
ANALYSIS OF BCL6 IN A GENETICALLY TRACTABLE HUMAN B CELL LINE

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By

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Declaration of originality

I Jennifer Zobel declare that the work presented in this thesis is my own and that it has not been submitted elsewhere for any award. Where appropriate, I have referenced all sources and clearly indicated the work of others.

Abstract

B cell lymphoma 6 (Bcl6) is a proto-oncogene functioning as a transcriptional repressor at the germinal centre (GC) stage of B cell development, regulating proliferation, differentiation and survival. Bcl6 is commonly dysregulated in GC-derived lymphomas and hence a promising therapeutic target. Although a number of agents targeting Bcl6 are being developed, there is currently no good model available for functional analysis and hence drug target validation. To address this issue, I have established a conditional *BCL6* knockout cell line for in-depth functional analyses of Bcl6. I have used homologous recombination to disrupt one *BCL6* allele in the human Burkitt's lymphoma cell line DG75 with a *zeocin* resistance cassette and have shown this line to be capable of supporting tetracycline (tet)-regulated Bcl6 expression from an integrated expression construct. The *zeocin* cassette used to disrupt the first *BCL6* allele was subsequently deleted by Cre recombination. This allowed me to reuse the same targeting construct to disrupt the second *BCL6* allele. I subsequently set out to characterise the resulting conditional knockout cell line and found that depletion of Bcl6 leads to less proliferation, characterised by a prolonged G₀/G₁ cell cycle phase. I went on to further characterise the effect of Bcl6 depletion in my system by cDNA microarray analysis, which revealed interesting insights into functions of Bcl6 such as the modulation of B cell receptor signalling and calcium signalling. The system I have developed is the first precisely regulated genetic model for studying Bcl6 function and will be of great benefit for research aiming to develop Bcl6 targeted drug therapy.

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Abbreviations

A	adenine
aa	amino acid
AID	activation-induced deaminase
amp	ampicillin
AP-1	activator protein 1
APC	antigen presenting cell
APE 1	apurinic/aprimidinic endonuclease 1
APML	acute promyelocytic leukaemia
ATR	ataxia telangiectasia and Rad3 related
ATRA	all-trans retinoic acid
B2M	β -2 microglobulin
BAZF	Bcl6-associated zinc finger protein
Bcl6	B cell lymphoma 6
Bcl6S	small Bcl6 isoform
BCoR	Bcl6-interacting co-repressor
BCR	B cell receptor
BER	base excision repair
BL	Burkitt's lymphoma
Blimp-1	B lymphocyte-induced maturation protein-1
BLNK	B cell linker
bp	basepair
BSA	bovine serum albumin
BTB	broad complex, tramtrack and bric-à-brac
C	cytosine
C segment	constant segment
CAMK	calmodulin/calcium-dependent kinase
CBP	CREB-binding protein
CD40L	CD40 ligand
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLP	common lymphoid progenitor
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
c-Myc	cellular myelocytomatosis
Cre	causes recombination
CSR	class switch recombination
CtBP1	C-terminal binding protein 1
C-terminus	carboxy-terminus
D segment	diversity segment
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dox	doxycycline
DUB	deubiquitinase
E. coli	Escherichia coli
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetraacetic acid
EJC	exon junction complex
ER	endoplasmatic reticulum
ETO	eight-twenty-one
Evi9	ecotropic viral integration site 9

FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
Fc	fragment, crystallisable
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
FL	follicular lymphoma
G	guanine
G418	geneticin
GC	germinal centre
gDNA	genomic DNA
GFP	green fluorescent protein
GOI	gene of interest
GPCR	G-protein-coupled receptor
HA	hemagglutinin
HAT	histone acetyl transferase
HDAC	histone deacetylase
HIV	human immunodeficiency virus
hr	hour
HRP	horseradish peroxidase
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IP ₃	inositol 1,4,5-trisphosphate
IRF4	interferon-regulatory factor 4
J segment	joining segment
K	lysine
kb	kilobase-pair
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani
LHA	left homology assay (PCR screen)
LHAF	left homology assay-F (primer)
LHAR	left homology assay-R (primer)
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MC promoter	polyoma enhancer/herpes simplex virus thymidine kinase promoter
min	minute
Miz-1	Myc-interacting zinc finger protein-1
MMR	mismatch repair
mRNA	messenger ribonucleic acid
mSIN3A	mammalian orthologue of the yeast SIN3 co-repressor
MTA3	metastasis-associated protein 3
MTC	major translocation cluster
NCoR	nuclear hormone receptor co-repressor
NEB	New England Biolabs
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end-joining
NLS	nuclear localisation signal
NMD	nonsense-mediated decay
nt	nucleotide
N-terminal	amino-terminal
OD	optical density
ORF	open reading frame
Pax5	paired box protein 5
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEST	sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T)

