the guide strand, selected by the argonaute protein by its 5' end stability; the passenger strand is destroyed within the RISC. Once integrated into the RISC miRNA bind to the 3'UTR of target mRNAs and this double stranded RNA eventually leads to translational repression (fig. 1-8). A more comprehensive review of miRNA biology can be found in Hammond (2015).

There is increasing evidence of miRNAs having roles in regulation of cell growth, development, differentiation and also in cancer and other diseases. Furthermore, recent evidence indicates that miRNAs can regulate multiple functionally related genes in specific biological pathways (Sirotkin et al. 2010). miRNAs have been reported as oncogenes or tumour suppressor genes, however some groups also highlight a number of miRNA as playing both roles making miRNAs an interesting field for diagnostic, prognosis and even therapeutic research that includes haematological malignancies (for further details Mazan-Mamczarz and Gartenhaus, 2013 and Di Lision et al. 2012). In B cell

malignancies, for example, it is known that some miRNAs are found upregulated in Burkitts and other B cell lymphomas (Metzler et al. 2004, Kluiver et al. 2005, Sandhu et al. 2011) . miR-29a is thought to target MCL1, myeloid leukaemia cell differentiation protein, and anti-apoptotic protein of the BCL2 family, and also the oncogene TCL1A which is found disrupted in many T cell leukaemias ((Pekarsky et al. 2006, Mott et al. 2007)

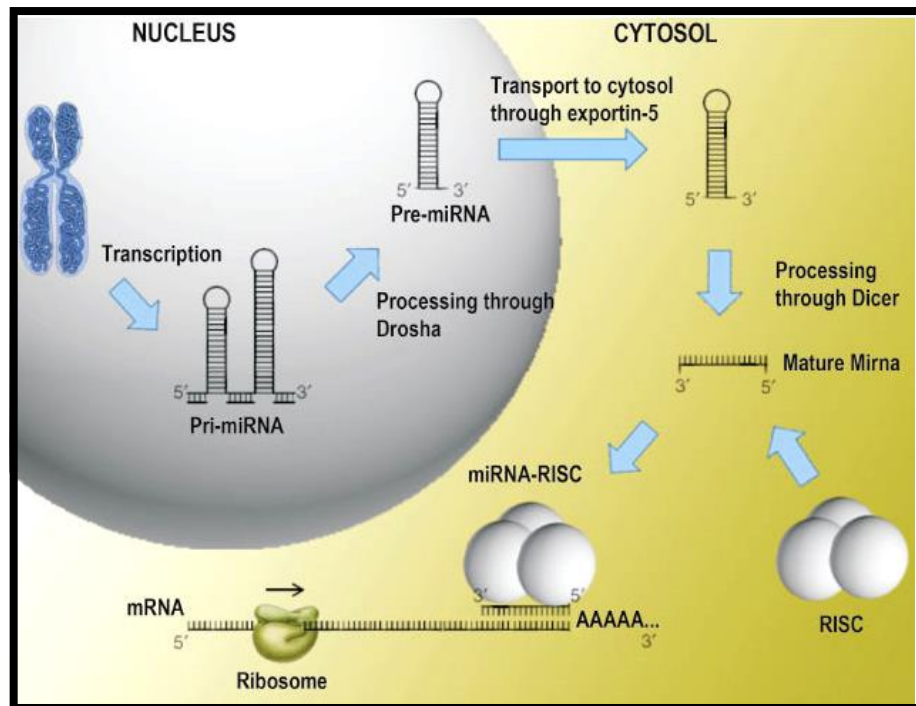


Figure 1-7 RNA silencing pathway.

Depiction of miRNA pathway leading to translational repression and mRNA cleavage. Pri miRNAs in nucleaous are exported to cytoplasm by protein exportin 5, in the cytoplasm the pre-miRNA is shortened by Dicers resulting in double stranded RNA of about 22 nucleotides long. These strands of these RNAs are separated and, after unwinding one strand will be incorporated into the RISC via Argonoute protein, and the other strand will be degraded. The miRNA-RISC is then ready to bind to specific sequences in the target mRNAs and induce cleavage or translational repression preventing the binding of ribososomes. **Image modified from Oliveira-Carvalho et al. (2012)**

Elements of the miRNA pathway might be needed for ZFP36 degradation of mRNA. Dicer was seen to be required for ZFP36 to induce degradation of *TNF α* mRNA. The same paper showed that miRNA16 could pair with bases of the ARE of *TNF α* mRNA and furthermore that this pairing of 8 bases was actually required for mRNA degradation to occur, absence of either ZFP36 or miR16 did not lead to degradation of *TNF α* mRNA suggesting that both RNA binding factors are needed for degradation to occur (Jing et al. 2005)

Some predicted miRNA targets can be found in ZFP36 genes indicating a potential inter-regulatory relationship (Asirvatham et al. 2009). miRNA profiling in breast cancer samples showed that miR-29 levels were increased in cells with ZFP36 loss, and in fact over expression of the miRNA resulted in suppressed ZFP36 expression (Gebeshuber et al. 2009); *ZFP36* mRNA was reported as a target for miR29a (Gebeshuber et al. 2009, Sanduja et al. 2011, Liu et al. 2013). miR29a, and miR29b,

could also possibly target ZFP36L1, since previous studies had shown that over-expression of both miRNAs decreased levels of ZFP36 and ZFP36L1 (Gebeshuber et al. 2009, Sinha et al. 2009).

In the simian immunodeficiency virus model ZFP36 is downregulated during infection and maintained at low levels in the asymptomatic phase, using this model regulation of ZFP36 by miR29a was confirmed although the negative correlation between miR29a was not observed in the brain (Liu et al. 2013). Another group examining the relationship between miRNAs and AU binding proteins (AUBPs) showed that HuR antagonised the binding of miR27 to the *zfp36* 3'UTR mRNA, and as a result hindered the suppression of ZFP36 by miR27 (Lu et al. 2014). Taken together, there is evidence that ZFP36 proteins and miRNAs may exhibit cross-regulation and that mRNA degradation pathways of ZFP36 proteins and miRNAs may be inter-related but the interplay between ZFP36 proteins and miRNAs is not yet clear.

1.1.7 Other RNA binding proteins

RBPs are key regulators of gene expression; they are involved in transport, stability, maturation and translation of RNAs. Eukaryotic genomes in vertebrates can code thousands of RBP (Glisovic et al. 2008) and the functional characterisation of the vast number of these proteins is still unknown. The diversity in RNA and RBP interactions is determined by the different RBPs and the variety of their RB motifs (often several RBDs are present in one RBP). That vast array of interactions poses a challenge to systematically study protein and RNA targets. The data available is still short but there is an increasing effort in identifying motifs and sequence preferences. Ray et al. 2013 aimed at profiling a small number of RBPs that would allow the assessment of sequence preferences of RBPs in eukaryotes. They analysed the sequence preferences of over 200 RBPs by incubating RBPs with a pool of RNAs in excess (RNAcompete) and recovering the proteins with its associated targets, these were then studied using microarrays. They concluded that RBPs do have sequence preferences, they also highlighted evolutionary conservation and selection of motifs in relation to function and ultimately they observed that closely related proteins have preference for similar targets (Ray et al. 2013). Overall, the motifs in RBPs can be used to infer the post transcriptional mechanism these proteins exert and the group created a compendium of motifs as a resource to aid in understanding interactions of RBPs and RNA transcripts, post transcriptional regulation, and health and disease processes. A web tool is available where users browse or bulk download motifs for all eukaryotic RBPs including direct measured motifs for more than 200 RBPs, as well as more motifs inferred by homology. Another group created a census of over 1500 RBPs manually curated and they analysed their interaction with different classes of RNAs, to what extent they have been conserved, their abundance and tissue expression (Gerstberger et al. 2014). In 2015 over 1300 genes that code for RBPs were also catalogued (Neelamraju et al. 2015). A web server for mapping RNA binding sites of RBPs has also been created and is freely available (Paz et al. 2014). A table with some of the web resources available regarding RBPs binding sites was published in a 2014 paper (Li et al. 2014).

mRNA transcripts containing AREs can be regulated by proteins binding to the ARE that may lead to mRNA degradation or to its stabilisation. These AUBPs have a key role in post-transcriptional gene regulation and hence their expression can impact the stability of gene products whilst impairment of their function can lead to diseases, such as, cancer (Audic and Hartley 2004). ZFP36 proteins are one family of AUBPs that mediate mRNA degradation and are the major focus of this thesis. Briefly, the role of other AUBP proteins that regulate the stability of mRNA transcripts, namely HuR and AUF will be considered here

HuR/ELAVL1 is an AUBP that can stabilize important mRNAs such as p53 or VEGF (Audic and Hartley 2004) and also BCL2 (Ishimaru et al. 2009), but has been reported to destabilise other mRNAs such as TNF α or STAT3 (Papadaki et al. 2009). HuR is also regulated by miRNAs, as miRNAs such as miR-16 repressed its translation (Xu et al. 2010). HuR is also known to play a positive role in cancer formation (Hacker et al. 2010). On comparing the binding preferences of ZFP36 and HuR (Mukherjee et al. 2014) found thousands of overlapping binding sites for both proteins ZFP36 binds and degrades mRNA via specific AREs that are actually a subset of the uracyl rich sequences that HuR interacts with to stabilise transcripts (Mukherjee et al. 2014).

AUF1 proteins also regulate BCL2 (Lapucci et al. 2002) and TNF α (Wilson et al. 2003) stabilising the mRNA transcripts. A key role of AUF1 is performed in hematopoiesis, the mice knockout for AUF1 show decreased T and B splenic cells, and this low number of cells was thought to be as a result of decreased pro-survival proteins like Bcl2 (Sadri et al. 2010).

Some AUBPs share the same mRNA targets as ZFP36 family members. The final balance in mRNA levels may be the result of the interplay of ZFP36 members and other AUBPs such as HuR which stabilise mRNA transcripts, leading to the idea of a network of AUBPs with inputs into the final balance in the levels of mRNA transcripts (Zanocco-Marani 2010). Targets mRNAs may be shared amongst the different families of AUBPs and this balanced regulation may be deregulated in disease.

1.2 B lymphocytes

B cells may be defined as a population of clones expressing clonally diverse immunoglobulins (Ig) aimed at the recognition of specific antigen epitopes. The initial landmarks in B cell discovery took place using animal models (chicken and mice) and studying immunodeficient patients (Good and Zak 1956)

The detection of immunoglobulin in serum led to the discovery of B cells (Tiselius and Kabat 1938), although the idea of an antibody producing plasma cell came about later on (Fagraeus 1948). The connection between B cells and antibody production was possible as technology advanced, and

eventually cell marker characterization technology allowed for a number of studies showing that immunoglobulin expression could serve as a marker to characterize normal and malignant B, and T, cells (Raff 1973)

1.2.1 B cell development

Normal B cell development starts in the foetal liver. Hemocytoblasts, multipotent hematopoietic stem cells in the embryo, develop into two types of progenitor cells, myeloid and lymphoid cells. B cells arise from a lymphoid progenitor cell that give rise to NK cells, T cells and B cells. From the foetal liver, as gestation advances, their site of production moves to the bone marrow (Tavian and Peault 2003). After birth B cell development continues in primary and secondary lymphoid organs. The bone marrow provides a specialised microenvironment for the initial stages of B cell development where stromal cells have a key role to the immature B cell stage (Nagasawa 2006). A series of interactions occur between stromal cells and B cells through adhesion molecules starting B cell proliferation. B cells in the bone marrow change the level of contact with stromal cells as they develop. The early stem cells are found within the bone marrow area closest to the inner bone surface, later they migrate within the marrow moving towards the centre of the marrow cavity. As development takes place the need for stromal cell contact decreases and eventually B cells leave the marrow as immature B cells to further develop into mature B cell in the peripheral lymphoid organs. Stromal cell IL7 signalling was discovered as a key cytokine in stromal cell marrow, needed in mice for B cell development for V to DJ rearrangement and transmitting survival and proliferating signals (Milne and Paige 2006).

In early B cell development the heavy (H) and light (L) chain of the immunoglobulin are rearranged (ALT et al. 1986). Recombination of the V, D and J genes H chain segments and V, J in the light chain (Brack et al. 1978) via RAG1/2 takes place mainly in the fetal liver and adult marrow leading to a diverse range of VDJH and VJL functional rearrangement to encode the B cell receptor (BCR) (Schatz et al. 1989, Oettinger et al. 1990). A surrogate light chain is first formed, a two protein heterodimer (Sakaguchi and Melchers 1986) that with the H chain form a pre-B cell receptor, characterizing the pre-B cell stage (Melchers 2005) following a pro-B cell stage. Pro-B cells do not express Ig or a pre-BCR. Pre-BCR is needed for B cell development as much as the BCR is needed for survival in the periphery (Lam et al. 1997, Bankovich et al. 2007)

B cells leaving the bone marrow acquire IgD and are CD21, CD22 positive (Chung et al. 2003) IgM IgD positive B cells enter the blood and migrate to the periphery, here and in the spleen the vast majority of B cells are mature IgM IgD positive B cells. At this stage these cells respond to T cell independent antigens, e.g. LPS, giving rise to a quick immune response lacking the intervention of MHC (major histocompatibility complex) class II T cells (Coutinho and Möller 1975). A range of transcription factors control early B cell development, importantly: E2A, EBF, Pax 5 in B cell lineage commitment and differentiation (Nutt and Kee 2007); a figure with indications to key transcription factors in B cell development can be found in the appendix. Amongst transcription factors with a

role in late B cell stages BCL6 has a key part in GC (GC) formation, BLIMP1 and STAT3 in plasma cell differentiation, Pax5 although not expressed in plasma cells has a key role in driving and maintaining B cell identity. BCL6 is suggested to negatively regulate other late B cell development transcription factors; BCL6 overexpression actually inhibits plasma cell differentiation suggestion that BCL6 inhibits STAT3 dependent expression of BLIMP1 (Reljic et al. 2000). And the inhibitory effect of overexpression of BCL6 was later observed in primary human B cells (Diehl et al. 2008). IRF4 is another factor present in late B cell stages, it is detected in plasma cells and centrocytes, not in centroblasts and GC B cell seem unable to differentiate into plasm in the absence of IRF4 (Klein et al. 2006). IRF4 ectopic expression in GC derived cell lines, Raji and Daudi, Burkitts lymphoma cells, has shown as a result an up regulation in the plasma cell marker studied in this project, CD38 (Teng et al. 2007).

Most B cells not residing in gut associated lymphoid tissue are found in lymphoid follicles and in the spleen and lymph nodes ready to encounter T cell dependent antigens bound to dendritic cells. B cells expressing CD40 interact and are activated by activated T cells through CD40L (CD154), proliferate and differentiate to plasma cells or form generate GCs which are composed of rapidly proliferating centroblasts. GCs are the main site for the generation of high affinity antibody secreting plasma cells and memory B cell generation (Coutinho and Möller 1975). Somatic hyper mutation (SHM) takes place in GCs and the highest affinity clones are selected to form either plasma cells or memory B cells (Jacob et al. 1991, Kelsoe 1996). B cells going through SHM of their Ig variable domains introduce microdeletions and single nucleotide modifications creating clones with different and varied antigen affinities. Class switching also takes place and B cells with the highest affinity antibody will survive with the remaining B cells apoptosing due to their lower antibody affinity and also B cells will express different classes (isotypes) of antibody (Nemazee et al. 1999).

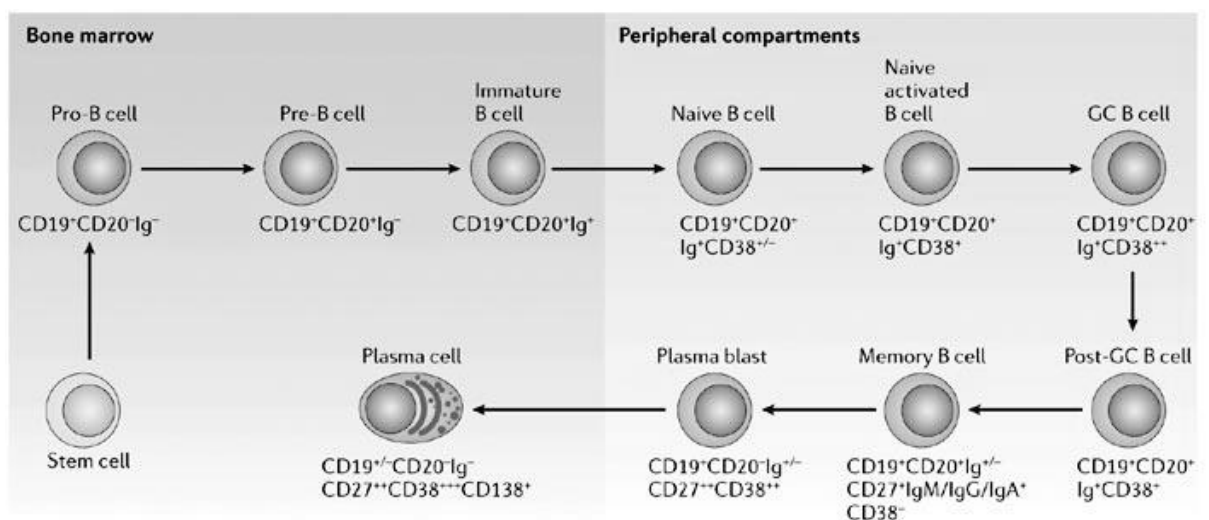


Figure 1-8 Stages in B cell development.

The figure shows the pathways in B cell development and expression of different cell surface markers and IgM and IgD. Imagemodified from Edwards and Cambridge (2006). A more detailed diagram can be found in the appendix.

B cells enter the spleen via the blood and are found in the white pulp follicles, these may have GCs if the B cells have actively engaged in an immune response. In the marginal zone, within the white pulp, there are marginal zone B cells, a different set of resting B cells that also express different markers to follicular B cells, these cells are somewhat similar to B1 cells. B-1 cells are B lymphocytes not involved in the humoral response, are not part of the adaptive immune system and nor have B cell memory function, they do produce antibodies and also act as antigen presenting cells. B1 cells are present in the peritoneal and pleural cavity (Tangye 2013). In the mantle zone of lymph nodes there are CD5+ or CD5- B1 cells (Hayakawa et al. 1983; Hardy and Hayakawa, 2003). B1 cells assist in the innate immune system by protecting against bacteria in naïve hosts. B1 CD5- cells have a role in long-term adaptive immune response to LPS and T independent antigens (Haas et al. 2005). Marginal zone B cells do not seem to need T cell involvement to become activated (similar to B1 cells which are T cell independent cells). Marginal Zone B cells are thought to possess antibodies with low antigen specificities and more affinity for self-antigens, they provide a rapid response for antigen entering via the bloodstream. As opposed to B1 cells which do express high levels of CD5, marginal zone B cells do not express this cell surface marker.

Lymph nodes regularly show follicles of B cells generating GCs upon activation. B cells enter the lymph node, also via the blood, passing through the T cell area to the follicles, here they proliferate, undergo somatic mutation and class-switching and go through affinity maturation and cell selection after encountering antigens held on follicular dendritic cells, then the cells become either memory B cells or plasmablasts. A highly affinity mutated BCR will fast react upon a second antigen exposure rapidly differentiating into antibody producing plasma cells (McHeyzer-Williams and McHeyzer-Williams 2005). Plasmablasts leave the lymph node via the efferent lymphatic back to the blood. Plasma cell development is tightly regulated by factors such as BCL6 and BLIMP1 (Shapiro-Shelef and Calame 2005). Plasma cells become resident in the bone marrow.

Some immune pathologies arise from errors in the B cell maturation process, for instance, defects in CD40;CD40L interaction result in Hyper IgM syndrome. Class switching and SHM are tightly controlled and errors in these processes can lead to activation of oncogenes, for instance Burkitts lymphoma, where there is over-expression of the c-Myc gene (Vale and Schroeder 2010). One of the factors that influence survival of a B cell is its pre-B cell receptor (BCR), its development and correct function is essential for the development of B cells pass the pre-B cell stage.

1.2.3 B cell Malignancies

The balance in B cells by the continuous production of new B cells and their loss ensures a healthy homeostatic state and a variety in the receptor possibilities for recognising new antigens.

The B cell development stages depicted in the previous section are those of a normal B cell; however, those development stages may present a variety of problems that include immune pathologies (e.g. Hyper IgM syndrome) or malignant versions of each stage where a dominant clone expands leading to pathologies such as leukaemia and lymphoma (fig. 1-10).

We have knowledge of gene expression due to profile studies in normal and malignant B cells (Staudt and Dave 2005); one key protein, which is the centre of a chapter in this thesis, is BCL2. In follicular lymphoma (FL) the anti-apoptotic BCL2 is translocated with IgH loci and BCL2 is overexpressed (Tsujimoto et al. 1984). The ZFP36 proteins have been reported to be involved in cancerous processes; a deletion in chromosome 14(q) has been reported to be present in different B cell Malignancies; this deletion affects the *ZP36L1* gene (Pospisilova et al. 2007).

Blood malignancies are usually grouped in three main categories: lymphomas, myelomas and leukaemias, although the boundaries within and between each category may not be clearly defined especially when patients show symptom across the three categories.

Lymphoma, specially non-Hodgkin, and leukaemia are common haematological malignancies in the UK. Myeloma is less commonly found but still prevalent (Cancer.gov, 2015). Lymphoma affects the lymphoid system, divided in non-Hodgkin (NHL) and Hodgkin lymphoma (HL) the latter type is the most common amongst the two. In both cases the number of lymphocytes is increased. NHL can be of T or B cell whereas HL is diagnosed if Reed-Steinberg cells are detected, these are malignant B cells that almost in all cases arise from GC B cells that cannot synthesize immunoglobulin (Bräuninger et al. 2006, Mathas et al. 2006)

Leukaemias affect white blood cells, B and T lymphocytes and are further classified depending on the aggressiveness of the disease, acute or chronic. The cell development stage at which leukaemia arises has an impact on the life course of the disease determining a slow, insidious progression or a rapid, acute onset of the disease. B-CLL is further discussed later on.

And finally **Myeloma**, or Multiple Myeloma (MM), affects terminally differentiated plasma cells and ultimately **Ig** production; the prevalence in the UK is less than that of lymphoma and leukaemia (Cancer.gov, 2015). The bone marrow is also thought to play an important role in the development and maintenance of this cancer, even on therapy resistance although a direct link and how this may happens remains to be known (Hideshima et al. 2007). The initial phase of myeloma is usually characterised by asymptomatic monoclonal gammopathy of undetermined significance (MGUS) followed by MM, and eventually the more aggressive plasma cell leukaemia. In myeloma plasma cells have accumulated genetic hits. The genetic aberrations are classified as initiation (IgH translocations) and progression events (chromosomal deletions and gains) (Boyd et al. 2012). The first genetic abnormalities are thought to occur at the time of SHM and isotype switch within GCs. Further genetic mutations take place on transformation of MGUS into a myeloma cells and on progression of the disease (Bianchi and Munshi 2015). Besides the low frequency of BCL2 translocation t(14;18) in MM patients the protein itself is actually found at increased levels in primary and cell lines of MM (Spets et al. 2002). Symptoms and survival varies from patient to patient, varying from 5 to 10 years. MM is treatable although there is no cure and the incidence is the

disease is low with about only 15000 to 20000 people suffering from the condition at a given time in the UK, about 4800 are newly diagnosed each year, making the second most common bone marrow cancer but only 1% of total cancers (myeloma.org.uk 2015).

Figure 1-9 shows cell type/development stage affected by malignancy and classification

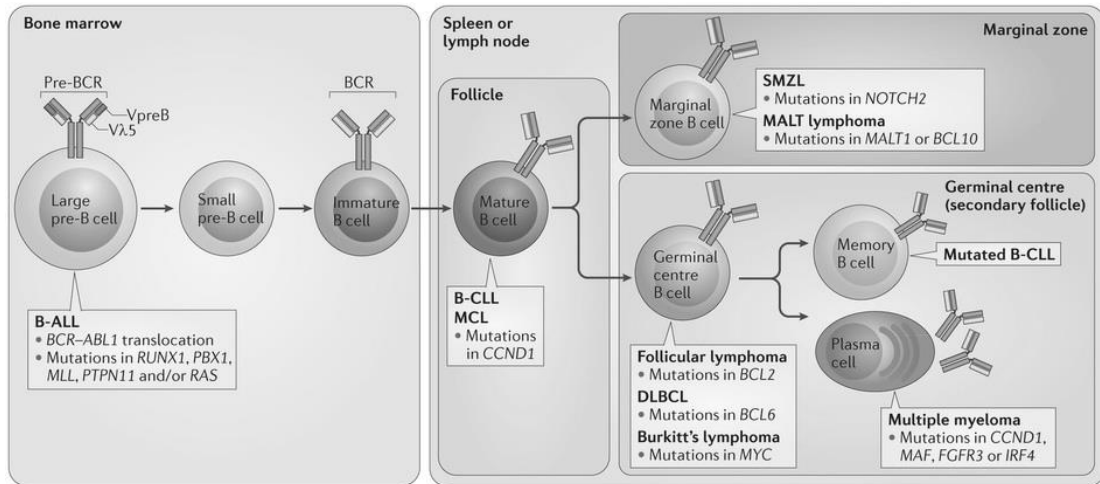


Figure 1-9 Malignancies characteristic of each B cell development stage.

Reproduced and modified from Rickert (2013) New insights into pre-BCR and BCR signalling with relevance to B cell malignancies, Nature reviews/Immunology

1.2.4 B cell chronic lymphocytic leukaemia

B-CLL is a common B cell malignancy, characterised by accumulation of anti-apoptotic mature B cells. It is the most common type of leukaemia in the Western world; with an incidence that rises alongside age it is usually seen in older patients and males (Hernández et al. 1995, Sant et al. 2010) although diagnosis in younger individuals is not uncommon (Hernández et al. 1995) Because B-CLL cells express mostly CD19+ B-lineage phenotype the disease is called B-CLL but CLL also affects T lymphocytes although just a small proportion compare to the incidence of B-CLL

This type of B cell malignancy is characterised by significant accumulation of apoptosis-resistant monoclonal B-lymphocytes in peripheral blood and lymphoid organs. Although there are B-CLL cases called familial, the pattern of inheritance is not clear although it is estimated that one in every 10 patients with B-CLL has either a family history of B-CLL or there are links to other lymphoproliferative disorders within the family, calculating a 30 fold increment in the risk of developing B-CLL with first degree relatives (Johnston JB 2009).

Diagnosis of B-CLL, as revised by the international workshop in B-CLL, 2008, is based on $5 \times 10^9/L$ monoclonal B lymphocytes peripheral blood for at least 3 months and the clonality of circulating B lymphocytes needs to be confirmed by flow cytometry. Typical immunophenotype of B-CLL lymphocyte is CD5+, CD23+, CD43+/-, CD10-, CD19+. CD20 dimly expressed in B cells is also characteristic in B-CLL, expression of this marker and of surface Ig assist in distinguishing B-CLL from mantle cell lymphoma in CD23 is not expressed. Whilst flow cytometry is essential for correct

diagnosis, bone marrow examination and a CT scan might be required for diagnosis and or staging (Hallek et al. 2008)

Two different staging systems are used in B-CLL. The Rai system (Rai et al. 1975) tends to be used in the United States and the Binet system (Binet et al. 1981) is commonly used in Europe. Both systems look at blood count and physical examination to subset patients in groups and establish median times of survival.

Table 1-3 Rai and Binet B-CLL staging systems

<i>Rai staging (1975)</i>				
Stage	Lymphadenopathy	Hepatomegaly or Splenomegaly	Hemoglobin (g/dL)	Platelets (Platelets/ μ L)
0	None	None	>11	>100,000
I	>1	None	>11	>100,000
II		>1	>11	>100,000
III			<11	>100,000
IV				<100,000
<i>Binet staging (1981)</i>				
Stage	Number of Involved Areas	Hemoglobin (g/dL)	Platelets (Platelets/ μ L)	
A	<3	>10	>100,000	
B	>3	>10	>100,000	
C		<10	<100,000	

Modified from Parker and Strout (2011) .

One system proposed looking at lymphadenopathy, organomegaly and cytopenias (anaemia and thrombocytopenia) to establish prognostic groups, stage 0 >150 months, stage I, 101 months, stage II, 71 months, stage III or IV, 19 months (Rai et al. 1975).The other proposed a system where patients are stratified in three stages looking at nodal areas and cytopenias (Binet et al. 1981).

Cancer heterogeneity and B-CLL

Cancers deriving of the same organ may be further classed in different subtypes and within these subtypes each cancer may contain different tumour cell populations that confer each cancer specific clinical characteristics such as invasive potential (Felipe De Sousa et al. 2013). Inter tumoural and intra tumoural heterogeneity are a challenge for successful cancer therapy. A number of factors (genetic, epigenetic, tumour microenvironment, origin of cancer) influence this diversity (Felipe De Sousa et al. 2013). Intra clonal diversity has been used to explain therapy resistance: selective pressure on cancer cells leads to resistant clones. Advantageous mutations- “driver” mutations- over “passenger” mutations alongside mutator lesions and microenvironment changes become key to explain clonal evolution (Greaves and Maley 2012). Passenger mutations are non function related somatic mutations occurring in cell division often found in cancer genomes, these mutations were present in ancestor cells when they acquired the driver mutations. Driver mutations grant a

growth advantage for the carrier cells, these cells are positively selected during the cancer evolution, surviving over the cells carrying passenger mutations (Stratton et al. 2009).

In efforts to make records of somatic mutations in the different types of cancers databases like COSMIC or ICG DCC have been created. COSMIC stands for Catalogue of Somatic Mutations in Cancer and now it even curates mutations that offer cancers resistance to drugs (Forbes et al. 2015). ICG DCC is the International Cancer Genome Consortium Data Coordination Centre and offers the opportunity to browse genes of interest or cancer types and retrieve information on mutation status (Zhang et al. 2011). For ZFP36L1 the web based tool shows that the gene was found to be mutated in malignant lymphoma and B- CLL samples from donors, the number of mutations and other details information may also be found in the site. A map of the gene with low, high and unknown mutation impact is also available, for ZFP36L1 about the same number of mutations are found to have a high functional impact on the protein than low impact, and about half that number is unknown. ZFP36 is also found mutated in malignant lymphoma and to a lesser rate in B-CLL. Malignant lymphoma donors also showed mutations in ZFP36L2 (ICGDCC 2016).

In B-CLL the clinical heterogeneity is great and leads to variability in the response to treatments with patients needing clinical intervention promptly but others not requiring treatment in years or not at all. As its names states it is a chronic condition, prognosis depends on subtype and stage and although the five year survival has increased in the last three decades to eight years (Molica and Levato 2001) the individual survival is greatly variable, again markedly due to the disease's heterogeneity. A number of markers have been found that assist in understanding the biology of B-CLL and classifying patients in different subtypes with similar clinical course and prognosis. Overall, this classification of B-CLL patients in subtypes aims at enabling a successful therapy choice. In broad terms the major and most studied subtype is that formed by patients with mutated and unmutated IgHV. Cytogenetic changes have also been linked to prognosis (17p del, 13q del) alongside CD38 positivity. The discovery of new prognostic markers were added to the staging systems in use: tables 1-3 and 1-4 show Rai and Binet systems and common prognostic markers in B-CLL. Recently it has been proposed that genetic variability within a tumour may lead to a higher aggressive disease course and in B-CLL it may explain tumour progression (Ecker et al. 2015). Ecker et al. (2015) showed that within the more aggressive type of leukaemia, IgHV non mutated, there is a higher gene expression variability; and in patients with less aggressive disease course the variability was lower. Overall, the group led by Alfonso Valencia highlighted that the coefficient of variation for gene expression in B-CLL can predict its aggressiveness and this variability may be use to from a classifier of subtype of B-CLL patients. The IgHV unmutated subtype of B-CLL was also found to have more driver mutations, some of these newly identified as putative cancer genes (Landau et al. 2015).

Table 1- 4 Common prognostic markers in B-CLL.

Prognostic Marker	Incidence, %	Median Survival, Years
IgHV mutated	55	24
IgHV unmutated	45	8
CD38 >30%	30	<10
CD 38 <30%	70	>15
ZAP-70 >20%	60	6-10
ZAP-70 <20%	30-40	>15
Deletion 13q	55	17
Deletion 11q	18	6-8
Trisomy 12	16	9-11
Normal cytogenetics	18	9-11
Deletion 17p	7	2-3

The table shows a list of commonly used markers, with incidence and survival time, to assess disease prognosis, CD38 positivity is linked to a poor outcome. Table modified from Parker and Strout (2011).

B-CLL prognostic markers, an overview

Lymphocyte doubling time is the time needed for lymphocyte to double the number at time of diagnosis; less aggressive diseases are characterized by a doubling time of more than 12 months, median survival of 5 years (Molica and Alberti 1987, Damle et al. 2010, García-Muñoz et al. 2012)

IgHV mutational status: mutated IgHV is linked to earlier stage disease and longer survival compared to unmutated IgHV ((Hamblin et al. 1999, Damle et al. 2010). **ZAP70** is normally expressed in T cells, ZAP70 expression in B-CLL cells was found to correlate with IgHV mutational status and poor outcome (Rosenwald et al. 2001, Crespo et al. 2003) however, discordance between both factors is also found in about a quarter of patients and ZAP70 was finally established as an independent marker (Rassenti et al. 2004). **CD38 positive clone**, explained in detail later, suffice to say that a B cell population is assessed as CD38 positive if 20% or 30%- depending on the lab-of the clone expresses the cell marker. CD38 positivity is indicative of a poor prognosis (Hamblin et al. 2002). The majority of B-CLL patients are found to have acquired **chromosomal abnormalities**, in fact as much as 80% of them; the most common abnormality is a deletion in 13q, found in about 55% of patients (Döhner et al. 2000). 11q deletion is found in around 18% patients and is associated with other poor prognosis factors such as unmutated IgHV (Donner et al. 2000). Trisomy 12 is found in a slightly less proportion of patients, whilst 18% of them are found to have normal cytogenetics (Döhner et al. 2000) the least common is a deletion in 17p, 7% of patients, and is associated with unmutated IgHV and a poor prognosis (Döhner et al. 2000), the deletion seems to affect p53 (Zenz et al. 2010).

For this cancer, as for any other cancer, the molecular mechanisms that lead these cells to behave in an aberrant manner are not fully understood. Although research is advancing in the discovery of how cells become cancerous, we are yet to understand its exact process and the need to find new therapeutic targets is still an imperative.

1.3 BCL2 protein family

BCL2 (B-cell lymphoma 2), encoded by the *BCL2* gene, is the founding member of the cell death regulators “BCL2 family”. Within the family some proteins have a pro-apoptotic function (Bax, Bak, BAD as examples) and others, like BCL2 an anti-apoptotic effect (Thomadaki and Scorilas 2006) (fig 1-10). Structurally the family members have conserved domains and their presence varies amongst the two groups, pro -apoptotic members of the family usually lack the BH4 domain and the anti-apoptotic members have usually the BH1 and BH2 domains at least, but the ones similar to BCL2 have the four domains (Adams and Cory 1998, Thomadaki and Scorilas 2006). The BCL2 family is divided into three subgroups: the multi domain/multi region pro-apoptotic proteins that directly permeabilise the mitochondrial outer membrane and the BCL2 homology domain 3 (BH3)-only members (also pro-apoptotic) that directly or indirectly activate the pore-forming class members and the anti-apoptotic proteins that inhibit this process at several steps (Shamas-Din et al. 2013).

The family of proteins is generally believed to be cytoplasmic and predominantly membrane-associated present in the outer membrane of the mitochondria, their main site of action, and the endoplasmic reticulum (ER). The pro survival BCL2 family members are generally found in the outer mitochondria membrane where they inhibit the pro-apoptotic BCL2 partners, and the pro-apoptotic proteins exert their activity by facilitating the permeabilisation of that membrane (Reed 2008).

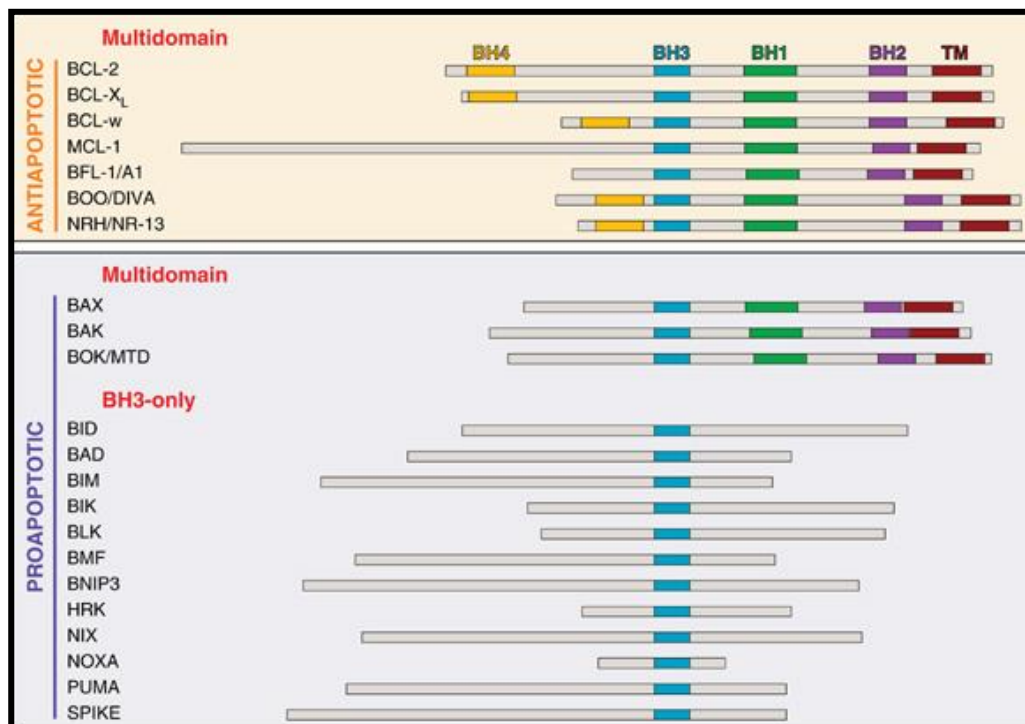


Figure 1-10 Selected proteins in the BCL2 family.

Pro- and anti-apoptotic proteins interactions influence cell survival, the proteins of the BCL2 family are important regulators of apoptosis and can act as pro apoptotic (Bax, BAD, Bak and Bok among others) or anti-apoptotic (including Bcl-2, Bcl-x_L, and Bcl-w, among others). Image modified from Walensky (2006) .

BCL2's name is linked to its discovery and role in the development of B cell lymphomas. It was first described in chromosomal translocations –chromosomes 14 and 18- in FLs (Tsujiimoto et al. 1984, Cleary et al. 1986) (Bakhshi et al. 1985). The t(14;18) is seen in 85% of FLs and 20% of diffuse B cell lymphomas (DLBCL), the result in the translocation is the positioning of *BCL2* at chromosome segment 18q21 juxtaposed with the heavy chain immunoglobulin gene leading to a deregulated expression of *BCL2* (Vaux et al. 1988a). The description of BCL2's anti-apoptotic and tumorigenic role resulted from a landmark piece of work by (Vaux et al. 1988b). Unbalanced and deregulated apoptosis plays a role in a number of pathologies: uncontrolled cell death is characteristic in diseases such as Alzheimers; whereas uncontrolled cell growth or lack of cell death determines cancerous processes.

This commonly termed “programmed cell suicide” is driven by caspases; cysteine proteases caspases are sequentially activated and lead to apoptosis in two pathways. The different initiator caspases in either apoptotic pathways lead to end point cleavage and activation of effector caspases (caspases 3, 6 and 7) which in turn can cleave cellular proteins critical for cell survival (Adams 2003, Shi 2006). Caspases 8 and 10 are activated after ligation of “death receptors” of the TNF family on the cell membrane: after a death signal, Fas associated death domain protein (FADD) attaches to caspase 8 and activates it, and further activate caspase 3 to lead to proteolytic activity, another option is that after caspase 8 attachment to FADD Bid is attached by caspase 8 and the cleaved truncated Bid protein translocates to mitochondria and acts with pro-apoptotic proteins (Bax, Bak) and induce release of cytochrome C which binds to Apaf1 and leads to a conformational change forming the apoptosome; the apoptosome triggers activation of caspase 9 which further activates caspase 3, caspase 7, caspase 6 ultimately resulting in death in the extrinsic apoptosis pathway (Adams 2003, Shi 2006). The extrinsic pathway operates independently of p53 and immune cells like cytotoxic T cells may be involved producing ligands that bind to the “death receptors” and induce the cascade of events delineated above: Cytotoxic signals translocate BCL2 pro-apoptotic members to organelles and there they bind to the anti-apoptotic counterparts inhibiting their action and triggering apoptosis (Adams and Cory 2001). The intrinsic apoptotic pathway is induced by a number of internal stimuli; in broad terms, the intrinsic pathway is induced by lack of positive signals for cell survival (e.g. lack of growth factors, cytokine starvation) or by internal signals of accumulation of oxidative signals, misfolded proteins, endoplasmic reticulum stress signals, DNA damage. If the positive signals are present e.g. trophic factors, they bind to a trophic receptor and that binding results in phosphorylation of pro-apoptotic Bad protein, this phosphorylated Bad cannot bind to mitochondrial membrane bound BCL2 and Bcl-XL complex to inhibit their survival action. In the opposite scenario, absence of trophic factors, Bad is not phosphorylated and binds to BCL2/BCL2-XL inhibiting their pro survival activity, Bax, (pro-apoptotic), is freed and permits an influx of ions and thus the release of cytochrome C into the cytosol, here cytochrome C binds to adaptor protein Apaf1 and pro caspase 9, forming the apoptosome, and containing activated caspase 9. Caspase 9 can cleave pro caspase 3 into its

active form and further cleaves downstream targets that lead to photolytic activity within the cells (targeting the nuclear lamina, the cytoskeleton etc.) if the intrinsic pathway is triggered by DNA damage, p53 stimulates the transcription of pro apoptotic BCL2 proteins and are released by the mitochondria (Adams 2003, Shi 2006).

The BCL2 family not only has residence and direct action on the mitochondrial membrane, it also resides and functions on the ER, members of the three subgroups have been identified at the ER regulating the induction of apoptosis (Zhu et al. 1996, Germain et al. 2002, Zong et al. 2003, Chen et al. 2004, White et al. 2005). When apoptosis signalling is induced by ER stress, cell death also depends on cytochrome C release by mitochondria into the cytosol and opening of the permeability transition pore which leads to imbalanced mitochondrial membrane potential due to Calcium intake after its release into the cytosol. Once cytochrome C is released the APF1 binding step and other subsequent events already described take place (Samali et al. 1999, Zou et al. 1999, Acehan et al. 2002).

A number of models have been proposed to explain the anti-apoptosis mechanism of pro survival molecules: the sequestration model, mitochondrial integrity model, and Caspase inactivation action where BCL2 proteins interact with other molecules and inhibit caspases activation (Chinnaiyan et al. 1997, Wu et al. 1997); a recent review of anti-apoptotic mechanism and cell survival can be found in Portt et al. (2011) where the authors describe in detail molecular mechanisms of cell survival in health and disease.

One model explaining how BCL2 and homologs exert their pro survival functions is that the anti-apoptotic BCL2 family members may stop the apoptosome complex from forming by sequestering the scaffold protein and/or by stopping apoptogenic factors from organelles such as the mitochondria (Cory and Adams 2002). BCL2 and homologs may sequester the pro-apoptotic proteins of the family therefore stopping their cell death signalling (Yin et al. 1994). This "kidnapping" takes place by binding of BH3 domain of pro-apoptotic counterparts; the BH3 α helix of the apoptogenic proteins can bind to a hydrophobic groove on the pro-survival proteins and preventing their pro cell death function (Adams and Cory 2001), concretely BAX and BAK mediated mitochondrial apoptosis (Emily et al. 2001). This type of anti-apoptotic pathway is the centre of recent approaches in cancer therapy, a number of BCL2 inhibitor therapies have been trialled, the bases if these therapies is that when BCL2 is antagonized by a BH3 mimetic molecule, the pro-apoptotic molecules are released to lead to death, (this is what can occur in B-CLL). This type of death is based on permeabilisation of the mitochondrial outer membrane by BAX or BAK, pro-death BCL-2 family proteins (Vogler et al. 2009).

Lack of cells death and hence oncogenesis can be the result of anti-apoptotic genes being over-expressed whilst pro-apoptotic genes are under-expressed. Different cancers have shown BCL2 being over expressed (Thomadaki and Scorilas 2006, Thomadaki et al. 2007), including NH

lymphoma (Waters et al. 2000) and B-CLL (Adachi et al. 1990). In leukaemic cells, that BCL2 over expression has been shown to lead to drug resistance in chemotherapy (Miyashita and Reed 1993). Although it is possible that in some cases, e.g. lymphomas, anti-apoptotic BCL2 over-expression in lymphocytes alone does not automatically lead to a oncogenesis; concomitant over-expression of BCL2 and the myc proto-oncogene can lead to aggressive B-cell malignancies, lymphoma being one (Otake et al. 2007).

Reviews by Reed and Pellecchia (2005) and more recent one by Zivny et al. (2010) list some of the advances in the knowledge of the regulation of apoptosis in cancer therapies. The review discusses some of the drug discovery targets identified and some of the progress to date towards translating the growing knowledge of these targets into new therapies for cancer and concretely leukemia. In both reviews, especially in the 2005 paper, the targeting of BCL2 with therapeutic potential is described at the level of gene expression, mRNA, protein, and endogenous activation of BCL2 antagonists, highlighting the key role of BCL2 in the research around the control of apoptosis and bringing up its relevance in this research project.

The 3'UTR of *BCL2* mRNA is a binding site for RNA binding factors that regulate BCL2 expression by promoting stabilisation as in the case of HuR (Ishimaru et al. 2009) or decay of the transcript like AUF1 (Ishimaru et al. 2010) or Tino (Donnini et al. 2004).

1.3.1 BCL2 and B lymphocytes:

BCL2 is an inhibitor of apoptosis in differentiating B cells and hence it is low or absent in stages where cells are lost by apoptosis and highly present in stages where cells are long lived. BCL2 is expressed in bone marrow B cells, in pro-B cells and in mature B cells, whereas pre-B cells and immature B cells lack its expression (Merino et al. 1994). GC B cells express BCL2 only after activation by signals that prevent their entry into apoptosis (Liu et al. 1991). BCL2 expression is not seen in proliferating centroblasts in the dark zone and centrocytes in GC light zone, they go through apoptosis when their antigen affinity is lowered by hyper mutation, as opposed to the B cells in the mantle which do express BCL2 GC B cells, centrocytes centroblasts lack this protection against apoptosis by BCL2 and are programmed to die unless rescued by high affinity interaction with the antigen. Normal resting peripheral B lymphocytes do not express BCL2 unless they receive mitogenic stimulation (Graninger et al. 1987, Reed et al. 1987).

1.3.2 In neoplasias the expression of BCL2 is altered:

Normally expressed in small B cells of the mantle and marginal zone (Meda et al. 2003) (and in some T cells) BCL2 is over expressed in FLs with the t(14:189 translocation, present in nearly 80% of these (Weiss et al. 1987, Pezzella et al. 1990a, Horsman et al. 1995) and in 20% of DLBCL (Weiss et al. 1987)- although BCL2 over expression is also seen in lymphomas without the translocation-. It is common that B cells in the centre of the neoplastic follicle express BCL2 at

higher levels than the cells in the mantle zone, but this pattern is variable, not only due to the innate differences between cases of the same neoplasm but also due to the different grades or type of the disease, as in the case of grade III FL where BCL2 positivity is not common (Nguyen et al. 1996); BCL2 expression is greater in grade I and grade II types

Neoplastic expression of BCL2 not only increases cell survival but also can confer resistance to chemotherapy induced cell death. c-Myc transcription factor is coded by *c-Myc* proto oncogene and it is at the same time a cell proliferation and apoptosis inducer (Askew et al. 1991). In B cells c-Myc effects proliferation (de Alboran et al. 2001). C-Myc expression sensitises the cells to a number of pro-apoptotic factors thought to happen via cytochrome c release (Juin et al. 1999), but in normal environment c-Myc over expression leads to apoptosis (Evan et al. 1992) and proliferation (Evan and Littlewood 1993).

The previously mentioned work by David L Vaux et al. in 1988 highlighted the cooperation between BCL2 and c-Myc promoting proliferation of pre-B cells and cell survival to immortalise pro-B cells; they observed how over expression of BCL2 led to neoplasia and this was synergised by c-Myc over expression concluding first that proliferation and survival are different pathways and are controlled by different signals and that inhibition of apoptosis is core in tumorigenesis. In general both proto oncogenes are found deregulated in lymphomas, in fact translocations of both genes have been reported in a number of cases which may have implications in identifying high risk patients (Johnson et al. 2009). A paper published from the "The International DLBCL Rituximab-CHOP Consortium Program" recently reported that co-expression of both proteins is linked to an aggressive course of the disease with an inferior prognosis for patients (Hu et al. 2013).

1.3.3 BCL2 in B-CLL:

In about 10% of B-CLLs the *BCL2* gene is translocated resulting in its known increased expression (Schena et al. 1992) but even without that translocation B-CLL has been shown to be consistently dependent on BCL2 (Moore et al. 2007) protecting B-CLL cells from apoptosis *in vitro* and *in vivo* (Jewell et al. 1994).

It is known that anti-apoptotic BCL2 is required for maintenance of leukemia (Letai et al. 2004) and recently, targeted BCL2 inhibition is proving effective in B-CLL (Roberts et al. 2012). Most of the research on the role of BCL2 in B-CLL has been done in its function of binding BH3 domain pro-apoptotic proteins and the majority of recent cancer therapies are aimed at BH3 mimetic molecules. BCL2 are bound to these mimetics and pro-apoptotic molecules are released to promote B-CLL cell death. Patients with relapse or refractory B-CLL were treated with navitoclax, a BCL2 inhibitor, it selectively binds to BCL2 and BCL-xL and induces the mitochondrial apoptotic pathway by avoiding BCL2 mediated sequestration and the inactivation of pro-apoptotic factors. The study showed that 90% of patients who had peripheral blood lymphocytosis showed a reduction of 50% just after day of administration of treatment; this was linked to increase apoptosis of circulating

leukemic cells. The disease free progression after the treatment was of 25 months. 31% of patients showed a partial response and nine of them did not respond to treatment. The positive outcome of the trial was not without side effects as patients reported thrombocytopenia, grade 4, probably mainly due to targeting of BCL/xL. An interesting piece of information arose from looking for biomarkers responsible for the response to the drugs, and they found that levels of BCL2 were not altered, no correlation between BCL2 levels and response was found but the researchers hypothesised that the drug also targets cells expressing another pro survival factor, MCL-1 via displacement of its inhibitor BIM from a complex with BCL2 (Roberts et al. 2012), overall establishing BCL2 as a potential and valid therapeutic target in B-CLL.

The BCL2 protein family not only controls mitochondrial permeabilisation during apoptosis, also controls cytoplasmic calcium levels (Rong and Distelhorst 2008). If BCL2 at the mitochondria engages the BH3 domain of pro-apoptotic proteins and inhibits their pro cell death function, at the ER BCL2 modulates calcium signalling leading to proliferation and resistance to apoptosis, but here BCL2 acts via its BH4 domain which binds to inositol 1,4,5 triphosphate receptor (IP3R), a membrane glycoprotein acting as a channel. IP3R is the main intracellular Ca²⁺ channel with a diverse biological role that include apoptosis; BCL2 can dampen Ca²⁺ oscillations that would induce apoptosis. Evidence of a complex between IP3R and BCL-2 provides the connection to BCL2 and physiologic control of calcium levels (Palmer et al. 2004, Zhong et al. 2006). Late research has aimed at targeting that BH4 domain triggering apoptosis in B-CLL cells and DLBCL. Zhong and colleagues report on a peptide that selective targets B-CLL cells via disruption of BCL2 interaction with IP3R (Zhong et al. 2011).

Deletion of miR15 and miR16 genes, which are responsible for encoding suppressors of BCL2, lead to over expression of the protein, these two genes are commonly deleted or down regulated in many B-CLLs. Both deletion and up regulation of expression of miR15 and miR16 are inversely correlated to that of BCL2, regulating BCL2 at post-transcriptional level. miR15 and miR16 have proven to have an antisense role to BCL2 including the induction of apoptosis by targeting BCL2 when the miRNAs are expressed (Calin et al. 2002, Cimmino et al. 2005) one of these miRNA, miR-16 is involved with ZFP36 in the post transcriptional control of other genes (Jing et al. 2005), as described in section 1.1.6 providing further ground to study the ZFP36 protein family as possible regulators of BCL2 expression.

1.4 Cluster of differentiation 38, CD38

CD38 (cluster of differentiation 38), also known as cyclic adenosine diphosphate (ADP) ribose hydrolase, is an ectoenzyme, CD38 was discovered as part of research done on T cell surface molecules by Reinherz and Schlossman, giving rise to a list of novel molecules (Reinherz et al. 1980). CD38 is a multifunctional protein, CD38 has receptor/receptorial functions and enzymatic functions; it is a type II transmembrane glycoprotein, with an L shape form and has two domains, the C domain with four parallel beta sheets is the extracellular and catalytic domain (Liu et al.

2005); the N terminal is formed by a number of alpha helices and two beta strands. A hinge region connects both domains, disulphide bonds further stabilise the structure (Lee 2006). CD38 has also been described in different intracellular organelles (Malavasi et al. 1994, Yamada et al. 1997, Sun et al. 2002), including the nucleus (Adebanjo et al. 1999, Khoo et al. 2000, Khoo and Chang 2002).

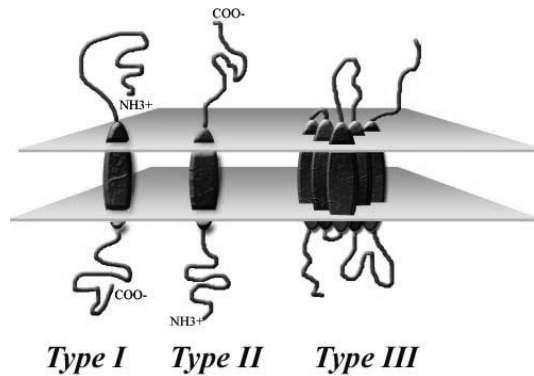


Figure 1-11 Schematic structure of transmembrane proteins.

CD38 is a type II transmembrane protein. Type I has a single transmembrane α -helix (bitopic membrane protein) and type III a polytopic transmembrane β -sheet protein. Image modified from proteinchemist.com (2015)

CD38 is part of a family of proteins. After CD38, CD157 was identified as a family member, they have sequence similarity and function analogy (Itoh et al. 1994). In murine cells CD157 expression is seen in synovial, vascular endothelial and follicular dendritic cells; the molecule is seen expressed in pre-T cells in the foetal thymus (Godfrey et al. 1993, Godfrey and Zlotnik 1993). In B cells it is observed in pre-B cells and circulating immature B cells that recently left the bone marrow (Ishihara et al. 1996). CD38 and CD157 are part of a nucleotide metabolising enzyme family with shared evolutionary history, other members of this family are CD26, CD39, and ADP ribosyl transferases (Malavasi et al. 2008).

One of the first groups to highlight the possible receptor activities of CD38 even before a ligand was found were Mehta et al. (1996). They concluded that its enzymatic and receptor function were independent after observing the effects CD38 ligation (blocking lymphopoiesis, inhibiting apoptosis as discussed in section "*CD38 expression: B lymphocytes*") without affecting the enzymatic ability of the molecule. Within these observations it was also seen that CD19 is part of the receptor signalling cascade of CD38 in B cell precursors acting as a docking site for kinases and activating the proteins of this type (Kitanaka et al. 1997). It seems that the CD38 channel is autonomous from other surface receptors, ligation of CD38 in circulating B cells induced CD25, HLA-II and mRNA of a number of cytokines leading to proliferation of the cells, this effect was also seen in B blast cells, but Ig production was never observed (Funaro et al. 1997). Interest in CD38 signalling is especially relevant as it is thought to be a co-receptor that has a key role in B-CLL as it will be discussed further on.

As an enzyme CD38 is involved in Ca^{2+} signalling, its enzymatic activity was first highlighted in research done on intracellular messengers in the *Aplysia* sea mollusc as a result of observing the

similarities between CD38 and the ADP ribosyl cyclase (ADPRC). Later the similarity between human lymphocyte CD38 and *Aplysia*'s ADPRC was further confirmed by DNA sequence analysis (States et al. 1992). This observation led to increasing research over the years in ectoenzymes and their catabolism of nucleotides and certainly CD38 enzymatic role. The enzymatic functions of CD38 contribute to the array of its immunoregulatory functions.

The main catalytic functions of CD38 are ADP-ribosyl cyclase and hydrolase activity:

A. CD38 acts as a glycohydrolase on nicotinamide adenosine dinucleotide (NAD⁺) inside the cell, catalysing it into ADP ribose and nicotinamide, molecules that have a role in Ca²⁺ signalling. CD38's role in calcium signalling has been widely studied, it is considered the main ADP ribosyl cyclase in mammals, CD38 transfers the ADP ribosyl moiety of NAD⁺ to different receptors (Bertheliet et al. 1998).

B. The production of cyclic ADP-ribose (cADPR, ADP-ribosyl cyclase activity) and to hydrolyse this cyclic metabolite into ADP-ribose (cyclic ADP-ribose hydrolase activity) (Bertheliet et al. 1998).

Stimuli lead to induction of cADPR activity by CD38 synthesis and hence calcium release from intracellular cytosolic compartments but also Ca²⁺ entry from the outer environment of the cell, all leading to a range of cellular events. Calcium mobilisation is involved in a variety of cell functions, cell proliferation and differentiation, neurotransmitter production, muscle function, and in lymphocyte activation and proliferation. A recent review of CD38's enzymatic role can be seen in Wei et al. (2014).

CD38 function as an enzyme has got a role in diabetes; cADPR and Ca²⁺ both induce insulin in cell free system of islet microsomes, further on using rodent studies confirmed a link between CD38 and diabetes, CD38 mRNA levels are reduced by 50% in islets (Takasawa et al. 1998), and genetically modified animals for increased or reduced expression of CD38 showed that mice over expressing CD38 had enhanced glucose induced insulin release whereas CD38 knockout mice had severe beta cell impaired function. In humans nearly 18% of diabetes patients in a Japanese study were shown to have autoantibodies for CD38 linking this data to one of the causes of major impairment in glucose induced insulin (Ikehata et al. 1998). High CD38 autoantibodies titres were also found in Caucasian patients in type 1 (Pupilli et al. 1999) and type 2 diabetes (Mallone et al. 2002).

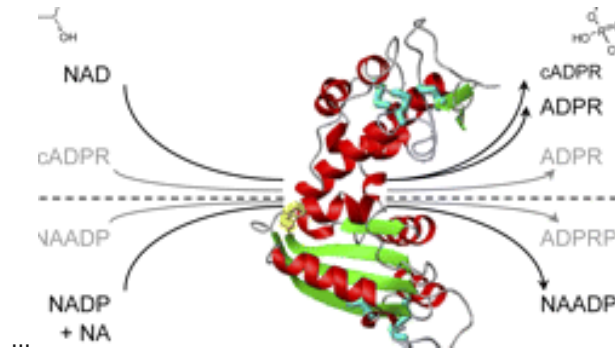


Figure 1-12 Enzymatic activities of CD38.

NAD exists as NAD⁺ and NADH, NAD with a key role in energy metabolism is also involved in signal transductions. NAD⁺ has a role in redox reactions, as a donor of ADP ribosylation and as a precursor of cyclic ADP-ribose, both are second messengers that lead to the release of Calcium from intracellular compartments when stores are low in the cytosol. The top reaction happens in neutral pH and the bottom one in acidic pH. The chemical structure of the product in the reaction are shown in the figure, image modified from Malavasi et al. (2008)

Before relating the relevance of CD38 in lymphocytes, and specifically in B cells and B-CLL, it is worth mentioning that CD38 is widely found in non-immune tissues, although the protein may regularly be found as a nuclear or cytoplasmic molecule, not as an ectocellular element. CD38 is found in brain, eye, prostate, gut, muscle, bone, kidney and other tissues, a table with further references found (Malavasi et al. 2008).

1.4.1 CD38 and B lymphocytes

CD38 is an important molecule in B cells; it is expressed differentially in B cell development. CD38 is found expressed in bone marrow precursor cells, down regulated in normal mature resting B cells. CD38 expression in B cells seems induced when naive B cells are activated, it increases upon entry to the GC, it is highly expressed just before these cells go into SHM, and decreases when centroblast/centrocytes differentiate and finally disappear in memory cells (although not the case for murine memory B cells) to finally being expressed again in plasma cells (Malavasi et al. 2008). This expression pattern highlights the relevance and need for CD38 at different stages in B cell development (Malavasi et al. 1994, Funaro and Malavasi 1998). The micro-environment of the cell and the impact of soluble ligands for CD38 have an important role in the functions of CD38 in B cells. CD31 is a ligand for CD38, CD31 is a platelet adhesion molecule that by binding to CD38 seems to control signalling to lymphocytes (Deaglio et al. 2001). Stimulated CD38 in immature B cells induces apoptosis (Kumagai et al. 1995), whereas in mature B cells (and murine) inhibits apoptosis (Santos-Argumedo et al. 1993, Zupo et al. 1994). In the stroma supported bone marrow microenvironment CD38 ligation stops lymphopoiesis, it inhibits growth of immature B cell precursors, and this is not done by altering the enzymatic action of CD38, thus indicating a possible regulatory role in B cell lymphopoiesis by a CD38 ligand (Kumagai et al. 1995). Inhibition of DNA synthesis and induction of apoptosis is what led to blocking differentiation in B cells. In tonsillar GCs the opposite occurs, CD38 binding by anti-CD38 monoclonal antibodies protects GC mature B cells against apoptosis mediating pro-survival signal but this cross linking fails to induce proliferation (Zupo et al. 1994), and in mature circulating B cells ligation of CD38 does induce proliferation

through promoting induction of cytokines, CD25 expression and MHC-II (Funaro et al. 1997, Malavasi et al. 2008).

1.4.2 CD38 and B-CLL:

B cell lymphomas, myelomas and leukaemias all generally express CD38. Expression of CD38 in B-CLL cells seems to increase signalling via B cell receptor (BCR) (Lund et al. 1996) and regulate IgM and IgD induced apoptosis (Zupo et al. 2000). The interaction between the microenvironment and B-CLL cells seems to be important in rescuing cells from apoptosis, in this microenvironment CD38 is said to play a key role (Chiorazzi et al. 2005, Deaglio et al. 2006) CD38 increases cell survival and proliferation, the latter possibly due to interaction between CD38 and CD31 (Deaglio et al. 2010).

The first indication of CD38 as a negative prognostic marker in B-CLL came from Damle et al. (1999) , they connected IgHV mutated status to CD38 expression. However, further research established that these two markers might be working independently and in fact CD38 itself may vary during the course of the disease (Hamblin et al. 2002), hence IgHV mutation and CD38 expression are now considered independent parameters for prognosis. The threshold at which a patient is considered clinically positive for CD38 is not clear (this varies from 10%, 20% to 30% of cells in the clone expressing CD38); an Italian group has suggested that the size of the population expressing CD38 is irrelevant and the mere presence of a CD38+ distinct population related to IgHV status is what is relevant as a prognostic tool (Ghia et al. 2003). In any case, a CD38 positive population identifies a poor disease prognosis notwithstanding the issue of CD38 varying expression through the course of the disease. Malavasi et al. (2008) have worked on the hypothesis that CD38 expression in B-CLL has pathogenic potential and have looked at CD38 ligation effects with monoclonal antibodies showing that CD38 ligation leads to proliferation and blast transformation on some B-CLL cells, thus indicating that CD38 may act as a signalling receptor too (Deaglio et al. 2003).

The first suggestion of CD38 acting as BCR co-receptor came from Deaglio et al. (2003) as they observed that the signalling abilities of CD38 are enhanced by IL2 acting via up regulation of CD38 expression, and that CD31 ligand, which is expressed in stromal cells and nurse like cells, bound to CD38 may activate CD38 signals, furthermore the interaction between CD38/CD31 upregulates CD100 (a survival receptor) that is involved in sustaining B-CLL growth and survival (Deaglio et al. 2005).

Combined expression of CD38 and cytoplasmic kinase ZAP70, another negative prognosis marker (Hamblin et al. 2002), indicates aggressive disease course. CD38 ligation is linked to activation of ZAP70, and a downstream survival pathway by tyrosine phosphorylation of ZAP70 in CD38/ZAP70 positive B-CLL cells (Deaglio et al. 2007). Their association and how they interact or act as co-receptors relaying signals is not clear. Besides B-CLL, CD38 is found in lymphoid tumours

(Malavasi et al. 1984), some AIDS associated lymphomas (Hoffmann et al. 2005) and many post-transplant lymphoproliferations (Garnier et al. 2002).

CD38 mRNA has an ARE in its 3' UTR, it contains a basic AUUUA sequence flanked by uridines. Using bioinformatics tools to identify mRNA relevant to plasma cells, *CD38*'s 3'UTR ARE was highlighted as a possible target for the ZFP36 proteins (Nasir et al. 2012). The ARE of *CD38* is a binding site for miRNAs and recent papers reported regulation of the *CD38* mRNA in airway smooth muscle cells by miRNA-708 (Dileepan et al. 2014) or miR-140-3p (Jude et al. 2012b). Other RNPB like Hur have been examined for their binding ability to the ARE of *CD38*, resulting in evidence indication that although HuR is able to bind to a nucleotide representing the *CD38*ARE, in fact the induction of *CD38* expression by TNF in asthmatic airway smooth muscle cells may be down to regulation by miR-140-3p, but not HuR (Jude et al. 2012b).

1.5 Aim and Objectives of the project

This overall aim of this project was to investigate role of ZFP36L1 protein in human B cell functions. Specifically, the project focussed on investigating the role of ZFP36L1 in normal and malignant mature B cell populations by studying its ability to post-transcriptionally regulate *BCL2* and *CD38* mRNAs.

The following were objectives of the project:

1. To measure levels of ZFP36L1 expressed in B cells; (A) representing different stages of B cell differentiation from early B progenitor to plasma cell stage. (B) A particular focus is on determining if there are heterogeneous levels of ZFP36L1 expression in different B-CLL populations that are associated with clinical and/or prognostic markers of disease.

2. To determine if the inferred *BCL2*, and *CD38* mRNAs encoding important regulatory proteins in B cells, are authentic ZFP36L1 mRNA targets and whether. ZFP36L1-mediated post-transcriptional regulation of these mRNAs plays important roles in mediating functional effects in B cells.

3. To analyse ZFP36L, BCL2 and CD38 protein expression levels in healthy lymphoid tissue and malignant lymphoma tissue sections by immunohistochemistry in order to see if the hypothesised negative regulation of *BCL2* and *CD38* mRNAs by ZFP36L1 is reflected in an inverse relationship in protein expression in these tissues.

Chapter 2

Materials and Methods

2.1 Materials and Methods

Throughout the different experiments performed in this project a variety of reagents and protocols were used. The following pages provide a list of materials used It includes reagents, equipment and cells information. The second half of this chapter provides a description of the methods used. When the protocol used as recommended by the manufactures this is stated, if a change has been implemented this is also noted; when protocols have been designed or considerably modified for the purpose of the research a description of the methods is provided. Most of the trade names of reagents, and kits, purchased from a provider are registered trademark and must be treated as such even if not stated within the main text of this thesis

Some key materials such as Hela cells, tissue slides and plasmids have been kindly donated by other researchers to whom I take this opportunity to declare my gratitude and whose names are mentioned .as appropriately in the text.

2.2 Materials:

This section lists the different materials used during the experiments performed in this project. The city and country of a supplier is only cited the first time

PCR materials

In all the PCR reactions performed a Biometra T Personal Cycler was used.

Master Mix, Promega, Southampton, UK. Prod M750B

RNAase free water, Promega, Prod P119A.

Restriction enzymes

Xba1, Promega Prod R6185

Xho1, Promega Prod R6165

DNA Ladders and Protein Markers

DNA 1kb Ladder, Promega, Prod no. G5711 and 100bp DNA Ladder, Promega, Prod no. G2101 were used in gel electrophoresis to estimate the size of unknown fragments of DNA in an agarose gel (2% or 1%). The loading dye used was 6X Blue Orange Dye from Promega, Prod no. G1881.

To assess the molecular weight of unknown proteins the Pre-stained Protein MW Marker from Pierce ThermoScientific, Paisley, UK was used, Prod no. 26612.

Primers used in PCR and Sequencing

The primers used in this project were custom made for each experiment and ordered from Invitrogen, Paisley, UK and Eurofins, Ebersberg, Germany.

Table 2-1 Primers.

Primer	Sequence	Company
<i>BCL2</i> ARE Forward	5'-CTCGAGAGTCAACATGCCT-3'	Invitrogen
<i>BCL2</i> ARE Reverse	5'-TCTAGAGGTGATCCGGCCAA-3'	
ZFP36L1 Forward	5'-GATGACCACCACCCTCGT-3'	Eurofins
ZFP36L1 Reverse	5'-CTGGGAGCACTATAGTTGAGCA-3'	
<i>CD38</i> ARE Forward	5'-ATCCTCGAGCCTGAGGATTCATCTTG-3'	Eurofins
<i>CD38</i> ARE Reverse	5'-GGCCTCTAGAGTTTACTATCTGTAGG-3'	

Plasmids

The plasmids used in this project are shown below; a map with their key features can be found in the appendix section.

Table 2-2 Plasmids.

Plasmid	Company
pGem T Easy vector	Promega, Prod no.A1360
pmirGLO	Promega Prod no. E1330
pcDNA6ZFP36L1 pcDNA6mutZFP36L1	Provided by Dr Christoph Moroni, University of Switzerland
pcDEST26ZFP36L2	SourceBioscience (Full ORF Expression clone)
pcSPORT 6 ZFP36	Donated by Dr Manfred Frick, Universität Ulm, Germany
pcDNA3 -empty vector-	Invitrogen, Prod no. K4800-01

Antibodies

The different antibodies used in Western blots and in immunohistochemistry are listed in table 2.3.

Table 2-3 Antibodies

Antibodies	Company
BCL2 Rabbit Western blot	Cell Signalling, Danvers, MA, USA, Prod no. 2876
BRF1/2 Rabbit Western blot	Cell Signalling, Prod no. 2119
TTP (ZFP36) (N-18) Goat Polyclonal IgG Western blot	Santa Cruz Biotechnology, Santa Cruz, CA, USA, Prod no. sc-8458
Donkey anti-rabbit-HRP	Pierce Thermo Scientific, Prod.no.31458
Donkey Anti Goat IgG HRP Western blot	Promega, Prod no. V805A
Anti-HSP90a/b (H-114) rabbit	Santa Cruz Prod no. sc-7949
Pan-Actin (DC18C11)	Cell Signalling, Cat no. 8456
CD38 FITC anti-human CD38, clone HIT2	Biologend, London, UK, Cat no. 303503
Rabbit anti-human ZFP36L1 lab made sera-9332 and pre-immunised sera as negative control for IHC	The sera was lab-made produced by immunizing a rabbit with a synthetic peptide (HSGSDSPTLDNSRR) corresponding to amino acids 313- 236 of human ZFP36L1 (J. Murphy)
Monoclonal Mouse anti-human BCL2	DAKO Cambridgeshire, UK, Prod no. M0887 for IHC
Rabbit anti-human CD38 IHC and Western blot	Abcam Cambridge, UK, Prod no. ab108403
HRP Polyclonal Goat anti-mouse	DAKO, Prod no. PO447 for IHC.
HRP anti-rabbit, IgG Goat	Perkin Elmer, Cambridge, UK, for IHC, Prod no. NEF812001EA

Kits

A number of kits were used in this project, detailed of these are given in the following table

Table 2-4 Kits

SuperSignalWestFemto Used to detect chemiluminescence using a UVP machine	Pierce ThermoScientific Prod no. 34095
Dual Luciferase Reporter Assay System	Promega Prod no. E1910
QIAprep Spin Miniprep Kit	QiAgen, Crawley, UK, Prod no. 27106
QIAquick Gel Extraction kit (50)	QiAgen Prod no. 28704
EndoFree Plasmid Maxi Kit (10)	QiAgen Prod no.12362
RNAeasy Mini kit	QiAgen, Prod no. 74104
Reverse Transcription kit for QRT-PCR	QiAgen, Prod no. 205311
Sybr Green PCR Kit	QiAgen, Prod no. 204143/ 204163

For SDS page and Western blot

Table 2-5 Western blot materials

Mini PROTEAN Tetra Cell	Biorad Herts, UK, Prod no. 65-8000
Mini Trans-Blot Electrophoretic Transfer Cell	Biorad Prod no.170-3930
Mini PROTEAN TGX Precast Gels, 10%,	Biorad Prod no. 456-1033

Oligos

The oligos used during the siRNA experiments were acquired from Invitrogen, details in table 2.6

Table 2-6 siRNA oligos

Stealth ZFP36L1 siRNA1	MSS236070(3-164089G05	Invitrogen
Stealth ZFP36L1 siRNA2	MSS236071(3-164089G07	Invitrogen
Stealth ZFP36L1 siRNA3	MSS236069(3-164089G03	Invitrogen

Transfection reagents

- FuGENE HD Transfection Reagent, Promega, for DNA transfection. Prod no. E231
- Hiperfect, QiAgen, Prod no. 301704
- Lipofectamine RNAiMax, Invitrogen, for RNA transfection. Prod no. 13778100

Cell Lines

Cell lines used in the project so far are shown in the following table:

Table 2-7 Cell lines used in the project

Name	Origin	Description	Source
HeLa cells	Human	Human cervical epithelial cell line	Dr Edward Wright, University of Westminster
HEK293T cells	Human	Human embryonic kidney epithelial cell line	Dr Marie Bijlmakers, Kings college London
Ramos cells	Human	Human Burkitts B lymphoma cell line	From ATCC, CatN CRL1596
Namalwa	Human	Human Burkitts B Lymphoma cell line	From ATCC CatN CRL1432
NALM6 cells	Human	Pre-B cell line	Dr Kwee Yong, University College Medical London
JJN3 cells	Human	Myeloma cell line	Dr Kwee Yong, University College Medical London
KMS27	Human	myeloma cell line	Dr Kwee Yong, University College Medical London
MM1S cells	Human	Myeloma cell line	Dr Kwee Yong, University College Medical London
KMS-28 cells	Human	Myeloma cell line	Dr Kwee Yong, University College Medical London
RPMI-8226 cells	Human	Myeloma cell line	Dr Kwee Yong, University College Medical London

Immunohistochemistry

A combination of kits and reagents were used in the immunohistochemistry investigations, the details are displayed in the following table

Table 2-8 Immunohistochemistry materials and reagents

Dinitrophenol (DNP) amplification	Tyramide Signal Amplification (TSA) DNP (HRP) from Perkin Elmer, Bucks, UK, Prod.NEL747B001KT kit reagents
SIGMADAST DAB	Tablets from Sigma Aldrich, Dorset, UK
Antigen Retrieval Solutions	100mmol/L of Tris and 10mmol/L of EDTA, pH 9 and pH 6 buffer (10mM Sodium Citrate, 0.05% Tween 20) 5 mins
TN (Tris and NaCl buffer) wash buffer	0.1 M of Tris HCl, 0.15 M NaCl: pH 7.5
TNB (Tris NaCl blocking buffer)	0.1 M of Tris, 0.15 M of NaCl plus blocking reagent supplied by the TSA kit

Tissue slides from human tonsils, reactive lymph nodes and FL were kindly donated by Dr. Anthony Warford (University of Westminster). Records of tissue sections are kept within the University of Westminster under his supervision and in accordance with the Human Tissue Act. Leica Application Imaging system was used to produce the histology photographs.

2.3 Methods:

Cell Culture and isolation

All mammalian cell culture was done under a class II laminar flow hood under sterile aseptic conditions.

For HEK29T cells Dulbecco's Modified Eagles Medium (DMEM) with 10% foetal calf serum (FCS, PAA, Cat. No. A15- 102), 50 U/ml penicillin/streptomycin/glutamine (Sigma Aldrich Cat. No.10378016) was used. HeLa cells were maintained using this media or RPMI1640 (Sigma, Cat No. 12633020)

Nalm6, JJN3, KMS27, KMS28, MM1S, RPMI-8226 and KMS-11 cells (all obtained from Prof. K Yong, Dept. of Haematology, University College London, UK) were maintained in RPMI 1640, 10% FCS, 50 U/ml penicillin/streptomycin and 2 mM L-Glutamine. Blood from B-CLL patients was obtained from the Haematology Department, University College London under the direction of Prof. Amit Nathwani (Research Ethics Committee reference number, 09/H07146). B Cells were isolated from B-CLL patients' blood using Histopaque (Sigma Aldrich) density gradient media; cells at 1×10^8 or more were used for the production of protein lysate for Western blot analysis. Protein lysates were prepared using standard lysis buffer and the protein content was quantified using Bicinchoninic Acid Assay and results were used to determine the appropriate protein loading amount for SDS and Western blot analysis.

Long term mammalian and bacterial cell storage

Mammalian cells were aliquoted and stored in FCS (85%), DMSO (15%) and stored in cryo-tubes in liquid nitrogen after being kept overnight at -80C.

Bacterial cells were stored in autoclaved sterilised 60% glycerol at -80C. A sterile loop was used to scarp the surface of the culture for recovering bacteria, the loop was then streaked onto an agar plate or to culture media for incubation at 37C.

Cloning of putative target mRNA AREs into luciferase reporter vectors

Human *BCL2*ARE sequences, wild type and mutant versions were amplified from plasmids PCRII/*bcl-2* ARE+ and PCRII/*bcl-2* mutARE (Lapucci et al. 2002) and cloned into pmirGLO luciferase reporter vector (Promega) to generate pmirGLOBCL2ARE and mutant pmirGLOBCL2ARE luciferase reporter vector. The amplified ARE DNA sequences were run on an agarose gel (2%), bands were extracted and purified (using the appropriate Qiagen kit, listed in previous pages and cloned into pGem T easy vector, from Promega, adding restriction sites for

Xba1 and Xho1. JM 109 *E. coli* cells were transformed, plated out and individual colonies were cultured and used to prepare minipreps that were then checked for positive colonies containing the wild type and mutant versions of the BCL2ARE pGemT Easy constructs. After the checks were done the inserts were amplified by PCR and used to clone into pmirGLO luciferase reporter vector. The DNA sequences of the constructs were checked by DNA sequence analysis (GACT Sequencing services, London, UK), and are shown in the appendix section. The same protocol was followed for cloning of CD38 3' UTR ARE; in this case the sequence was amplified from pmirTarget CD38 reporter vector (Origene cat no. SC206546) with primers designed to introduce restriction sites for Xho1 and Xba1. The DNA sequence of this vector construct was checked by DNA sequence analysis (GATC Sequencing services) and is shown in the appendix section.

Recovery of plasmids that were spotted on filter paper

The area on the filter paper was cut out and placed in an eppendorf tube with sterile water at room temperature. After 5 minutes incubation the tube was then centrifuged and the supernatant containing the diluted plasmid used for subsequent experiments.

Transformation of competent JM109 *E. coli* cells

The pGem T Easy protocol was followed. Competent JM109 in 50µl aliquots were purchased from Promega (cat. no. L2005) and used for transformation. These cells were shown to be the best option in the transformation experiments, the number aliquots tubes were defrosted on ice and cells were used to proceed with transformation.

Plasmid DNA Minipreps and Maxipreps

Qiagen kits were used for maxipreps and minipreps of plasmid DNA and, manufactures instructions were followed in each case.

Restriction enzyme digestion

Minipreps of plasmids were checked by enzymatic digestion using Xba1 and Xho1 restriction enzymes. The reaction was prepared adding 10 units of each enzyme per 1µg DNA in a total of 20µl reaction. The appropriate buffer from Promega was buffer D (R004A), where both enzymes work at 100-75%. Water was added and the reaction was put in a 37°C water bath for 1h. After the hour the samples were run in a 2% agarose gel and viewed under UV light.

Gel purification of DNA fragments

DNA bands were extracted and DNA purified using the Qiagen kits mentioned above according to the manufacturer's instructions.

DNA ligation

Ligation to pGemT Easy vector and pmirGLO were done following the protocol for pGem T Easy

For ligation into pmirGLO several agarose gels were run to maximise the number of DNA and therefore the amounts of DNA extracted, the concentration and purity of DNA was key in the success of ligation into pmirGLO. Vector to insert DNA ratios of 1:3 and 1:6 showed the best results. Ligations were, in all cases, left at 4°C incubating overnight.

Polymerase chain reaction (PCR)

PCR was carried out using PCR Master Mix (Promega). 25µl reaction volume was prepared as per instructions: 12.5µl of Master Mix, 1µl of forward primer (10µM), and 1µl of reverse primer (10µM), 1µl of template DNA and 9.5µl of RNAase free water. A Biometra T Personal thermocycler was used to run a PCR cycling program as described here: Denature step, 95°C for 2 minutes, then 30 cycles of 95°C for 30sec, 50°C for 30 seconds, and 72°C for 1 minute in 30 cycles with a final extension step at 72°C for 5 minutes.

Agarose Gel electrophoresis

0.5% to 1% agarose gels were prepared by dissolving the appropriate amount of agarose powder (Invitrogen, Cat. N. 16500100) in 50ml of TBE buffer (Trizma base, 54g, Boric acid, 27.5g and 20ml of 0.5M EDTA in 1L of distilled water). A microwave was used to heat up the solution to dissolve the agarose and then cooled before adding it to a gel tray with an appropriate comb. The gels were left to set for 30 min to 45 mins. After setting, the DNA samples were loaded on to the gel after adding 6X Blue Orange Dye from Promega (Prod no. G1881) to the DNA. Electrophoresis was performed by running gels were run at 100 volts for 1 hour. The gel was then bathed in *Ethidium bromide* solution (0.5µg/ml final concentration in distilled water) for 10 minutes and visualised using a UVP transilluminator.

DNA Sequencing

DNA sequencing services were GATC, London, UK and SourceBioScience, Nottingham, UK, sequencing services. Instructions from each company to prepare samples were followed. Samples were delivered to the company with appropriate primers.

SDS polyacrylamide gel electrophoresis (PAGE)

Proteins from different B cell populations were separated using SDS PAGE with precast gels from Bio-Rad, 10% Acrylamide, 30µl (cat. no.456-1033). 30-40µg of protein samples were loaded after mixing with the equivalent volume of Laemmli buffer (Sigma) and heating the samples for 3 min at 95°C. The gels were run at 100-120v for 1 hour.

Western blot analysis of protein expression

Using a Bio-Rad SDS-Western blot tank the proteins were transferred from the gel to a nitrocellulose membrane (Protran). After 1h at 100v the membrane was removed from the transfer reaction and left in blocking buffer (5% milk powder, 0.1% Tween 20 in PBS) at 4°C overnight. The membrane was incubated with the primary antibody for 1h in a shaking tray at room temperature.

Then the membrane was washed 3 times for 5 minutes each with PBS 0.1% Tween 20. After the washes the membrane was incubated with the HRP conjugated secondary antibody. Finally the membrane was washed 3 times (5min each) with PBS 0.3% Tween 20, and another 3 times (also 5 min each) with PBS 0.3% Tween 20.

Enhanced chemiluminescent detection of protein expression on Western blots

SuperSignal West Femto chemiluminescence substrate (Pierce Thermo Scientific) was used to detect the horseradish peroxidase conjugated secondary antibody bound to membranes using an AutoChemi System UVP tool from Bioimaging systems. The reagents and UVP Auto Chemi equipment alongside Labworks 4.1 software were prepared, set up and used as per their protocol/instructions.

HEK 293T cells transfections and 3'UTR Luciferase Reporter Assays

The HD Fugene kit and manufacturers protocol were used to carry out transfection of HEK293T cells with the wild type and mutant constructs of pcDNA6ZFP36L1, pcDNA6 ZFP36L2 and pcSPORT6-ZFP36 and the pmirGLOBCL2ARE wild type, the mutated version of the *BCL2ARE* and the pmirGLOCD382ARE. Another control, pcDNA3, the empty vector was also used in transfection experiments. 24 h prior to transfection 1 ml of HEK 293T cells (2×10^5 cells/ml) were seeded per well of a 12 well plate. Cells were then transfected with 100 ng of pmirGLOBCL2 3'ARE or its mutant version, or pmirGLOCD38 3'ARE Luciferase reporter vector along with 200 ng of human expression vector for each ZFP36 protein family member (pcDNA6ZFP36L1 or its zinc finger mutated version, pcDNA6ZFP36L2, pcSPORT6-ZFP36). An empty vector control, pcDNA3, was also transfected. 3.3 μ l of Fugene was used per transfection reaction with 50 μ l of serum free medium. Cells were left incubating at 37°C for 24h after transfection in the case of pcDNA6ZFP36L1 and pcDNA6ZFP36L1mut, then cell lysates were prepared by the passive lysis protocol (Promega), and luciferase and renilla signals measured using the Dual luciferase reporter assay system (Promega) on a Fluostar Optima (BMG, Labtech) plate reader.

For ZFP36L2 and ZFP36 the cells were lysed at least 48hours after transfection. For pmirGLOCD382ARE experiments the optimum time for preparing cell lysates was found to be 48-72 hours after transfection.

Overexpression of ZFP36L1 in HEK293T cells

A cDNA encoding the ZFP36L1 open reading frame was cloned into the pcDNA6 plasmid (pcDNA6ZFP36L1) and this or empty pcDNA3 was co-transfected with the pmirGLOBCL2ARE wild type or mutant version into HEK 293T cells. After 24h to 72h following transfection, cells were lysed and protein samples were prepared and analysed using SDS Page and Western blot as described above.

Densitometry analysis

A gel imaging scan and software were used to perform densitometry analysis of Western blot results. The GS-800 Calibrated Densitometer from Bio-Rad was used, the software was Quality One, also from Bio-Rad

siRNA transfection using Lipofectamine siRNAiMax

HeLa cells were cultured in RPMI or DMEM complete media and plated in 12 wells plates or 6 wells plated at $1-2 \times 10^5$ /ml concentration reaching 80% confluency on the day of transfection. A total of 20nM siRNA was used and the manufacturer's instructions were followed for each transfection reagent tested. Incomplete media RPMI or DMEM was used to dilute transfection reagents and siRNAs. The transfection complex was added to the wells and they were left to incubate for 24, 48 and 72 hours to determine the right time for harvesting cells and follow on analysis.

Flow cytometry analysis of immunofluorescence stained cells

Cell sorting and flow cytometry analysis was done using Cyan ADP Summit. Cells were incubated on ice with staining buffer (PBS and 0.01%FCS) and an appropriate amount of antibody was added and incubated as per manufactures instructions for 30 min at 4°C. Cells were washed twice or three times in staining buffer to eliminate unbound antibody and re suspended in 0.5 ml of staining buffer ready to measure using a cell sorter.

Statistical Analysis and Graphs

A students T test was performed to analyse statistical significance of differences between datasets wherever applicable. The p values are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and exact values are given in legends. The statistical analysis tool in GraphPad Prism was used to obtain p values. The same software was used to design the graphs shown in this thesis.

Immunohistochemistry

Healthy lymphoid tissue sections were used to analyse the expression of ZFP36L1 and BCL2 and CD38 protein. 4 slides per case were used so each slide would be stained for either ZFP36L1 or BCL2 or CD38 and a fourth slide used as a negative control for ZFP36L1, which was the pre-immunised serum. A total of $n=3$ (3 individual cases) were analysed for healthy lymphoid tissue and also $n=3$ for FL. Protein concentrations of rabbit anti-huZFP36L1 and pre-immunised serum were calculated and staining amounts adjusted to ensure that equal concentrations of antibodies were used on each slide. The following dilutions were used for each antibody: ZFP36L1, 1/50, pre immunised serum, 1/37, BCL2 and CD38, 1/100.

Tissue slides were processed for dewaxing, dehydration and clearing (Incubating the slides for 30 minutes in at oven at 60°C degrees and bathing slides for at least 3 minutes in: xylene 1, xylene 2, then in 99% alcohol (industrial methylated spirit was used) 99% alcohol again, then 75% alcohol and a second incubation in 75% alcohol, to finally wash in running tap water. Endogenous

Peroxidase Activity was blocked by immersing the slides in fresh made solution of 0.3% hydrogen peroxide (0.5 ml of 30% of H₂O₂ and 49.5 ml of methanol) for 10 minutes.

Antigen Retrieval was performed for restoration/de-masking of epitopes by heat induced epitope retrieval (HIER) using a pressure cooker using the appropriate pH HIER buffer (details of buffers are shown in Materials section-Immunohistochemistry-. With slides for ZFP36L1 and pre-immunised serum staining pH9 buffer was used and pressure cooking for 10 mins was used, for BCL2 and CD38 pH buffer 6 was used and 5 minutes pressure cooking. Slides were cooled down in running water and then washed 3 times for 5 min in TN buffer (details in Materials-Immunohistochemistry-)

Sections were dried around the tissue section and the area was circled with a DAKO pen. 100µl of prepared (diluted) primary antibody was added onto each slides fully covering each section. The slides were then incubated in a moist chamber overnight at 4°C. All primary antibodies were incubated overnight.

After overnight incubation the slides were washed 3 times with TN buffer for 5 minutes and horseradish peroxidase labelled anti-rabbit or goat secondary antibody diluted 1/100 in TN buffer was added and left for 30 min at room temperature in a moist chamber. Then the slides were washed again 3 times with TN buffer for 5 minutes.

TyramideSignal Amplification detection

Tissue sections were covered with freshly prepared DNP amplification reagent and incubate 10 min and then washed in TN buffer for 3 x 5 min. Slides were covered in anti-DNP-HRP diluted 1/100 in TNB and incubate for 30 min. slides were washed again in TN buffer for 3 x 5 min.

Chromogen development

Chromogen development was carried out using SIGMAFAST DAB with (cobalt) metal enhancer, Prod no. D0426, Sigma Aldrich. The solution was prepared fresh by dissolving one diaminobenzidine and one hydrogen peroxide tablet in 5 ml of pure water. As soon as the tablets fully dissolved, the sections were covered with the substrate solution and incubated for a maximum of 10 minutes monitoring blue - black substrate development microscopically for optimal results.

Finally, the slides were rehydrated and mounted by reversing rehydration steps and using DPX and coverslips.