PI	propidium iodide
PIP ₂	phosphatidyl 4,5-bisphosphate
PLC	phospholipase C
PLZF	promyelocytic leukaemia zinc finger protein
PNK	polynucleotide kinase
POZ	poxviruses and zinc finger
PRDM1	PR domain containing
PTC	premature stop codon
RACE	rapid amplification of cDNA ends
RAG	recombination activating gene
Rb	retinoblastoma
RHA	right homology assay (PCR screen)
RHAF	right homology assay-F (primer)
RHAR	right homology assay-R (primer)
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNAi	RNA interference
S region	switch region
SAPE	streptavidin-phycoerythrin
SDS	sodium dodecyl sulfate
sec	second
SHM	somatic hypermutation
siRNA	small interfering RNA
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
sq RT-PCR	semiquantitative reverse transcription polymerase chain reaction
SSC	sodium chloride sodium citrate
STAT	signal transducer and activator of transcription
T	thymine
TAE	tris-acetate ethylene diamine tetraacetic acid
TBS	tris-buffered saline
TBS/T	tris-buffered saline/Tween20
T _d	doubling time
TdT	terminal deoxynucleotidyl transferase
TE	tris-ethylene diamine tetraacetic acid
tet	tetracycline
TLS	tumour lysis syndrome
TRE	tetracycline responsive element
TRP	tetracycline responsive promoter
tTA	tetracycline transactivator
U	uracil
UDG	uracil-DNA glycosylase
V	volt
V segment	variable segment
v/v	volume per volume
w/v	weight per volume
Xbp-1	X-box binding protein-1
Y	tyrosine
zeo	<i>zeocin</i> (Sh bleomycin-resistance)
ZF	zinc finger

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CHAPTER 1 – Introduction

The focus of this thesis is the functional analysis of the B cell lymphoma 6 (Bcl6) protein. Bcl6 is a master transcription factor and potent oncogene involved in normal immune responses and germinal centre-derived B cell lymphomas (Jardin et al., 2007). I will therefore commence with an overview of the role of B cells in the adaptive immune response and particularly the germinal centre (GC) reaction. I will then describe what is known about the role of Bcl6 in lymphomas and outline the regulation of Bcl6, its interactions with co-repressors via its various domains and the potential of Bcl6 as therapeutic target. Following this literature review, I will discuss the limitations of models currently available to study the function of Bcl6 and propose a novel cell line-based *BCL6* knockout model that will greatly facilitate functional analysis of the Bcl6 protein.

1.1 Human immune response and B cell development

The human immune system is a multi-layered defence mechanism, aiming to prevent and fight infection by microorganisms. The first layer of protection prevents the infectious agent from entering the human body. The innate immune system is the first barrier to infectious agents. Macrophages and neutrophils recognise a variety of microbial constituents and mount an immediate innate immune response (Janeway and Medzhitov, 2002). They can engulf and degrade invading pathogens and trigger local inflammation leading to the recruitment of additional cells and molecules of the adaptive immune response that help clear the infection. Local inflammation eventually also recruits cells of the adaptive immune system, which then trigger a more specific response and establish immunologic memory (Carroll, 2004, Fearon and Carter, 1995, Fearon and Locksley, 1996). B and T lymphocytes mediate the adaptive immune response. B cells are responsible for antibody production to defend against extracellular pathogens. T cells in turn, have roles in fighting

intracellular pathogens and regulation of the B cell response. As the current thesis focuses on Bcl6, a master transcriptional regulator in B cell development, it is essential to draw special attention to the development of B lymphocytes.