Chapter 3

“Investigating *BCL2* 3' UTR ARE mRNA as a target of the ZFP36 family of proteins”

3.1 Introduction and background

BCL2 is the founding member of the apoptosis regulator family of proteins with the same name. The three subgroups of the family, categorised by their structural domains, exert regulatory functions on apoptosis and pro-survival as described in the introduction of this thesis. BCL2 is an anti-apoptotic protein known to have an important role in cell survival and carcinogenesis (Adams and Cory 2007). Over expression of BCL2 protein is reported in many types of human cancers (Sánchez-Beato et al. 2003). Its role in haematological malignancies is widely studied and it is known to play a major part in B-CLL. A hallmark of malignant, mostly non-dividing, B-CLL cells is the over expression of the anti-apoptotic protein BCL2 (Vaux et al. 1988b, Dyer et al. 1994, Kitada et al. 1998, Letai et al. 2004, Adams and Cory 2007, Moore et al. 2007, Otake et al. 2007, Majid et al. 2008). The discovery of BCL2 was linked to the finding of a translocation in FLs, t(14;18) leaving the BCL2 gene under the control of the Ig heavy chain (IgH) promoter and resulting in over transcription by the 4 enhancers in the 3' end of the IgH gene. The outcome is an overproduction of anti-apoptotic BCL2 (Tsujimoto et al. 1984, Tsujimoto et al. 1985a, Heckman et al. 2003). However, in most malignancies the t(14;18) translocation is not present, including B-CLL where only 5% of cases show that juxtaposition of genes (Adachi et al. 1990), and hence BCL2 over production which drives the accumulation of mature leukaemic lymphocytes (Majid et al. 2008) is not down to that genetic re-arrangement, it must rather be as a result of deregulation at another level, be it transcriptional (Young and Korsmeyer 1993, Alkayed et al. 2001) or post-transcriptional (Dyer et al. 1994, Harigai et al. 1996, Sherrill et al. 2004).

Clarifying transcriptional and post-transcriptional mechanisms in the control of BCL2 protein levels in B cell malignancies, with or lacking t(14;18)(q32;q21), can have therapeutic implications. The BCL2 family and namely BCL2 protein is a main target in cancer therapy (Thor Straten and Andersen 2010). In recent times manipulation of BCL2 family members' function and levels has been the focus of new drugs developed with varying success, recent reviews can be found in (Kang and Reynolds 2009, Thomas et al. 2013, Vogler 2014). BCL2 functions through heterodimerization with pro-apoptotic members of the BCL2 family to prevent mitochondrial pore formation and prevent cytochrome C release and initiation of apoptosis. Many of the new drugs act on the BCL2 protein: inhibitors called BH3 mimetics are small peptides that inhibit the function of BH3 BCL2 members, including BCL2 itself, by binding to their BH3 groove which in turns blocks heterodimerization and inhibits protein activity (Besbes et al. 2015). There are also attempts to develop a BH3 profiling tool to use as a biomarker to predict response to the BH3 mimetics and identify resistance mechanisms to new agents being studied in B-CLL (Davids et al. 2013).

The 3'UTR of *BCL2* mRNA has a 107 nucleotide ARE, it contains a type II ARE with a series of AUUUA repeats, it includes a series of pentamers near a nonamer (Schiavone et al. 2000) and it serves as a regulatory sequence for the mRNA transcript. Several AUBPs are known to bind to the ARE and thus post-transcriptional ARE led regulation of BCL2 expression requires analysis of its AUBPs; the complexity of the regulatory network is even more intricate since affinity for AREs may

change due to post-translational modifications (Ishimaru et al. 2010). TINO and AUF1 destabilise the *BCL2* transcript while Nucleolin stabilises it (Donnini et al. 2001, Lapucci et al. 2002, Ishimaru et al. 2009, Ishimaru et al. 2010). TINO interacts with *BCL2* mRNA (Donnini et al. 2004), this protein was initially seen to degrade a chimeric reporter construct containing the *BCL2* ARE. AUF1 (Lapucci et al. 2002, Ishimaru et al. 2010), Mex3D (Tino)(Donnini et al. 2004) are other AUBPs have been shown to mediate *BCL2* mRNA *in vitro* downregulation, other proteins like HuR promote stabilisation of mRNA (Ghosh et al. 2009, Ishimaru et al. 2009). AUF1 was one of the first AUBPs researched in relation to BCL2 and it was shown that this protein bound *in vitro* and *in vivo* to the *BCL2* ARE (Lapucci et al. 2002). HuR, another AUBP, also was shown to have a role in *BCL2* mRNA regulation via its ARE; downregulation of HuR reduced endogenous BCL2 levels but the opposite was seen for ectopic BCL2 (Ghisolfi et al. 2009, Ishimaru et al. 2009), but besides this, BCL2 itself was seen to overcome the activity of HuR and hence take the lead role in the regulation of its own mRNA in a dose-dependent manner (Ghisolfi et al. 2009). Other groups have reported on the role of Nucleolin (Ishimaru et al. 2010). Nucleolin has been shown to bind to murine *Bcl2* mRNA increasing its half-life (Ishimaru et al. 2010). Over expression of nucleolin and altered localisation leads to augmented *BCL2* mRNA stability in B-CLL cells (Otake et al. 2007), EBP1 has been shown to destabilise the transcripts *in vitro* using a chimeric construct in HL-60 leukaemic cells (Bose et al. 2006).

As already introduced, the ARE motif is also a regulatory site for miRNAs, miR16 and miR15 induce apoptosis by targeting BCL2, they are able to recognise and bind to its 3'UTR ARE mRNA (Cimmino et al. 2005). In B-CLL up regulation of BCL2 is thought to be due to deletion or downregulation of miR16 and miR15 which are found deleted in the majority of B-CLLs (about 70%) (Calin et al. 2002). The level of miR15a/16-1 and BCL2 expression were found inversely correlated in B-CLL where increased BCL2 relates to down regulated miR16 (Cimmino et al. 2005). This miRNA regulation of BCL2 brings up a link to the ZFP36 proteins as this family of proteins has been reported to be involved in miR16 function (Jing et al. 2005). The prototype, ZFP36 protein, and miR16 are needed to recognise and bind to ARE in *TNF α* 3' UTR: mRNA decay mediated by the ARE in 3'UTRs of unstable mRNAs is dependent on the presence of miR16 and requires ZFP36's presence, the binding of ZFP36 is not directly to miR16, rather via ZFP36 association with Ago/eiF2C family members so a complex with miR16 is formed for the targeting of the ARE (Jing et al. 2005).

The ARE of *BCL2* mRNA is a class II ARE, a type of ARE that ZFP36L1, ZFP36L2 and ZFP36 are able to bind to via their tandem ZFD to promote transcript degradation (Lai et al. 2000). Using a gene expression microarray dataset of 206 cell line, representing cell types of diverse histological origin, Bioinformatics research identified possible mRNA targets for the ZFP36 protein family. One of the possible targets identified was *BCL2* mRNA as a candidate for ZFP36L1 and ZFP36 (Zekavati et al. 2014). Another group also reported *BCL2* mRNA as a candidate for ZFP36L2 (Hudson et al. 2004). In particular for ZFP36L1, induction of apoptosis had already been reported in cell lines such as Ramos (Burkitts lymphoma cells) (Ning et al. 1996, Johnson et al. 2000, Johnson

and Blackwell 2002), and an interaction with *BCL2* ARE had also been shown in REMSA assays (Zekavati 2009, Zekavati et al. 2014).

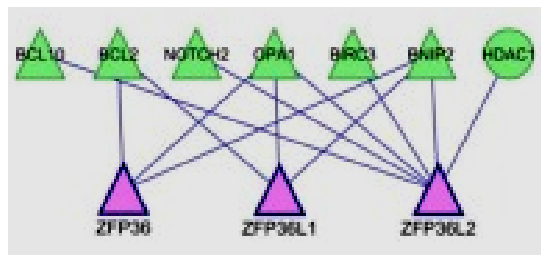


Figure 3-1 *BCL2* mRNA identified as a target for ZFP36 proteins.

Network diagram showing bioinformatic interactions established using reverse engineering gene regulatory network for the ZFP36 family. *BCL2* appears as a possible target for ZFP36L1. Almage adapted from Zekavati et al. (2014)

The post-transcriptional regulation (or rather deregulation) of *BCL2* mRNA could be key in the oncogenic role of the protein (Capaccioli et al. 1996, Bevilacqua et al. 2003, Capaccioli 2015). Understanding the interaction between ZFP36L1, and the other ZFP36 family members, and *BCL2* mRNA could provide important information of ZFP36L1's role in cell fate regulation. The research reported in this chapter is especially relevant to this topic and adds further evidence to the complicated regulatory network of *BCL2*.

In order to assess the relationship between ZFP36 proteins, especially ZFP36L1 and *BCL2*, two strategies were followed; part of the investigation was done at mRNA level, and a second part was done at ZFP36L1 and *BCL2* at protein level. The latter looked at *BCL2* and ZFP36 proteins by Western blot analysis of primary leukaemic cells, and cell lines representing B cell development stages (see chapter 6) Immunohistochemistry analysis of ZFP36L1 and *BCL2* in healthy tonsil tissue and FL tissue was also done and these findings are described in chapter 5.

This results described in this chapter show the experimental data from analysing the direct interaction of ZFP36L1, ZFP36L2 and ZFP36 to the 3' UTR ARE of *BCL2* mRNA by using a 3'UTR dual luciferase reporter assay (DLR assay). The following was analysed:

- If the members of the ZFP36 protein family were able to bind to the 3'UTR containing the ARE region of *BCL2* mRNA and induce its degradation.
- If the ARE core sequence was key for recognition, binding and degradation of the 3' UTR.
- If the binding to the 3' UTR UTR ARE via ZFP36L1's dependent on the integrity of the ZFD.

The 3'UTR DLR assay has high sensitivity and its dual reporter quality allows data to be normalised to successful transfection and cell viability. The DLR assay uses the different biochemical luminescence properties of luciferase proteins from firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) (Sherf et al. 1996). Firefly luminescence occurs due to an oxidative reaction, and no posttranslational processing is needed, nor is it needed for Renilla's luminescence. Different substrates are used in each case (oxigen and coelenterazine respectively), this avoids cross activation permitting distinguishable luminescence signals from each gene which are sequentially

measured. The pmirGLO dual reporter vector allows for the insertion of a sequence of interest, in this case a 3'UTR ARE, downstream of the luciferase stop codon. Firefly luciferase is the primary reporter gene, to monitor mRNA stability, and renilla luciferase acts as control, luminescence readings from this gene are used for normalisation for transfection efficiency and cell viability. A reduction in firefly luciferase luminescence indicates binding to the cloned sequence of the RNA binding protein that has interacted with the 3' UTR sequence and therefore caused a reduction in firefly expression (vector map provided by Promega can be seen in appendix).

3' UTR luciferase reporter assays have previously been successfully used to analyse the relationship between another putative target, also part of the ARACNe list of possible mRNA targets of the ZFP36 family: BLIMP1 (Nasir et. 2012). BLIMP1 is an important and key transcriptional repressor, it has been identified (with other factors such as Bach2) to form an important regulatory network for the terminal differentiation of B cells to plasma cells (Igarashi et al. 2007). The luciferase assay has also been used as part of the research done to validate targets like Stat5b. (Vignudelli et al. 2010) demonstrated that ZFP36L1 and also ZFP36 are able to directly bind the 3' UTR of *Stat5b* mRNA, and thereby triggering its degradation. A luciferase assay was also used for investigating ZFP36L1 and ZFP36L2 interaction with the 3'UTR of *MAPK* mRNA, binding of the ZFP36 proteins degraded the *MAPK* 3'UTR mediated reduced luciferase activity (Lin et al. 2012). (Adachi et al. 2014) used a luciferase reporter assay to validate *LDLR* mRNA as a target for ZFP36 proteins.

In the present study the interaction between ZFP36 proteins and *BCL2* 3'UTR ARE and its possible degradation effect was investigated. HEK293T cells were transfected with the following constructs following the experimental outline detailed below:

Table 3-1 *BCL2* 3'UTR ARE luciferase assay experimental layout.

<u>ZFP36L1 vs <i>BCL2</i> 3'UTR ARE</u>	<u>ZFP36L2 vs <i>BCL2</i> 3'UTR ARE</u>	<u>ZFP36 vs <i>BCL2</i> 3'UTR ARE</u>
wtZFP36L1 vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE	wtZFP36L2 vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE	wtZFP36 vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE
muZFP36L1 vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE		
Empty vector vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE	Empty vector vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE	Empty vector vs wt <i>BCL2</i> ARE vs mut <i>BCL2</i> ARE
Untransfected cells	Untransfected cells	Untransfected cells

For ZFP36L1 the mutation consisted in a single nucleotide change within its ZFD; for *BCL2* ARE the mutation consists in a deletion of a core AU sequence, figure 3-4, Transfection combinations were performed in a minimum of n=3 independent experiments. Cells were transfected with 100 ng of pmirGLO*BCL2* ARE luciferase reporter vector, or its mutant version, and with 200 ng of human ZFP36L1 expression vector (pcDNA6ZFP36L1) or ZFP36L1 with a zinc finger domain mutation (mutZFP36L1) or empty vector (pcDNA3). pcDNA6.ZFP36L2 or pcSPORT 6.1 ZFP36 were also transfected using 200ng of constructs in each case. Wild type (wt); mutated (mut).

Since ZFP36 proteins are reported to recognise and bind to 3'UTRs of mRNAs via their ZFD, in this study, a mutated ZFP36L1 variant was used, the mutation consist of a single nucleotide modification within the ZFD that leads to an aminoacid change. A mutated *BCL2* ARE was also used where the core AU rich sequence was deleted (Lapucci et al. 2002). These mutated constructs would provide information regarding the specificity of the interaction between ZFP36 family protein ZFDs and AREs and the importance of their integrity.

In order to perform the 3' UTR DLR assays, human *BCL2* ARE sequences from plasmids PCRII/*BCL2*ARE+ and PCRII/*BCL2* mutARE (Lapucci et al. 2002) were cloned into the pmirGLO luciferase reporter vector. Wild type and mutated versions of *BCL2* 3' UTR ARE were amplified using primers to insert restriction sites for Xho1 and Xba1, the products were then cloned into the dual reporter vector pmirGLO. Once successful cloning was confirmed in pmirGLO by PCR, restriction enzyme analysis and DNA sequencing the assay was performed. HEK293T cells were used for transfection experiments as these cells are commonly known for easy culture and transfection. Importantly this cell line does not express ZFP36 proteins and neither expresses *BCL2*. The strategy, results and their discussion are described in the following pages.

3.2 Validation of *BCL2* ARE as a target for the ZFP36 protein family using a 3' UTR dual luciferase reporter assay

3. 2. 1 Cloning of the *BCL2* 3' UTR ARE into dual reporter vector, pmirGLO

In efforts to determine whether *BCL2* mRNA is a direct target for ZFP36L1 protein, we cloned a *BCL2* ARE wild type, identified in these set of experiments as (+), and a mutant *BCL2* ARE into the pmirGLO luciferase reporter vector (Lapucci et al. 2002).

3. 2.1.1. Polymerase Chain Reaction (PCR) and restriction enzyme analysis

In order to confirm the success of cloning different tests were carried out: restriction enzyme digestion of plasmid minipreps (fig. 3-2) and PCR analysis (fig. 3-3).

Figure 3-2 A and B show bands corresponding to the right size AREs for *BCL2*ARE "+" (396bp) and its mutant version (289bp) released from the pmirGLO vector after enzyme restriction; this confirmed the cloning of the inserts in the vector. Figure 3-3 shows amplicons of the right size again for both wild-type and mutant AREs showing PCR amplification of the inserts was successful and confirmed their presence in the vector.

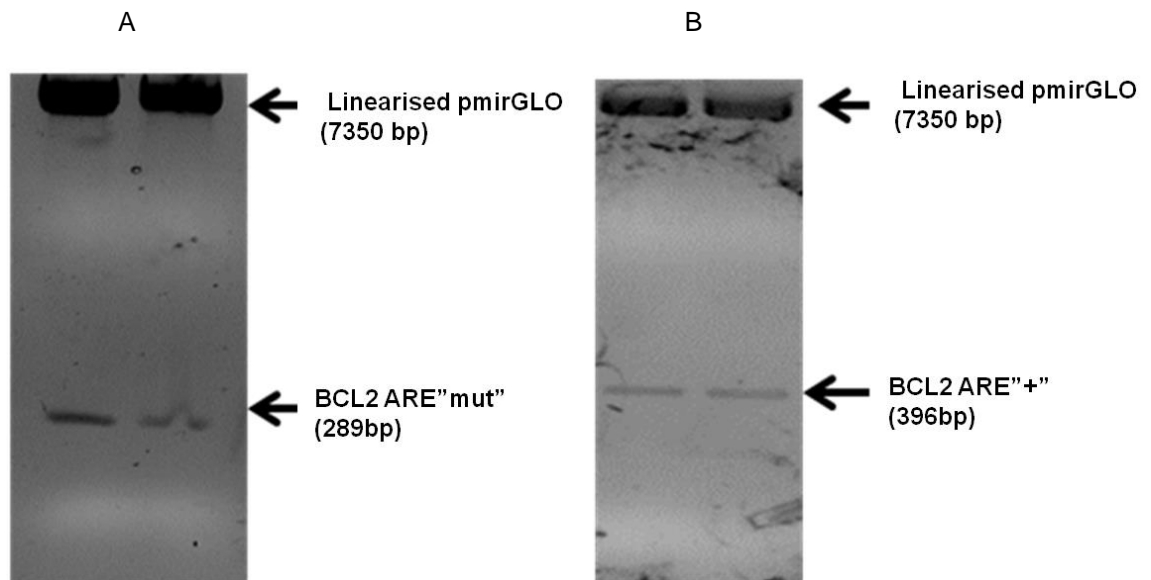


Figure 3-2 Analysing the cloning of *BCL2* 3'UTR ARE into pmirGLO vector.

Restriction enzyme digestion of pmirGLO vector after cloning of *BCL2* AREs (A) Gel shows in both lanes linearised pmirGLO from one colony and digested product *BCL2* ARE mutant after restriction digestion with Xho1 and Xba1, the band correspond to the right size for *BCL2* ARE mut. The second gel (B) shows *BCL2* ARE insert of correct size also after digestion of pmirGLO with Xho1 and Xba1, in both lanes. *BCL2* mut is a modified version of *BCL2* "+". *BCL2* mut lacks the core ARE sequence.

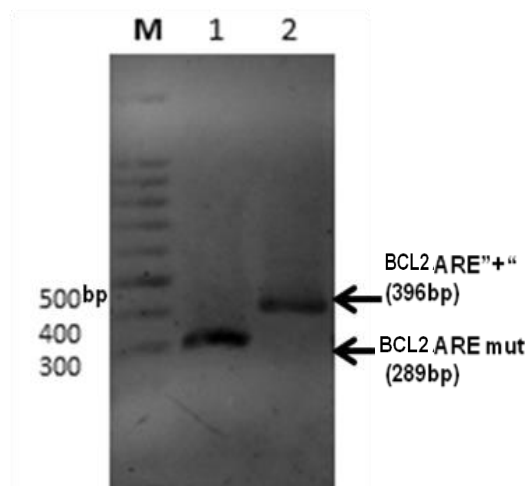


Figure 3-3 PCR analysis to detect cloned *BCL2* 3'UTR ARE in pmirGLO vector.

PCR of pmirGLO AREs constructs to detect inserts cloned into of pmirGLO. The figure shows agarose gel with correct size PCR amplified bands for *BCL2* ARE mut -Lane 1- and *BCL2* ARE+ -Lane 2- amplified from pmirGLO plasmid minipreps. Clones were selected from colonies on LB agar plates. The same colonies were minipreped and digested with Xho1 and Xba1 obtaining restriction products shown in figure 3-2.

3. 2.1.2 DNA Sequencing results to confirm the correct DNA sequence of clones used in these studies.

Clones for both the wild-type and mutant *BCL2* AREs were DNA sequenced using appropriate primers. The ZFP36L1 insert in pcDNA6ZFP36L1 and its zinc finger mutant version (pcDNA6ZFP36L1 mut) were also DNA sequenced. DNA sequencing results confirmed the right DNA sequences and expected mutations for *BCL2* ARE and ZFP36L1 constructs were all correct. DNA sequences and their modifications are shown in the appendix section.

BCL2 ARE wild type and its mutant version were successfully cloned into pmirGLO, a luciferase reporter vector. The success of cloning was assessed in different ways; minipreps of pmirGLOBCL2ARE and mutant *BCL2* ARE were tested using Xba1 and Xho1 restriction enzymes- whose recognition sites had previously been introduced designing the appropriate primers and amplifying the ARE from a stock plasmid by PCR-. This restriction reaction gave bands of the right size for both AREs (wild type *BCL2*ARE -396bp- and mutant *BCL2*ARE -289bp-) and for the linearised vector, pmirGLO vector (7350bp). The same miniprep plasmids were used in a PCR using primers to amplify these inserts, gel electrophoresis after PCR showed amplicons of the right sizes for both *BCL2* ARE sequences. These constructs were transfected into HEK293T cells alongside pcDNA6ZFP36L1 and its mutant version.

3.2.2 Over expression of ZFP36L1 and effect on HEK293T endogenous BCL2 expression

BCL2 ARE constructs were transfected along with pcDNA6ZFP36L1 into HEK293T cells. Western blot analysis was carried out to confirm over expression of ZFP36L1, samples transfected with the plasmid containing the gene showed the right size bands for the ZFP36L1 protein (fig. 3-4.A).

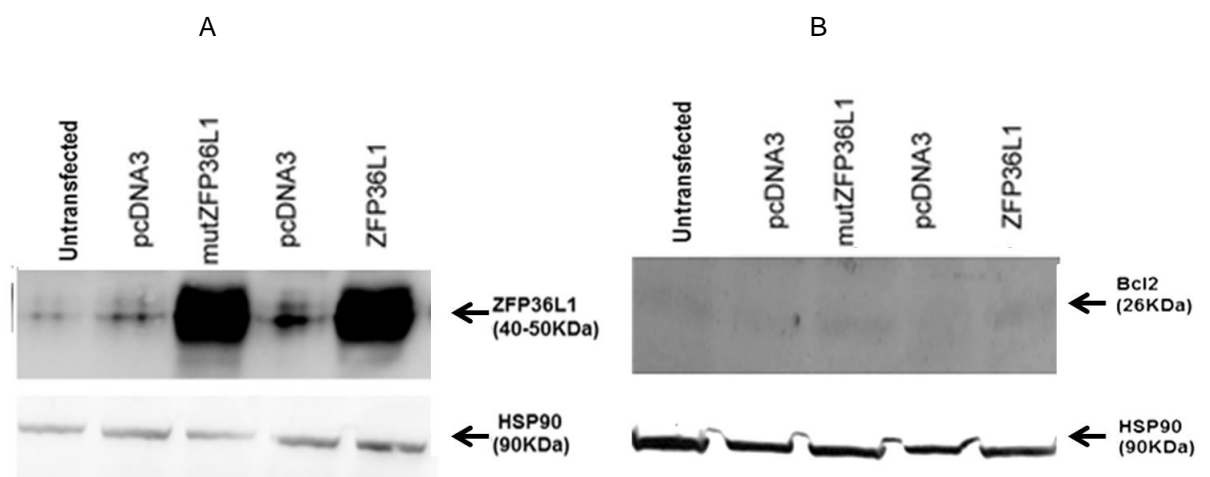


Figure 3-4 Western blot analysis of over expression of ZFP36L1 in HEK293T cells

(A) Western blot showing bands for over-expression of ZFP36L1 in HEK293T cells transfected with pcDNA6ZFP36L1 and its mutant; controls used were untransfected cells and pcDNA3 empty vector transfected cells. Anti-ZFP36L1/2 antibody was used to probe the membranes. (B) Blot showing very faint bands for endogenous expression of BCL2 in all HEK293T cells (transfected with pcDNA6ZFP36L1 and controls) Anti-BCL2 antibody was used to detect the 26KDa protein. Anti-HSP90 antibody was used to detect the constitutively expressed protein HSP90 as a loading control.

Western blots of lysates from the transfected HEK293T cells confirmed over-expression of ZFP36L1 (fig. 3-4 A), bands for ZFP36L1 were detected in samples transfected with wild type ZFP36L1 construct and for its mutant ZF version.

HEK293T cells are reported to express low/ no BCL2 (HPA, Cell Atlas CAB000003) but the expression profile of cell lines may vary and since Western blot analysis had to be done to assess

the over expression of ZFP36L1, BCL2 expression was also tested. BCL2 bands were very faint or not detected in all samples either transfected HEK293T cells with pcDNA6ZFP36L1 or controls (fig. 3-4B). The very low amounts of BCL2 detected made it difficult to assess if a decrease in endogenous BCL2 in these cells had taken place due to over-expression of ZFP36L1. For the luciferase assay, these results support the use of HEK293T cells in the experimental model since they do not express BCL2 and have very low/absent expression of ZFP36L1.

Once it was confirmed that endogenous BCL2 and ZFP36L1 were not expressed or were very faintly expressed in HEK293T cells, and that cells transfected with the ZFP36L1 vector over-expressed the protein, transfection of HEK29Tcells with the different combinations of plasmids as laid out in table 3-1 was carried out. For further details and reagents used in the transfection please refer to Materials and Methods chapter.

3. 2.3 ZFP36 protein family targeting *BCL2* ARE

3. 2.3.1 ZFP36L1 and *BCL2* 3'UTR ARE luciferase assay results

HEK293T cells were transfected with pmirGLOBCL2ARE, pcDNA6ZFP36L1, and mutant versions for both (mutations as described in figure 3- 5). pcDNA3 empty vector was also transfected as a control empty vector. After 24 hours cells were harvested and luminescence for firefly and renilla measured with a FluostarOptima plate reader. Luciferase to renilla ratios were calculated and plotted in a bar chart, as seen in figure 3-5.

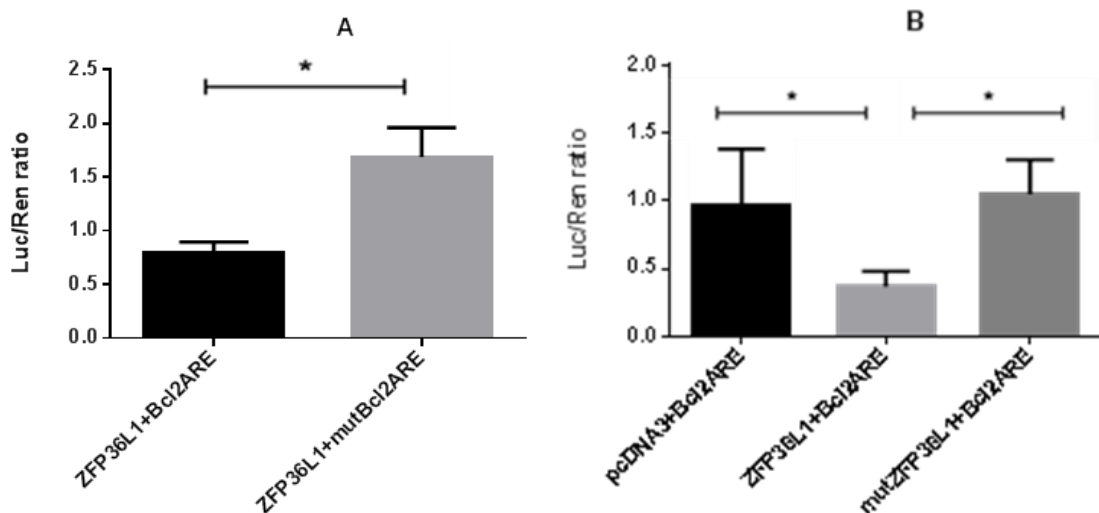


Figure 3-5 ZFP36L1 binds and degrades *BCL2* ARE 3'UTR.

Figures showing ratios of luciferase/renilla readings from FluoStar Optima. (A) HEK293T cells were transfected with pcDNA6ZFP36L1 and pmirGLOBCL2 ARE or a mutant version, *BCL2*AREmut, which lacks the core ARE region in the middle of its sequence. This vector acts as a control for specificity of recognition and binding of ZFP36L1 to the *BCL2* ARE. (B) HEK293T cells transfected with either empty pcDNA3, pcDNA6ZFP36L1, mutant pcDNA6ZFP36L1 along with pmirGLOBCL2ARE. The mutant version of pcDNA6ZFP36L1 with a modification in the zinc finger region was used to test the role of ZFP36L1 intact zinc finger domains in recognition and binding to the *BCL2* ARE. Mean and standard error shown for three independent experiments (n=3) in both A and B. T-test was used to determine the significance of the difference between the means as shown. A:*P value=0.02 B:*P values=0.01 and 0.03

Figure 3-5A shows a decrease in luciferase activity in HEK293T cells transfected with the wild type ZFP36L1 and wild type *BCL2* ARE compared to those where a mutated version of the ARE was used. Reduced firefly luciferase expression indicates binding of ZFP36L1 to the cloned target sequence, the wild type *BCL2* ARE. The lack of an effect of ZFP36L1 on mut *BCL2* ARE showed that the core ARE sequence was required for ZFP36L1 binding and degradation of the *BCL2* ARE. Figure 3-5 B. shows that the level of luciferase activity was lower in cells transfected with both wild type constructs (pmirGLOBCL2ARE and pcDNA6ZFP36L1) compared to the level seen in cells transfected with a mutated ZFP36L1 (zinc finger domain mutation) and to that of empty vector (pcDNA3) transfected cells. These results indicated that ZFP36L1 mediates degradation of the 3' UTR ARE of *BCL2* and that for this effect functional ZFP36L1 zinc finger domains are needed. These results were statistically significant.

3.2.3.2 ZFP36L2 / ZFP36 and *BCL2* 3' UTR ARE luciferase assay results

The members of the ZFP36 family may differ in their functions and may possibly recognise and post-transcriptionally regulate different mRNAs. In order to gather further information to characterise the targets recognised by each of the proteins this study it was also tested if ZFP36L2 and ZFP36 bound and led to degradation of *BCL2* 3' UTR ARE.

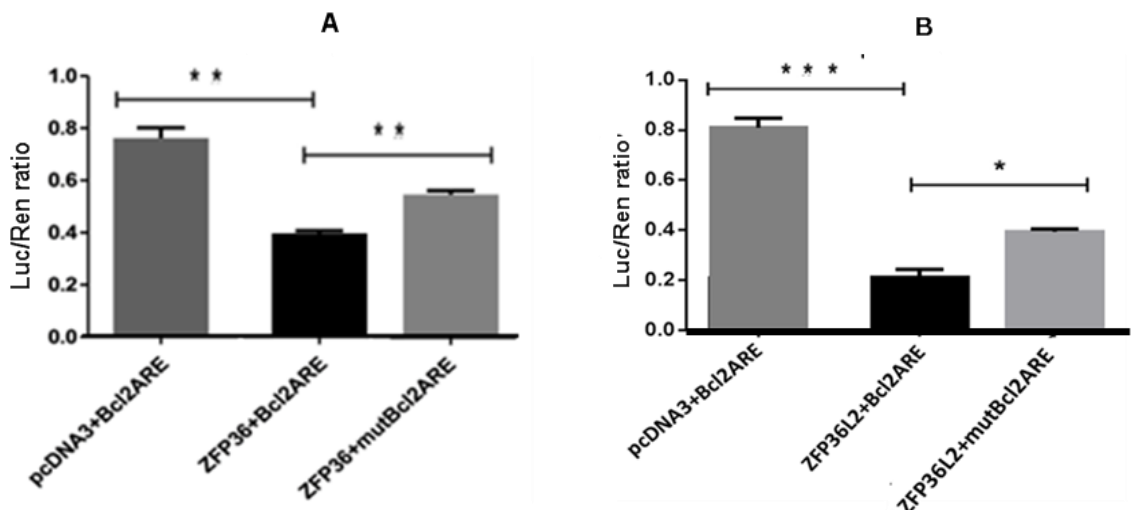


Figure 3-6 ZFP36L2 and ZFP36 also target *BCL2* 3' UTR ARE.

The ratios of firefly luciferase/renilla readings from FluoStar Optima are shown. HEK293T cells were transfected with pcSPORT6ZFP36 (A) and pDEST26ZFP36L2 (B) and pmirGLOBCL2 ARE or the mutant version –*BCL2*AREmut which lacks the core ARE acting as a control for specificity of recognition and binding of the ZFP36 proteins. HEK293T cells were also transfected with empty pcDNA3 and pmirGLOBCL2ARE as a control. Mean and standard error shown for three independent experiments (n=3) in both A and B. A T-test was used to determine the significance of the difference between the means as shown; A: **P values= 0.0084 and 0.0024 respectively; B: ***P=0.0003; *P=0.02

The *BCL2* ARE was further tested as a possible target for the other two human family members, ZFP6L2 and ZFP36, using the 3' UTR DLR assay. The same *BCL2* 3' UTR ARE constructs were used, wild type and mutant versions. HEK293T cells were transfected and samples were read 48 hours for ZFP36L2 and 72 hours later in the case of ZFP36, as these times were assessed as the optimal ones for each set of experiments. The Material and Methods followed were those described for ZFP36L1. It is worth highlighting that due to the consistent results obtained from ZFP36L1 on

BCL2 3' UTR ARE these constructs were deemed a good positive control for transfection and luciferase assay and were hence also transfected in these experiments (data not shown)

Luciferase assay results indicated binding of both proteins, ZFP36L2 and ZFP36, to the wild type *BCL2* 3'UTR but not the mutant *BCL2* 3' UTR ARE version lacking the core AU rich sequence; the firefly to renilla luminescence ratios for each of the ZFP36 and ZFP36L2 proteins with wild type *BCL2* 3' UTR ARE showed very significant decreased levels compared to empty vector and the mutated *BCL2* ARE (fig. 3-6).

3. 3 Discussion

Results from 3'UTR DLR assay showing a decrease in luciferase luminescence readings for the wild type ZFP36L1 and wild type *BCL2* mRNA indicated that ZFP36L1 recognised and bound to *BCL2* ARE. Following mutant ZFP36L1 transfection luciferase to renilla ratio readings were similar to those from pcDNA3 empty vector transfected cells, indicating recognition of *BCL2* ARE had been inhibited by the mutation introduced in one of its ZFD -a single nucleotide mutation, a Cysteine to Arginine change (Lai et al. 1999; Stoecklin et al. 2002). These results further support the role of the integrity of the ZFDs in this protein's function as a post-transcriptional regulator. These findings are in line with previous work indicating the importance of the integrity of the zinc finger domains in the post-transcriptional roles of the ZFP36 proteins (Lai et al. 1999, Lai et al. 2002, Stoecklin et al. 2002, Fabian et al. 2013, Lai et al. 2014). The luciferase assay results also further support the hypothesis that the ARE sequence is the specific binding site for this protein. Binding by ZFP36L1 took place to the non-mutated *BCL2* ARE and did not happen with the mutated version of the *BCL2* ARE where a section of the core AU-rich region sequence is missing. ZFP36L2 and ZFP36 did not bind the mutated *BCL2* ARE either, but did so in the case of the wild type *BCL2* ARE.

These results from the 3'UTR luciferase reporter assays extend previous that showed an interaction between ZFP36L1 protein and *BCL2* mRNA ARE using RNA electrophoretic mobility shift assays (REMSA) (Zekavati, 2009). REMSA results showed the protein and the target formed a complex; ZFP36L1 interacted with the ARE of *BCL2* mRNA but not if the core AU-rich sequence of the *BCL2* ARE was deleted. These experiments already highlighted the AU rich region of the ARE as the binding site (Zekavati, 2009; Zekavati et al. 2014).

The core AU rich region of the sequence of *BCL2* ARE was needed for the ZFP36 proteins to bind the *BCL2* ARE. AREs are present in about 5-8% of human mRNAs; AREs serve as regulatory sequences of mRNA transcripts by means of RNA binding factors (AUBPs or miRNAs), that determine the fate of the transcript affecting stability, localization and translation (Bakheet et al. 2001). An early paper was the starting point for looking at the ARE of *BCL2* as a regulatory sequence in charge of its own downregulation (Schivone et al. 2000). The group showed that the destabilisation of *BCL2* and subsequent downregulation during apoptosis was linked to its own ARE in the 3'UTR of the mRNA transcript. The *BCL2* ARE also serves as a binding site for stabilising factors, overexpression of Nucleolin in B-CLL cells induces stabilisation of murine *Bcl2* mRNA

(Otake et al. 2007) . Recently it has been reported that deletion of AU-rich elements within the Bcl2 3'UTR reduces protein expression and B cell survival *in vivo* (Díaz-Muñoz et al. 2015). This study demonstrates that the binding of RBPs to the *Bcl2* 3'UTR ARE is directly linked to the stabilization of the its mRNA and regulates BCL2 protein expression with functional consequences for B cell maintenance (Díaz-Muñoz et al. 2015). The results described in this paper further support the conclusion that the ARE in *BCL2* 3'UTR is an essential factor in the regulation of the protein and that perhaps a major part of this regulation is done via that ARE by virtue of AU binding factors, such as the ZFP36 protein family.

There is much still to find out in regards to establishing the targets that each protein of the family binds to and regulates. A recent paper studying structural characteristics of ZFP36 pointed at features in each ZFP36 protein that confer specific modes of interacting with RNA (Lai et al. 2014), this would explain part of the diversity in the targets the each ZFP36 protein may bind to. However, the ZFP36 proteins do also share some mRNA targets. TNF α is one of the most researched targets for the ZFP36 family and it indeed a target shared by all three ZFP36 family proteins (Carballo et al. 1998, Lai et al. 1999, Lai et al. 2000). It was also reported that different members of ZFP36 family can act redundantly as co-over-expression of ZFP36L1 and ZFP36 mediated a cumulative effect on Stat5b down-regulation (Vignudelli et al. 2010). Vignudelli et al. 2010, showed the protein negatively regulates immature red blood cells differentiation by binding to the 3' UTR of *Stat5b* encoding mRNA. But not only ZFP36L1, also ZFP36 was shown to directly bind the *Stat5b* 3' UTR and trigger its degradation. More recent evidence from Adachi et al. 2014 also reported on an mRNA target that was shared between ZFP36L1 and ZFP36L2 (LDLR mRNA, encoding a lipoprotein). Taken together, the evidence is consistent with the ZFP36 family proteins may target overlapping mRNA.

For ZFP36 other research further supports the results reported in this chapter: ZFP36 bound *in vitro* to *BCL2* mRNA as shown by UV Cross-Linking assays RNA and Immunoprecipitation Assay (Bevilacqua et al. 2007) and more recent literature has provided more evidence for this interaction and its functional consequences. BCL2 expression in cancer cells provides resistance to chemotherapy drugs like Cisplatin. Head and neck cancer cells with resistance to Cisplatin had high levels of BCL2 and low levels of ZFP36 and the opposite was seen in Cisplatin sensitive cells (Park et al. 2014). siRNA inhibition of ZFP36 led to higher BCL2 levels and lower sensitivity to Cisplatin, but if ZFP36 was over expressed BCL2 levels decreased. ZFP36 was therefore linked to downregulate BCL2 expression in response to Cisplatin (Park et al. 2014). The interaction between ZFP36 and the *BCL2* 3'UTR ARE evidenced from the DLR assay results in this thesis may be providing part of the missing link between ZFP36 and BCL2.

The same group reporting that ZFP36 is needed for Cisplatin sensitivity had already published in a previous paper that ZFP36L1 was also required for the chemotherapy action of Cisplatin also in human head and neck squamous cell carcinoma cell lines (Lee et al. 2005). Using cDNA microarrays they found that ZFP36L1 was highly expressed in cells sensitive to Cisplatin. Furthermore inhibiting ZFP36L1 led to a decrease in the Cisplatin-sensitivity whereas over expression of the protein increased it. Elevated expression of ZFP36L1 decreased the expression

level of enhanced green fluorescent protein linked to a 3' UTR ARE of *cIAP2* mRNA. In short, they demonstrated that ZFP36L1 expression enhanced Cisplatin sensitivity by reducing the levels of apoptosis protein-2 (cIAP2), another anti-apoptotic regulator (Lee et al. 2005).

The presence of ZFP36L1 is also needed for the action of another cancer drug, a monoclonal antibody; Rituximab is used in breast cancer treatment and is the standard of care for patients with B cell NHL. Rituximab mediates cytotoxicity of CD20 positive B cells, it also sensitises B cell NHL to cytotoxic chemotherapy and has direct apoptotic and anti-proliferative effects (Stolz et al. 2008). ZFP36L1 is needed for induction of apoptosis in B-CLL cells in the presence of Rituximab (Baou et al. 2009b) although the molecular target/s that would lead to mediate the pro-apoptotic effect of Rituximab by ZFP36L1 are not known, however there is a group reporting that the monoclonal antibody down regulates BCL2 in AIDS derived lymphoma cells (Alas et al. 2002). In previous research, Rituximab treatment showed selective down-regulation of the BCL2 protein in B cell NHL cell lines, and induced sensitivity to chemotherapy drugs through down-regulation of anti-apoptotic IL-10 (Alas et al. 2001), which in turn happens to be an mRNA target for ZFP36 (Stoecklin et al. 2008, Tudor et al. 2009, Gaba et al. 2012).

The 3'UTR luciferase reporter assay results described for the three human ZFP36 family members, and especially for ZFP36L1, indicate a direct interaction with *BCL2* ARE, suggesting that ZFP36 protein may lead to the degradation of BCL2 mRNA. These results may add further light to explain the mechanism of apoptosis induction in cells mediated by ZFP36 family proteins (Ning et al. 1996; Johnson et al. 2000; Baou et al. 2009).

3.4 Conclusion

There are important implications in elucidating the complex mechanism by which anti-apoptotic proteins like BCL2 are regulated. The level of BCL2 is determined by transcriptional and post-transcriptional mechanisms. In this chapter, evidence has been presented on the three members of the ZFP36 family of post-transcriptional regulator proteins and their ability to mediate *BCL2* mRNA destabilisation and decay via their highly conserved zinc-finger domains and through binding to the AU-rich core sequence of the *BCL2* ARE.

Some of this work was incorporated in a paper where other experimental data further supported the hypothesis that *BCL2* mRNA is a target for ZFP36L1 (Zekavati et al. 2014). ZFP36L2 and ZFP36 were also seen to bind and degrade the BCL2 ARE indicating they may also post-transcriptionally regulate BCL2 at the mRNA level. None of the ZFP36 family proteins bound to a mutated version of the BCL2 ARE lacking a core AU rich sequence in this *in vitro* experiments. Recent *in vivo* evidence from other researchers further supports the AUBP binding site on BCL2 as the ARE (Díaz-Muñoz et al. 2015). These results presented here may assist in explaining the role of ZFP36L1 and other ZFP36 proteins in induction of cell apoptosis.

Chapter 4

“Investigating the *CD38* 3' UTR ARE mRNA as a target of the ZFP36 family of proteins”

4.1 Introduction

The overall aim of the PhD research project was to investigate two of the putative mRNA targets identified for the ZFP36 family of proteins; *BCL2* has already been introduced in chapter 3. Aiming to expand the research on the list of candidate mRNAs for the ZFP36 protein family and confirm them as authentic targets or discount them, the second 3' UTR ARE chosen for analysis was that of *CD38* mRNA, which is the focus of this chapter. Both targets, *BCL2* and *CD38* are of special relevance for B cells and B cell malignancies, and notably for B-CLL.

CD38 is a molecule with a role in signalling and cell adhesion, a transmembrane protein essential for the regulation of intracellular Calcium. In B cells its expression is developmentally regulated, with high levels of *CD38* expression found in plasma cells. *CD38* is used as a marker for human leukaemias and myelomas. In B-CLL it is involved in its pathogenesis and outcome, its expression signifies a poor prognosis. In the last few years research has placed *CD38* as part of a network signalling for growth and survival in B-CLL cells, facts that lead to considering *CD38* as a potential therapeutic target in B cell malignancies (Deaglio et al. 2008, Malavasi et al. 2008).

Previous *in silico* analysis to find putative targets for the ZFP36 family resulted in a list of potential targets for ZFP36L1 (Nasir et al. 2012). The analysis using the ARACNe algorithm (Margolin et al. 2006, Basso et al. 2010) looked for candidate mRNA targets in mature B cells related to plasmacytoid differentiation. A gene regulatory network was reconstructed based on microarray datasets terminating in a list that included targets significantly up regulated in plasma cells and 23 candidate target mRNAs were identified such as *GAS6* (growth arrest) and *GFI1* (transcriptional repression). Importantly, the list also included *BLIMP1*-plasmacytoid differentiator, *SEL1L*- a key ER protein- and *CD38 mRNA* (Nasir et al. 2012). All these possible mRNA targets contain canonical 3' ARE elements that are targeted by the ZFP36 protein family (Baou et al. 2009a). *BLIMP1* was confirmed as a mRNA target for ZFP36L1 in that same study (Nasir et al. 2012).

Computational analysis of the 3'UTR of *CD38* gene revealed the presence of an ARE sequences located within the last 400-bp region of the 3'UTR. The human *CD38* 3' UTR has a copy of the DNA motif UUAUUUUAU that can also be found in the 3'UTR of some inflammatory mediators such as GMC-SF and TNF α , examples which are in turn confirmed targets for some or all the ZFP36 family members (Carballo et al. 1998, Carballo et al. 2000, Lai et al. 2000).