1.1.1 B cell development in the bone marrow

The earliest B cell (pro-B cell) develops from the common lymphoid progenitor (CLP) and this differentiation step is dependent on the specialised microenvironment of the bone marrow (Nagasawa, 2006). Signals supplied by specialised stromal cells in the bone marrow trigger somatic rearrangement of the immunoglobulin receptor genes. The human heavy chain gene on chromosome 14 contains three groups of gene segments that require genetic recombination in order to produce a functional pre-B cell receptor (Matsuda and Honjo, 1996). Figure 1.1 shows a schematic representation of the steps involved in V(D)J recombination i.e. the generation of a functional pre-B cell, and mature B cell receptor (BCR) gene. On a molecular basis, V(D)J recombination requires the recombination activating genes 1 and 2 (RAG1 and RAG2) (Mombaerts et al., 1992, Shinkai et al., 1992). RAG1 and RAG2 form a complex with the recombining genomic region and introduce a nick in the DNA (deoxyribonucleic acid) strand. These nicks then get processed resulting in double strand breaks that are subsequently joined by non-homologous end-joining (NHEJ) (Puebla-Osorio and Zhu, 2008). Due to the fact that NHEJ often results in the loss of nucleotides (nt) at the break region, two out of three recombinations between these protein coding sequences will be out of frame and thus non-functional. If recombination does not achieve a functional open reading frame, recombination will be re-attempted using the second allele of the heavy chain locus. If recombination of the second allele repeatedly yields a non-functional reading frame, the cell will undergo apoptosis (Mostoslavsky et al., 2004). In case of a successful in-frame recombination of the first or second allele, transcription through the newly recombined VDJ segments and the downstream constant (C) gene segment produces a messenger ribonucleic acid (mRNA) coding for a functional heavy chain of the immunoglobulin (Jung et

al., 2006). Subsequently, the heavy chain of the receptor binds to a transiently expressed surrogate light chain, forming the pre-B cell receptor (Nishimoto et al., 1991). The mature BCR is created when the final light chain replaces the surrogate light chain after rearrangement of the light chain locus (Figure 1.1).