This chapter reports on experimental work that examined whether *CD38* mRNA is a target for ZFP36L1, it also includes data obtained for ZFP36L2 and ZFP36. Two strategies were followed, one consisted in directly testing if each ZFP36 protein interacted, bound to the *CD38* 3' UTR ARE using a dual luciferase reporter assay (DLR assay). The DLR assay and the rationale for its choice have already been introduced in chapter 3; suffice to add that luciferase reporter assays in relation to *CD38* have already been used to determine RNA binding factors recognising and degrading *CD38* mRNA, examples are miR-140-3p binding to the *CD38* 3' UTR (Jude et al. 2012b) and

miR108 (Dileepan et al. 2014). The second strategy aimed to manipulate expression levels of ZFP36L1 by down regulating it and to analyse correlated changes in CD38 expression with the hypothesis being that if ZFP36L1 regulates CD38 expression down regulation of ZFP36L1 protein will lead to increased CD38 levels. To further research the relationship between ZFP36L1 and CD38, immunohistochemistry (IHC) analysis of lymphoid tissue and FL was also performed, this constitutes the content of chapter 5 where the results from these analyses are reported.

4.2 Validation of CD38 3'UTR ARE as a target for the ZFP36 protein family using a 3' UTR dual reporter luciferase assay

The strategy chosen to investigate CD38 3'UTR ARE as a target for the ZFP36 proteins aimed at obtaining evidence for the ZFP36 protein family directly interacting, binding and leading to the degradation of the 3'UTR ARE of CD38 mRNA. For ZFP36L1 a mutated version of the protein was used to test the role of the integrity of the zinc finger domains (ZFD) in binding to the ARE. A DLR assay was performed after cloning the 3'UTR ARE of CD38 mRNA into the dual reporter vector, pmirGLO. This experiment required the cloning steps seen for BCL2 3'UTR ARE in chapter 3 in order to insert the CD38 3' UTR ARE sequence downstream of the firefly luciferase reporter gene in the plasmid. This construct was transfected into HEK293T cells, chosen here again for similar reasons as in the case of the BCL2 3' UTR ARE investigation: HEK293T cells are easy to culture, to transfect and do not constitutively express ZFP36 proteins nor CD38. On this occasion the layout of the experiments was:

Table 4-1 CD38 3'UTR ARE luciferase assay experimental layout:

<u>ZFP36L1 vs CD38 3' UTR ARE</u>	<u>ZFP36L2 vs CD38 3' UTR ARE</u>	<u>ZFP36 vs CD38 3' UTR ARE</u>
wtZFP36L1 vs CD38 3' UTR ARE	wtZFP36L2 vs CD38 3' UTR ARE	wtZFP36 vs CD38 3' UTR ARE
muZFP36L1 vs CD38 3' UTR ARE		
Empty vector vs CD38 3' UTR ARE	Empty vector vs CD38 3' UTR ARE	Empty vector vs CD38 3' UTR ARE
Untransfected cells	Untransfected cells	Untransfected cells

Independent experiments were minimum of 3, n=3. ZFP36L1 vs wt BCL2 3'UTR ARE was used as a positive control on initial experiments. This experiment would test if the ZFP36 proteins were able to recognise, bind and degrade the CD38 mRNA 3' UTR.

Analysis to confirm over expression of ZFP36L1 in HEK293T transfected cells is reported in chapter 3, figure 3-4.

4.2.1 Cloning of *CD38* 3' UTR ARE into dual reporter vector, pmirGLO

pmirGLO vector is a plasmid with a reporter gene, luciferase and a control gene, renilla, the transfection of this vector alongside human ZFP36 proteins expression vectors in HEK293T cells would result in two types of readings: luminescence signals given by the luciferase gene (reporter gene) and another luminescence signal for the renilla gene. The renilla gene serves as a control for transfection and cell variability, renilla's luminescence serves as a normaliser for the resulting data. For further details and discussion regarding this choice of assay please see the information in the introduction to Chapter 3.

Briefly, human *CD38* 3' UTR ARE sequence was amplified from pmirTarget (Origene), using designed primers to insert restrictions sites for XhoI and XbaI enzymes for cloning into pGEMT easy vector and pmirGLO vector. The target sequence, *CD38* 3'UTR ARE, was cloned downstream the luciferase gene in the dual reporter pmirGLO plasmid. To assess the success of the cloning, polymerase chain reaction, restriction enzyme digestion analysis and DNA sequencing were performed.

4.2.1.1 Polymerase chain reaction analysis and restriction enzyme analysis

PCR analysis to determine if the *CD38* 3' UTR ARE had been successfully cloned onto pmirGLO showed amplicons of the expected size for the product, 493bp (fig. 4-1). For PCR cycle settings please see Materials and Methods. The presence of amplicons for the target insert in tests samples and not in controls indicates that the primers were able to amplify the right insert since the size of the bands correspond to that of the *CD38* 3'UTR.

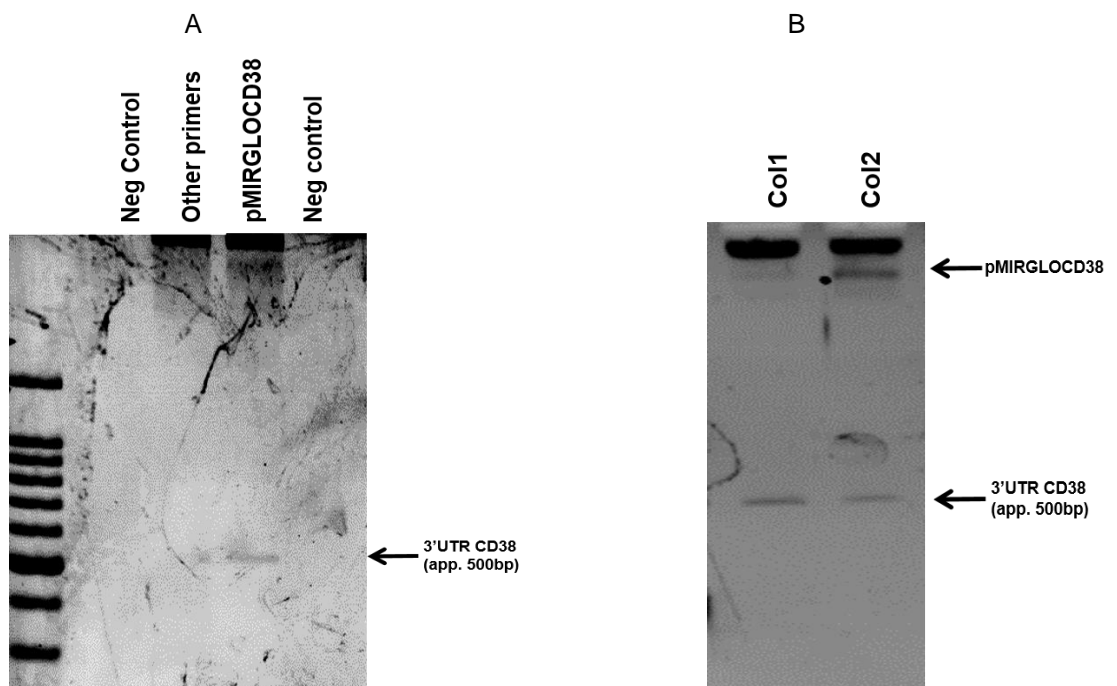


Figure 4-1 *CD38* 3'UTR PCR products amplified from pmirGLO.

A. Agarose gel showing PCR products and various negative controls (no DNA) and using different non-specific primers, a band for *CD38* 3' UTR ARE was amplified from a miniprep of a colony culture after transformation with pmirGLO*CD38ARE*.

B. PCR products from amplifying *CD38* from 2 colonies after transformation with pmirGLO*CD38* construct correct size bands of approx. 500bp.

The analysis performed by PCR using primers to amplify *CD38* 3'UTR ARE, indicated that the sequence was cloned onto pmirGLO vector. As controls different non-specific primers were used and also samples where no DNA was added were run. Primers for *CD38* 3'UTR were those designed for the amplification of the target sequence from pmirTarget, and to add restriction sites for Xho1 and Xba1. Restriction enzyme analysis of pmirGLO*CD38*ARE was attempted but bands were very faint and not easily visualised. Restriction enzyme analysis would have shown a band for the right size of the UTR, around 500bp, confirming an insert of the right size was released from the pmirGLO cloned vector.

4.2.1.2 DNA sequencing to confirm cloning of *CD38* 3' UTR ARE into pmirGLO

PCR analysis indicated that the right size of insert had been cloned into the pmirGLO vector and in the right direction, however, DNA sequencing was carried out to ensure that the correct DNA sequence for the 3' UTR ARE of *CD38* had been cloned. A clone for pmirGLO*CD38*ARE was DNA sequenced using appropriate primers for the human *CD38* 3' UTR ARE (see Materials and Methods). Sequencing results confirmed that *CD38* 3'UTR was cloned onto pmirGLO vector. Sequencing results and ARE motif are shown in the appendix.

4.2.2 ZFP36 protein family targeting *CD38* ARE- Dual luciferase reporter assay results

Once data confirmed cloning of the *CD38* 3'UTR ARE onto pmirGLO vector HEK293T cells were transfected with the constructs as laid out in table 4-1.

Cells were transfected with 100 ng of pmirGLO*CD38*ARE luciferase reporter vector and with 200 ng of human ZFP36L1 expression vector (pcDNA6ZFP36L1) or ZFP36L1 with a zinc finger domain mutation (mutZFP36L1), pDEST26.ZFP36L2 or pcSPORT6ZFP36 or empty vector (pcDNA3) using Fugene (Promega).

Luminescence measures were taken in a plate reader 48-72 h later using a DLR assay system and a FluoStar Optima plate reader- details in Materials and Methods-. Data was analysed and plotted as a bar chart as seen in figure 4-2. A Student T- test was also performed to evaluate statistical significance:

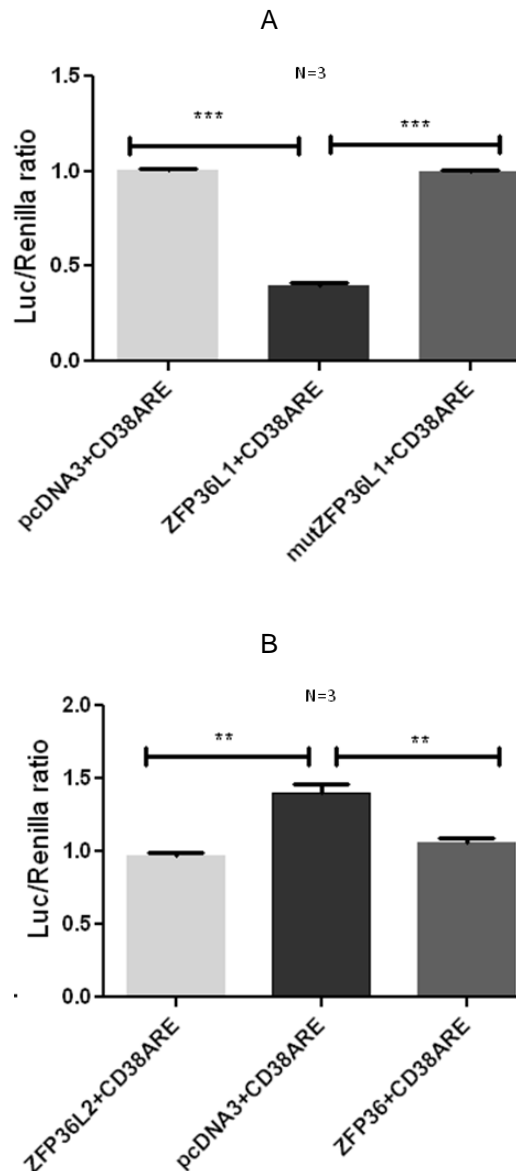


Figure 4-2 ZFP36L1 mediates degradation of *CD38* 3'UTR ARE and ZFP36L2 and ZFP36 also target *CD38* 3' UTR ARE for degradation.

A. HEK293T cells were transfected with pcDNAZFP36L1 or its mutant version and pmirGLO*CD38ARE*; mutant ZFP36L1 has a nucleotide modification that leads to an amino acid change in its ZFD. The firefly luciferase/renilla luminescence readings compared to empty vector pcDNA3, showed that wild type ZFP36L1 led to the degradation of the *CD38 ARE*; zinc finger mutant ZFP36L1 did not bind and degrade *CD38 3' UTR ARE*. Mean \pm SEM shown for independent experiments (n=3). T-test determined the significance of the difference between the means (empty vector control Vs. sample; mutant control Vs. sample), T Test:***P value =0.0006 and 0.00058 respectively. B. HEK293T cells were transfected with ZFP36L2 and ZFP36 and pmirGLO*CD38ARE*. HEK 293T cells were also transfected with empty pcDNA3 and pmirGLO*CD38ARE* as control. Figure shows ratios of firefly luciferase/renilla luminescence readings from FluoStar Optima. Luc/renilla ratios of ZFP36L2 and ZFP36 compared to empty vector pcDNA3 indicated that both proteins also lead to the degradation of *CD38 3' UTR ARE*. Luminescence readings for untransfected samples not shown. T-test determined the significance of the difference between the means, Mean \pm SEM shown for three independent experiments (n=3). B:**P value =0.009 and 0.0088 respectively.

The luminescence readings normalised to the control renilla gene's readings showed a very significant decrease in luciferase/renilla ratio indicating ZFP36L1 bound to the *CD38 3' UTR ARE* (fig. 4-2A). Higher luciferase/renilla luminescence ratio indicated that the mutated version of the

protein, with a change in an amino acid in the zinc finger region, did not lead to the protein interacting with *CD38* 3' UTR and readings were very similar to pcDNA3 empty vector control (fig. 4-2A). Luciferase/renilla ratio luminescence readings were also significantly reduced in the case of ZFP36L2 and ZFP36 compared to empty vector controls, indicating that each of these ZFP36 family proteins had also bound to the 3' UTR ARE of *CD38*.

In all cases luc/renilla ratios were significantly lower in the tests samples compared to those from an empty plasmid control (fig. 4-2B) and the individual results from each independent experiment did not vary much from one and another as shown by the errors bars indicating SEM.

4.3 Down regulation of ZFP36L1 and effect on CD38 level of expression

This second strategy in the investigation to research *CD38* mRNA as a target for ZFP36L1 tested if the interaction of *CD38* 3' UTR observed in the DLR assay experiments would also lead to changes at the level of CD38 protein expression in situations where ZFP36L1 expression levels were manipulated. Other studies have already reported on the manipulation of ZFP36 proteins for research of target mRNA validation (Jalonen et al. 2006a, Baou et al. 2009b, Lin et al. 2012, Nasir et al. 2012, Adachi et al. 2014). The working hypothesis was that if ZFP36L1 targets *CD38* mRNA, and hence controls *CD38* gene expression post transcriptionally, knocking down the expression of ZFP36L1 protein would lead to increase CD38 levels.

The objectives of this part of the project were: to manipulate the expression of ZFP36L1 by down regulation with siRNAs in an appropriate cell model, and measuring levels of CD38 protein to analyse if they were affected by this down regulation and the direction of this effect. Down regulating ZFP36L1 would require several siRNAs and their validation as effective in knocking down ZFP36L1 protein levels. This part of the project would also involve the detection of CD38 expression by flow cytometry on live cells and Western blot analysis of ZFP36L1 and CD38 protein levels in cells lysates.

RNA interference is a highly conserved mechanism in multicellular organisms. RNA oligonucleotides of about 20-23 in length regulate gene specific silencing via RISC. Briefly, long double stranded RNAs are cut into smaller double stranded strands by the enzyme Dicer; these shorter dsRNAs about 21-24 nucleotides long are called small or short interfering RNAs (siRNAs) and are incorporated into the RISC a multimeric protein that will be led by one of the strands of the siRNA to a complementary RNA sequence in the target mRNA; after binding to the transcript it is spliced, cleaved and translation has been inhibited (Kurreck 2006). RNAi has long been a research tool in laboratories; it is a commonly used technique to identify gene functions. In the case of siRNAs, these small oligos function by incorporating a sequence specific strand onto the RISC, in this way, a gene can be targeted and silenced and the effects of this silencing studied. If a given protein is thought to be a regulatory factor on the synthesis of a second protein the siRNA model

may be used to assess that relationship by means of silencing the regulatory protein and observing the effect on the target protein.

A key part in this experimental work was choosing an appropriate cell model:

Ideally the knockdown experiment would have been performed in primary leukaemic cells choosing patients with some expression of CD38 and where ZFP36L1 would have also been confirmed as expressed (e.g. by Western blot analysis). A B-CLL patient is classed as positive for CD38 when flow cytometry analysis shows CD38 is detected in 20% of malignant cells (or 30% at times, it will depend on the health authority of the territory and the clinical guidance issued to this respect) of the cell population (Dürig et al. 2002); this experiment was attempted. Spanish groups had reported successful transfection of B-CLL cells using Hiperfect (Redondo-Muñoz et al. 2010, Ugarte-Berzal et al. 2012), other groups using Lipofectamine (Baou et al. 2009b), but the notorious difficulty of transfecting primary cells and optimising the experimental conditions for this objective was leading to contradictory results in the present project and no consistent data was produced that could allow to move this approach forward. Electroporation was not available to be used and due to the time limits of this project a second strategy was attempted. This second approach consisted in using B cell lines which were reported positive for CD38 expression and confirmed from tests in the laboratory by using Western blot analysis and immunofluorescence and flow cytometry. H929 myeloma cells proved a good cell line for this purpose, but again, these cells as suspension cells, proved a challenge for reliable transfection.

Finally, a cell line model that would be easy to transfect and that would have detectable expression levels of ZFP36L1 and CD38 was sought. HeLa cells were confirmed to express CD38 despite contradicting literature, and also were shown to express ZFP36L1 and ZFP36L2; in fact HeLa cells have previously been used to study CD38 (Numata et al. 2012) and the same cells have also been used to knockdown the expression of ZFP36L1 and ZFP36L2 (Adachi et al. 2014). Nevertheless, considering the contradictory reports on the literature regarding CD38 expression in HeLa cells a number of key authors who work on CD38 and, or in HeLa were contacted, advice was requested from experts in CD38 like Prof. Malavasi (University of Torino Medical School, Torino, Italy) and Prof Okada (Graduate University for Advanced Studies, Kanagawa, Japan), before moving ahead. HeLa cells were kindly donated by Dr E. Wright at the University of Westminster and tests were run to confirm CD38 expression and also ZFP36L1 expression using Western blot analysis. The HeLa cells to be used in these experiments were confirmed to constitutively express ZFP36L1 and CD38. They were therefore found to be a suitable model to perform the knockdown experiments and analyse the effect on CD38 expression of down regulating ZFP36L1.

The cells were transfected with 3 different siRNAs targeting ZFP36L1 expression (please refer to Materials and Methods) and appropriate controls (Scramble RNA, untransfected cells); knockdown efficiency for each siRNA (referred to as siRNA1, siRNA2 and siRNA3 from this point on) was assessed using Western blot analysis, and effects on CD38 expression was analysed by immunofluorescence and flow cytometry of live cells and by Western blotting analysis.

4. 3.1 Western blot analysis of down regulation of ZFP36L1 by siRNAs

This part of the project aimed at analysing the effectiveness of 3 different siRNAs and different transfection reagents to down regulate the expression of ZFP36L1 in HeLa cells.

Briefly, HeLa cells were plated and transfected following manufacturer's instructions for each transfection reagent tested (only Lipofectamine RNAiMax –LifeTechnologies- and Hiperfect- Qiagen- results are shown here). Three siRNAs targeting different parts of the *ZFP36L1* gene were tested (from Life Technologies). A final concentration of 20nM siRNA was transfected into HeLa cells in exponential growth. Cell harvest time was estimated as optimal 48-72 hours after transfection.

One problem encountered was obtaining enough lysate from an optimised numbers of cells in order to detect expression of CD38. This was solved by plating HeLa cells in 12 wells plates and transfecting a number of wells and/or using 6 wells plate. Another factor that shows to be important in order to be able to assess an effect on CD38 was the time of incubation until harvesting the cells. Once the optimal time to harvest the cells was assessed, siRNA transfected HeLa cells were prepared for flow cytometry and cells lysate for Western blot analysis, for methods please refer to Chapter 2.

Western blots were performed to confirm the down regulation of ZFP36L1 by the siRNAs. Figure 4-3 shows ZFP36L1 expression levels in HeLa cells transfected with siRNAs: siRNA1 and siRNA2; Scramble siRNA, and in untransfected HeLa cells using two different reagents: Lipofectamine RNAiMAX and Hiperfect.

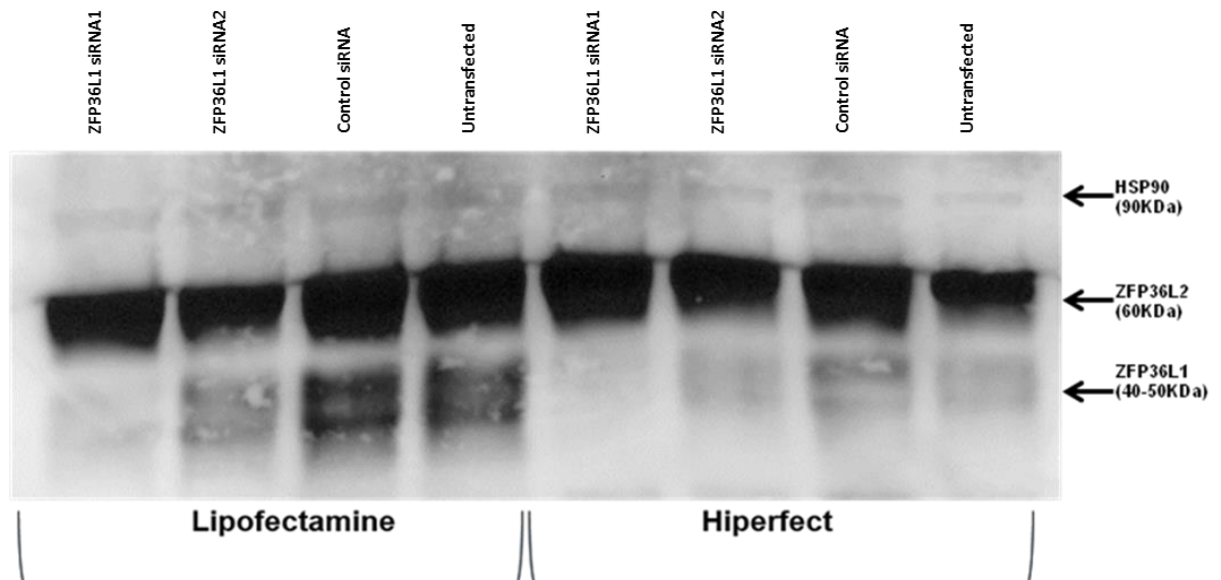


Figure 4-3 ZFP36L1 knockdown in HeLa cells analysed by Western blot.

Western blot analysis of ZFP36L1 expression to assess knockdown efficiency for two different siRNAs and two different transfection reagents. siRNA1 is more efficient in knocking down ZFP36L1 expression than siRNA2 and compared to controls, scramble RNA and untransfected cells. Of the 2 reagents, Lipofectamine RNAiMAX seems to be the best as a transfection reagent as shown by stronger band intensity and levels of ZFP36L1 knockdown. Transfections were done in HeLa cells which express ZFP36L1, and CD38, and serve as a good model for this experiment. Cells transfected were lysed in NP40 buffer and processed for Western blot analysis. Anti-human ZFP36L1/ZFP36L2 antibody was used and the blot was developed using chemiluminescence substrate in a UVP viewer.

Western blot analysis of ZFP36L1 expression knockdown efficiency for two different siRNAs and two different transfection reagents showed that, with both transfection reagents used, siRNA1 was more efficient in knocking down ZFP36L1 expression than siRNA2 but siRNA2 still was able to reduce ZFP36L1 expression levels considerably. Down regulation was seen with both reagents used (Lipofectamine RNAiMAX and Hiperfect) and in both cases the same pattern of down regulation was observed for each siRNA, higher down regulation in siRNA1 than in siRNA2 transfected cells. Expression of ZFP36L2 was unaffected. For scramble RNA (control RNAi) and the untransfected HeLa cells the level of expression of ZFP36L1 was roughly equivalent and higher than those of the siRNA transfected samples.

4.3.2 Analysis of CD38 expression after ZFP36L1 down regulation

This was investigated using two different experimental strategies: A. Immunofluorescence and flow cytometry of cells and B. Western blot analysis of cell lysates.

The previous pages described how successful down regulation of ZFP36L1 was confirmed in HeLa cells using *ZFP36L1* specific siRNAs. For the same cells the levels of CD38 expression was analysed by immunofluorescence and flow cytometry and also by Western blot analysis. Harvested cells were stained with FITC labelled anti-human CD38 antibody; fluorescence levels of all samples were read using a flow cytometer (CyAn ADP Analyzer, from Beckman Coulter). For detailed description of this experiment and reagents please see Materials and Methods. Cell samples where flow cytometry analysis had shown a histogram shift in fluorescence intensity indicating an increase in CD38 expression were lysed and used for Western blot analysis alongside controls.

A. Flow cytometry analysis of CD38 levels in ZFP36L1 knockdown HeLa cells

CD38 is an ectoenzyme, the molecule is a cell membrane protein and its expression can be easily analysed by flow cytometry. In flow cytometry the cells are stained with a fluorescently labelled antibody and the fluorescence signal is detected following excitation with a laser. The cells are initially distributed in a density plot by size and granularity and an area can be chosen (gating) to further analyse fluorescence in a histogram plot. This approach was chosen to analyse, in HeLa cells, the expression of CD38 in siRNA transfected cells, scramble siRNA transfected cells and untransfected cells. Testing the hypothesis that ZFP36L1 targets and degrades *CD38* mRNA, the samples where ZFP36L1 is down regulated would show an increase in CD38 fluorescence indicating higher CD38 expression compared to controls if the hypothesis were correct.

After siRNA transfection and knockdown of ZFP36L1 expression, HeLa cells were prepared for flow cytometry analysis of CD38. The cells were stained with FITC labelled anti-human CD38 antibody; in each experiment the density plot was gated where the largest population of cells was concentrated, considering size and granularity to gate for live cells. Where possible samples were run so that a similar number of cells would be counted within the area originally gated and so a similar number of cells would therefore be compared. Figure 4-4 shows a representative density plot with gating where the populations of cells is denser; figure 4-5 shows histograms comparing

CD38 fluorescence signals for the 3 different siRNAs, scramble transfected cells and untransfected HeLa cells.

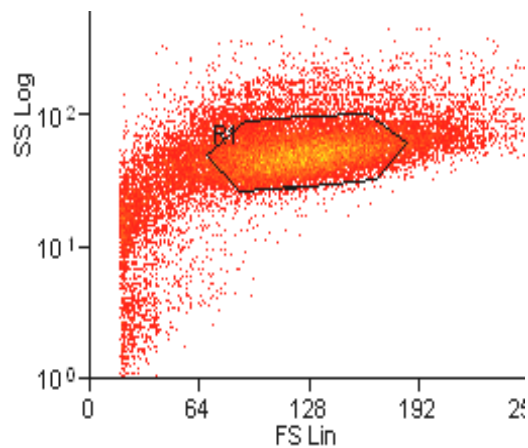


Figure 4-4 siRNA transfected HeLa cells density plot.

Representative density plot with gated area for analysis. Gates were chosen in control samples and placed where the population of cells was densest so the largest number of cells could be compared across samples. Size (FS LIN) and granularity (SS LOG) were considered in order to gate so live cells would be placed within the area. Samples were run trying to achieve an equal number of cells within the original gate so an even number of cells will be compared across samples.

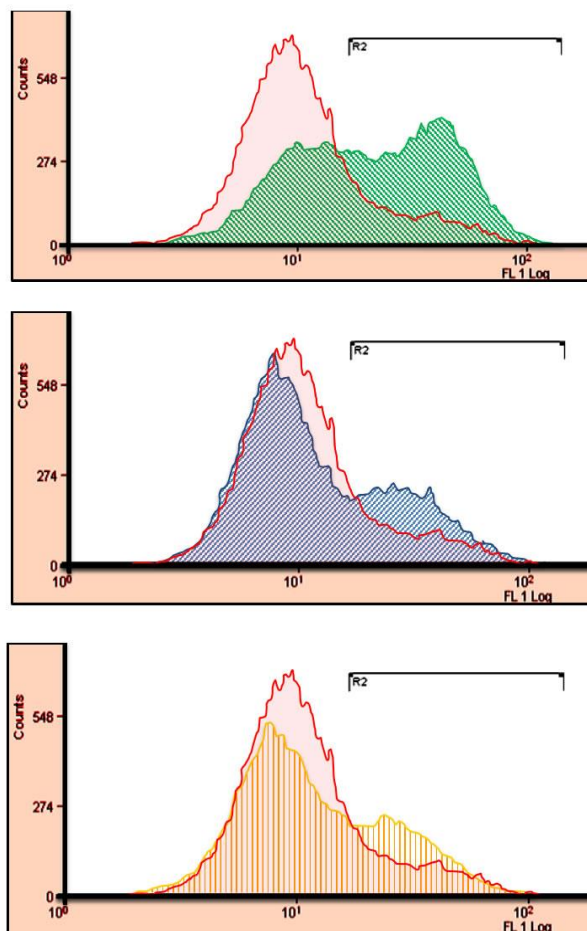


Figure 4-5 CD38 fluorescence histogram in ZFP36L1 down regulated HeLa cell samples and controls.

Overlay log fluorescence intensity histogram with siRNA transfected samples and controls with gated cell area for % of cells and MFI analysis. Red: Scramble; Green=siRNA1, Blue= siRNA2, Yellow=siRNA3. A shift in fluorescence towards the right was observed in all siRNA transfected samples with down regulated ZFP36L1, that shift indicated an increase in CD38 expression levels. Representative of n=5 for siRNA1, n=3 for siRNA2, and n=4 for siRNA3

Flow cytometry analysis of HeLa cells transfected with the 3 different siRNAs showed that all siRNA transfected cells resulted in a shift in the histogram curve for CD38 fluorescence, the shift is indicative of increased CD38 levels compared to controls. This was true for the three siRNA transfected samples silencing ZFP36L1 but with different degrees of CD38 detection for each siRNA. There was a clear difference observed in the case of siRNA1, which is also the siRNA leading to the highest ZFP36L1 knockdown and transfection of cells with siRNAi1 led to the highest increase in CD38 expression.

Table 4- 2 Percentage of CD38 positive cells in R2 gate (as shown in Fig 4-5) in different experiments

n	SiRNA1	SiRNA2	SiRNA3	Scramble RNA
1	60.79		53.35	33.27
2	47.66			25.3
3	56.58	23.12	27.68	23.07
4	62.06	31.26	35.05	20.84
5	47.33			43.03
6		37.23	40.21	36.69

Data shaded blue was analysed for statistical significance.

Paired T Test analysis of siRNA1 and Scramble RNA transfected cells produced a value of P=0.014 showing a statistical difference in the % positive CD38 cells in siRNA1 transfected vs scramble siRNA transfected cells (Table 4-2).

Figure 4-6 shows normalised mean CD38 fluorescence levels for siRNA transfected samples. CD38 mean fluorescence from samples with down regulation of ZFP36L1 were increased compared to scramble transfected cells where no down regulation of ZFP36L1 was seen:

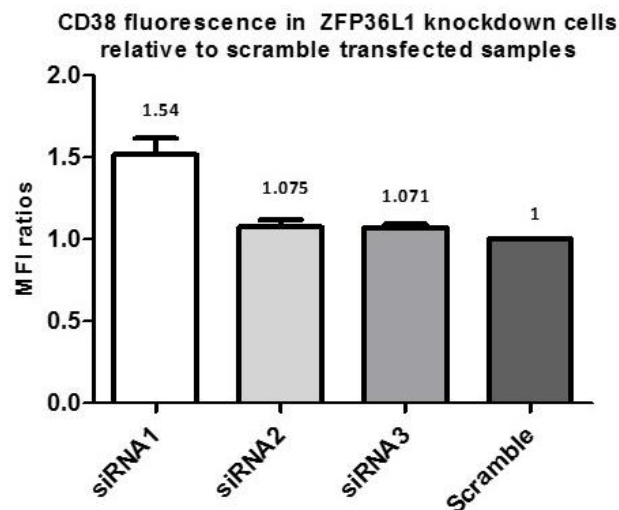


Figure 4-6 CD38 Mean fluorescence intensity ratios normalised to scramble transfected cell mean fluorescence intensity levels.

CD38 mean fluorescence intensity in samples where ZFP36L1 had been down regulated by each siRNA relative to CD38 fluorescence detected in scramble RNA transfected samples where ZFP36L1 had not been knocked down. n=5 for siRNA1; n=3 for siRNA2; n=4 for siRNA3. Mean/SEM shown

Normalised mean fluorescence intensity for each set of transfected cell samples, showed that siRNA1 transfected cells, where down regulation of ZFP36L1 was most effective, led to an increase in CD38 fluorescence levels compared to scramble transfected cells (fig. 4-6).

To summarise, flow cytometry analysis of CD38 expression in HeLa cells transfected with the 3 different siRNAs against ZFP36L1 indicated that for the siRNA that had shown the most efficient ZFP36L1 knock down by Western blot, siRNA1, CD38 levels increased the most compared to the scramble controls where no down regulation of ZFP36L1 occurred. SiRNA2 lead to the second largest increase in CD38 levels which also correlated to its effects down regulating ZFP36L1 less efficiently than siRNA1. siRNA3 transfected cells showed the least increase in CD38 levels. The results from flow cytometry analysis for CD38 levels inversely correlate to level of decrease in ZFP36L1 expression and are consistent with the ZFP36L1 knock down levels seen by Western blot figure 4-3.

B. Western blot analysis of CD38 expression in down regulated ZFP36L1 samples

To further assess the possible increases in CD38 levels detected by immunofluorescence and flow cytometry following ZFP36L1 down regulation, cell lysates of siRNA transfected HeLa cells used in the previous set of experiments, were used in Western blotting to detect CD38 expression levels (these same samples were used to assess ZFP36L1 knockdown by Western blots in figure 4-3). On this occasion samples were Western blotted for CD38 analysis, -the results are shown using Lipofectamine RNAiMax transfected cell lysates-.

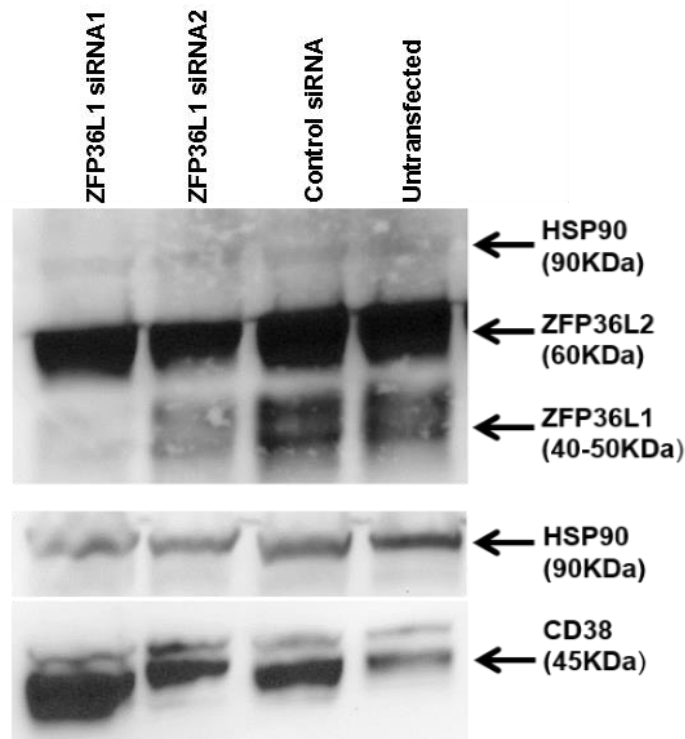


Figure 4-7 Western blot analysis of CD38 expression in ZFP36L1 down regulated HeLa cells.

Figure shows bands detected for CD38 in samples where ZFP36L1 had been down regulated (siRNA1 and siRNA2) and control; it shows a combined image of blots analysing down regulation of ZFP36L1 and the corresponding CD38 expression levels.

Western blot analysis of CD38 expression showed bands for CD38 in all samples, with the highest expression corresponding to samples where siRNA1 had been used to knockdown ZFP36L1 protein levels leading to the highest down regulation of ZFP36L1. The band detected for CD38 in the samples transfected with siRNA2 was not of an intensity or size that could determine an increase in CD38 as it should be expected from the results obtained by the flow cytometry analysis, these bands were measured in order to semi quantitate the blot results and further analyse expression levels.

C. Semi-quantitative analysis of expression bands detected for ZFP36L1 knockdown and CD38

The bands detected by Western blot analysis to confirm down regulation of ZFP36L1 and increased expression of CD38 (fig. 4-7) were semi quantified using GS-800 Calibrated Densitometer from Bio-Rad. Figure 4-8 shows densitometry results normalised to expression levels of HSP90.

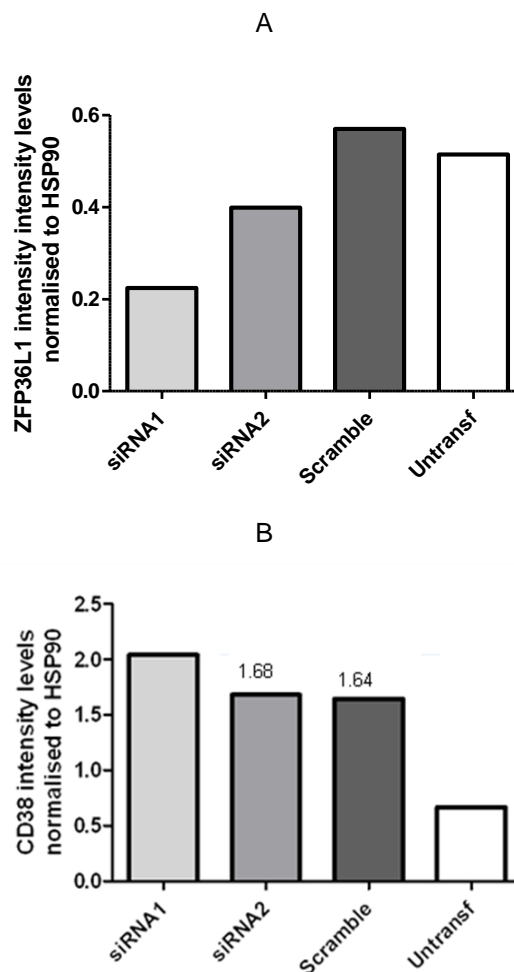


Figure 4-8 ZFP36L1 bands intensity normalised to HSP90 and CD38 bands intensity normalised to HSP90 levels.

A. The intensity of the bands for siRNA1 downregulated ZFP36L1 was 60% less to that of scramble transfected samples; intensity of band for ZFP36L1 knock down using siRNA2 was of 30% less to scramble samples confirming a considerable level of downregulation of the protein. .B. CD38 bands intensity normalised to HSP90. Intensity levels normalised to HSP90 indicated that CD38 expression increased, CD38 bands in the samples with highest ZFP36L1 downregulation were 20 to 30% increased in intensity compared to scramble siRNA transfected cells with no ZFP36L1 downregulation. The increase in CD38 band intensity was only of 2.5% in siRNA2 transfected cell where the level of downregulation of ZFP36L1 was the least.

Semi quantitative analysis of protein bands detected in blots confirmed that the highest knockdown of ZFP36L1 is achieved with siRNA1 which corresponds to the highest increase in CD38 expression. Normalisation of protein expression to HSP90 levels for ZFP36L1 downregulated samples showed that siRNA1 was able to knockdown the expression of the ZFP36L1 protein to half the level than that found in siRNA2 transfected cells (fig. 4-8). For siRNA2 transfected samples the intensity values normalised to HSP90 expression determined that CD38 expression was higher than in scramble or untransfected but the increase was minimal.

4.4 Discussion.

The luminescence readings from the DLR assay showed a very significant decrease in luciferase/renilla ratios indicating ZFP36L1 interacted with *CD38* ARE (fig. 4-2A) and suggesting it may mediate *CD38* mRNA degradation. Readings from the samples transfected with the mutated version of ZFP36L1 showed increased luminescence (fig. 4-2A); the *CD38* ARE was not targeted by the mutated version of ZFP36L1 with a zinc finger base pair modification leading to an amino acid change, indicating that intact zinc fingers motifs are required for ZFP36L1 targeting the *CD38* ARE.

This result is in line with previous evidence shown in this thesis for BCL2, and in the REMSA assays (Zekavati 2009, Zekavati et al. 2014) already discussed in chapter 3 for BCL2. The same ZFP36L1 mutant construct was also used to study ZFP36L1 interaction with BLIMP1, in a luciferase assay where they also tested the functionality of the ZFD showing that the target ARE was not bound by the altered ZFD protein (Nasir et al. 2012). The interaction between the proteins and the ARE does take place via the protein's ZFD and functional ZFDs are required for the degradation of the mRNA 3' UTR ARE.

A mutated version of *CD38* ARE was not available for use in this study, however, research looking at the post transcriptional regulation of *CD38* provides information not only on other factors that bind to its 3' UTR but also on the specificity of that ARE for that interaction. A 2014 paper showed that *CD38* mRNA is negatively regulated via its ARE by miR709 and reports on findings validating the binding of miR-708 to the 3' UTR of *CD38* (Dileepan et al. 2014). The study used a mutated version of the UTR target sequence, this mutant hindered, reversed the inhibitory effect of miRN-708 on luciferase activity, and this observation led to confirming the specificity of its target binding at the 3' UTR ARE of *CD38*.

The interaction between *CD38* and ZFP36 proteins has been suggested by other authors looking to determine the ARE relevance in the regulation of this protein. *CD38* ARE is not a binding site for HuR, over expression of HuR in airway cells did not alter its mRNA decay (Jude et al. 2010). In a subsequent review by the same author he proposed that other RNA binding proteins like ZFP36 may have a counteracting role in TNF α induced *CD38* expression (Jude et al. 2012a). The regulatory web of *CD38* expression might involve the ZFP36 family in two ways, by direct regulation via its ARE and by regulation of TNF α in a dual pathway; TNF α is itself a confirmed

target for the three ZFP36 proteins and, on the other hand, it also regulates CD38 post transcriptionally, TNF α regulation by another miRNA, miR140-3p, induces CD38 expression (Jude et al. 2012b).

The DLR assay provided evidence indicating that the other ZFP36 family members also recognise CD38 ARE (fig. 4-2B) but for these proteins no further investigations were pursued. These results are in agreement with what was obtained for BCL2 and with other research showing the proteins might share some targets. In previous work it has been confirmed that the 3 ZFP36 family proteins bound and degraded the mRNAs of not only TNF α (as already mentioned in chapter 3) but also GMCSF, or IL3 (Carballo et al. 1998, Carballo et al. 2000, Stoecklin et al. 2000, Lai and Blackshear 2001, Stoecklin et al. 2002). More recently, ZFP36L1 and ZFP36L2 were shown to bind to the 3'UTR of *MAPK* mRNA and downregulate its 3'UTR mediated luciferase activity (Lin et al. 2012). The redundancy in their role as post transcriptional regulators proposed by Vignudelly et al. 2010 has been discussed in chapter 3.

Manipulation of gene expression via RNA interference or over expression has also been a key strategy in the quest to elucidate gene function in general, and concretely to validate putative targets for this family of proteins. Past and recent groups have demonstrated by RNAi the role of ZFP36 proteins in mRNA regulation; double siRNA-mediated knockdown of ZFP36L1 and ZFP36L2 has been used previously showing that silencing of the proteins could efficiently be achieved in HeLa cells (Adachi et al. 2014) and provided supporting evidence to the experimental strategy followed in this thesis. The Adachi group used antisense nucleotides against ZFP36L1 and ZFP36L2 to investigate the relationship between these proteins and LDLR. They demonstrated that downregulation of ZFP36L1 and ZFP36L2 resulted in up regulation of *LDLR* mRNA and LDLR protein, they used a luciferase reporter assay, cloning the 3'UTR of *LDLR* mRNA, and finally assessed knockdown efficiency by QRT-PCR and Western blot analysis.

The same siRNAs used to knockdown ZFP36L1 expression in this thesis were used in previous studies (Zekavati et al. 2014); of the 3 siRNAs tested, siRNA1, and siRNA2 yielded the most efficient knockdown (no Western blot data is shown for siRNA3). Knockdown for ZFP36L1 expression was optimally achieved by siRNA1; siRNA2 knockdown was less efficient compared to siRNA1 but still achieved a high downregulation of ZFP36L1, and the same result was seen when either reagent, LipofectamineRNAiMax or Hiperfect, was used. For scramble RNA (control RNAi) and the untransfected HeLa cells, the expression of ZFP36L1 was evident and higher than that observed in the siRNA transfected samples. The different knockdown efficiency between siRNA1 and siRNA2 may be down to the fact that these siRNAs target different exons in the *ZFP36L1* gene; however, this was not further investigated.