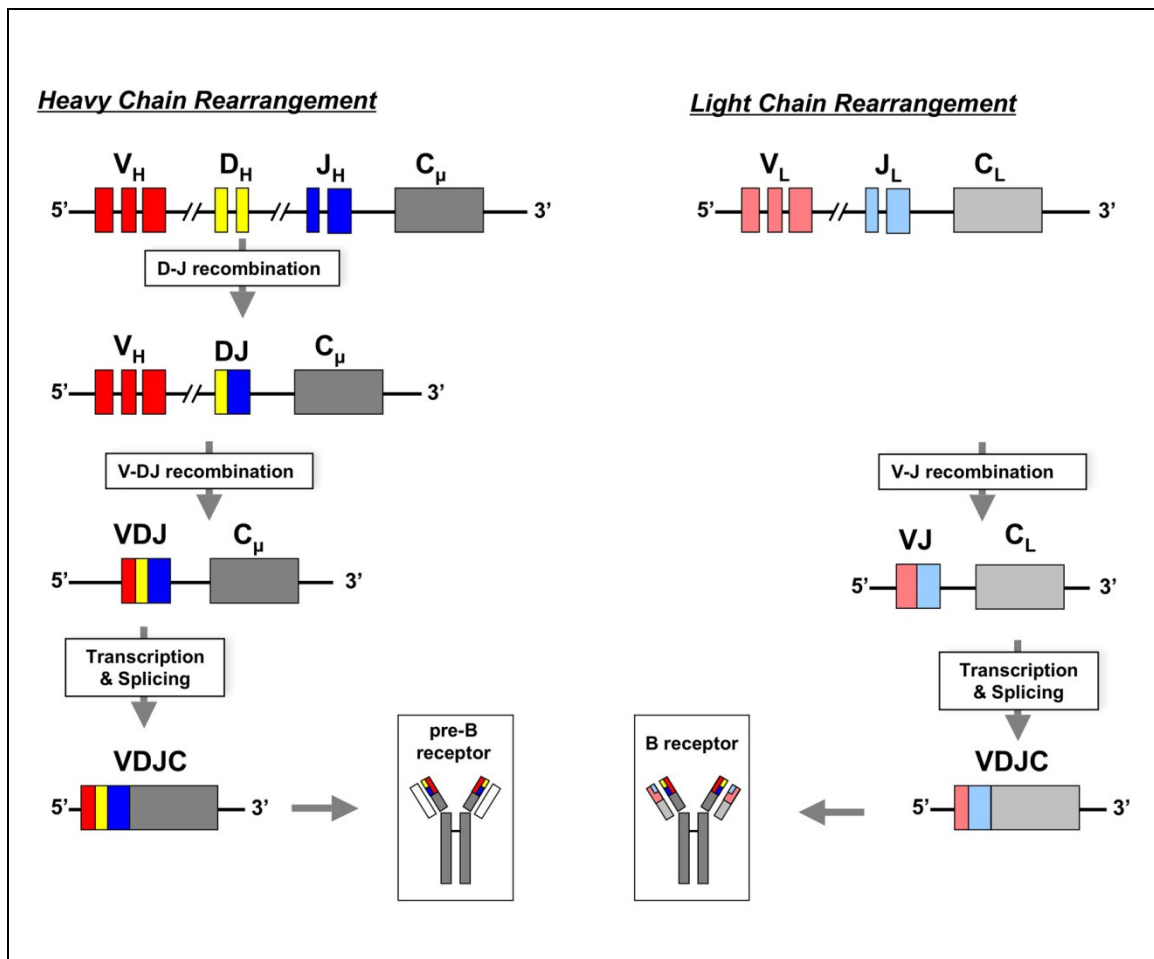


Figure 1.1: Gene rearrangement at the heavy and light chain loci

Left: Heavy chain rearrangement follows the somatic and sequential joining of D-J and V-DJ. In this process, a diversity (D) gene segment (yellow box) is initially recombined with a joining (J) segment (blue box), deleting all genomic sequences in between (D-J recombination). This DJ segment is then joined to a variable (V) gene segment (red box) to complete rearrangement of the heavy gene locus, again leading to the loss of all DNA between the two segments that are being joined (V-DJ recombination). Transcription through the recombined heavy chain locus and mRNA processing generates a transcript encoding a complete immunoglobulin heavy chain. The heavy chain then associates with a transient, surrogate light chain (white) that completes the pre-B receptor. Right: Rearrangement of the light chain locus does not include a diversity segment and hence V-J recombination (pale red and pale blue boxes), followed by transcription and mRNA processing results in the formation of a functional light chain that replaces the transient light chain of the pre-B cell receptor, thereby generating the final B cell receptor.

1.1.2 B cell activation in the periphery

The contact of a B cell with its antigen in the periphery is usually mediated through the interaction of the B cell with an antigen-presenting cell (APC) that has taken up and processed antigen at a site of infection. When a mature B cell recognises its cognate antigen displayed by an APC, the B cell upregulates its chemokine receptors and this will help it to find its way to T-cell rich areas within secondary lymphoid organs (Batista and Harwood, 2009). Once the activated B cell arrives in T cell zones, it becomes fully activated in response to the interaction with additional APCs and CD4+ T cells. These fully activated B cells then follow one of the two distinct routes to antibody production. They can either proliferate as plasmablasts (MacLennan et al., 2003) or alternatively enter the germinal centre reaction which will enable them to improve the affinity of their BCRs for their corresponding antigen and also enable them to produce antibodies with diverse effector functions.

1.2 Germinal centres and affinity maturation of immunoglobulin receptors

1.2.1 Germinal centres

GCs constitute a complex microenvironment that supports affinity maturation of rapidly proliferating B cells. They can form in secondary follicles of antigen-stimulated lymph nodes, B cell areas of the white pulp of the spleen and the mucosa-associated lymphoid tissue (MALT). Figure 1.2 displays a graphic overview of GC B cell development. The developmental stages and selection processes involved will be described in more detail in the following sections.