Efficiency and specificity in downregulation are important assessments for RNA interference, a problem that might be encountered is "off target" effects (Jackson et al. 2003). The off target effects arise from the sense strand of siRNAs having homology to a non-target gene which is incorporated to the RISC; an unwanted gene is in consequence silenced which can interfere the interpretation of

results. Unwanted silencing effects on ZFP36L2 and/or ZFP36 could influence CD38; these proteins were also shown to interact with the 3'UTR ARE of *CD38* mRNA in the DLR assay. Off target effects may happen even between sequences of low homology since only a small region of base pairing is required and not only genes from a homologous family but other genes can be affected by the siRNA oligos. Considering that avoiding all off target effects is pretty much impossible, different methods and techniques have been developed to reduce them. The siRNAs used in the present study were designed to not recognise sequences in ZFP36L2 and ZFP36 transcripts; expression of ZFP36L2 was unaffected, as seen in figure 4-3, which provides evidence to confirm that siRNA1 and siRNA2 acted with specificity for silencing ZFP36L1.

Advanced specificity alignment of Stealth siRNA ("Life technologies"), the type used in these experiments, limits potential for off-target effects. In classic siRNAs both strands could enter the RNAi pathway but the siRNA used for these experiments are modified so only the antisense strand is able to enter the silencing pathway, get incorporated into the RISC and engage in the interference and knockdown of gene expression, hence limiting very much the off target events. This modification to stop the sense strand producing off target effects is done on the most modern siRNAs (Chang et al. 2012). The Stealth siRNAs used in the CD38 investigation are also more stable with a longer half-life. Their stability is an advantage for the study of some targets as in the case of CD38, where in order to observe an effect due to knockdown of the regulator protein (ZFP36L1) incubation of the cells had to be longer than the usual 24-48h.

Within the efforts to ensure that the phenotype observed in the siRNA experiments described in this chapter is reliable, controls were put in place, such as a scramble version of siRNA and the use of different siRNAs to target ZFP36L1. A strategy to ensure specificity in RNAi experiments and that knockdown of a gene leading to a phenotype is not due to off target effects consists in using multiple siRNAs to the same target. The basis of this is that their off-target effects will be different but their effect on the target mRNA would be expected to be the same, and so an important requirement to validate the specificity of a knockdown experiment would be confirming the observed phenotype with more than just one siRNA which would target different regions (Cullen 2006). The standard rule is to use at least two siRNAs, Western blot data is shown in figure 4-3 for two siRNAs confirming downregulation of ZFP36L1. Downregulation by siRNA1 and siRNA2 was observed even when using two different transfection reagents, furthermore the downregulation reproduced the pattern of reduction in ZFP36L1 for each siRNA (highest downregulation by siRNAi1, followed by siRNA2) further confirming the reproducibility of the results.

These results or rather these differences in the level of downregulation of the protein by each siRNA were later observed to be consistent with the results obtained by flow cytometry analysis of CD38 expression. Overall, analysis of flow cytometry results indicated that different levels of downregulation of ZFP36L1 inversely correlated to different levels of increased CD38. Flow cytometry analysis of HeLa cells transfected with each siRNA showed a shift in the histogram curve for CD38 fluorescence indicating increased levels of CD38 compared to controls (fig. 4-6). Cells transfected with siRNA1, where the level of ZFP36L1 had been downregulated the most, showed

the biggest increase in CD38 fluorescence compared to scramble RNA samples (fig. 4-6). siRNA2 led to the second largest increase in CD38 levels which also correlated to its effect downregulating ZFP36L1 less efficiently than siRNA1. The third siRNA tested, siRNA3 was not as efficient in leading to an increase in CD38 mean fluorescence. Further analysis of siRNA transfected cells for CD38 levels showed that there were differences between % CD38 positive cells compared to scramble controls in all siRNA transfected cells, for siRNA1 transfected cells showed that there was also a strong statistical difference in the % of CD38 positive CD38 compared to scramble siRNA transfections (table 4-2).

Densitometry analysis of ZFP36L1 and CD38 bands (fig. 4-7 and 4-8) resulted in data showing that ZFP36L1 band intensity was about 60% lower than in scramble RNAi control, and that for those samples CD38 band intensity increased 20-30%; for siRNA2, these numbers were 30% reduction in ZFP36L1 band intensity and 2.5% CD38 intensity increase. Although no further comment may be made on this analysis since it was performed just once, the intensity levels would seem to indicate that siRNA1 downregulated ZFP36L1 double than siRNA2 and although siRNA2 achieved a less efficient downregulation of the protein it still knocked down ZFP36L1 considerably; the correlated level of CD38 in siRNA2 samples was lower than that seen in samples with the highest ZFP36L1 knockdown (fig. 4-8 A and B). From the data obtained, and within the limits imposed by densitometry measures, a question may be made to the possibility that perhaps a certain level of ZFP36L1 downregulation is needed for a considerable and clearly detectable increase in CD38 expression to be measurable.

Lastly, a comment on data shown for untransfected cells: the blots show how the lysate of these cells gave CD38 bands that do not match that of scramble transfected cells (fig. 4-7 and 4-8) although flow cytometry has shown a more similar result when untransfected and scramble are compared. Scramble siRNAs activate the RNAi machinery in order to determine baseline reference for the effect of the siRNAs. When it comes to comparing results it must be taken into account that siRNA transfected samples and scramble siRNA transfected samples are more equally treated as both have had the same transfection reagent and equal amounts of RNA added, and that is not the case for untransfected cells. It is also known that transfection may cause the cells to stress, may be immunogenic or the transfection reagent may cause toxicity (O'Keefe 2013). There is one last possible explanation for the stronger CD38 band detected in the scramble siRNA transfected samples and that would be a partial complementarity of the scramble oligo with the ZFP36L1, however the protein levels do not seem downregulated. Off target effects of the control siRNA are also a possibility, but, as already discussed, these oligoes are new generation siRNAs and have been validated to avoid secondary effects. Therefore the seemingly augmented expression of CD38 in samples transfected with scramble siRNA are most possibly down to the manipulation, reagent and transfection process (O'Keefe 2013). To further assess this possibility more than one scramble siRNA could be used in future experiments, also other transfection reagents and even a different cell line could be used. Measuring mRNA levels would also assist in evaluating any effect of scramble siRNA on either protein studied. In addition, the difference observed between Western blot and flow cytometry results may also be due to the sensitivity of the methods and complexity -of

blotting in this case-, but also due to the different independent samples analysed; where Western blot analysis just done once, flow cytometry analysis was performed in at least n>5 samples, making the latter results more reliable

It is impossible to quantify the direct relationship between level of downregulation of ZFP36L1 and up regulation of CD38 by the experiments shown here alone, although flow cytometry is more sensitive and can often have a better quantitation range. Western blot is, at most, a semi-quantitative method, measuring a protein relative to total protein from cell lysates where variations in loading and transfer rates between samples in the lanes influence the detection of the proteins in the membrane (Dragowska et al. 2000). Because of these variables other methods such as QRT-PCR might be also useful to help quantitate down regulation/or up regulation of a gene. However, Western blot analysis with controlled and normalised results provides, like in this case, evidence of increase or decrease expression and a trend. Some argue that analysing knockdown and up regulation at the protein level by Western blot rather than solely by QRT-PCR is a more reliable way to assess downregulation, especially in mammalian cells because only part of mammalian RISCs are capable of performing siRNA-directed RNA cleavage, for the rest the expression is suppressed by actually interfering with protein synthesis, if knockdown is only analysed by measuring RNA levels (be it by quantitative PCR or northern blotting) it can overestimate knockdown for long-lived proteins, as it is the case of CD38, or underestimate knockdown caused by contributions of non-catalytic RISCs (Chang et al. 2012). Hence analysis of downregulation of ZFP36L1 (and later expression of CD38) by Western blot serves in this project as an appropriate method demonstrating that ZFP36L1 expression had been downregulated by 2 different siRNAs, and CD38 had increased in the same samples.

CD38 is a plasma cell marker and its regulation by ZFP36L1 may fit with a role for the ZFP36L1 protein in mature/plasma B cells. Other researchers (Wegmüller et al. 2007, Vignudelli et al. 2010) had already reported a role for ZFP36L1 in the negative regulation of cell differentiation; when the protein was downregulated by shRNAs cardiomyogenesis was enhanced (Wegmüller et al. 2007); and targeting of *Stat5b* mRNA by the protein negatively regulates erythroid differentiation (Vignudelli et al. 2010). Results from this chapter provide further information on the regulatory framework of plasmacytoid differentiation by ZFP36L1, they fit into the work done indicating that ZFP36L1 may function as a negative regulator of plasma cell differentiation

BLIMP1 contains several pentameric AUUUA motifs (Nasir 2012), just like CD38, and its validation as target for ZFP36L1 (Nasir et al. 2012) provided evidence of ZFP36L1 being implicated in B cell lymphopoiesis by negatively regulating the mature B cell to plasma cell stage. B cell line models and murine primary B cells showed ZFP36L1 expression but it was not detected in plasma cells (Nasir et al. 2012). ZFP36L1 knockdown experiments using shRNA led to enhanced cytokine-induced plasma cell differentiation but enforced expression of ZFP36L1 in the murine BCL1 cell line showed blocked cytokine-induced plasma cell differentiation (Nasir et al. 2012). The BLIMP1 study used a similar experimental strategy to that described here for CD38. Their gene expression manipulation results also show a similar pattern to those described in this chapter. They also used a

luciferase reporter assay to confirm ZFP36L1 bound to *BLIMP1* 3'UTR, silencing of ZFP36L1 resulted a significant increase in levels of blimp1 mRNA, that increase was smaller with another shRNA, and those results translated to Western blot analyses with highest ZFP36L1 knockdown cells expressing higher levels of BLIMP1 protein compared to control cells (Nasir et al. 2012).

The relevance of ZFP36 proteins regulating CD38 may be extended to mature B cell malignancies where several reports show that ZFP36 genes are altered in MM by being either deleted or mutated (Davies et al. 2003, Chapman et al. 2011). Deletions in a regulatory intron of ZFP36L1 have been found in a significant minority of primary MM cases, a malignancy classically CD38 positive (Chapman et al. 2011); the functional relevance of this finding is not yet known but results from this chapter could be relevant in this regard. The mRNA degradation machinery seems also to be impaired in myeloma by loss of exosome function, the exosome is one of the components involved in the ZFP36 led degradation pathway (Chen et al. 2001), that impairment may be as a result of another mutated gene found in 11% of myeloma cases studied, Rrp44 gene (encoding exosome component 11), which is mutated within its coding region (Chapman et al. 2011).

In an earlier paper, clinical data, cytogenetics and microarray expression analysis to compare healthy versus malignant gene expression in monoclonal gammopathy of unknown significance (MGUS) and MM were used to develop molecular profiles associated with plasma cell malignancy. The resulting transcriptome model evidenced genes differentially expressed between plasma cells from healthy donors and individuals with MGUS or MM. Post-transcriptional regulatory proteins shown to be downregulated included members of the zinc finger family, namely ZFP36L1 and ZFP36 (Davies et al. 2003). On the other hand, in MM a number of suspected and confirmed targets for ZFP36 proteins are up regulated and involved in developing and sustaining cell malignancy, e.g. TNF α , GM-CSF and IL6 supporting the growth and survival of MM cells both by stimulating proliferation and inhibiting apoptosis (Hussein 2002)

4.5 Conclusion

The DLR assays showed that *CD38* 3' UTR ARE is bound by ZFP36L1 via its ZFDs, ZFP36L2 and ZFP36 results also indicated they interacted with the *CD38* 3' UTR ARE. Downregulation of ZFP36L1 by siRNAs correlated to increased CD38 expression, further providing evidence to establish a post transcriptional regulatory role of ZFP36L1 over *CD38* mRNA. In order to improve these experiments a siRNA phenotype rescue could be performed, and also measurements of mRNA levels by QRT-PCR could be studied.

The findings could also be extended by performing ZFP36L1 knockdown in primary B cells, such as B-CLL cells or human tonsil B cells. Mutant versions of the ARE for *CD38* and ZFD mutant versions of ZFP36L2 and ZFP36 could also be used in other luciferase assays to extend the studies presented here. The expression of these other two ZFP36 family proteins could also be manipulated to assess any changes in *CD38* expression and QRT-PCR measurements taken alongside Western blot analysis in order to validate if they also regulate CD38.

Chapter 5

**“Immunohistochemistry analysis of ZFP36L1, BCL2 and CD38 expression
in normal and malignant lymphoid tissue”**

5.1 Introduction

This part of the thesis describes IHC experiments in healthy lymphoid tissue (tonsil and lymph nodes sections) and FL to test for patterns of expression of ZFP36L1 in relation to the putative targets under study. Based on the results described in previous chapters, the hypothesis behind this set of experiments was that positive and high levels of ZFP36L1 expression in cells may be associated with absent/low levels of BCL2 and CD38. These experiments would add another dimension to this project since they look at protein expression at tissue level. In IHC analysis antibodies bind to antigen in a specific manner and provide spatial localisation, also giving information of particular cells and proteins.

Tonsils are defined as accumulations or mass of lymphoid tissue located in the upper respiratory and gastrointestinal tract. Lymph nodes are small, bean shaped structures and part of the lymphatic system distributed around the body. They serve as a filtering point of the lymph fluid brought in by lymphatic vessels. Their structure is formed by cortical and medullary regions, and like tonsils they have primary follicles (lymphoid follicles without a GC) and secondary follicles (lymphoid follicles with a GC).

Whilst lymph nodes are distributed throughout the body, tonsils are present in the aerodigestive tract. Pharyngeal tonsils situated at the roof of the nasopharynx, tubal tonsils posterior to the opening of the Eustachian tube in the ear, palatine tonsils in the oropharynx, and lingual tonsils on the posterior tongue constitute the Waldeyers ring, protecting the entry to the respiratory systems; it is a ring of lymphoid tissue involved in the production of immunoglobulins and the development of both B cells and T cells. Tonsils are part of mucosa associated lymphoid tissue (Kenna et al. 2009). Palatine tonsils are covered by stratified squamous epithelium that invaginates to form blind ended tunnel like structures, these are networks of branched crypts, allowing for a larger internal surface area and hence, increased contact between the lymphoid tissue resident immune cells and environmental influences; the crypts serve as a first contact to pathogen entry route. The tonsils are formed by four lymphoid compartments: reticular crypt epithelium, the extra follicular area, the mantle zones of lymphoid follicles, and the follicular GCs. In tonsils, foreign particulate or bacteria may enter the crypts and pass to the follicles (transcytosis) by the epithelial cells within the crypt lining starting an immune response. The GC may be polarised with cells forming two zones, a light zone (with centrocytes, CCs) nearer the antigen entry site and a dark zone (proliferating centroblasts, CBs), that will move to the light zone away from the site of antigen entry at the epithelial surface; a mantle of cells also forms surrounding the GC (Knowles 2001, Natkunam 2007); in the case of the lymph node polarisation can also be seen in the GC but the antigen entry happens from the lymph fluid via lymph vessels (Janeway et al. 2001).

Tonsil and lymph nodes contain B cells that represent different stages of B cell differentiation that include GC B cells, and memory and plasma B cells. The primary follicle mostly contains mature naive B-cells, that upon activation of resident T cells, will develop into a GC formed by those B cells undergoing clonal expansion, B-cell receptor affinity maturation, positive selection of B cells

according to receptor affinity for antigen and differentiation to B memory cells and plasma cells (Brandtzaeg et al. 2003), Therefore, tonsil and lymph node sections can be very helpful for this study since they house different B cell populations.

GC formation, independently of whether it is in tonsillar tissue or lymph nodes, starts with IgM⁺IgD⁺ naive B cells receiving stimulatory signals from helper cells, they are activated by antigen receptor stimulation and B cells transform into CBs and start to proliferate, they form the dark zone of the GC. SHM inducing single nucleotide changes in the IgV regions takes place modifying the antibody affinity to the antigen and the cells become CCs, forming the light zone of the GC. As CCs the B cells now go through clonal selection based on the ability of their antibodies to recognise the antigen held on follicular dendritic cells and at this stage many CBs undergo apoptosis so the highest antibody affinity containing CCs survive. Those CCs with the highest affinity antibody are selected for survival and eventually differentiate into plasma cells or memory cells. Some CCs also change IgM and IgD to other Ig isotypes through class-switching using somatic DNA recombination and generating different effector antibodies. Those high-affinity antibody containing memory B cells leaving the GC will be long lived cells and are able to rapidly differentiate into Ig-secreting cells upon secondary immune responses (Klein et al. 2003).

GC B cells are involved in the pathogenesis of many types of human B cell malignancies, including DLBCL, FL, and Burkitt Lymphoma (Stevenson et al. 1998, Epstein et al. 1999). The counterpart to healthy tonsil tissue and healthy lymph nodes is here represented by FL tissue, viewed as a GC-derived neoplasm (Leich et al. 2009). Lymphomas are subdivided depending on the type of cells that are involved; they are mainly categorised as HL and non-HLs and in IHC analysis this division is made based on the presence of Reed Steinberg cells. The symptoms tend to be similar for both categories of diseases, tiredness, fever, night sweats, itchiness, and at times swelling in key nodal areas. Under the category of non-HL there are B and T cells lymphomas, with B cell lymphomas making 85% of cases (Dotan et al. 2010, Mitchell et al. 2012). B cell lymphomas of high grade include Burkitt or DLBCL. FL is the second most frequent non-HL in the Western world and is usually an indolent disease with a 7 to 12 median survival. It affects older adults, has no cure and can transform into an aggressive version of the disease (Pulte et al. 2013). FL is considered a low-grade neoplasm but within FL the histologic grading can be from I to III. At grade II the disease starts to show similarities with DLBCL although cytogenetic characteristics differ (no BCL2 translocation). Grading of FL is recommended to be done based on the counting methods by Mann and Berard as per World Health Organisation 2008 guidance and classification system (Harris 2008, Pileri et al. 2008) where FL cases can be defined to be in one of the 3 stages depending on number of CBs/high power field (Jaffe 2009); details of this grading system alongside Ann Arbor staging system can be found in appendix. Grade 3 is divided into 3A and 3B after observing CCs (3A) or solid sheets or entire follicles made of CBs (3B) (Jaffe 2009). The staging is based on morphological features so IHC analysis is at the core of the assessment. Whereas in many cases the indolent nature of the disease permits patients to live without the need for drug treatments, patients with grade III FL, behaving like or transforming into DLBCL, are usually treated (Ott and Rosenwald 2008). Treatment will depend on assessment of each patient and may include

radiotherapy, chemotherapy, monoclonal antibody therapy, e.g. Rituximab (Doss et al. 2012), and even surgery. Immunohistologic markers typically used in the assessment of FL are CD10, BCL6 and BCL2 (Fouad-Younes et al. 2010). BCL2 is typically found in the GC-like structure at the centre of the follicle.

In a neoplastic follicle components of a normal reactive GC can be found, CCs and CBs are present but in abnormal ratios; the degree to which the follicle structure is maintained varies and the follicular pattern can be lost towards a diffuse form although some partial follicle structure might still be identified (Ott and Rosenwald 2008). Histologically, FL can present different structural patterns that range from complete effacing of the normal follicle structure to partial conservation of some typical features, it can consist of sheets of lymphoid cells arranged either diffusely or in a follicular formation although either way no real GC formation is present besides these cell aggregates resembling lymphoid follicles (Fouad-Younes et al. 2010). The more aggressive versions are characterised by diffuse lymphomas where the pattern taken by the cells does not show a follicle structure formation, or follicle aggregation, uniform sheets of neoplastic lymphocytes are present instead of the histologic features of a normal lymph node (Elmore 2006). In FL with some conserved structure neoplastic cells aggregate into follicles, irregular, larger and more variable in size than normal follicles presents an intermediate stage in the disease; it is also possible to find partial FL involvement where some GCs show non malignant characteristics and others do display FL features (Armitage et al. 2009, Ioachim and Medeiros 2009, Jegalian et al. 2011)

Therefore due to its pathogenicity FL can be said to biologically represent the neoplastic version of a normal GC (Ott and Rosenwald 2008). It is also interesting because of some of its molecular clinical characteristics, especially considering one of the proteins researched in this thesis, BCL2. BCL2 expression in FL is well characterised and has a particular profile: about 90-95% of FL have BCL2 expression, it is known to be found inside the GC/follicle cells as opposed to non-malignant GC cells which do not express BCL2. In normal tissue BCL2 is detectable in mantle zone cells and outside the GC. Another protein, CD38 is not a marker that is used in FL, but this malignancy would still provide a good model for the aims of the present study. CD38 is a marker for plasma cells, and some of these may be found in GC/follicles in cells undergoing plasmacytoid differentiation.

Based on the hypothesis outlined in the first paragraph of this text, this part of the project looked at the relationship between expression of ZFP36L1 and BCL2 and CD38 expression in healthy lymphoid tissue and also in malignant lymphoid tissue. The experiment would potentially provide important information regarding the regulation of BCL2 and CD38 expression in relation to ZFP36L1. ZFP36L1 expression has only been reported upon once, in normal tonsils (Nasir et al. 2012), and never in malignant tissue. CD38 is a well characterised protein but the information available regarding its expression in lymphoma is also limited.

A custom antibody against human ZFP36L1 was produced by immunising rabbits with a synthetic peptide (J.M. Unpublished) was used (see Materials and Methods section for full description),

alongside pre-immunisation serum as negative control, (the same batch was used throughout the experiments, number annotated in materials list, to avoid variability).

Anti-BCL2 and anti-CD38 antibodies were purchased (see Materials and Methods section for full details). Human tonsil, lymph node and FL slides were kindly donated by Dr Anthony Warford (Department of Biomedical Sciences, University of Westminster). Handling of slides and their storage was done in accordance with the Human Tissue Act, 2004. All slides were paraffin sections. For further information on methodology please refer to Materials and Methods (chapter 2). Three cases (n=3) of non-malignant lymphoid tissue and three cases (n=3) of FL were analysed, four slides of each case were used for each antibody and in all cases the slides were consecutive cuts.

In all the experiments, reported here, one antibody known to be positive for each tissue was used as positive control, this was either BCL2 or CD38 as appropriate. Advice throughout the IHC process and on interpretation of results was sought from Dr Anthony Warford, a senior pathologist. The focus of this analysis was to assess if cells expressing ZFP36L1 are also positive for BCL2 or CD38; the positive staining is reported here in a range of 0 to 3: 0 = no staining, 1 = scant/ faint staining, 2 = moderate staining and 3 = abundant/strong staining. Scoring was assessed by Dr Anthony Warford as experienced senior pathologist and as an impartial figure.

5.2 Immunohistochemistry analysis of expression of ZFP36L1 and BCL2 in healthy tonsil tissue and lymph node tissue and in FL

5.2.1 Immunohistochemistry analysis of ZFP36L1 and BCL2 in normal tonsil tissue.

Tonsil and lymph node tissue slides were stained with ZFP36L1, pre immunised serum and anti-BCL2 antibodies using appropriate antigen retrieval methods and Tyramide Signal Amplification, results representative of n=3 are shown below. Detailed methods and list of materials can be found in chapter 2.

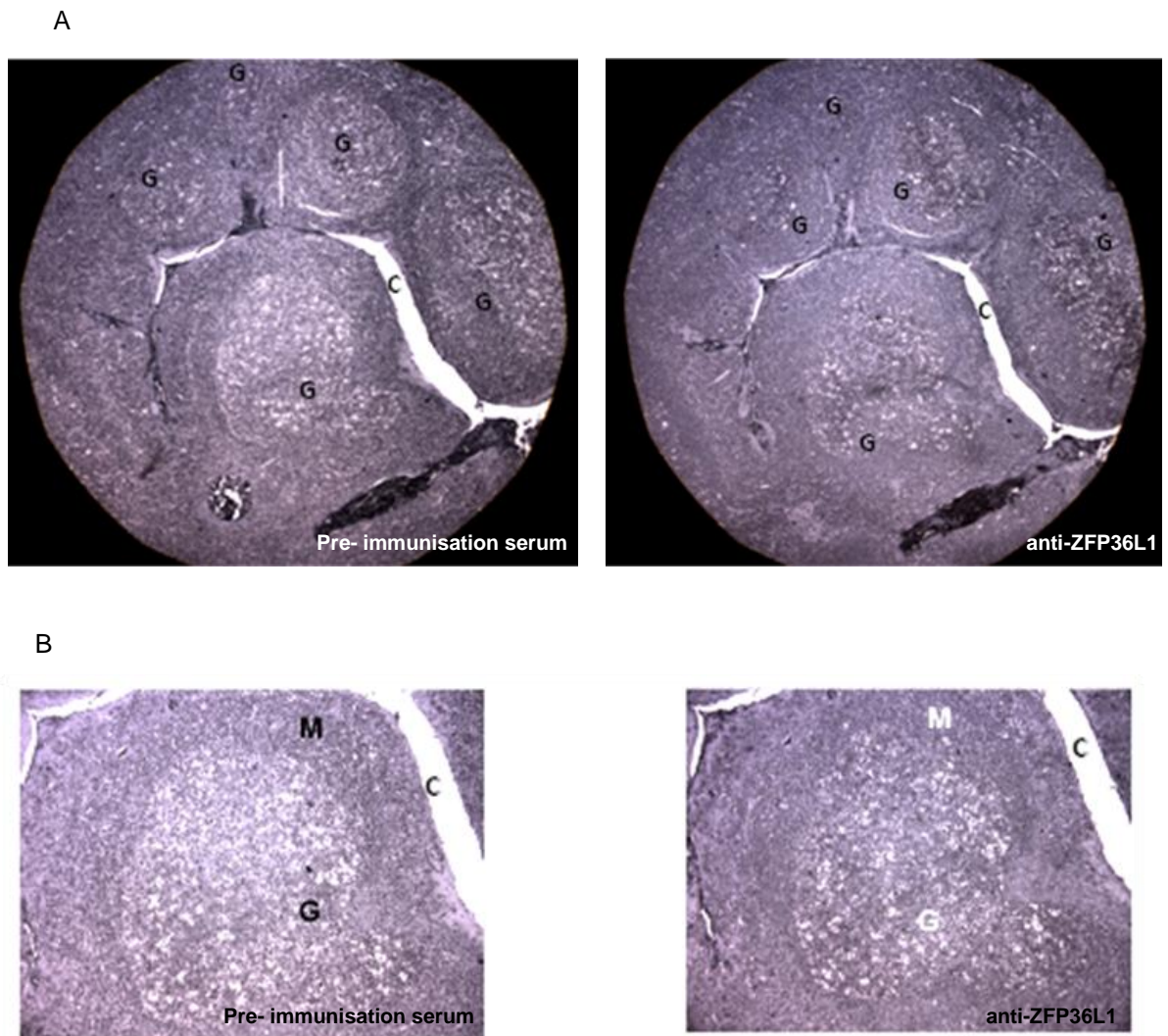


Figure 5-1 ZFP36L1 expression in human tonsils sections.

Human tonsil sections stained with pre immunisation serum (left) as control, and lab made anti human ZFP36L1 raised in rabbit (right) using equal concentrations of antiserum. A. Area shown: GC with nearby crypt. A.Images taken at x10, showing several GCs and crypt. B.Images show enlarged GC in the mid region to appreciate pattern and difference in the staining. Slightly intense staining is detected in the lower part of the GC in left and right images, with slightly stronger stain in the right figure- could be due to possible concentration of ZFP36L1 expressing cells within the dark zone of the GC .A.and B. images are from the same case but representative of n=3. M=mantle; G= GC; C=Crypt.

Healthy tonsil tissue was stained using the lab made anti-ZFP36L1 and pre-immunised serum after calculating and equalising protein concentrations of the two different antisera. The GCs were

clearly defined and identified with surrounding mantle zone. The area shown in figure 5-1 shows a GC with nearby crypt and some polarisation, typical of normal GCs and not distinguishable in malignant tissue. Results showed that there was weak to moderate positive staining in GCs for ZFP36L1; the staining was cytoplasmic. High background was observed for anti-ZFP36L1 antiserum and control serum which was not optimal for the assessment of staining but a closer photograph of the signal aided in further confirming a difference between pre immunised serum and anti-ZFP36L1 antiserum staining. Interestingly, close assessment of the staining indicated the possibility of a definition of a dark zone and light zone and it appeared that ZFP36L1 expression was predominantly located in the dark zone (fig. 5-1).

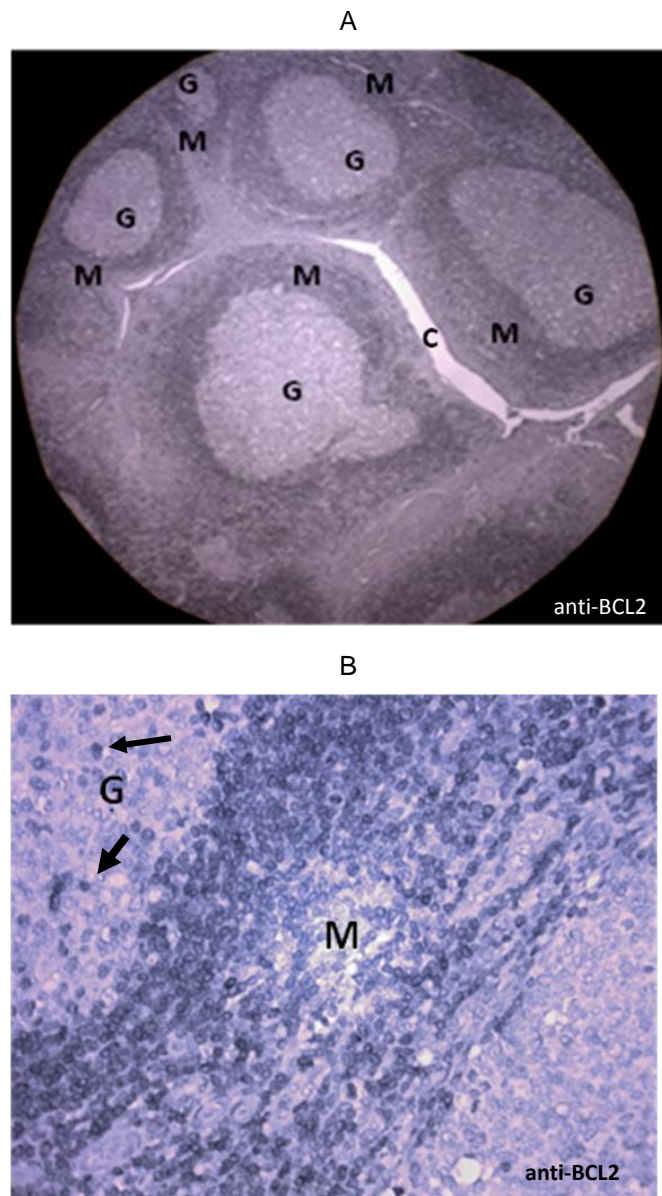


Figure 5-2 BCL2 expression in human tonsil sections.

Human tonsil tissue stained with anti BCL2 antibody, left. The same case shown for ZFP36L1 staining in figure 5-1 is shown here for BCL2 staining. A. Image taken at x10 showing several GCs and crypt. B. enlarge area of central GC to show detail of cells stained by anti-BCL2 antibody; detail image of staining for BCL2 in germinal centre where only a few cells are positive for BCL2, indicated with arrows. Staining is strong in the mantle and cytoplasmic. Representative of n=3. M= mantle; G= GC; C=Crypt.

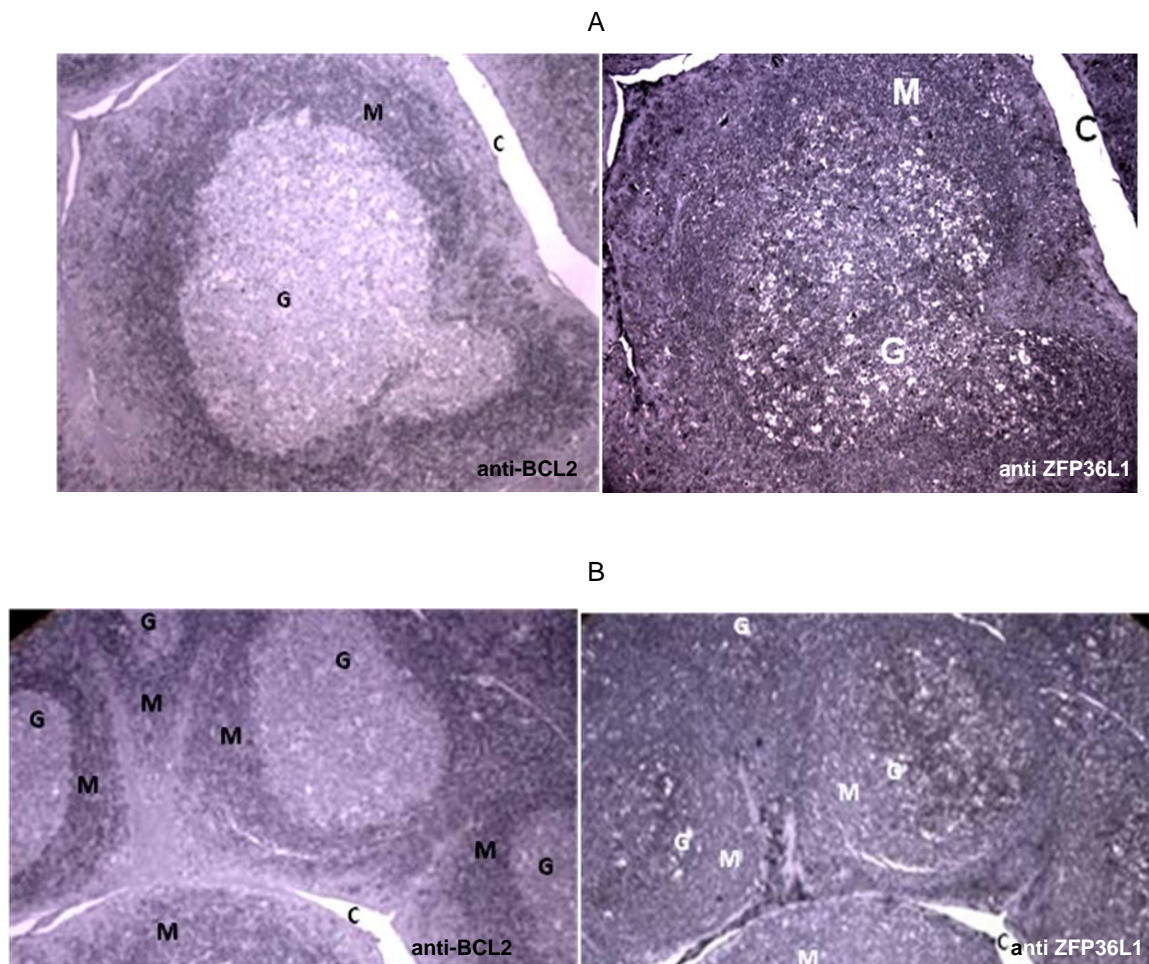


Figure 5-3 ZFP36L1 and BCL2 expression in human tonsils.

A and B. Comparing staining locations of BCL2 and ZFP36L1 expression in human tonsil sections; expression from consecutive cut slides of the same area and case. X10 Both antibodies showed cytoplasmic staining; representative of n=3

BCL2 staining showed the protein was moderately to strongly detected in mantle zone B cells, BCL2 was not seen at large in GC cells (fig. 5-2). Only a few cells (fig. 5-2B, indicated by arrows) stained for BCL2 within the GC as opposed to ZFP36L1, which showed a great many cells within the GC do express the ZFP36L1 protein.

A summary of IHC results on healthy tonsil tissue is shown in table 5-1. BCL2 and ZFP36L1 detected in cells in spatially different locations, BCL2 not seen in GC and ZFP36L1 signals indicated cells within the GC did express the protein.

Table 5-1 Immunohistochemistry results for ZFP36L1 and BCL2:

Antibody	Cells stained	Staining *	Comments
Anti-ZFP36L1	GC	1-2 weak to moderate	High background, but shows stronger signal in GC, in agreement with Nasir et al. 2012.
Pre-immunised serum			High background
BCL2	Mantle	2-3 moderate to strong	Consistent with literature; low/not detected in GCs

*Staining classified as 1- weak, 2-moderate, 3-strong

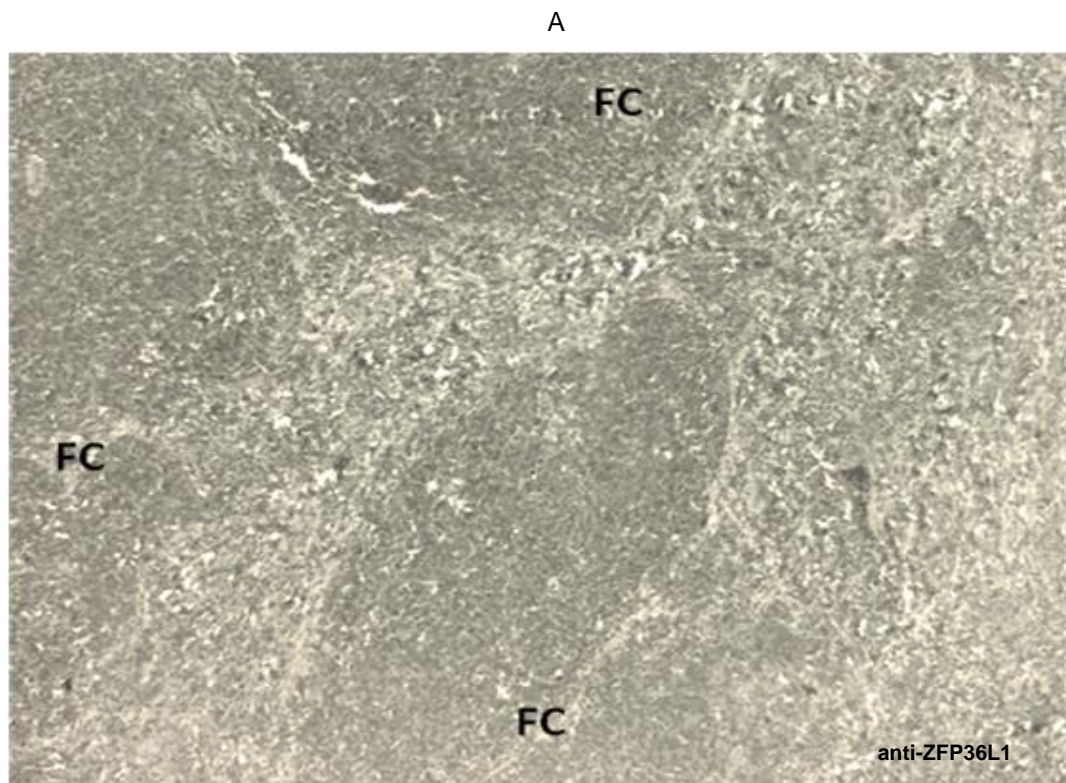
IHC analysis of ZFP36L1 expression showed positive detection of the protein in GCs, where BCL2 is low/not expressed. BCL2 was, as expected, not present in the majority of normal GC cells, whereas its detection is observed in the mantle zone and interfollicular areas; the results shown above are supported by the literature and by what is known about BCL2 function as a pro-survival protein, B cells undergoing SHM and affinity maturation die within the GC unless rescued by survival signals and are then primed by BCL2 for survival.

In samples stained for ZFP36L1 a high background was observed in the sections stained with the negative control antiserum and anti-ZFP36 antiserum, most likely due to the unprocessed and unfiltered state of these antisera, which had not been purified. After obtaining these results consideration was done as to purifying both, control serum and anti-ZFP36L1 antiserum, however the relatively small amounts available for the present study did not allow for this possibility. A close examination of the staining results indicated the presence of ZFP36L1 stained cells was observed in the GC cells. This is in agreement with a previous report where the same anti-ZFP36L1 lab made antiserum was used, and localisation of ZFP36L1 expression in GCs was observed (Nasir et al. 2012).

Overall, when staining results of both proteins were looked at and compared, results indicated that ZFP36L1 was present in the GC, whereas BCL2 was only detected in very few cells within the GC.

5.2.2 Immunohistochemistry analysis of ZFP36L1 and BCL2 in FL

FL slides were stained with anti ZFP36L1 antibody and pre-immunised serum, and BCL2 antibody. A total of n=3 cases were studied.



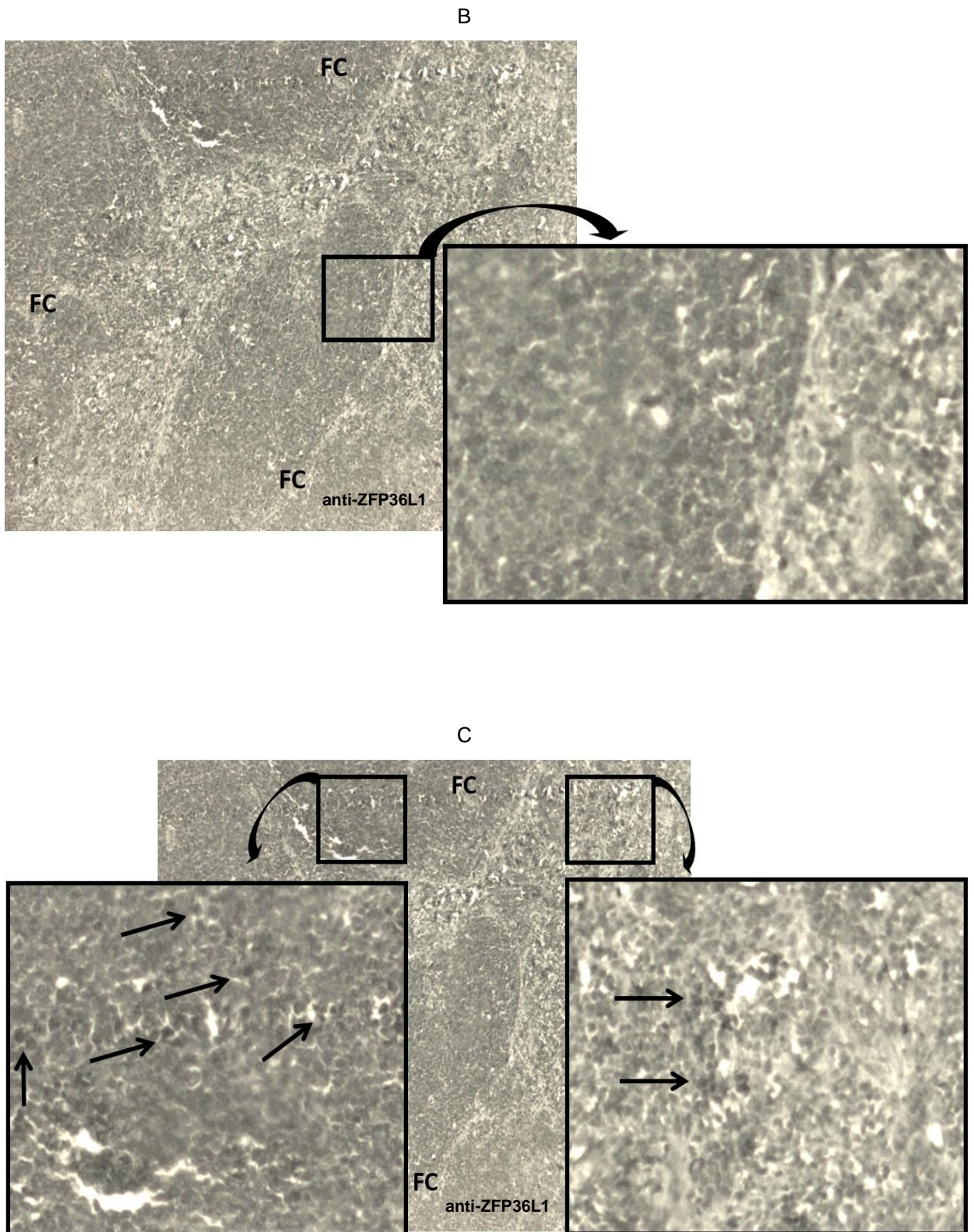


Figure 5-4 ZFP36L1 in FL sections

Case 2; FL with progressive effacing of follicle centre structure although some conservation can be seen with concentration of cells. Image shows ZFP36L1 staining in cells in 3 neoplastic follicles. A. taken at x10, B. 3 areas selected for amplification to show detail of staining, are shown here demonstrate difference in the FC border. C. the other 2 selected areas, left image shows positive staining within FC cells compared to right image of staining outside the FC (right), arrows in the right boxed image indicate some strong positivity for ZFP36L1 comparable to inside the FC. There is also background staining due to the unpurified nature of the antibody. FC= follicle centre. Case shown is the same as in the images that will be presented further on for CD38 in figure 5-11.

FL slides stained with anti-ZFP36L1 showed that the protein was detected in cells forming the misshaped follicles characteristic of FL although cells stained with the antibody can be also be seen outside the follicle centres too (C, bottom right). Generally stronger staining was observed in the follicle areas although maximum staining is moderate. Within the FC cells are not as clearly defined as in normal tissue but some cells can be seen with darker stain for ZFP36L1 (arrow, figure C, left). The detection of ZFP36L1 does not seem to change in relation to non-malignant tissue and remains largely present within the centre of follicles –GC in non-neoplastic cases-. Overall the high background signal made difficult the assessments but overall results would indicate that follicle centre cells expressed ZFP36L1 protein.

The same cases using consecutive tissue slides were stained with anti-BCL2 antibody, however on this occasion no results were obtained, the antibody did not produce any staining. These runs on FL tissues were done over a year after the images shown in the previous section; the most likely reason for the antibody failing to provide a signal was thought to be its expired date and/or storage. These samples were processed at the same times as other samples e.g. anti-CD38 stained samples, which gave positive results and effectively worked as a positive control.

The literature has a large number of papers and images where BCL2 is reported to be detected in follicle centres cells in FL. A number of images are given here from different sources, duly referenced, in order to facilitate the discussion further on. BCL2 staining distinguishes non - malignant reactive follicles from FL, IHC analysis of its expression can be used to differentiate FL from e.g. follicular hyperplasia although for a clinical diagnosis of FL other markers must be analysed. BCL2 is an anti-apoptotic protein, a pro-survival inner mitochondrial membrane protein and the *BCL2* gene is translocated in most cases of FL from chromosome 18 to chromosome 14 placing the expression of the BCL2 protein under the control of the promoter and enhancers of the *IgH* chain gene leading to over expression of BCL2. The highest percentage of cases showing a translocation of *BCL2* are seen in grade I and II FL but it may also be present in grade III FL, and furthermore increased over-expression of BCL2 may be observed even when that translocation is not present (Ott and Rosenwald 2008).

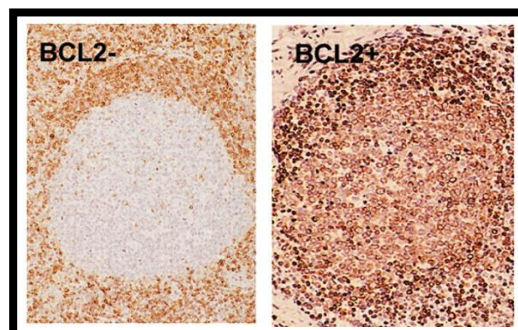


Figure 5-5. Reference image for BCL2 staining in FL

Image modified from (Natkunam 2007) to demonstrate typical staining pattern for BCL2 in FL cells; it shows how BCL2 expressing cells are detected in the GC on the right image of FL. BCL2 protein was detected by using an IHC technique that produces a brown stain. In reactive follicles BCL2 is present in mantle zone cells but not follicular centre B cells. Left image is normal GC, right image is GC/follicle centre in lymphoma: FL stained for BCL2 showing positivity of the follicle centre.

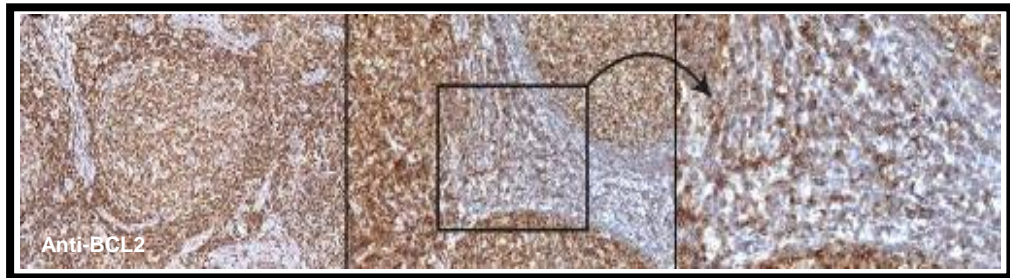


Figure 5-6 Reference image of follicular and inter follicular BCL2 staining in FL

Typical BCL2 staining pattern in FL . Left follicular pattern, middle and right show inter follicular pattern, adapted from (Fouad-Younes et al. 2010). For this FL case the structure of the follicle centres is still fairly preserved.

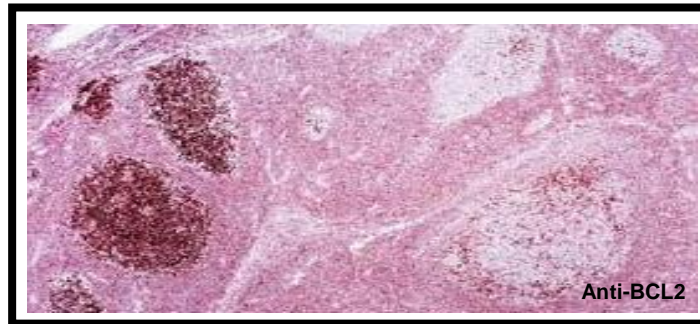


Figure 5-7 Reference image of FL stained for BCL2,

Follicle centres show cells strongly positive for BCL2. In this case some follicles do not have BCL2 positive cells or the population is sparse, the FL partially involves the follicle. From (Fletcher 2007). Extensive research reports BCL2 detected in follicle centres in FL., as opposed to BCL2 expressing cells in healthy tonsils and lymph nodes (fig. 5-1.B) but FL may show only partial involvement of the lymph node and therefore some follicle centres may have less BCL2 expressing cells.



Figure 5-8 Reference image of FL follicle centres strongly positive for BCL2

The follicles have partially lost their normal structure and cells are strongly positive for BCL2. Adapted from (Fletcher 2007), as opposed to non-malignant follicles where in GCs BCL2 is restricted to the mantle and to portions of the light zone implicated in the selection and maintenance of plasma cells and memory B cells. Some positivity for BCL2 in normal GC is actually reported to found in GC T cells (Hockenbery et al. 1991, Schenka et al. 2005)

Table 5-2 shows a summary of IHC data of FL samples stained for ZFP36L1 and BCL2.

Table 5-2 ZFP36L1 and BCL2 staining in FL:

Antibody	Cells positive	Staining*	Comments
Anti-ZFP36L1	Follicle centre cells	2	High background, but shows stronger signal in neoplastic follicle- never reported before.
Pre-immunised serum			High background
BCL2	Follicle centre cells	3	As per literature- references stated after each image shown

*Staining classified as 1- weak, 2-moderate, 3-strong

BCL2 immunostaining shows that BCL2 is expressed mainly in the neoplastic follicle centres although may be found in inter follicular neoplastic B cells. ZFP36L1 is detected also in the neoplastic centre cells. Abnormal follicles show no apoptosis, positive for BCL2, no phagocytosis and no/ reduced mitotic features and lack a well defined mantle, as it can be seen in the representative images from diverse sources. Although BCL2 is found in cells within the neoplastic follicle centres, some centres may not have cells expressing the protein if the FL involvement is partial (illustrated in fig. 5-7); the detection can be moderate to strong, depending on the stage of the FL.

5.3 Immunohistochemical analysis of ZFP36L1 and CD38 expression in normal tonsil tissue and lymph node and FL

IHC analysis of ZFP36L1 and CD38 expression in healthy tonsil and lymphoma sections was performed to further assess the relationship between both proteins and analyse if there is an inverse relationship between them, in terms of their levels of expression, at tissue level.

FL samples had been used to study the expression of ZFP36L1 and BCL2 as described at the beginning of this chapter and IHC analysis of the second putative ZFP36L1 target studied in this project, the *CD38* mRNA, may also provide useful information. Since CD38 is a well characterised plasma cells marker in normal lymphoid tissue, and although CD38 is not a marker commonly studied in FL, it may still present an interesting scenario for the aim of these experiments. Some plasmacytic differentiation can be seen in a number of FLs. For example, within the morphological variations of FL a small number of follicle centre cell lymphomas show plasma cell differentiation with either intra or inter follicular monoclonal plasma cells (Gradowski et al. 2010).

In order to assess the expression of ZFP36L1 and CD38 in B cells, normal lymphoid tissue was used for IHC analysis as described in materials and methods (chapter 2). The same analysis was later performed on FL sections. To compare CD38 expression with that of ZFP36L1 the results obtained for the latter shown in figure 5- 1 will be referred to in the results and discussion.

5.3.1 Immunohistochemistry analysis of ZFP36L1 and CD38 in healthy tonsillar tissue.

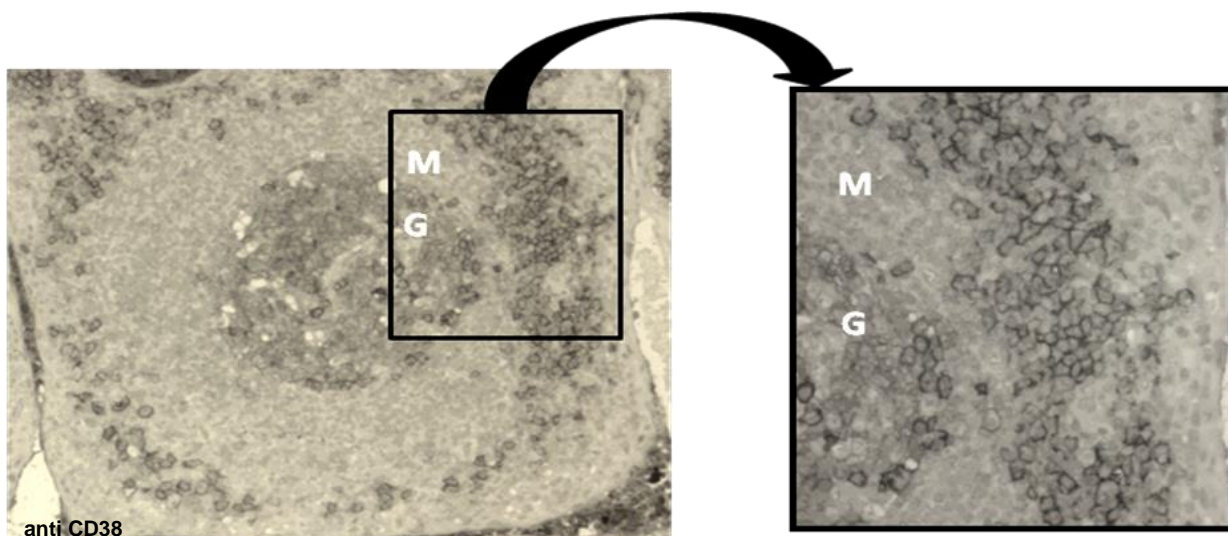


Figure 5-9 Detail of CD38 staining in human tonsils GCs

GCs of normal tonsils stained with anti CD38. Left, image is at x40. Right:, part of GC enlarged shows cell membrane staining of CD38 positive cells. The images shown are representative of analysis carried out on three separate cases (n=3). M=mantle; G= GC.

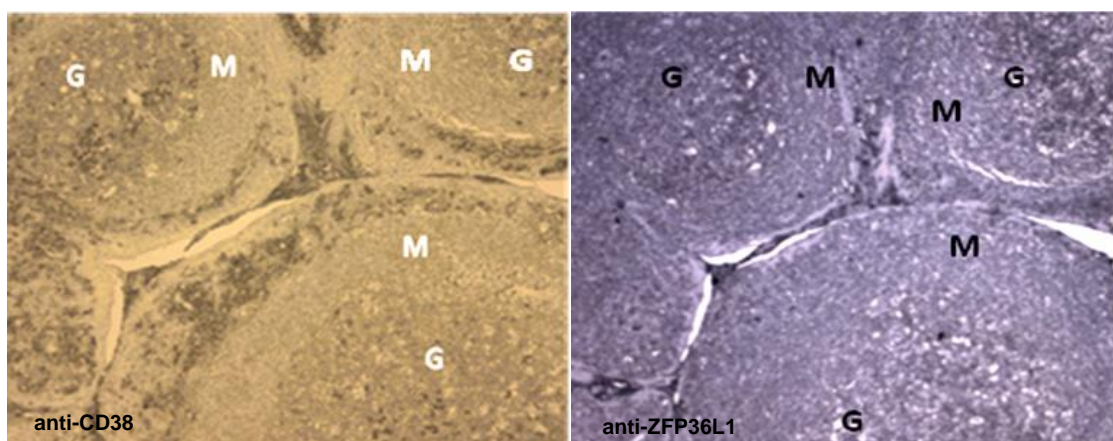


Figure 5-10 CD38 staining and ZFP36L1 on human tonsils

GCs of tonsil stained with anti-CD38 antibody some cells in the GC stained for CD38, but stronger staining is shown outside the GC. The majority of CD38 positive cells appear in the subepithelial region. There are few CD38 positive cells with moderate staining in GC and M. Right, same area stained with anti-ZFP36L1 antibody, cells positive for ZFP36L1 are seen mainly in GC, Only a few cells may be considered to show overlapping expression for both proteins inside the GC but due to the dense staining of ZFP36L1 this is difficult to assess. Magnification is at x10. G= GC; M= mantle zone.

CD38 and ZFP36L1 were detected in cells generally in spatially different locations, a few CD38 stained cells in GC, with moderate staining whereas ZFP36L1 was detected mainly within GC cells; results are summarised in table 5-3.

Table 5-3 Immunohistochemistry summary results ZFP36L1 and CD38:

Antibody	Cells positive	Staining*	Comments
Anti-ZFP36L1	GC	1-2 weak to moderate	High background, but shows stronger signal in GC, in agreement with Nasir et al. 2012
Pre-immunised serum			High background
CD38	Mainly outside of GC; and at lower level GC	Overall stain is weak in GC cells, where a few cells show level 2 staining. Larger number of cells outside GC and M with level 3 staining	Membrane staining Localisation and type of staining is consistent with literature.

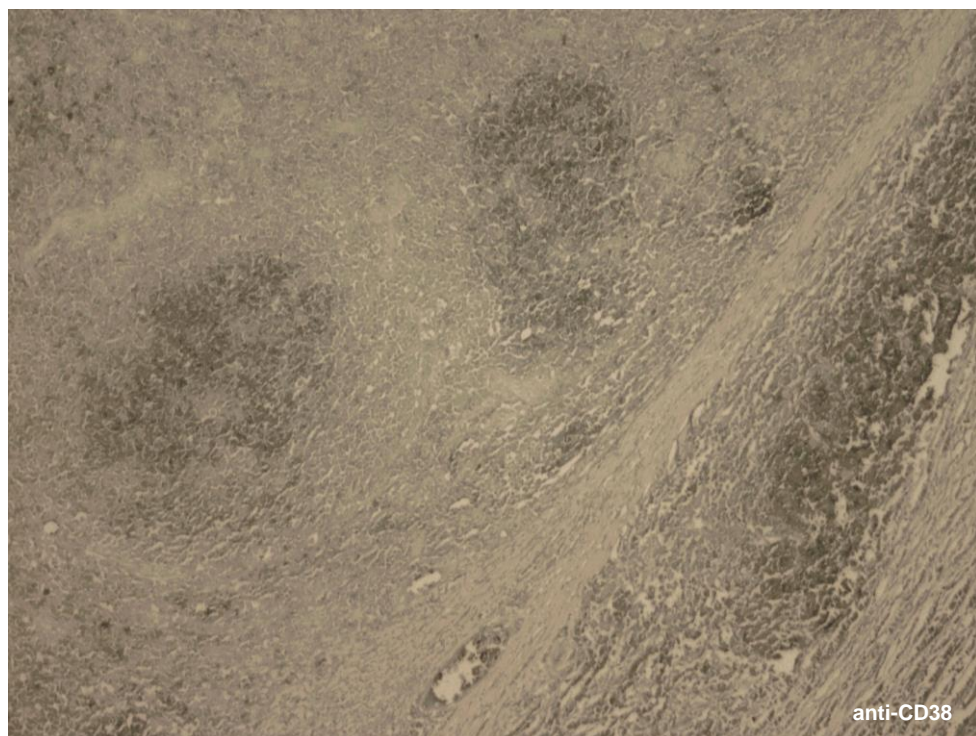
* Staining classified as 1- weak, 2-moderate, 3-strong

CD38 was mainly detected in cells outside the GC and outside the mantle zone with stronger staining than cells in those found within GC, here a few cells resulted positive for CD38 with moderate staining as it can be seen in figure 5-9 and 5-10.

Compared to GCs stained for ZFP36L1, figure 5-10 images showed that where CD38 is highly detected, outside GC and mantle zone, in the sub epithelial region, no clear or obvious staining for ZFP36L1 was detected there. In the GC, staining for CD38 is mostly weak or moderate and there are also a few cells with high level CD38 staining. In contrast, ZFP36L1 expression is mainly located in GC cells. CD38 membranous staining is clearly seen in figure 5-9.

5.3.2 Immunohistochemistry analysis of ZFP36L1 and CD38 staining in FL

A



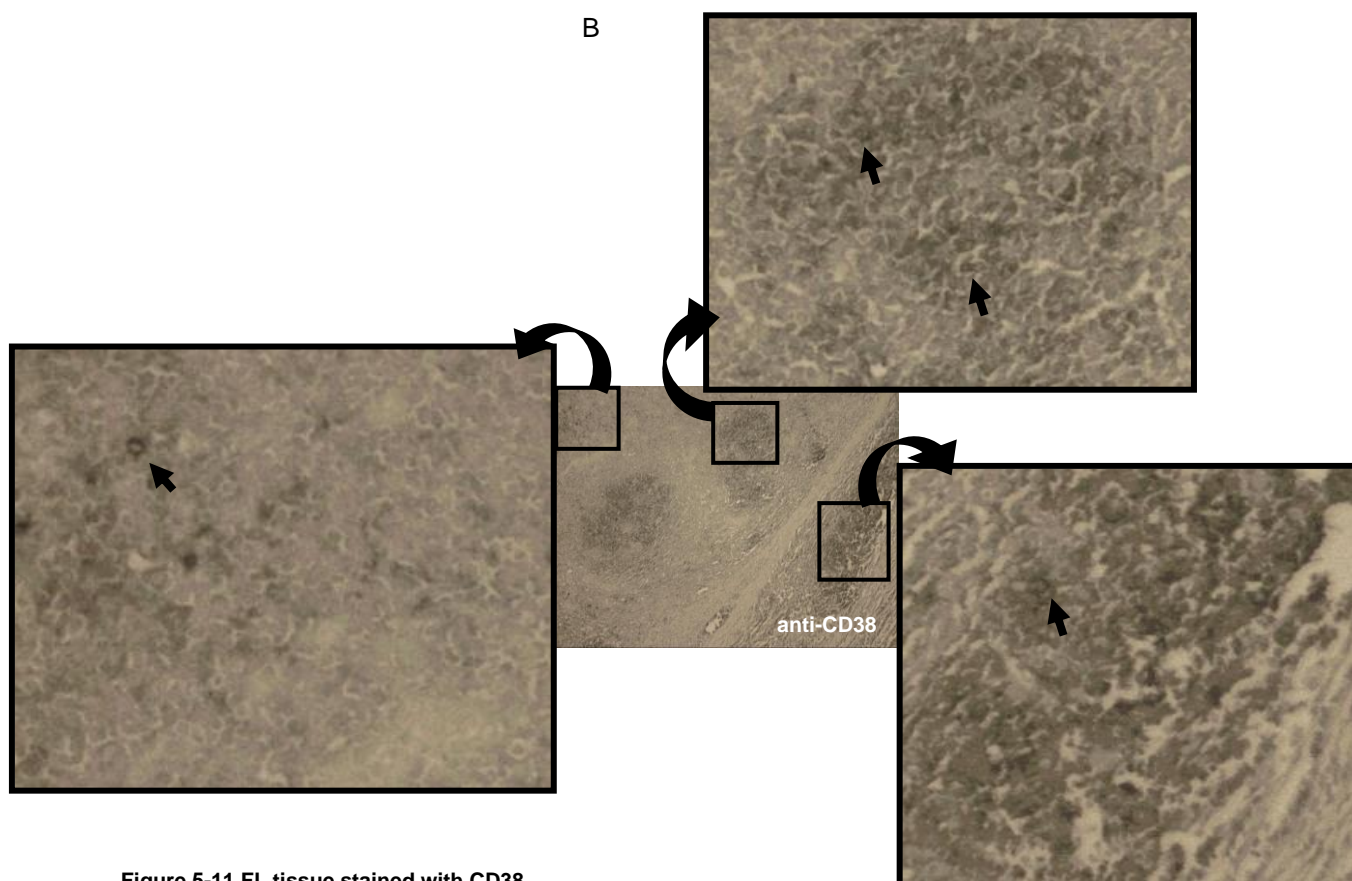


Figure 5-11 FL tissue stained with CD38,

FL tissue stained for CD38, magnification at x10. With areas selected and enlarged. FL case showing little conservation of normal follicle centre features and showing progressive sheet appearance of neoplastic cells. For CD38, it showed moderate to strong staining within the centre cells, and some staining seen outside the partially effaced follicle centres. Arrows show single cells positive for CD38 with strong membrane staining observed in scattered plasma cells. Intrafollicular plasmacytoid differentiation is characteristic of high grade FL (Gradowski et al. 2010). Top and right boxes show detail of CD38 positive cells in progressively effacing follicle centres

A summary of staining results for ZFP36L1 and CD38 in FL are shown in table 5-4

Table 5-4 ZFP36L1 and CD382 staining in FL

Antibody	Cells positive	Staining*	Comments
Anti-ZFP36L1	Follicle centre cells	1 to 2 low/weak	High background, but shows stronger signal in GC, consistent with Nasir et al. 2012
Pre-immunised serum			High background
CD38	Follicle centre cells, some outside the centres	Strong staining observed in scattered plasma cells-C. In the follicle centre staining is moderate with some cells showing strong staining	FL may show some plasmacytoid differentiation, and CD38 would detect plasma like cells, hence plasma cells outside follicle provide the strongest positivity for this marker

*Staining classified as 1- weak, 2-moderate, 3-strong

5.4. Discussion

5.4.1 Analysis of ZFP36L1 and BCL2 expression

Results from IHC have indicated that ZFP36L1 is detected in GC cells in normal lymphoid tissue, and in FL it is found in follicular centre cells. In normal tissue there may be indication that ZFP36L1 could be able to differentiate the light and dark zones within the GC. In the GC naive B cells get activated and start proliferating rapidly, clonal expansion of these CBs within the dark zone takes place and during this stage SHM occurs leading to modified antigen receptors. Differentiated into CCs in the light zone these cells are not as active and not proliferating; CCs are selected based on the binding affinity to the given antigen and the remaining cells will die by apoptosis (Klein and Dalla-Favera 2008). The increased proliferating rate within the dark zone may explain the darker stain observed for ZFP36L1, indicating a higher expression of the protein consistent with the cycling state of these cells. There is rapid interchange of CCs and CBs between the zones to the point of these cells being considered transient states of a same development step and that are very much indistinguishable in their morphology with much overlap in their gene expression, although they do have differences in action, chemokine, proliferation and DNA damage genes (Bannard et al. 2013). This opens a route to perhaps further study of ZFP36L1 detection in these different B cells (CBs and CCs) in different locations within the GC.

As widely reported in the literature BCL2 is also seen in the follicle centres of FL, as opposed to non-malignant tissue where GC cells generally do not express the protein (fig. 5-2). In normal lymphoid tissue, e.g. tonsils, as depicted in figure 5-1 BCL2 is observed in cells within the mantle zone, no BCL2 or low levels of BCL2 expressed in isolated cells within the GC are detected: in normal tissue BCL2 is needed to maintain selected B cells that will move on to performing a normal function in immune responses, it develops its anti-apoptotic role in resting B cells of the mantle zone and post follicular B cells resulting in long-lived memory cells and plasma cells, but in the GC, cells do not have BCL2 expression and go through apoptosis unless selection for survival takes place by antigen specificity.

The opposite scenario is seen in malignancy, as represented in figures 5-5, 5-6, 5-7 and 5-8. BCL2 is a key marker to differentiate a neoplastic follicle from a non-malignant hyperactive one; its detection distinguishes follicular hyperplasia or a reactive lymph node from FL (Knowles 2001) (although sometimes BCL2 T cell positivity within GC may be observed, which complicates diagnosis, a pan T cell marker may be used in these cases (Knowles 2001)). About 80-90% of FL cases have a BCL2 translocation - t(14;18)(q32;q21)-, more common in low grade FL (Bakhshi et al. 1985, Tsujimoto et al. 1985b). The translocation leads to over expression of the BCL2 protein and these FL show reduced mitotic features and a mantle cell layer definition loss. In FL without the translocation, BCL2 may be over expressed by other genetic aberrations (Ott and Rosenwald 2008, Wahlin et al. 2012). The over expression of BCL2 leads to an accumulation of B cells in the GC “falsely” rescued from apoptosis, on top of the BCL2 translocation their increased life span also means an opportunity for other genetic changes to take place that accompany the formation of FL (Ott and Rosenwald 2008), in fact, some argue that the mere presence of that translocation is not

sufficient for the development of the malignancy but other added genetics hits accompany the neoplasia. Other secondary chromosomal aberrations happen in FL, and the accumulations of these and greater complexity are features of higher grade FL (Yunis et al. 1987, Ott et al. 2002). One of these secondary translocation involves another oncogene: *Myc*, a t(8;14)/*MYC* (De Jong et al. 1988) that is usually linked to a Burkitt like appearance; in these cases diagnosis can be done by observing the morphology and additional detection of the *BCL2* translocation (Ott and Rosenwald 2008).

The altered regulation of *BCL2*, and even the t(14;18)(q32;q21), is also present in other B cell malignancies: in about 10-40% of DLBCLs (Gascoyne et al. 1997) and even in a small number B-CLL cases (Elyamany et al. 2014). But even if the translocation in present B-CLL may still show an increase in *BCL2* expression (Dyer et al. 1994, Majid et al. 2008).

With regard to ZFP36L1 detection, the antibody raised in rabbit had already been used in the only report to date showing IHC data of ZFP36L1 in lymphoid tissue (Nasir et al. 2012). Nasir et al. 2012 reported that they mainly found the protein within cells of the GC in normal lymphoid tissue. The results of n=3 non-malignant sections reported in this chapter are consistent with their findings as besides a high background a structural delineation of the GC with signs of an increased signal from within their cells was observed (fig. 5-1). In the FL cases stained for ZFP36L1 it was seen that the protein continued to be detected in the follicle centres, now neoplastic GCs, its expression was, therefore, concurrent with that of *BCL2*.

The results of ZFP36L1 detection in FL within the follicle centre would not necessarily oppose the hypothesis of ZFP36L1 as a negative regulator of *BCL2* expression. One possibility is that ZFP36L1 expression has not changed- or not changed compared to the increase in *BCL2* expression- but certainly *BCL2* levels have increased abnormally be it because of the presence of that translocation that leads to the juxtaposition of *BCL2* gene with enhancer sequences of the IgH gene promoter region, or by other mechanisms in the small number of FL cases without that translocations, but where the over expression is at equivalent level to cases with the cytogenetic abnormality (Pezzella et al. 1990b). The result is an augmented, out of control over production of *BCL2* in cells where no change in ZFP36L1 expression has occurred, therefore a normally expressed protein (ZFP36L1) – or perhaps even a down regulated or mutated non-functional ZFP36L1- would not be able to down regulate the abnormally produced *BCL2* at least not to the same level that would be seen in normal cells, especially if other pathways that may interact and assist ZFP36L1 in the targeting and down regulation of *BCL2* may also be altered. This possibility is only a hypothesis to explain the results obtained in FL and would need of course further research, starting from ensuring that ZFP36L1 itself is or is not abnormally produced in FL. There are reports that indicate that in some cancers the level of ZFP36L1 is down regulated or even mutated (Davies et al. 2003, Chapman et al. 2011). In general other tests could be done to improve and further investigate the findings reported here such as enhanced IHC with a purified antibody for ZFP36L1, comparing mRNA levels of normal and malignant cells and investigating possible ZFP36L1 mutations in FL.

FL can present itself in a range of forms, from low grade to high grade, its presentation can be morphologically different; as seen, structurally, the neoplasia can show complete effacing of follicle centre structure or misshaped follicles but where centres can be clearly seen. Partial involvement of FL (fig. 5-7) may show only some neoplastic follicles and others with normal phenotype; in fact the partial nodal involvement by a FL can be missed if neoplastic follicles constitute a smaller proportion of all follicles. As represented in figure 5-7 (Fletcher 2007), it is possible to see BCL2 expression within the follicle centres in a varying degree within the same case of FL. It would have been interesting to stain one case of partial involvement of FL for ZFP36L1 to observe if those centres lacking, or with small number, of BCL2 expressing cells also showed staining for ZFP36L1 and to what level.

Having discussed the above, based on the differential staining pattern for each antibody, there are indications in the results from this set of analyses to support the hypothesis of ZFP36L1 targeting the pro-survival *BCL2* mRNA. BCL2 in normal GC would not be detected within the GC since B cells there are going through apoptosis unless rescued by a survival signal after SHM and affinity maturation. After this rescue, BCL2 expression would prime the selected cells for survival so they can carry out their immune effector functions. If ZFP36L1 negatively regulated BCL2, it is reasonable to deduce that both proteins would not be highly expressed together in the same cells at the same time unless some normal regulatory mechanism has gone wrong as it happens in the development of FL.

The IHC evidence gained in the experiments and discussed in this chapter provides further information on the relationship between ZFP36L1 and BCL2 closer to *in vivo* expectations. These IHC results, especially in the case of non-malignant tissue, showing ZFP36L1 expressed in GCs where BCL2 is very lowly/not expressed, must be considered alongside the luciferase assay results discussed in chapter 3 which showed the interaction of the ZFP36 proteins, specially ZFP36L1 with the *BCL2* 3'UTR ARE. In addition, further evidence from RNA electrophoretic mobility shift assays, provided in Zekavati et al. 2014 and also the evidence recently published on the role of the ARE in the regulation of BCL2 from *in vivo* studies (Díaz-Muñoz et al. 2015) must also be taken into account. Taken together, accumulating evidence strengthen a working model that is consistent with ZFP36L1 targeting *BCL2* mRNA *in vivo*.

5.4.2 Analysis of expression of ZFP36L1 and CD38

The GC is a complex formation with diverse cell populations; the expression patterns of cell markers, as in this case CD38, is connected to the diversity and the biology of the B cells in the follicles. GC formation is the result of antigenic stimulation of B cells; B cells cycle through the GC, and a small percentage survive to become plasma cells or memory B cells. Naive B cells residing in primary follicles start proliferating after stimulation forming GCs; within the GC a light zone populated with CCs, and a dark zone, populated with CBs that are highly proliferative and may undergo SHM, can be observed. These two zones are not distinguishable in neoplasms. Around the dark/light zones naive resting B cells that are not proliferating are pushed outwards and form the mantle area (may be considered an extension of the primary follicle). Once selection and maturation

of CB has happened the B cells leave the GC as plasma or memory cells. And thus, peripheral B cells can be divided into into naive, GC, memory, and plasma cell populations and in consequence a number of subpopulations of B cells can be found in, and, around a GC with different phenotypes determined, or rather, explained by the biology of the GC; in the case of CD38 the marker is reportedly expressed by GC B cells, plasma cells (and immature B, pre-B cells). Mantle zone, naive B cells are recognised as IgD⁺IgM⁺CD38⁻/resting small B cells; the GC starts with the clonal expansion of a few antigen-specific founder B cells, these are IgD⁺IgM⁺CD38⁺ (and low levels of BCL2); GC cells are IgD⁻CD38⁺ CBs and CCs; plasmablasts (IgD⁻CD38⁺⁺), these are early plasma cells that will leave the secondary lymphoid organ and migrate to the bone marrow or inflamed tissues and memory cells are IgD⁻CD38^{low} (Lebecque et al. 1997).

Considering the above, overall, CD38 detection results observed in figures 5- 10 are the expected results for B cells in GCs: positive for a number of cells within the GC with weak/moderate staining, CD38 positivity is stronger and in increased number of cells outside the GC, in plasma cells, or rather plasmablasts. Plasmablasts have transformed from naive mature B cells into a GC B cell and finally into a terminally differentiated B cell. The weak staining cells in the GC may be those that after, antigen capture via BCR-and Th interaction, have the B cells phenotype IgD⁺ and low/no CD38 which further develop into IgD⁻ and CD38⁺ GC founder B cells (Pascual et al. 1994).

As seen also in figure 5-9 CD38 positivity was strongest outside the GC cells and the mantle zone; the literature reports widely that although within the B cell follicles, CD38 is present on GC B cells, and not expressed by mantle B lymphocytes, committed plasma cells express higher levels of CD38 in the GC, in fact it is reported as the strongest positivity in the B cell compartment (Mehta et al. 1996, Funaro et al. 1997, Oliver et al. 1997, Funaro and Malavasi 1998, Mehta and Malavasi 2000, Malavasi et al. 2008).

The CD38 pattern of expression is useful in delineating B cell subsets and in understanding the signalling events involved in the development of these B cells (Oliver et al. 1997) but previous studies had defined tonsil B cell subsets by their expression of IgD and CD38 as “yes or no” category (Berek et al. 1991) (Choi 1997), however, the CD38 positive cells have also clear differences in CD38 expression levels that allow for other sub-classifications; other studies extend the “yes/no” classification by demonstrating that CD38⁺ tonsillar B cells can be divided into novel populations of CD38^{low} and CD38^{intermediate} cells by flow cytometry (Grammer et al. 1999), and therefore showing a lower level of staining within the GC cells compared to those outside. This is demonstrated in figure 5-9 consistent with the grading in CD38 expression which is probably highest in terminally differentiated plasma cells. Further studies showed that plasma cell differentiation can be dissected in distinct, developmental stages of CD38 low and CD38 high positive cells, including a CD38 low plasma cell precursor population present in tonsillar GCs (Arce et al. 2004). This supports the idea that IgG expressing cells upregulate CD38 as time passes and cells grow/differentiate, but that initial IgG producing cells express no or low CD38. Tissue sections in their study provided a similar result to the result shown in this chapter; cells containing high quantities of IgG in their membrane and expressing only little CD38 are located in tonsillar follicles.

In contrast, CD38 high IgG-containing plasma cells are mainly present outside the follicular areas. The group established that during their course of differentiation, CD38 low plasma cell precursors possibly leave the follicles and increase the expression of CD38, also supported by other studies (Fairfax et al. 2008, Oracki et al. 2010).

So, overall, CD38 expression is detected in cells within the GC, but at weak/moderate level and number and is highly expressed, or highest expressed in plasma cells outside the GC and mantle zone. The highest CD38 surface density among human cells is found in pathological terminally differentiated plasma cells and their healthy version (Malavasi et al. 2008) which is also seen in figure 5-11.

When compared to the detection of ZFP36L1 (fig.5-1 and 5-2, left), it is seen that, besides the background and the difficulty in assessing cell staining, it would seem that ZFP36L1 protein is mainly detected within the GC cells, here it would coincide with some cells expressing CD38 but further comments on this cannot be done due to the quality of the staining; what seems fair to conclude is that where CD38 expression is highest expressed ZFP36L1 is lowest/not detected: outside the mantle, within interfollicular space by plasmablasts (fig. 5-9 and 5-10) and that also the opposite is true, where ZFP36L1 is highest detected (in GC, fig. 5-1) CD38 is lowest. This would actually be consistent with evidence of other ZFP36L1 mRNA targets associated with plasma cells. Previous chapters and specially chapter 4 have already discussed the evidence of ZFP36L1 targeting BLIMP1 as reported by Nasir et al. 2012 (Nasir et al. 2012). Suffice to highlight in this chapter that BLIMP1 is a protein strongly linked to inducing plasmacytoid differentiation (Shaffer et al. 2002). The regulator/target relationship between ZFP36L1 and BLIMP1 shown in Nasir et al. 2012 would suggest that in plasma cells, BLIMP1 (and CD38) would be detected but ZFP36L1, as their negative regulatory protein, would not be present or it will be present at low levels. Evidence on early plasma cell commitment within the GC, shows CD38 positive cells/ IgG early secreting cells are committed to plasma cell differentiation in accord with the observation of BLIMP1 being expressed in about of 4–15% of GC B cells (Arce et al. 2004). In fact, and perhaps not surprisingly, BLIMP1 revealed a similar staining pattern to that of CD38 with some positive staining within GC cells and most staining in number and intensity in cells outside the GC near the epithelium (Arce et al. 2004). In tonsils, BLIMP1 protein is strongly expressed by plasma cells present in GCs, although staining is scattered, and is also seen in sub epithelial areas (Garcia et al. 2006). It is worth commenting that in B cell lines blots (fig. 6-1, chapter 6) ZFP36L1 was not detected in mature, plasma cells of MM further supporting the hypothesis that this protein is not expressed or very lowly expressed in plasma cells. Other regulators of differentiation are also found in GC and have a link to ZFP36L1. Certain cytokines are found in GCs that function to modulate cell differentiation, growth and signalling. A number of cytokines, already reported as mRNA targets for ZFP36L1, were detected outside the GC cells or reported as very lowly expressed within GC cells. These include GM-CSF which is only produced by mantle zone cells and not within the GC cells (Pistoia and Corcione 1995), TNF α , IL-6 or IL-10, IL2, are synthesized by both B cells in the GC and mantle zone, but at different levels with low/no detection within the GC cells (Butch et al. 1993, Toellner et al. 1995, Lisignoli et al. 1998). Another reported mRNA target for ZFP36L1, cIAP2 is detected in

plasma cells in reactive lymph nodes and bone marrow plasma cells (Jourdan et al. 2009), whereas in FL its expression is observed in most cases (Akyurek et al. 2006).

Whilst CD38 was detected in plasma cells and in the GC cells, in the case of FL, the phenotype only partially seemed to resemble its non-malignant counterpart. CD38 was found in the neoplastic follicle centres (fig. 5- 11), The staining in cells within the neoplastic centres indicated a denser population of cells expressing CD38 compared to a normal non-malignant GCs, this indicate a higher concentration of CD38 expressing cells. CD38 is not a marker for FL as BCL2 is, and its expression is rarely studied but some authors have suggested its analysis can distinguish between follicular hyperplasia and FL (Mantei and Wood 2009) by determining the positivity and abundance of CD38 cells in the malignant follicle.

The results observed in figure 5-11 are consistent with the results shown in one of the few reports found demonstrating CD38 expressed by the neoplastic follicles in all cases studied and it was down regulated in the interfollicular B-cell component in the vast majority of cases (Dogan et al. 1998). The results reported in this chapter are consistent with this description, and overall are markedly opposed to the phenotype found in normal tonsil tissue, where the highest CD38 detection is found in the interfollicular regions, outside the GC and mantle zone (fig. 5-9).

An important point must be highlighted, the phenotype of cells described against their location in FL cases is difficult to assess, since the various morphologies that it can present may impact the description of where in the tissue cells are found. In low grade FL GCs may be completely effaced and sheets of neoplastic cells can replace the non-malignant structure in the follicle, there are cases with partial involvement of FL, and also abnormal GC structures These are only examples of the morphological variety that may be found and hence, the results from the present study could be extended by analysing a more extensive number of cases for each grade of FL.

5.5 Conclusions

IHC analysis of non-malignant lymphoid tissue has shown that the presence of BCL2 and ZFP36L1 is in different locations, ZFP36L1 mainly in GC, BCL2 in mantle zone and interfollicular areas. For CD38, its expression is detected in GC cells but not as high as in cells outside the GC, where the highest staining is observed in most likely plasma cells.

For BCL2 and CD38 there is a bulk of literature confirming these findings, but this is the first time that ZFP36L1 expression has been analysed so extensively in normal lymphoid tissue and also in malignant sections (other than results reported by Nasir et al. 2012 on normal tonsil sections, in the supplement information of their paper).

BCL2 is an anti-apoptotic protein that would not be detected in the majority of GCs cells which undergo apoptosis if not clonally selected; CD38 is highest expressed in plasma cells largely outside the GC and M zones.

For the purpose of this research, the relationship between BCL2 and ZFP36L1 seems straightforward since in non-malignant tissue results show that where one protein is found the other is not, or at most that in cells where the highest expression of one is detected the other protein does not provide a signal. This inverse relationship would support the hypothesis of a negative regulatory role of ZFP36L1 on BCL2 expression.

For CD38, the literature reports GC cells as positive, however, the level of positivity varies, within different sub-populations of GC B cells, which would be consistent with findings reflected in figure 5-9 and table 5-4. The data shows the detection of CD38 within the GC at a lower level of intensity to that of cells outside the GC (likely plasma/plasmablasts that have left the GC). The existing literature shows a grading in the expression level of CD38 in lymphoid cells positive for this marker and confirms that the highest expression is seen in plasma cells and not the GC (Grammer et al. 1999, Arce et al. 2004). All these considerations would not contradict the idea of a negative regulatory role of ZFP36L1 on CD38, however, further investigations should be made to elucidate the relationship between ZFP36L1 and CD38 expression. Particularly helpful, would be a single cell analysis examining individual cells within lymphoid tissue for their expression of both these proteins.

ZFP36L1 detection within GC cells is consistent with a role for the protein as a negative regulator of its two proposed mRNA targets studied in this chapter, *BCL2* and *CD38*. The ZFP36L1 protein has been reported to be a key regulator of differentiation (Vignudelli et al. 2010, Nasir et al. 2012) and differentiation is a key feature within the GC, as GC B cells develop into plasma B cells or memory cells. As seen, the expression of other reported ZFP36L1 targets that modulate differentiation, growth and signalling -BLIMP1 (Nasir et al. 2012), GM-CSF (Pistoia and Corcione 1995) TNF α , interleukin 6 (IL-6) or IL-10, IL2 (Butch et al. 1993, Toellner et al. 1995, Lisignoli et al. 1998) cIAP2 (Jourdan et al. 2009, Akyurek et al. 2006) - may well also be found in highest levels in cells where ZFP36L1 expression is lowest although this was not further investigated in the present study and remains to be determined.

It is worth mentioning that some of the positive cells detected by the antibodies used could be from T cells producing the target epitope proteins. In this regard, GCs primarily consist of B cells along with a small number of T cells (5 to 10%) and follicular dendritic cells (FDC) (< or = 1%) (Butch et al. 1993).

Finally, the results shown in this chapter for BCL2 and CD38 expression in relation to ZFP36L1 expression in lymphoid tissue, must also be taken into account alongside the luciferase assay results described in chapter 3 and 4, that provided evidence of a direct interaction and functional interaction of ZFP36L1 protein with *BCL2* and *CD38* mRNA 3'UTRs leading to their degradation. This will be explored further in chapter 7.

Chapter 6

“Analysis of ZFP36 family protein, BCL2 and CD38 protein expression in B cell lines and primary B leukaemic cells”

6.1 Introduction

This chapter focuses on the analysis of ZFP36 proteins expressed in different B cell populations. It aimed at identifying how the ZFP36 proteins were expressed in cell lines representing B cell stages and in primary cells of B-CLL origin. The analysis of protein expression was done by Western blot. In some blots levels of expression of BCL2 were also analysed in order to assess the relationship between ZFP36L1 and BCL2. CD38 expression were also measured in a smaller sample of B-CLL cells.

6.2 Western blot analysis of ZFP36L1 protein expression in different B cell lines

6.2.1 Cell lines representing different stages of B cell development

SDS PAGE and Western blot analysis were performed on lysates from different B cell lines representing various stages of B cell development. Antibodies for ZFP36L1/ ZFP36L2 and BCL2 were used to detect protein expression. Bands were seen for ZFP36L2 and BCL2 but no bands were detected for ZFP36L1 (fig. 6- 1). ZFP36L2 showed different levels of expression amongst the B cell lines; all myeloma cell lines showed bands for ZFP36L2 except in the case of MM1S. At increased exposure MM1S presented only a faint band for the protein (fig. 6-1C). The expression levels were observed to be diverse amongst the ZFP36L2 expressing cells. Of the two lymphoma cell lines analysed, Ramos showed a ZFP36L2 band at different exposure levels, but in the Namalwa cells a band was only seen after increased exposure indicating low level expression. The same pattern was seen with pre-B-cell cell line Nalm 6 where a ZFP36L2 band was observed after increasing exposure time. BCL2 bands were clearly detected in all the cell lines tested, however the strength of the bands was slightly variable indicating that for BCL2, expression levels were generally low and showed some heterogeneity. Table 6-1 shows a summary of Western blot protein expression results for the B cell lines used in this study.

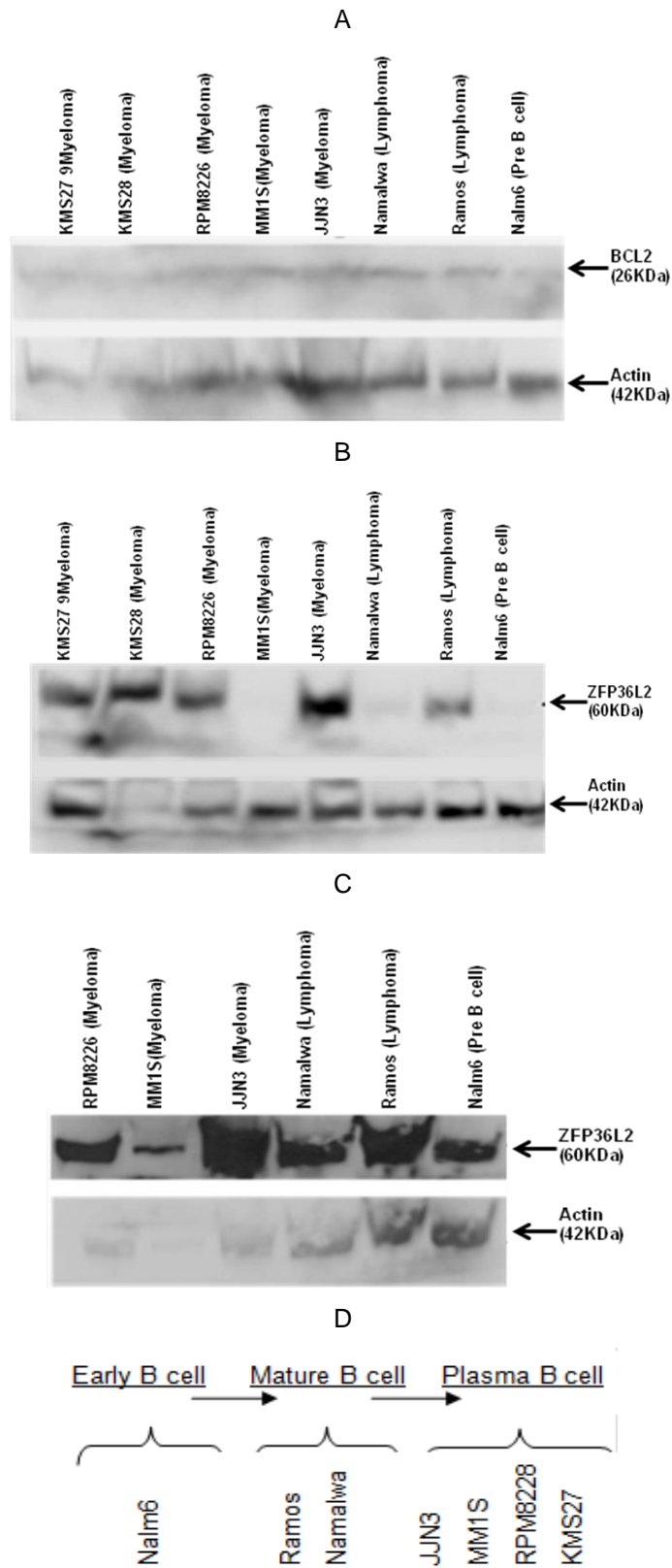
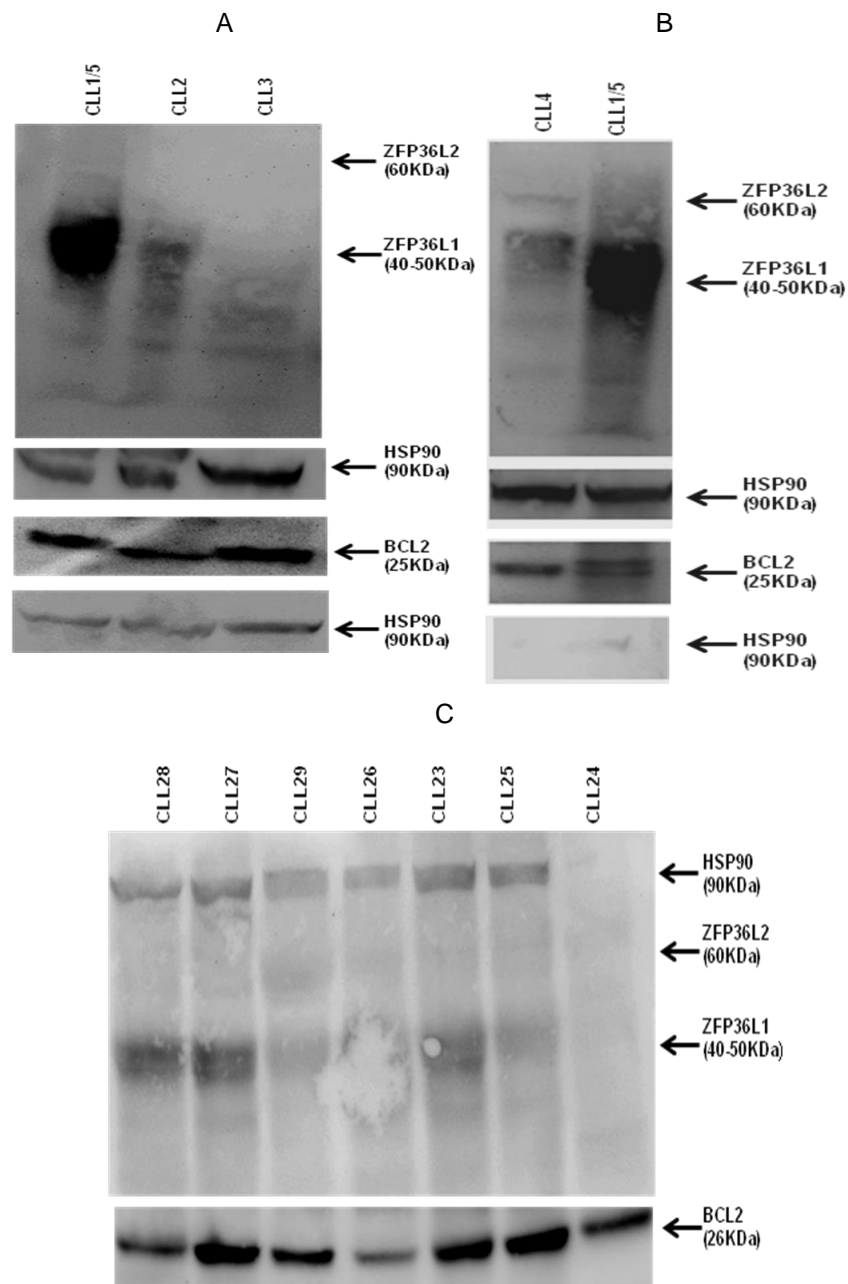


Figure 6-1 Western blot analysis of ZFP36L1 and ZFP36L2 expression

Western blot analysis of ZFP36L1 (40KDa) and ZFP36L2 (60KDa) and BCL2 (26KDa) in various cell lines representing different B cell development stages. BCL2 and ZFP36L2 were detected with anti-BCL2 and anti-ZFP36L1/2 antibodies, bands were seen for ZFP36L2 and BCL2, No signal was detected in any blot for ZFP36L1. ZFP36L2 and BCL2 showed heterogeneity in their expression patterns and levels. Loading control was carried out by probing with anti- Actin antibody after membrane stripping. D. Indicates the stages of development represented by cells studied in these blots.

6.3 Western blot analysis of ZFP36 protein family expression and BCL2 expression in primary B-Chronic Lymphocytic Leukaemia cells

Western blot analysis of ZFP36L1 expression in B-CLL cell populations indicated mostly low levels of ZFP36L1 (40kD) expression but there was some heterogeneity in levels of ZFP36L1 expression in different B-CLL populations (fig. 6-2). In contrast, the higher molecular weight protein ZFP36L2 (60kD) was not at all or barely expressed (fig. 6-2). BCL2 expression was also detected for the same samples, its expression was heterogeneous with different levels of BCL2 expression in the various B-CLL populations. Anti-ZFP36 antibody was also used to analyse expression of ZFP36 in B-CLL cells and homogeneous bands were present in all the samples tested (fig. 6-2). Table 6-2 shows bands detected and expression levels for ZFP36L1/L2 and BCL2 in the different B-CLL tested.



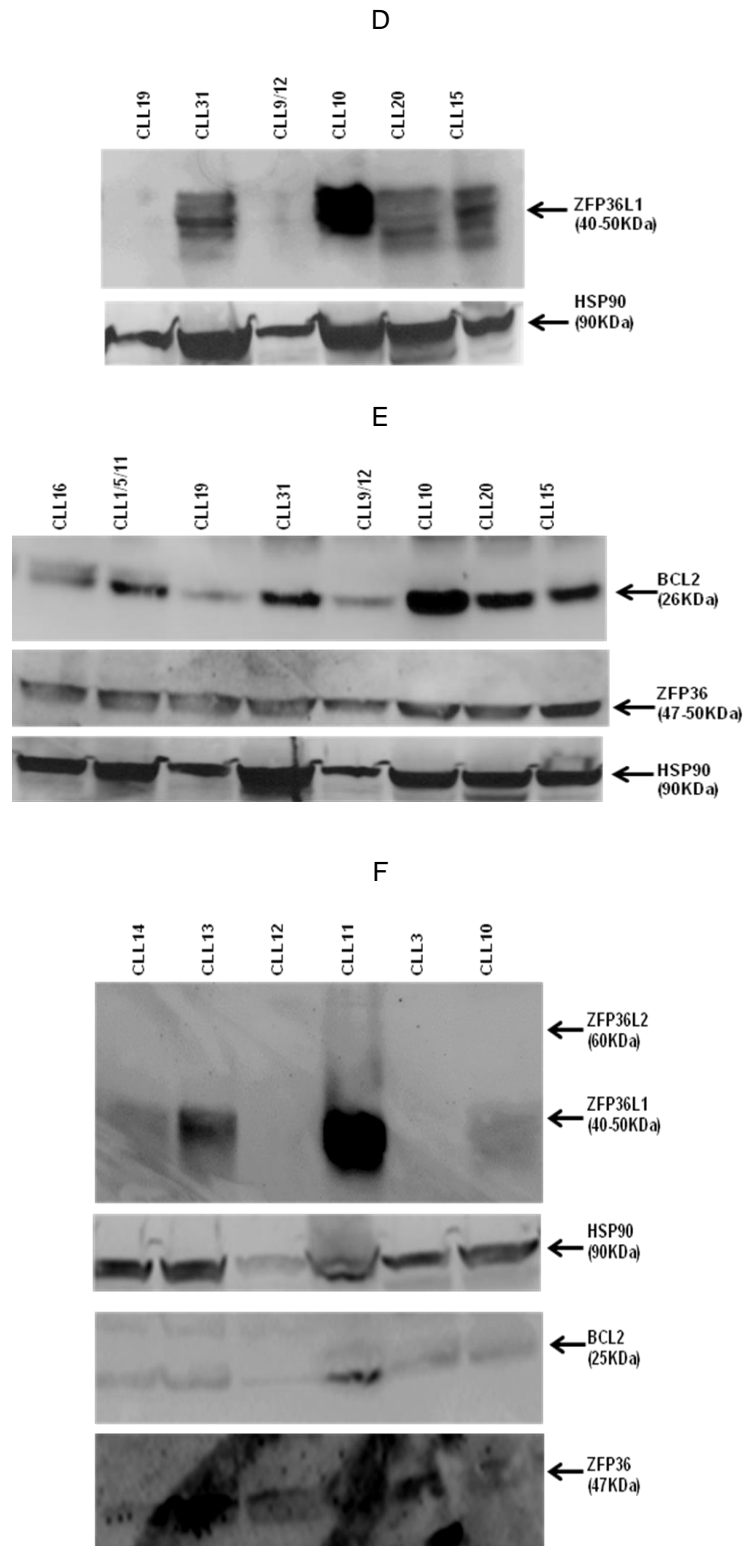


Figure 6-2 Western blot analysis of ZFP36, ZFP36L1, ZFP36L2 and BCL2 expression in B-CLL cells.

A.B.C Show Western blots results for ZFP36L1 and ZFP36L2 alongside BCL2. D. Shows different B-CLL cells probed for ZFP36L1 and ZFP36L2; the same cells, and 2 other populations are shown in another blot probed for ZFP36 and BCL2 in figure E. F Shows membranes probed with anti-ZFP36L1/ZFP36L2, anti-BCL2 and anti-ZFP36. Results showed heterogeneity in the expression of the proteins, especially for ZFP36L1. ZFP36 was expressed at low to medium levels in the different B-CLL populations. ZFP36 was observed to be the only ZFP36 family member to be expressed homogeneously in all B-CLL populations. An antibody able to detect both proteins, ZFP36L1 and ZFP36L2 was used. Anti-HSP90 antibody was used to detect the pan-protein HSP90 as loading control (CLL= B cell Chronic lymphocytic leukaemia sample).

Table 6-1 A summary record of relative levels of expression of different proteins in different cell lines

Cells	ZFP36L2	ZFP36L1	BCL2
Nalm 6, pre-B cell	*	0	*
Ramos, lymphoma	**	0	**
Namalwa, lymphoma	*	0	*
JJN3, myeloma	***	0	**
MM1S, myeloma	*	0	*
RPMI8226, myeloma	***	0	*
KMS28, myeloma	**	0	*
KMS27, myeloma	***	0	*

0 = Band not detected

* = Faint band/Low expression

** = Medium band/ Medium expression

*** = Strong band/High expression

Table 6-2 A summary record of relative levels of expression of different proteins in different B-CLL populations.

B-CLL population	ZFP36L1	ZFP36L2	BCL2	ZFP36
CLL1/5/11	***	0/*	**	**
CLL2	**	*	**	ND
CLL3	0	0	**	ND
CLL4/14	*	0/*	**	**
CLL6	0	0/*	*	ND
CLL7	0	0/*	*	ND
CLL8	*	0/*	*	ND
CLL9/12	0	0/*	*	**
CLL10	**/**	0	***	**
CLL13	**	0	*	**
CLL15	*/**	0	**	**
CLL16	**	0	***	**
CLL17	***	0	**	**
CLL18	**	0	*	**

Cont.

B-CLL population	ZFP36L1	ZFP36L2	BCL2	ZFP36
CLL19	*	0	**	**
CLL20	*/**	0	**	**
CLL21	0	0	*	**
CLL22	**/**	0	*	**
CLL23	**	*	**	ND
CLL24	0	0	**	ND
CLL25	*	0	**	ND
CLL26	*	*	*	ND
CLL27	**	0	**	ND
CLL28	**	0	*	ND
CLL29	*	*	*	ND
CLL30	**	*	*	ND
CLL31	**	0	**	**

0 = Band not detected ND=Not done

* = Faint band/Low expression

** = Medium band/ Medium expression

** = Strong band/High expression

6.4. Western blot analysis of ZFP36L1 and CD38 expression in B cell chronic lymphocytic leukemia cells

B-CLL cells were used to analyse the expression pattern of CD38. The total number of B-CLL populations used was smaller than those used to study ZFP36 proteins and BCL2, this was due to the reducing number of samples available in the last 18 months and the lack of time at the end of the project. Here a Western blot is shown for different B-CLL cells populations demonstrating that the pattern of expression of CD38 in these primary cells does not seem to directly correlate with the expression of ZFP36L1, a similar result to that seen in the analysis of BCL2 expression in relation to ZFP36L1.

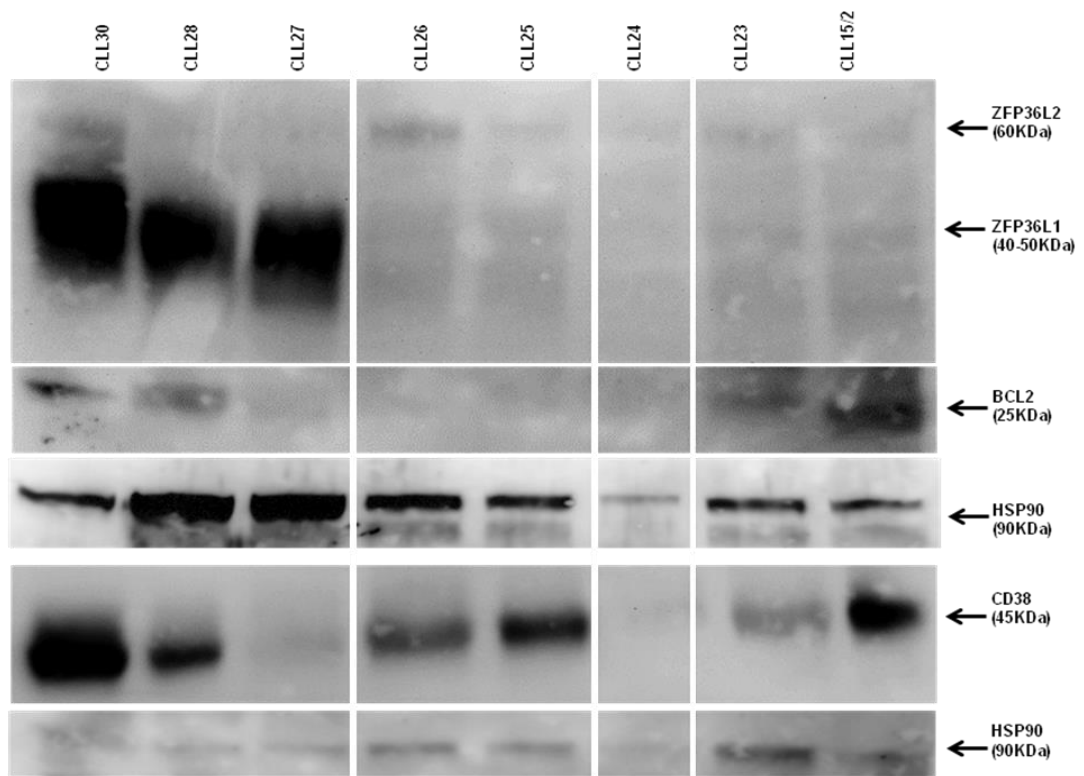


Figure 6- 3 Western blot analysis of CD38, ZFP36L1, ZFP36L2 and BCL2 expression in different B-CLL populations. Results indicate a heterogeneous expression pattern amongst the different B-CLL populations, analysis of ZFP36L1 expression indicated that high expression levels of the protein correlated with different expression levels of CD38 or even no expression (CLL30, CLL28, CLL27). In samples where no ZFP36L1 was detected or was detected at low levels, CD38 expression also varied (CLL23, CLL15/2 and CLL26, CLL25). These results are similar to those observed after the analysis of BCL2 expression in relation to ZFP36L1 in previous sections, figures 6-1 and 6-2.

The number of samples used in these blots was very small, just eight BCLL cells populations were tested , and this is one limitation that must be considered in this analysis, a larger set of samples could be tested to further confirm the results observed in the Western blot shown in figure 6-3. These results are consistent with the pattern of expression found during the analysis of BCL2 expression in relation to ZFP36L1, that a number of samples with similar expressions levels of ZFP36L1 showed varying degrees of CD38 expression. Clinical data available for the patients samples was not complete, but some extra information was gathered by flow cytometry/immunofluorescence analysis of CD38 performed by Nadeeka Rajakaruna (PhD student at the University of Westminster) who has kindly agreed referring to her data in this thesis. Flow cytometry analysis of CLL30 indicated mean fluorescence intensity of CD38 at 98.78% corresponding to the largest CD38 band in figure 6-3. The other samples analysed in the blot show different expression levels for CD38 alongside a heterogeneous expression pattern of ZFP36L1 in the same samples; CLL30 and CLL28 are positive for ZFP36L1 and CD38 but the level of CD38 is different between the two populations of cells whereas CLL27 has a high level of ZFP36L1 but not detectable levels of CD38. The remaining samples still have detectable levels of CD38 but low/no expression of ZFP36L1.

In summary, analysis of ZFP36L1/ZFP36L2 and CD38 expression revealed heterogeneity in the pattern of expression of the proteins. Samples with no or low ZFP36L1 showed positivity for CD38 at low to high levels or even no positivity at all. This pattern of expression seems similar to what was observed for BCL2, where no direct relationship is observed-inverse or proportional- between ZFP36L1 and CD38 expression in B-CLL cells.

6.5 Clinical data

An attempt was made to analyse clinical data provided for each B-CLL sample in order to assess if the ZFP36 family protein expression pattern observed related to clinical features that could explain the results obtained. The clinical data available included information on: cytogenetics, IgHV mutation, CD38 positivity but for any given sample not all this information was completed so a complete analysis was not possible.

Table 6-3: Western blot analysis summary data from all proteins analysed and comparison with clinical data obtained for all B-CLL samples:

B-CLL population	ZFP36L1	ZFP36L2	BCL2	ZFP36	CD38	IgHV	Zap70	CD38	Cytogenetics	YD	CD38 pos %
CLL1/5/11	***	0/*	**	**		unm		pos		2003	nm
CLL2	**	*	**		**				11q		
CLL3	0	0	**			unm				2011	
CLL4/14	*	0/*	**	**							
CLL6	0	0/*	*								
CLL7	0	0/*	*			mut			13q	1999	20.1
CLL8	*	0/*	*					neg		2001	
CLL9/12	0	0/*	*	**					17q		7.7
CLL10	**/**	0	***	**					17q		6.36
CLL13	**	0	*	**					Yes(1and2)	1998	
CLL15	*/**	0	**	**		unm		neg		2007	95.8X
CLL16	**	0	***	**		unm		pos		2005	
CLL17	***	0	**	**		mut	pos	pos		2007	
CLL18	**	0	*	**		mut		neg		2004	22.4
CLL19	*	0	**	**							
CLL20	*/**	0	**	**					17p 13q	2012	
CLL21	0	0	*	**		unm		pos	gain 12	2011	
CLL22	**/**	0	*	**					13q	2011	nm
CLL23	**	*	**		*						36.65
CLL24	0	0	**		0	mut		neg			nm
CLL25	*	0/*	**		**						
CLL26	*	*	*		*						47.28
CLL27	**	0	**		0						4.25
CLL28	**	0	*		**	mut		neg	13q		97.06X
CLL29	*	*	*							2009	9.39
CLL30	***	*/**	*		***						98.78
CLL31	**	0	**	**						1996	86.6

0= no detection or very faint band detected; Blank spaces: no info provided, or no tests done ; YD=Year diagnosis

Boxes blue: Blot results; Boxes green: clinical data provided by UCLH London hemato-oncology clinic Boxes pink: CD38 % of cells positive lab measured, % data measured by University of Westminster PhD student Nadeeka Rajakaruna nm=not

measured, no % given. The two samples marked with an X are queried samples, the clinical data says these are negative for CD38 but the data provided by immunofluorescence analysis performed by Nadeeka Rajakaruna) gave a large positive, this may be due to measuring CD38 at a different time, it is known that CD38 positivity may vary throughout the disease course.

Further analysis of clinical data and protein expression results could not be pursued due to incomplete information. Data on percentage of positive cells for CD38 produced by Nadeeka Rajakaruna was requested in the hope that this could provide some more information but this attempt was also unsuccessful as data was available only for a small number of samples, however it was noticed that data from Western blots and flow cytometry analysis were highly consistent for those samples that were tested for CD38 expression by these methods.

The seemingly contradiction between clinical data stating CD38 negative status for CLL28 may be explained by a number of factors. For clinical diagnostic purposes, CD38 positivity is set at 20-30% of positive cells within a clone, so a negative sample may still express CD38 that could be detected by Western blot analysis, especially with lysates produced from a high concentration of cells. Also, samples with a low % of positivity are demonstrated to show CD38 expression in figure 6-3, such as CLL23. Finally, flow cytometry data indicated that CLL28 CD38 positivity was 97.06% but clinical data classed the patient as negative, besides human error, a clinical explanation may clarify this; the levels of CD38 can vary throughout the course of B-CLL within a given patient (Malavasi et al. 2008) and the clinical data may state the negative status of the first clinical test when the patient attended the clinic (flow cytometry analysis performed at the University of Westminster was done immediately after receiving the samples every week).

Overall, an heterogeneous expression can be determined for CD38 in different B-CLL populations that has not direct or inverse relationship with ZFP36L1 expression, as seen by the blot data (fig. 6-3) and by the percentage of CD38 positive cells data (table 6-3). Some samples with a small percentage of cells positive for CD38 seemed to have also low expression levels of the other proteins analysed (CLL7, CLL29), other low percentage CD38 positive samples showed medium expression levels of the ZFP36L1 (CLL18 and CLL10). Without a more complete clinical data the analysis could not be pursued further.

6.6 Discussion

This study has looked at the presence of ZFP36L1 and its role regarding one of its putative targets, *BCL2* mRNA ARE. The *BCL2* gene codes for BCL2, a known oncogenic protein with regulatory functions in promoting cell survival and preventing programmed cell death, apoptosis. Dysregulation of this gene is associated with B-CLL and other cancerous processes (Thomadaki and Scorilas, 2006).

The expression of the ZFP36 protein family in cell lines representing different B cell development stages showed that ZFP36L1 was low/not detected and ZFP36L2 was expressed at higher levels although at different levels in different cell lines. Results showed heterogeneous patterns and levels

of expression of ZFP36L2 and BCL2. Nasir 2012 and Nasir et al. 2012 looked at *ZFP36L1* mRNA levels in cell lines representing different B cell stages, similarly to table 6.1 they used cell lines representing pre-B cells to plasma cells. QRTPCR showed that *ZFP36L1* mRNA levels were higher in early B cell stages and generally lower in mature and plasma cell stages, myeloma cells had lower (JFN3) or no levels (MM1S) detected. Western blot analysis results in table 6-1 did not detect bands for the ZFP36L1 protein in any of the lines including Ramos, a cell line used by Nasir et al. 2012 that resulted in the highest levels of *ZFP36L1* mRNA. The discrepancy in results could be due to further modification at mRNA level that may lead to handicapping ZFP36L1 protein viability but is possibly due to the different sensitivities of the assays and further protein expression level analysis should be done. Absence of ZFP36L1 in the later stages of B cell development, but its expression in early, non differentiated B cells suggests down regulation of the protein once differentiation signal have been received. ZFP36L2 was detected in varying levels within cell lines representing the same developmental stages but although further analysing would be needed, the summary table could indicate that ZFP36L2 levels are lower at early stages of B cell development and generally increase in mature B cells. To confirm this affirmation further blots should be performed and QRTPCR assessment carried out for ZFP36L2 levels. However it may be possible that the expression levels of ZFP36L2 in different cell lines representing the same stage of B cell development is variable since the clones may differ in their genetic/clinical profile, for multiple myeloma the heterogeneity is widely reported (Bianchi and M Ghobrial 2014, de Mel et al. 2014, Bianchi and Munshi 2015), just as it happens with B-CLL cases. Also as Nasir et al. 2012 have shown for BLIMP1 and ZFP36L1, it may be possible for ZFP36L2 that varying levels of the protein in different B cell stages are explained by the regulatory function of the protein on targets with a key role in B cell maturation or differentiation. Cells lines are useful tools for research but they may not represent the original tumours profiles due to continued culture, passaging and accumulated mutations; it would be useful to perform the analysis of ZFP36 proteins in primary B cell representing different B cell development stages.

In B-CLL cells expression of ZFP36L2 was not observed or faintly detected, and expression of ZFP36L1 was detected in a heterogeneous pattern with different levels of ZFP36L1 found in different B-CLL cells populations. Analysis of ZFP36 expression showed bands in all B-CLL cells populations with more homogeneity in its expression levels amongst the B-CLL cells populations compared to ZFP36L1. Some heterogeneity in expression levels was also detected for BCL2 in the B-CLL cells and although in some B-CLL cell populations an inverse relationship in the expression levels of ZFP36L1 and BCL2 was apparent; this was not a consistent finding overall in the different B-CLL populations studied. ZFP36 was found expressed at low/medium level in all B-CLL its pattern of expression was very homogeneous across all B-CLL populations.

Analysis of CD38 revealed similar results to those of the ZFP36 proteins and BCL2 expression analysis, different levels of CD38 were detected in different B-CLL samples, and within these samples levels of ZFP6L1 varied. Samples with high levels of expression of ZFP36L1 could have high, medium or low/no levels of CD38, the same was observed for samples where ZFP36L1 was not detected.

The characteristic heterogeneity of B-CLL could account for the different levels of ZFP36L1 found in different B-CLL populations. Clinical data was looked at to assess if there are clinical features shared amongst patients that relate to results obtained from protein expression analysis and may provide further information on ZFP36L1 expression levels in the different B-CLL cells populations. This study was attempted but the clinical data provided for the B-CLL samples (table 6-3) proved insufficient to draw any conclusion.

In summary, no clear inverse relationship is seen between levels of expression of BCL2 or CD38 and ZFP36L1 protein.

Further experiments could be done such as QRT-PCR to compare mRNA levels of ZFP36, ZFP36L1, ZFP36L2 and BCL2 in different B-CLL cell populations. One area that could be looked at is miRNAs in B-CLL cells. Micro RNAs signatures not only distinguish normal B cells from malignant B-CLL cells, their profile also gives information on the indolence of the disease, patients with high miR21 and miR15 have a higher risk of death compared to those with low expression; miR181b detection serves as a time to treatments predictor, and non-responders to treatment also offer a specific miRNA profile, expression of miR148a, miR122, miR21 (Balatti et al. 2015). Of the microRNAs that are involved in B-CLL two are connected with the ZFP36 proteins. miR15a and miR16-1, and both of these are frequently deleted or down regulated in B-CLL cells with 13q14 deletions (Calin et al. 2002). Cimmino et al. 2005 revealed a role for the miR15/16 family negatively regulating BCL2 expression and promoting B-CLL cell apoptosis and also provided evidence of the miRNAs targeting *BCL2* mRNA by binding of its 3' UTR. About 60% of B-CLL cases showed evidence of an inverse correlation between these miRNAs levels and BCL2 level of expression (Cimmino et al. 2005). Alterations in miRNAs in B-CLL cells, with regard to miR-15a and miR-16-1 deletions, contribute to malignant transformation by up-regulating BCL2. In addition, there is evidence of cooperation between microRNAs and the ZFP36 proteins in degradation of mRNAs, as reported for miR16 and ZFP36 where the destabilising effect of ZFP36 on TNF α mRNA was not observed if miR16 was absent (Jing et al. 2005). All this makes for an interesting research question directed at analysing the profile of expression of the ZFP36 proteins and selected microRNAs, specifically miR16 and 15a in B-CLL, the analysis could be done using different experimental strategies including; FISH, PCR or an RNA sequencing method available.

Chapter 7
General Discussion
and
Final Conclusion

7.1 General Discussion

This chapter will start by summarising the results discussed in this thesis aiming to bring them together to a final general conclusion.

This thesis has investigated two putative mRNA targets chosen for their relevance in B cell biology, specifically B cell malignancies. The results have extended and further strengthened the findings investigating the putative targets identified by *in silico* ARACNe analysis (Nasir et al. 2012, Zekavati et al. 2014). The previous chapters have described the experiments done to investigate *BCL2* mRNA and *CD38* mRNA as possible targets for ZFP36L1 and have also shown experimental data for ZFP36L2 and ZFP36. The project has also measured the expression of ZFP36 family proteins in primary cells B-CLL cells and in B cell lines representing different developmental B cell stages.

The role of ZFP36L1 in promoting cell apoptosis has been reported by several groups (Ning et al. 1996, Johnson and Blackwell 2002, Baou et al. 2009b), a possible route by which this pro-apoptotic function may function might be the regulation of key pro-survival proteins like those of the BCL2 family. The prototype, BCL2, has an ARE in its mRNA which has been highlighted as a possible target for the proteins (Zekavati et al. 2014). A role for ZFP36L1 in negative regulation of B plasmacytoid differentiation by targeting of the *BLIMP1* mRNA was also previously reported (Nasir et al. 2012).

This project has provided evidence that the three ZFP36 proteins interact with the 3' UTR ARE of *BCL2* mRNA. For ZFP36L1 a mutant version, with an amino acid change in the zinc finger domain, did not lead to the degradation of the 3' UTR ARE of *BCL2* mRNA. The ZF mutant form of ZFP36L1 therefore served as a negative control in the 3'UTR luciferase reporter assays and also confirmed that intact ZF domains are required for ZFP36L1 to bind to the *BCL2* ARE. Another strategy used to study the interaction of the ZFP36 family proteins with the ARE in *BCL2* mRNA was to use a *BCL2* ARE where the core ARE sequence was absent in a mutated form of the *BCL2* ARE. ZFP36L1 was unable to interact with the mutant *BCL2* ARE indicating that ZFP36L1 recognition of the *BCL2* 3'UTR is via the AUUUA sequences in the ARE. Results from analysing the interaction of the other two family members, ZFP36L2 and ZFP36, also indicated that they degraded the *BCL2* 3'UTR ARE and not its mutated version. No ZF domain mutants of the ZFP36L2 and ZFP36 proteins were available; however, using mutant versions of these proteins in further 3'UTR luciferase reporter assays would be useful to further strengthen the findings of the study and support that all three ZFP36 family proteins can interact with the *BCL2* ARE. REMSA assays had already shown the interaction between ZFP36L1 and the *BCL2* ARE (Zekavati 2009, Zekavati et al. 2014). REMSA experiments could also be carried out to analyse the interaction of ZFP36L2 and ZFP36 proteins with the *BCL2* ARE to possibly further strengthen the results of the present study.

The possibility that *CD38* mRNA is a target for ZFP36 proteins has never been studied before although *in silico* analysis had previously highlighted it as a putative ZFP36L1 target mRNA (Nasir et al. 2012). Using a similar strategy to that put in place with *BCL2* 3'UTR ARE, this project looked at investigating if there was a direct and functional interaction between ZFP36 proteins and the

CD38 3' UTR ARE. The 3'UTR luciferase reporter assays described in this thesis have provided the first *in vitro* experimental evidence of a direct interaction between ZFP36L1 and the 3'UTR ARE of *CD38* mRNA. ZF domain mutant ZFP36L1 was unable to bind to the 3' UTR ARE but wild type ZFP36L1, ZFP36L2 and ZFP36 led to reduced luminescence levels of the reporter gene containing the *CD38* 3' UTR ARE. A mutant *CD38* ARE was unavailable. The 3'UTR luciferase reporter assays could be expanded and strengthened by using mutant versions of the ZFP36 family proteins and the *CD38* 3' UTR ARE target.

To further analyse the possible regulation of *CD38* mRNA by ZFP36L1, the expression level of the ZFP36L1 protein was down regulated in HeLa by siRNAs, and the levels of *CD38* expression were assessed by immunofluorescence and flow cytometry and by Western blot analysis. In cells with a down regulation of ZFP36L1 expression, *CD38* expression levels were increased and there was an indication that the lower the level of ZFP36L1 expression in the cells, the higher the level of *CD38* was observed. One limitation to take into account in the case of these results is that a phenotype rescue (expressing a siRNA resistant form of the gene that has been knocked down, rescue of the knockdown phenotype indicates siRNA specificity) would have added another layer of certainty to the siRNA effect. Also QRT-PCR measurements of mRNA for ZFP36L1 and *CD38* would provide additional quantitative information regarding changes in both mRNAs that could potentially support the Western blot protein level analyses that was carried out. To further extend the study to immune cells siRNA-mediated knockdown of ZFP36L1 expression could be performed in primary B cells or B cell lines that are positive for ZFP36L1 and *CD38* expression. Knockdown experiments were attempted in B-CLL cells (data not shown) but the results regarding successful siRNA transfection were variable and inconsistent and therefore this approach was abandoned.

Analysis of expression of ZFP36L1, *BCL2* and *CD38* in lymphoid tissue by IHC showed that detectable levels of ZFP36L1 expression were confined to GC. *BCL2* expression was absent or very low in GCs of normal tonsils but more highly expressed in the mantle zone. The same strategy was used to analyse the proteins in malignant lymphoid tissue. Results obtained from immunohistochemistry analysis of FL showed that ZFP36L1 was expressed in follicle centres where *BCL2* is reportedly also highly expressed. Unfortunately, *BCL2* expression was not detected in FL in the present study due to a technical problem. In the vast majority of FLs *BCL2* is translocated (t(14:18), translocation) placed under the control of the IgHV promoter and is therefore highly up regulated. ZFP36L1 expression was detected at the same level in normal tissue GCs as in the malignant follicular centres. One possibility is that since the translocation would mean that the level of *BCL2* is highly up regulated in FL B cells, the normal negative regulatory control of ZFP36L1 over *BCL2* expression would be overcome by the abnormal increased expression of *BCL2*.

These IHC results, especially non-malignant tissue showing ZFP36L1 detected in GCs where *BCL2* is very lowly/not expressed, must be considered alongside the luciferase assay results discussed in chapter 3. Chapter 3 evidences the interaction of the ZFP36 proteins, especially ZFP36L1, with the *BCL2* 3'UTR ARE. In addition, data recently published on the role of the ARE in

the regulation of BCL2 from *in vivo* studies (Díaz-Muñoz et al. 2015) and the evidence provided in (Zekavati et al. 2014) are consistent with ZFP36L1 targeting *BCL2* mRNA *in vivo*.

CD38 is a marker with relevance in B cell development and is an important plasma cell marker. It has already been shown ZFP36L1 targets BLIMP1 (Nasir et al. 2012), to negatively regulate B cell plasmacytoid differentiation. In the study, *CD38* mRNA was also highlighted by *in silico* analysis as a putative ZFP36L1 mRNA target (Nasir et al. 2012). The IHC results in chapter 5 show that there is a low level CD38 expressing cells in GCs where ZFP36L1 is mainly expressed, but in tissue areas where CD38 was highly expressed, there was no detectable ZFP36L1 expression.

For CD38, the IHC results, together with the 3'UTR luciferase reporter assay results and the upregulation of CD38 expression after down regulating ZFP36L1 in HeLa cells, support the hypothesis that ZFP36L1 targets *CD38* mRNA and thereby negatively regulates its expression by transcriptional mechanisms.

The last results chapter of this thesis showed the analysis of expression of the ZFP36 family of proteins in B cell lines representing B cells of different developmental stages and primary B-CLL cells. The results showed that across the B cell lines, ZFP36L1 was expressed at low levels or not detected, but ZFP36L2 was generally higher expressed although at different levels in different B cell lines. In B-CLL cells the opposite was seen, ZFP36L2 was not expressed, or very faintly detected, and the expression level of ZFP36L1 varied amongst the different B-CLL cell populations, the protein was markedly heterogeneous in its level of expression. The ZFP36 protein showed fairly homogeneous expression levels in all B-CLL populations tested. For the B-CLL populations, the expression of BCL2 was also analysed and the protein was detected at different levels in different B-CLL cell populations. There did not seem to be an inverse relationship between levels of ZFP36L1 and BCL2 in B-CLL. The lack of a direct inverse relationship seen in Western blots in B-CLL cells could stem from the deregulation of BCL2 in B-CLL (Tzifi et al. 2011). Furthermore, in B-CLL the clinical stage and disease markers vary between different patients (Kampalath et al. 2003, Rodríguez-Vicente et al. 2013). This clinical variability in B-CLL may account for the different levels of ZFP36L1 and BCL2 expressed in different B-CLL populations. Clinical data available for the B-CLL patients was incomplete in the present study and therefore could not be used to make further assessments that may explain or provide insights into the reasons for the heterogeneity of ZFP36L1, ZFP36L2 and BCL2 expression levels in B-CLL.

CD38 expression was also found to be heterogeneous and without an evident direct or inverse relationship with ZFP36L1 levels. B-CLL cells with high ZFP36L1 showed high levels of CD38 and low/no CD38 expression, the same was seen for B-CLL cells with no ZFP36L1 expression.

Cell lines representing mature B cells and plasma cells such as myeloma did not express or expressed very low detectable levels of ZFP36L1. This could be seen as reasonably consistent with immunohistochemistry findings, where ZFP36L1 was detected in GCs of normal tissue and in FL but at a relatively low level of expression. The finding of absent or low level of ZFP36L1 in myeloma

cells has previously been reported although a more extensive analysis was done in the present study (Nasir et al. 2012).

As in previous studies other methods to assess the level of expression of ZFP36L1 in B-CLL cells and in B cell lines could be used (Nasir et al. 2012), e.g. measuring mRNA levels by QRT-PCR. An optimised histological analysis such as fluorescence *in situ* hybridisation could also be done to compare data with the results obtained in the present study, this could provide clearer assessment of which B cells are positive for ZFP36L1 in tissue sections.

Further extensions of the studies presented here could involve attempting to isolate mRNA targets from B cells for ZFP36L1 (and possibly ZFP36 and ZFP36L2) on a global scale using newly described protocols such as iCLIP (individual nucleotide resolution cross linking and immunoprecipitation) (König et al. 2012) or PARclip (Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) (Spitzer et al. 2014). These methodologies are potentially very powerful and important novel mRNA targets identified using these strategies could be further validated by similar approaches to those presented in this project. In the light of studies presented here and also the evidence that the promoters of the ZFP36 and ZFP36L1 genes are targeted for transcriptional repression by the BCL6 oncogene in GCs (Basso et al. 2010) it would also be useful to examine in more detail GC formation and B cell subpopulations alongside the formation of plasma cells in the GC in ZFP36L1 mouse knockout animals.

7.1. 2 Model of ZFP36L1 function in late B cell development

Overall, the evidence is indicating a role of ZFP36L1 in the regulation of mRNAs key in immunobiology, although the role may be shared with the other ZFP36 family members to the point that it has been reported that the loss of one protein may be compensated by another family member. There is evidence that in mice with deficient ZFP36L1, ZFP36 may compensate for its loss. ZFP36L1 is induced upon infection, but deficient ZFP36L1 mice have not indicated changes of lung cytokine production, bacteria clearance is not altered either as well as there is not a change in increased inflammatory lung injury. Data from ZFP36 deficient mice seem to indicate that ZFP36 and not ZFP36L1 is the major negative regulator of cytokine expression in macrophages. The authors concluded that ZFP36 may compensate for the loss of ZFP36L1 in these experiments (Hyatt et al. 2014).

The role of the ZFP36 proteins as tumour suppressors in immunological malignancies was initially reported in mast cell tumours (Stoeklin et al. 2003). ZFP36 is downregulated in a number of cancers (Brennan et al. 2009). Lack of ZFP36 destabilising function on mRNAs led to overproduction of TNF α , VEGF and overall tumour progression (Suswam et al. 2008). VEGF mRNA is also regulated by ZFP36L1 (Bell et al. 2006). Mice lacking ZFP36L1 and ZFP36 developed T-ALL and increased levels of NOTCH1 are observed (Hodson et al. 2010). Genome sequence analysis of MM samples has shown the presence of a mutation affecting the *ZFP36L1* gene (Chapman et al. 2011).

Differentiation might be controlled by ZFP36L1 at early stages as evidenced by data from mESCs where ZFP36L1 seems able to negatively regulate factors such as Nanog, a transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells (Tan and Elowitz 2014). In B cells, ZFP36L1 negatively regulates plasmacytoid differentiation by targeting BLIMP1 (Nasir et al. 2012) and evidence obtained in this research project suggest that ZFP36L1 might extend its post-transcriptional regulator role to CD38 and BCL2 in late B cell development; a model of ZFP36L1 regulatory function in late B cell development is given in figure 7-1.

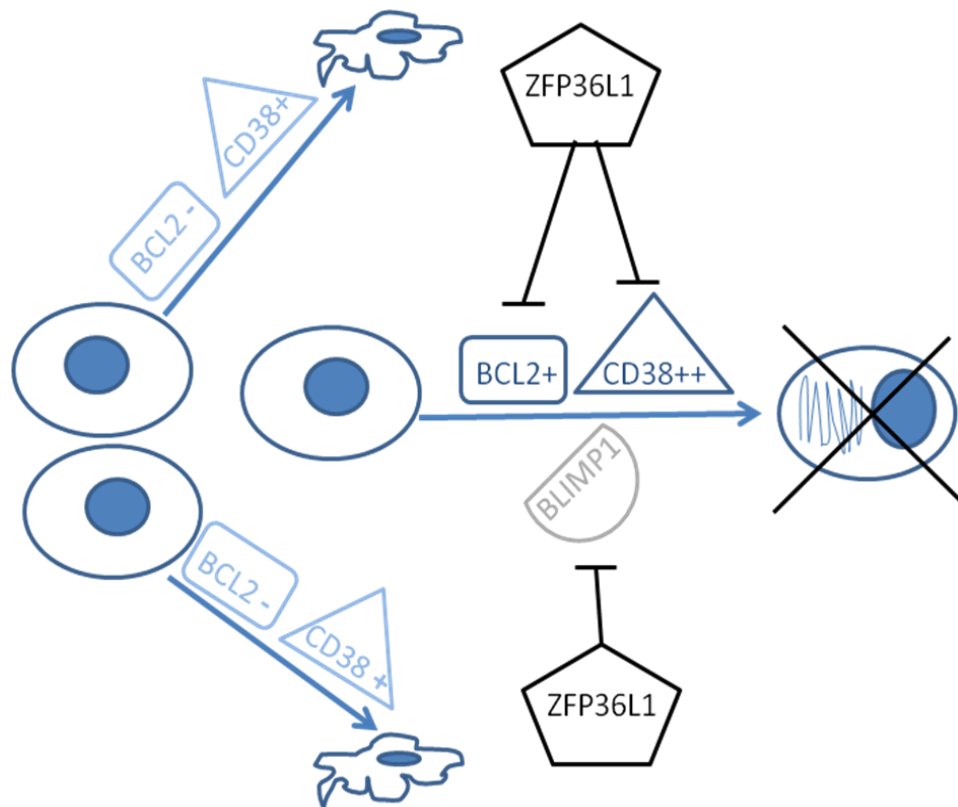


Figure 7-1 Proposed model of ZFP36L1 function in late B cell development

B cells can differentiate to plasma cells, primed for survival by BCL2 and expression of CD38 will increase in plasmablasts/plasma cells. The remaining of B cells die by apoptosis. BLIMP1 was shown to be a target for ZFP36L1 (Nasir et al. 2012). BLIMP1 expressions starts after activation of a naive B cell and increases driving differentiation into plasma cells. ZFP36L1 targets the 3'UTR ARE of *BCL2* and *CD38* mRNAs leading to degradation and downregulation of the proteins. BCL2 cannot induce anti-apoptotic effects and cells will not survive to differentiate to plasma cells. CD38 will also be downregulated and affect BCR signalling and cell survival. BLIMP1 is also negatively regulated by ZFP36L1, lack of BLIMP1 hinders terminal plasma cell differentiation

7.3 Final Conclusion

This project has studied two putative mRNA targets for the ZFP36 family of post-transcriptional regulator proteins. BCL2 and CD38 are important proteins in B cell development and B cell malignancy: Using a 3'UTR luciferase reporter assay, ZFP36L1 was found to interact with *BCL2* ARE and *CD38* ARE constructs. The zinc finger domains of ZFP36L1 are required for this interaction. ZFP36 and ZFP36L2, the two other members of the protein family, were also shown to interact with *BCL2* ARE and *CD38* ARE constructs. Down-regulation of ZFP36L1 expression in HeLa cells by siRNAs was accompanied by increased expression of CD38. In B-CLL, no obvious inverse expression relationship was found between ZFP36L1 expression levels and BCL2 expression levels or ZFP36L1 expression levels and CD38 expression levels. IHC analysis of normal tonsils and FL showed that ZFP36L1 was generally expressed at a detectable level in normal GC, where BCL2 expression is absent or very low. But ZFP36L1 expression was also found in the abnormal GC/follicle centres found in FL where BCL2 levels are reported to be highly expressed. CD38 was generally highly expressed in areas of lymphoid tissues where ZFP36L1 expression was very low.

Taken together, the evidence presented in the project, supports a role for the ZFP36 family proteins as post-transcriptional regulators of *BCL2* mRNA and *CD38* mRNA. These observations also support the view that the ZFP36 protein family mediated post-transcriptional control mechanisms have an important role in regulating normal and malignant B cell development and differentiation, particularly at the later stages in B cell development from the mature to plasma cell stage.

Appendix A mRNA targets for ZFP36 proteins

A-1 Reported mRNA targets for the ZFP36 family of proteins, as referred to in section 1.1.5
A review by (Baou et al. 2009a) had previously summarised and published a list of possible and confirmed targets for the three ZFP36 proteins. The list is shown below

Table A-1 Reporter mRNA targets for ZFP36 proteins

Protein	Reported mRNA targets		Reference
ZFP36,	TNF	mRNA stability	(Carballo et al. 1998)
	GM-CSF	mRNA stability	(Carballo et al. 2000)
	IL-3	mRNA stability	(Stoecklin et al. 2000)
	IL-6	mRNA stability	(Stoecklin et al. 2001)
	cyclooxygenase	mRNA stability	(Sawaoka et al. 2003)
	PAI type 2	mRNA stability	(Yu et al. 2003)
	Pitx2	mRNA stability	(Briata et al. 2003)
	TIS11	mRNA stability	(Brooks et al. 2004)
	IL-2	mRNA stability	(Ogilvie et al. 2005)
	1,4galactosyltransferase	mRNA stability	(Gringhuis et al. 2005)
	IL-12	?	(Jalonen et al. 2006a)
	Ccl2	mRNA stability	(Sauer et al. 2006)
	Ccl3	mRNA stability	(Sauer et al. 2006)
	c-Myc	mRNA stability	(Marderosian et al. 2006)
	cyclin D1	mRNA stability	(Marderosian et al. 2006)
	Fos	mRNA stability	(Patino et al. 2006)
	Ier3	mRNA stability	(Lai et al. 2006)
	Genome analysis 250 mRNAs	mRNA stability	(Lai et al. 2006)
	MIP-2	?	(Jalonen et al. 2006a)
	p21	mRNA stability	Patino et al. 2006)
	E47	mRNA stability	(Frasca et al. 2007)
	VEGF	mRNA stability	(Essafi-Benkhadir et al. 2007)
	IL-10	mRNA stability	(Stoecklin et al. 2008)
	Genome analysis 137 mRNAs	mRNA stability	Stoecklin et al. 2008)
	polo-like kinase 3	mRNA stability	(Horner et al. 2009)
ZFP36L1	TNF	mRNA stability	(Lai et al. 2000)
	GMCSF	mRNA stability	(Lai and Blackshear 2001)
	IL-3	mRNA stability	(Stoecklin et al. 2002)[
	VEGF	mRNA stability	(Ciais et al. 2004)
	c-IAP2	mRNA stability	(Lee et al. 2005)
	VEGF	translation	(Bell et al. 2006)
	STAR	mRNA stability	Duan et al. 2009)
ZFP36L2	TNF	mRNA stability	Lai et al. 2000)
	GM-CSF	mRNA stability	Lai and Blackshear 2001)
	IL-3	mRNA stability	Lai and Blackshear 2001)

Some targets have been validated using knockout animals, these are in bold

A-2 Inferred target mRNAs for ZFP36 family members.

(Zekavati et al. 2014) provided a list of possible targets for each ZFP36 family member, the list is not reproduced here due to its length but it may be found in the paper's supplementary data. The authors highlight BCL2 as a possible target. The paper also highlights other anti-apoptotic possible targets for the ZFP36 proteins such as those shown below:

Table A-2 Suggested anti-apoptotic targets by (Zekavati et al. 2014), including BCL2

ZFP36L1

TARGETS

Gene_symbol	Description
BCL2	B-cell CLL/lymphoma 2
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2

ZFP36 TARGETS

Gene_symbol	Description
BCL2	B-cell CLL/lymphoma 2
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2

ZFP36L2 TARGETS

Gene_symbol	Description
BCL10	B-cell CLL/lymphoma 10
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2
BIRC3	baculoviral IAP repeat containing 3
NOTCH2	notch 2
HDAC1	histone deacetylase 1
OPA1	optic atrophy 1 (autosomal dominant)

A-3 ZFP36L1 targets involved in B cell late development inferred from ARACNe analysis

(Nasir et al. 2012) looked at possible targets for ZFP36L1 in B cell differentiation. In the following list from this paper CD38 is highlighted as a possible target.

Table A-3 Suggested ZFP36L1 targets involved in late B cell development

Gene	Description
SEL1L	sel-1 suppressor of lin-12-like (C. elegans)
PLOD1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1
CD38	CD38 molecule
ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9
LMF1	lipase maturation factor 1
GAS6	similar to growth arrest-specific 6; growth arrest-specific 6
TIMP2	TIMP metalloproteinase inhibitor 2
APOE	hypothetical LOC100129500; apolipoprotein E
GFI1	growth factor independent 1 transcription repressor
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor
PPT2	palmitoyl-protein thioesterase 2
HMGA1	hypothetical LOC100130009; high mobility group AT-hook 1
DTX3	deltex homolog 3 (Drosophila)
BLIMP1	PR domain containing 1, with ZNF domain
AMPD1	adenosine monophosphate deaminase 1 (isoform M)
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1
DPEP1	dipeptidase 1 (renal)
ZCCHC24	zinc finger, CCHC domain containing 24
C12orf47	chromosome 12 open reading frame 47
MED27	similar to cofactor required for Sp1 transcriptional activation, subunit 8, 34kDa; mediator complex subunit 27; CRSP8 pseudogene
MVD	mevalonate (diphospho) decarboxylase
LMNA	lamin A/C
NCOR2	nuclear receptor co-repressor 2

Appendix B B cell development

As referred to in section 1.2.1 a range of transcription factors control early B cell development, a figure with indications to key transcription factors in B cell development can be found here

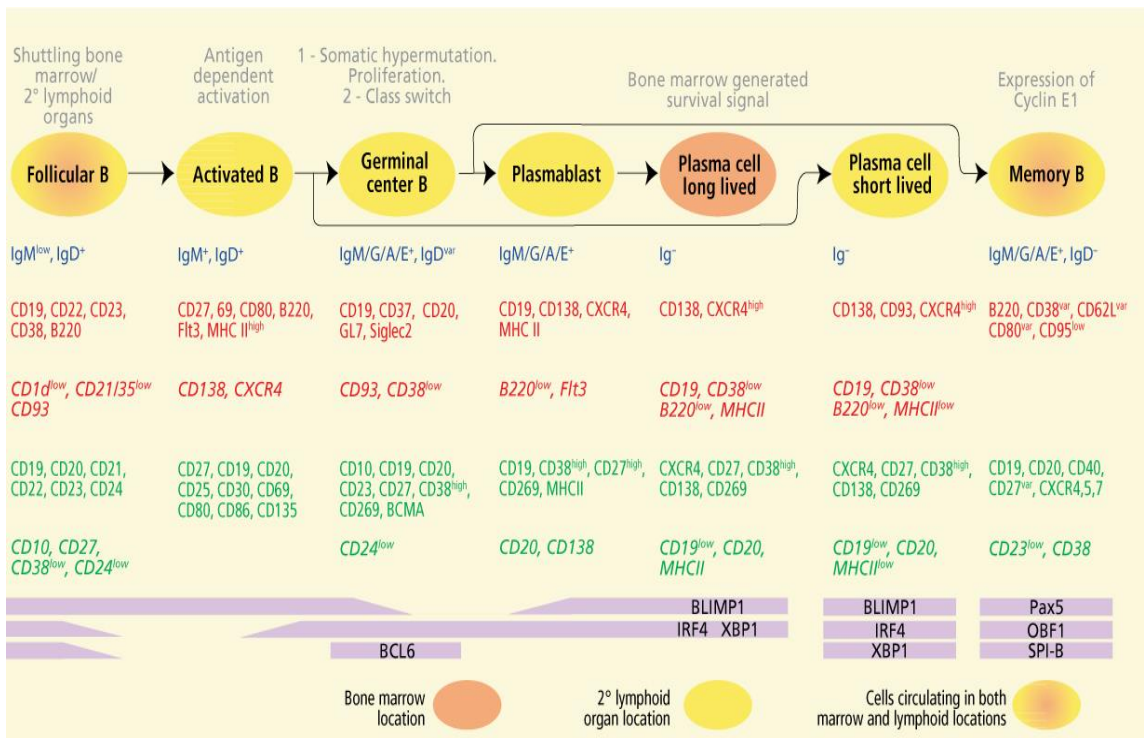
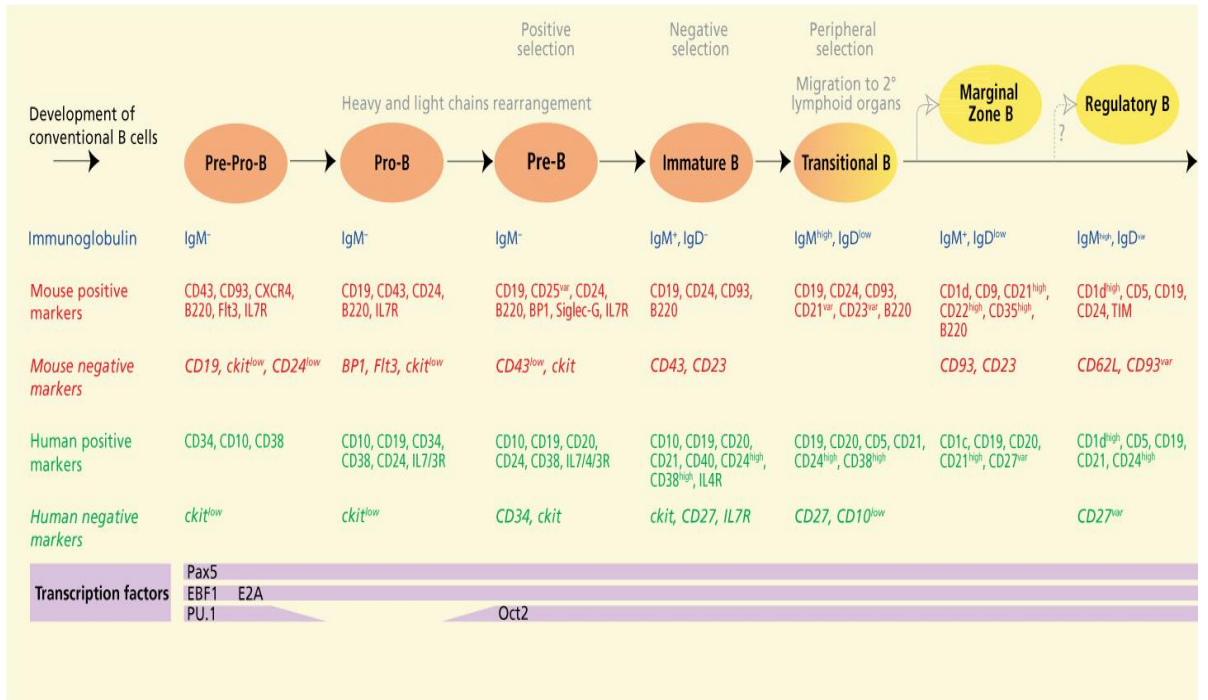


Figure B-1 B cell development stages with Ig, transcription factors and cell markers expression.

Figure adapted from www.bdbiosciences.com

Appendix C Plasmids Information

This appendix section shows the maps of the plasmids used in the experiments of this research

C-1 pGemT Easy vector map

The amplified ARE (BCL2 and CD38) from stock plasmids were initially cloned into the pGem T Easy vector, from Promega. The pGemT Easy constructs were then analysed for positive clones and the AREs were further processed for cloning into pmirGLO as described in Materials and Methods.

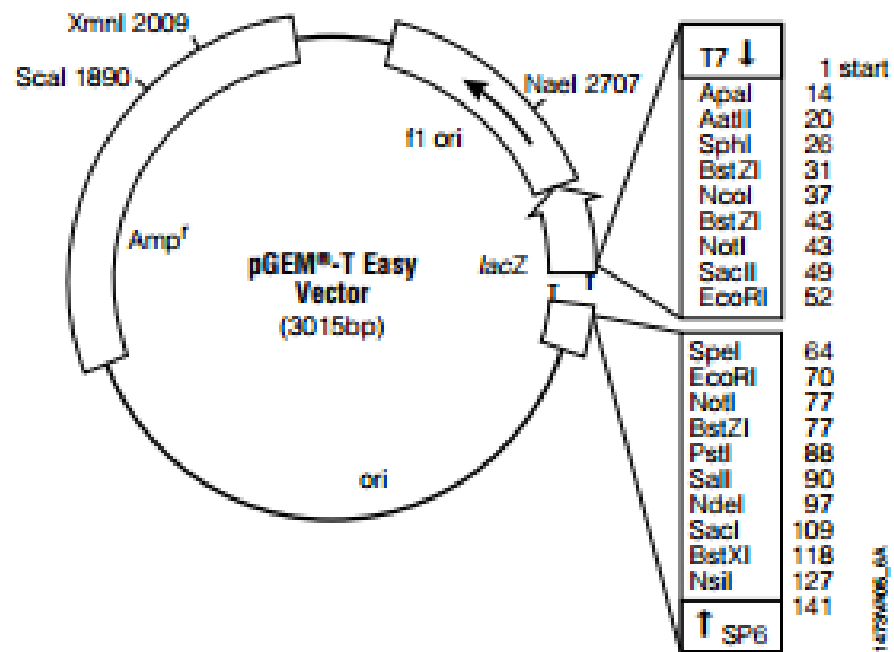


Figure C-1 pGem T Easy vector map

From Promega pGemT Easy vector Technical manual

C-2 pmirGLO Vector

The pmirGLO Vector is designed with a Firefly luciferase as the primary reporter gene; the vector was originally designed to study miRNAs function. Reduced firefly luciferase expression indicates the binding of endogenous or introduced miRNAs to the cloned miRNA target sequence. Firefly luciferase (*luc2*) is the primary reporter and renilla luciferase (hRluc-neo) acts as a control reporter for normalization and selection (Image from Promega, 2013 a). Instead of miRNA, the 3'UTR of putative targets can be cloned into the reporting vector as shown in chapter 3 and 4 of this thesis for the *BCL2* 3'UTR ARE and *CD38* 3'UTR ARE.

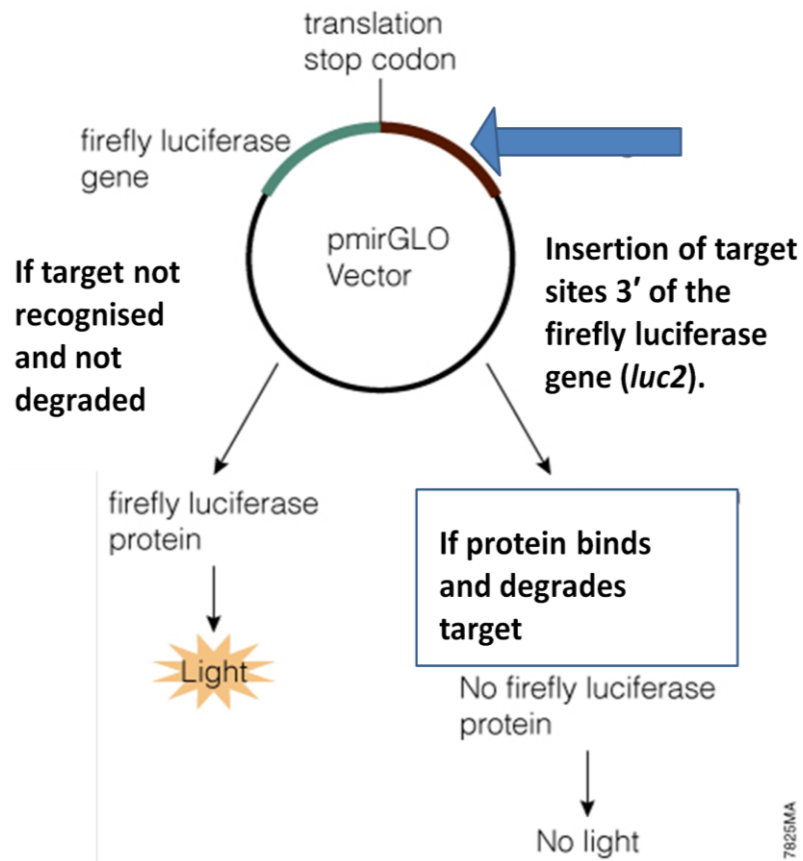


Figure C-2 Mechanism of action of pmirGLO reporter vector.

Putative ZFP36L1 targets have been cloned onto pmirGLO /Dual reporter vector to analyse recognition/binding of target (Image modified/adapted from Promega website, 2013).

The map of the vector is shown below:

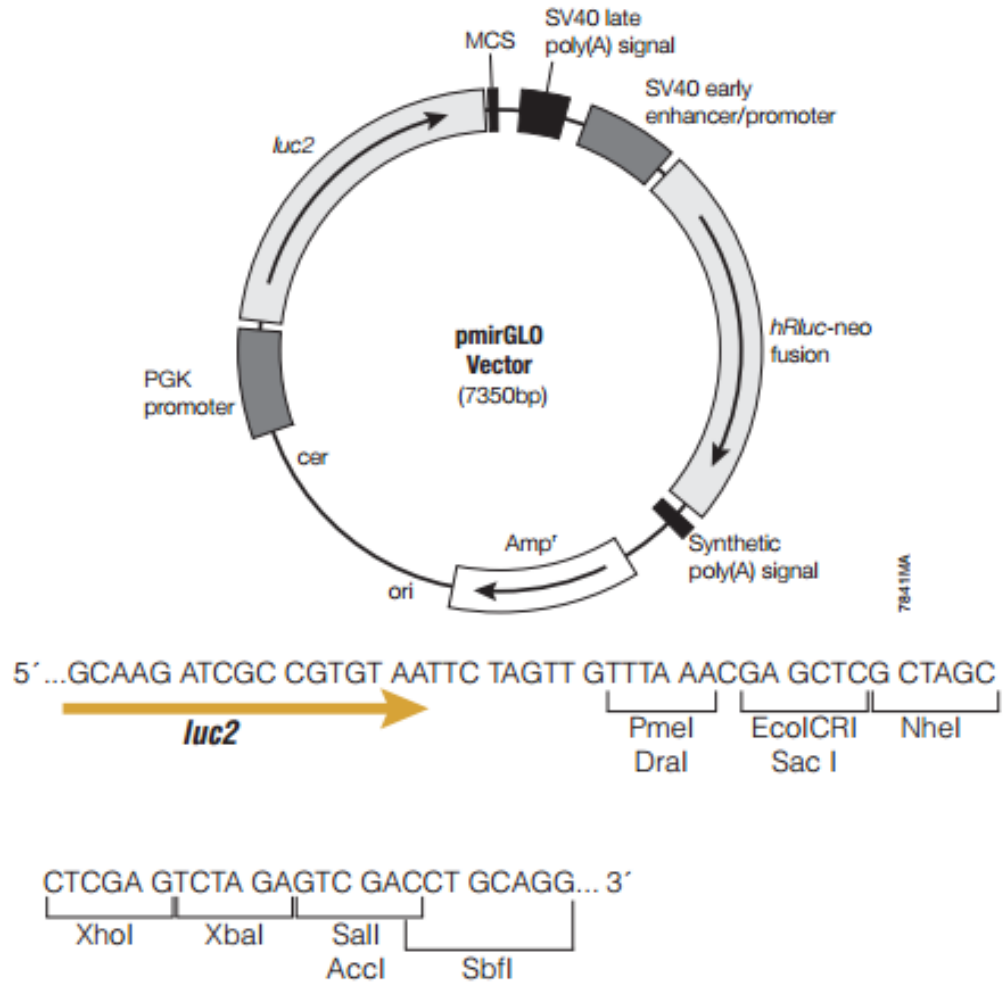


Figure C-3 Map of pmirGLO vector and key features

pmirGLO has a reporter gene and a control gene (luciferase and renilla respectively). MCS=Multiple cloning site. Image from Promega pmirGLO manual

C-3 pcDNA6ZFP36L1 and pcDNA6mutZFP36L1

The pcDNA6/His.ZFP36L1 and the ZFP36L1 mutant expression construct was kindly provided by Dr. Christoph Moroni, University of Basel, Switzerland and constructed by inserting DNA sequences corresponding to the human ZFP36L1 mRNA ORF region into *BamHI*/*EcoRV* sites of the plasmid pcDNA6/His.A (Invitrogen, Cat. No. V22220). The pcDNA6/His.ZFP3636L1 mutant which was constructed by site directed mutagenesis (changing the first cysteine residue of either zinc finger domain to an arginine). Details on these constructs, pcDNA6/His.zfp36l1 and pcDNA6/His.zfp36l1 referred as bsdHisBRF1WT and bsdHisBRF1C120R, can be found in(Stoecklin et al. 2002). The constructs were used in the dual luciferase reporter assays as seen in chapters 3 and 4.

The sequences for pcDNA6/His™ A, B, and C are available in www.invitrogen.com.

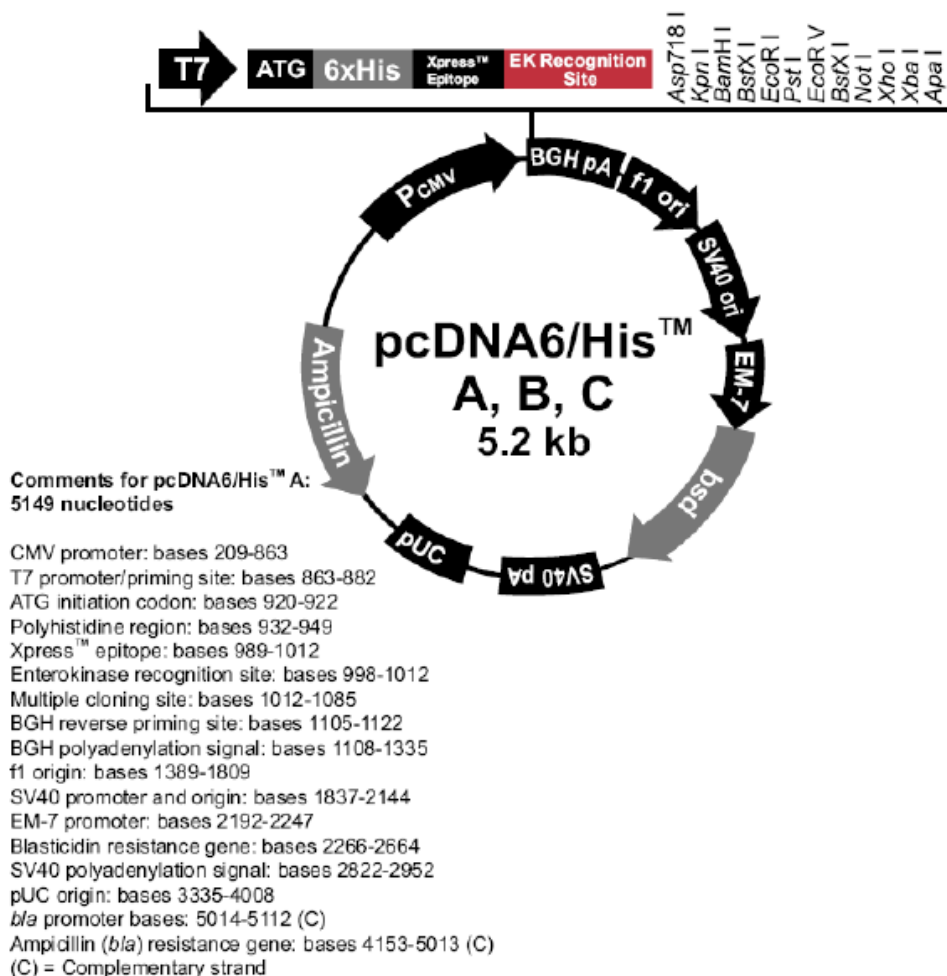


Figure C-4 Map of pcDNA6/His from Invitrogen.com

Table C-1 Features of pcDNA6/His

pcDNA6/His A (5150 bp), pcDNA6/His™ B (5151 bp), and pcDNA6/His™ C (5149 bp) contain the following elements.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987)
T7 promoter/priming site	Allows for in vitro transcription in the sense orientation and sequencing through the insert
N-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™
Xpress™ epitope tag	Allow detection of your recombinant protein with the Anti-Xpress™ Antibody (Catalog no. R910-25)
Enterokinase cleavage site	Allows removal of the N-terminal polyhistidine tag from your recombinant protein using an enterokinase such as EnterokinaseMax™ (Catalog no. E180-01)
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in frame with the Xpress™ epitope and N-terminal polyhistidine tag
BGH reverse priming site	Permits sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
fl origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM-7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the blasticidin resistance gene in E. coli
Blasticidin resistance gene (bsd)	Selection of transformants in E. coli and stable transfectants in mammalian cells (Kimura et al., 1994)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in E. coli
Ampicillin resistance gene (β-lactamase)	Selection of transformants in E. coli

Data extracted from pcDNA6His manual, pcDNA6/His™ A, B, and C, Catalog no. V222-20, Version C, 051302, 25-0237

C-4 pDEST26.ZFP36L2

pDEST26ZFP36L2 was purchased from Source Bioscience, the construct was used in the dual luciferase reporter assays as seen in chapter 3 and 4. The plasmid map for pDEST26 is shown below:

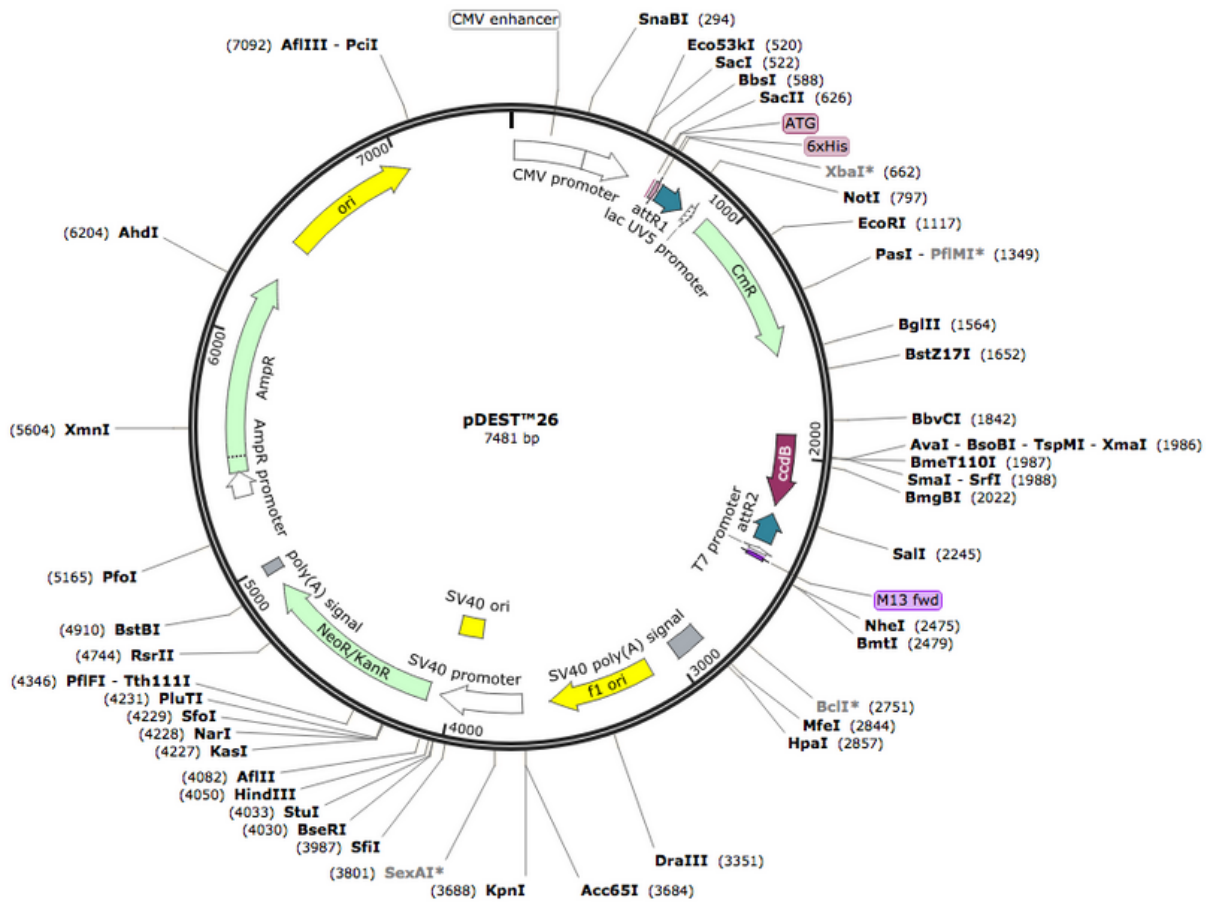


Figure C-5 pDEST26 vector map.

Image and Information from SourceBioscience

C-5 pcSPORT6 ZFP36

pcSPORT6ZFP36 construct was donated by Dr Manfred Frick and used in the dual luciferase reporter assays as seen in chapters 3 and 4. The pcSPORT6 plasmid map is shown below.

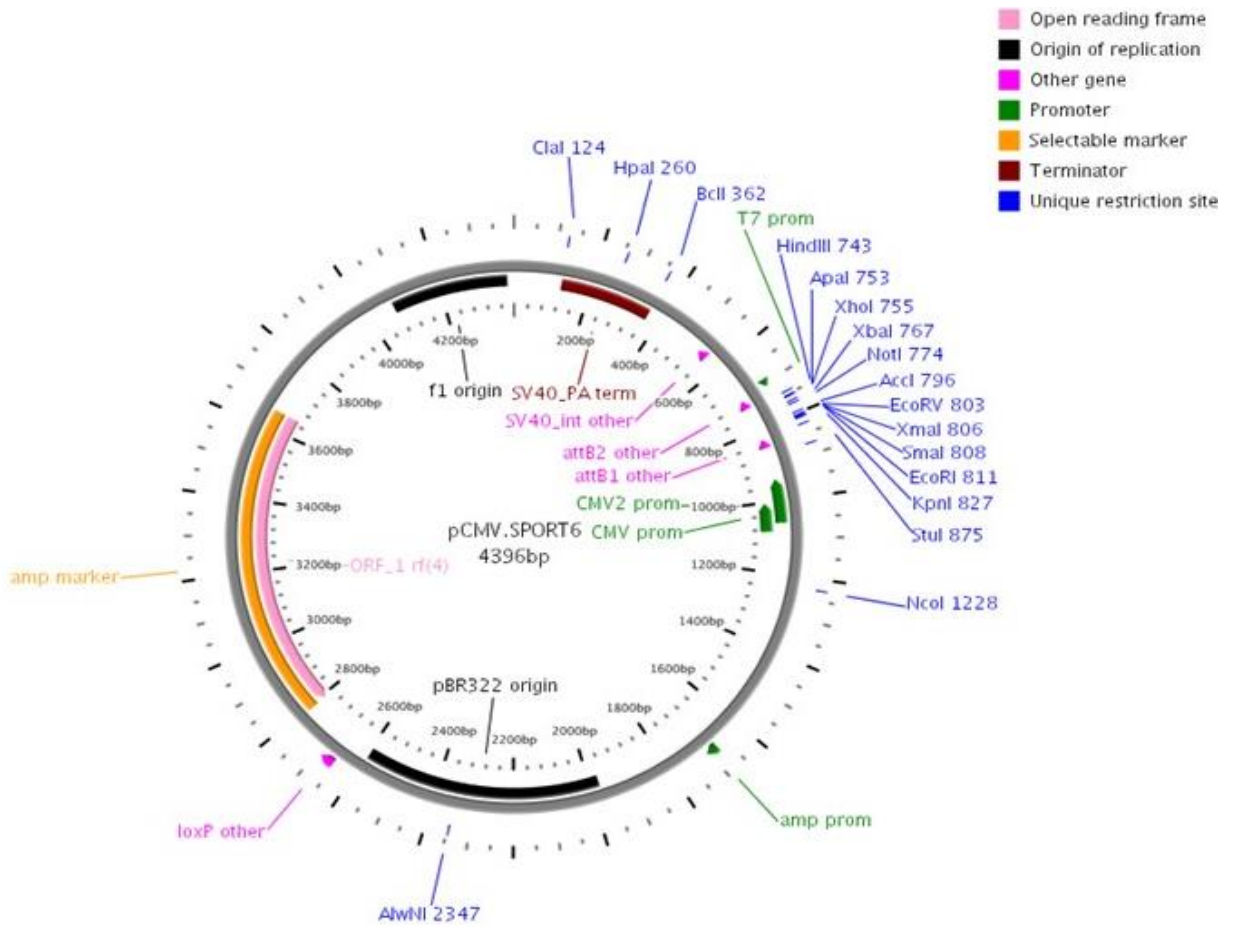


Figure C-6 pcSPORT6 vector map
Image from Source Bioscience

Appendix D DNA Sequences

DNA Sequencing results to confirm the correct DNA sequence of clones used in the dual luciferase reporter assays, chapters 3 and 4. The sequences have mutations highlighted in red.

D-1 pcDNA6 ZFP36L1- wild type sequence:

```
NNNNNNNNN NNNNNNNNN NGGGGNNNC NNCNCCATCN TCATCATGGT ATGGCTAGCA      60
TGACTGGNGN NCAGCAAATG GGTCCGGGATC TGNACGACGA TGACGATAAG GTANNNNNNA      120
TCCCCACCAC CCTCGTGTCT GCCACCNTCT TCTACTTGAG NNAANTTTTA TGCAAGGGTA      180
ACAAGATGCT CAACTATAGT GCTCCANNNG NANGGGGTTG CCTGCTGGAC AGAAAGGCAG      240
TGNNCACCCC TGCNNNNNNN GGCTTCCCTC GGAGGCACTC ANTCACCTG CCCANCTCCA      300
ANTTCCACCA NAACCAGCTC CTNNCAGCC TNNNGGGTGA GCCAGCCCC GCTCTGAGCT      360
CGNGAGACAG CCGCTTCCGA GACCGCTCCT TCTCGGAAGG GGGCGAGCGG CTGCTGCCCA      420
CCCAGAAGCA NCNNGGGGGC GGCCAGNTCA ACTCCAGCCN CNACAANACG GAGCTGTGCC      480
NCCCCTTTGA NGAAAACGGT GCCTGTAAGT ACGGGGACAA GTGCCNNNNN NCACACGGCA      540
TCCACGAGCT CCGCAGCCTG ACCCGCCACC CCAANTNNA GACGGAGCTG TGCCGCACCT      600
TCNACACCNN NNGCTTTTGC CCCTACGGGC CCCGCTGCNN NTNNNCCAN AACGNTGAAN      660
ANCGCCNTGC CCTGNCCGGG GCCCGGNACC TCTCCGCTGA CCGTNNNNN CTCNNNATA      720
NCTTTANCTT TGTTGGGTTT NN                                          742
```

D-2 pcDNA6 ZFP36L1 – mutant, showing base pair change in zinc finger region causing a mutation from Cysteine to Arginine:

```
NNNNNNNNN NNNNNNNNN NGGGGNTNN NTCNCCNTNN TNATCATGGT ATGGCTAGCA      60
TGACTGGTGG ACAGCAAATG GGTCCGGGATC TGTACGACGA TGACGATAAG GTACCTANGA      120
TCCCCACCAC CCTCGTGTCT GCCACCATCT TCGACTTGAG CGAANTTTTA TGCAAGGGTA      180
ACAAGATGCT CAACTATAGT GCTCCAGTGC CAGGGGGTTG CCTGCTGGAC AGAAAGGCAG      240
TGGGCACCCC TGCTNNNGNN GGCTTCCCTC GGAGGCACTC ANTCNCCCTG CCCAGCTCCN      300
AGTTCCACCA GAACCAGCTC CTCAGCAGCC TCAAGGGTGA GCCAGCCCC GCTCTGAGCT      360
CGCGAGACAG CCGCTTCCNA NACCGCTCCT TCTCGGAAGG GGGCGAGCGG CTGCTGCCCA      420
CCCANAAGCA GCCCGGGGGC GGCCAGGTCA ACTCCAGCCG CTACAAGACG GAGCTGTGCC      480
NCCCCTTTNA NGAAAACGGT GCCTGTAAGT ACGGGGACAA GTGCCNNTTC NCACACGGCA      540
TCCACGAGCT CCGCAGCCTG ACCCGCCACC CCAANTACAA GACGGANCTG CGCCGCACCT      600
TCNANNNNAT CGGNTTTTGC CCCTACGGGC CCCGCTGCNN NTTTCATCCAC AANNNTNAAN      660
AGCGCCGTGC CCTGGCCGGG GCCCGGGNCC TCTCCGCTGA CCGTCCCCGC CTCNNCATA      720
NCTTNANCTT TGCTGGNNTT TCCCAGTGCC GCTGCNNCCG CCGTGCNAN CNNGGCTGCN      780
GGACAGCCCC ACNCTNATCA CCCACCCNC NATTCTGAGC GCNATGANNT CNNGNGCTCA      840
```

Figure D-1 ZFP36L1 plasmids sequences show a single nucleotide change in a the zinc finer mutant

pcDNA6ZFP36L1, a gift from DR Christoph Moroni and was produced by introducing the ORF region of the human ZFP36L1 mRNA within BamH1-EcoRV in a pcDNA6/His.A from Invitrogen, Cat. N. V22220. The mutated version of the construct was also gifted, the mutation, a cysteine to arginine change in the zinc finger region of ZFP36L1 was constructed by site directed mutagenesis.

D-3 pmirGLO BCL2- wild type sequence:

```
NNNNNNNNN NNNNTNNNA NGCNGTAGNA ATAATATGCA TTGTCNGTGA TGTACCATGA      60
AACAAAGCTG CAGGCTGTTT AAGAAAAAT AACACACATA TAAACATCAC ACACACAGAC      120
AGACACACAC ACACACAACA ATTAACAGTC TTCAGGCAAA ACGTCGAA TC AGCTATTTAC      180
TGCCAAAGGG AAATATCATT TATTTTTTAC ATTATTAAGA AAAAAAGATT TATTTATTTA      240
AGACAGTCCC ATCAAAACTC CTGTCTTTGG AAATCGACC ACTAATTGCC AAGCACCCT      300
TCGTGTGGCT CCACCTGGAT GTTCTGTGCC TGTAAACATA GATTCGCTTT CCATGTTGTT      360
GGCCGGATCA CCTCTAGAGT CGACCTGCAG GCATGCAAGC TGATCCGGCT GCTAACAAAG      420
CCCGAAAGGA AGCTGAGTTG GCTGCTGCCA CCGCTGAGCA ATAACAGCA TAACCCCTTG      480
GGGCGGCCGC TTCGAGCAGA CATGATAAGA TACATTGATG AGTTTGACA AACACAAC      540
AGAATGCAGT GAAAAAATG CTTTATTGTG GAAATTTGTG ATGCTATTGC TTTATTGT      600
ACCATTATAA GCTGCAATAA ACAAGTTAAC AACACAATT GCATTCATTT      650
```

D-4 pmirGLO BCL2- mutant sequence.

The area where the sequence highlighted above is missing has been left blank in the image below

```
NNNNNNNNN NNNNTNNNA NGCNGTAGNA ATAATATGCA TTGTCNGTGA TGTACCATGA      60
AACAAAGCTG CAGGCTGTTT AAGAAAAAT AACACACATA TAAACATCAC ACACACAGAC      120
AGACACACAC ACACACAACA ATTAACAGTC TTCAGGCAAA ACGTCGAA      180
TGCCAAAGGG AAATATCATT TATTTTTTAC ATTATTAAGA AAAAAAGATT TATTTATTTA      240
                                     CGACC ACTAATTGCC AAGCACCCT      300
TCGTGTGGCT CCACCTGGAT GTTCTGTGCC TGTAAACATA GATTCGCTTT CCATGTTGTT      360
GGCCGGATCA CCTCTAGAGT CGACCTGCAG GCATGCAAGC TGATCCGGCT GCTAACAAAG      420
CCCGAAAGGA AGCTGAGTTG GCTGCTGCCA CCGCTGAGCA ATAACAGCA TAACCCCTTG      480
GGGCGGCCGC TTCGAGCAGA CATGATAAGA TACATTGATG AGTTTGACA AACACAAC      540
AGAATGCAGT GAAAAAATG CTTTATTGTG GAAATTTGTG ATGCTATTGC TTTATTGT      600
```

Figure D-2 DNA sequencing analysis of plasmids.

Sequencing results are shown in this figure. *BCL2* ARE wild type and mutant *BCL2* ARE differ in a section in the centre of their sequence, this section is not present in the mutant *BCL2* ARE (Lapucci et al. 2002). PcDNA6ZFP36L1 and its mutant version differ in their zinc finger region, the mutant ZFP36L1 has a change in its sequence, the first Cysteine (TGC) in the zinc finger changes to an Arginine (CGC) (Stoecklin et al. 2002).

D-5 pmirGLOCD38 sequence:

```
NNNNNNNNG AGCTCANAAT TCAAGNATC GCCCTGAGGA TTCATCTTGC ACATCTGAGA      60
TCTGAGCCAG TCGCTGTGGT TGTTTAGCT CCTTGACTCC TTGGGTTTA TGTCATCATA      120
CATGACTCAG CATACTGCT GGTGCAGAGC TGAAGATTTT GGAGGGTCTT CCACAATAAG      180
GTCAATGCCA GAGACGGAAG CCTTTTCCC CAAAGTCTTA AAATAACTTA TATCATCAGC      240
ATACCTTTAT TGTGATCTAT CAATAGTCAA GAAAAATTAT TGTATAAGAT TAGAATGAAA      300
ATTGTATGTT AAGTTACTTC ACTTTAATTC TCATGTGATC CTTTATGTT ATTTAATAT      360
TGTAACATC CTTTCTATTG AAAAATCACC ACACCAAACC TCTTTATTA GAACAGGCAA      420
GTGAAGAAAA GTGAATGCTC AAGTTTTTCA GAAAGCATT AATTCCAAA TGAATGACCT      480
TGTTGCATGA TGTATTTTGG TACCCTTCTT ACAGATAGTC AAACCACGCG TAAGCGGCNN      540
CNGCATCTAG ATTCGAANNN AANNNNNN      568
```

Figure D-3 CD38 3'UTR sequence cloned into pmirGLO.

The figure shows the sequence of 3'UTR of *CD38* mRNA cloned into the pmirGLO vector; ARE motifs are highlighted in red and shaded. The sequence shown matches the reference sequence of NM_001775 for *CD38* 3' UTR SC206546 3'UTR clone of NM_001775. The ARE binding sequence is highlighted in grey, the pentamer AUUUA with surrounding Us.

Appendix E FL grading systems

This appendix section provides details on the different grading system used for FL, as mentioned in section 5.1

E-1 World Health Organisation Classification of Tumours of Haematopoietic and Lymphoid Tissues

The World Health Organization (WHO) classification of lymphoid neoplasms updated in 2008 represents a worldwide consensus on the diagnosis of these tumours and is based on the recognition of distinct diseases, using a multidisciplinary approach (Swerdlow et al. 2008).

The WHO classification of FL (non-Hodgkin) recommends that grading be carried out according to the counting method proposed by Mann and Berard to define 3 grades based on the number of centroblasts (large or small) per 40x high-power field. Patients are categorised in groups and start of treatment and type of therapy is influenced by this categorisation. A classification of FL grade 3 suggests that the patient is at high-risk for disease progression. FL grade 3 is divided into grades 3A and 3B based on the presence of centrocytes, it signifies a high risk for disease progression, treatment guidelines state that treatment guidelines for DLBCL should be followed (bioncology.com 2015)- World Health Organisation guidelines-.

Table E-1 WHO classification of histological grades grades

Grading	Definition
1-2 (low grade)	0-15 centroblasts per hpf
1	0-5 centroblasts per hpf
2	6-15 centroblasts per hpf
3	>15 centroblasts per hpf
3A	Centrocytes present
3B	Solid sheets of centroblasts
Reporting of pattern	Proportion follicular
Follicular	>75%
Follicular and diffuse	25%-75%*
Focally follicular	<25%*
Diffuse	0%†

Diffuse areas containing >15 centroblasts per hpf are reported as DLBCL with follicular lymphoma (grades 1 to 2, grade 3A, or grade 3B).*

hpf = high power field of 0.159 mm² (40× objective, 18 mm field of view ocular, count 10 hpf and divide by 10).

Table from

E-2 Ann Arbor staging system

It is primarily based on the distribution of lymphatic involvement and presence of extra lymphatic organ involvement. It does not reflect spread of disease and it does not account for extra nodal involvement

Table E-2 Ann Arbor staging system for FL

Stage	Definition
I	Lymphoma in 1 lymph node region or a single localized extranodal site, ie, thyroid
II	Lymphoma in 2 or more lymph node regions situated either above or below the diaphragm
III	Lymphoma in lymph node regions situated both above or below the diaphragm
IV	Lymphoma in 1 or more extralymphatic organs with or without associated lymph node involvement (diffuse or disseminated)

Table and information from bioncology.com (2015)

Key papers discussion the 2008 HWO publications: (Jaffe 2009, Campo et al. 2011)

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