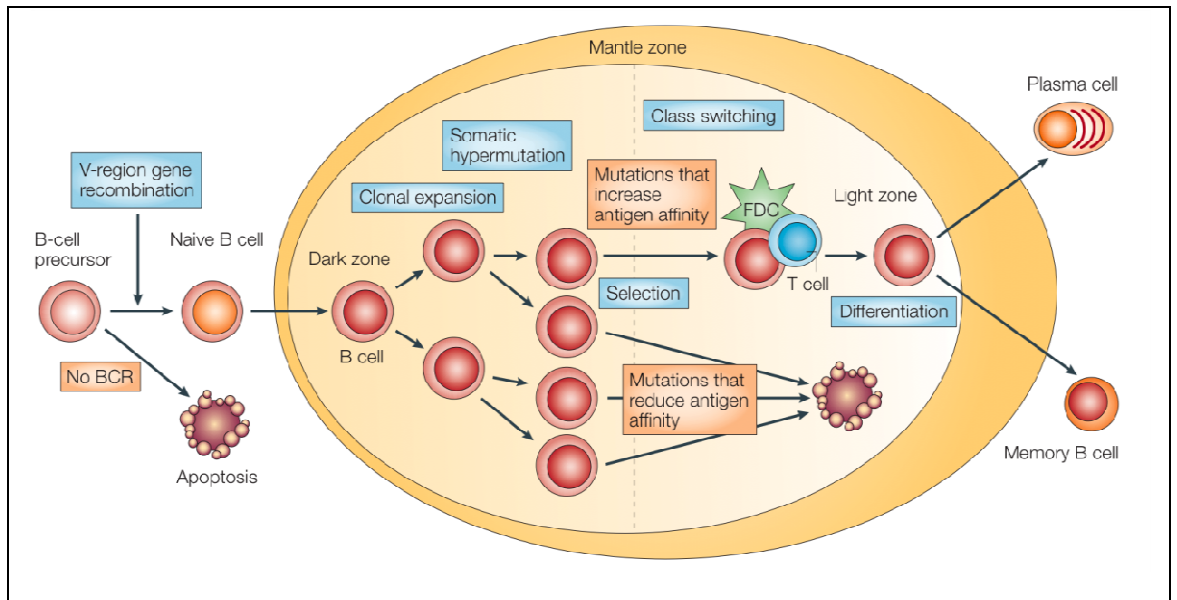


Figure 1.2: Schematic organisation of the germinal centre reaction (Kuppers, 2005)

Following rearrangement of the immunoglobulin genes in the bone marrow (V-region recombination), naïve B cells are activated in the periphery when they encounter an antigen that their BCR can recognise. These activated B cells migrate into B cell follicles in secondary lymphoid organs where they proliferate extensively, thereby establishing germinal centres. GCs can be divided into three separate areas: the dark zone, the light zone and the mantle zone (dark yellow). The dark zone (left half of pale yellow area) is the site of rapid proliferation of B cells (clonal expansion) and the site where immunoglobulin genes are subjected to somatic hypermutation (SHM). The binding affinity of the mutated BCR is tested in the light zone of the germinal centre (right half of pale yellow area). If a BCR has acquired disadvantageous mutations in the process of SHM, the B cell is destined to undergo apoptosis. Only if SHM improves the affinity of the BCR for antigen binding will the B cell survive in the GC light zone through interaction with follicular dendritic cells (FDCs) and T cells. Selected GC B cells can also remodel the effector function of their BCR by class switching and finally differentiate into memory B cells or plasma cells that leave the GC.

1.2.2 Initiating the GC reaction - Centroblasts and somatic hypermutation

The GC reaction is initiated when GC-precursor B cells migrate to primary follicles in secondary lymphoid organs. Within the primary follicles, the B cells interact with a network of follicular dendritic cells (FDCs) and differentiate into highly proliferative centroblasts. Centroblasts can complete a cell cycle within 6-8 hours, the shortest known for eukaryotic cells (MacLennan, 1994). The dramatic proliferative capacity of centroblasts is an effect of the strong upregulation of genes that promote cell proliferation and the downregulation of genes that impose a negative effect on cell cycle progression (Klein et al., 2003). Furthermore, centroblasts express telomerase, thereby securing limitless replicative potential (Hu et al., 1997). Thus, within only a few days, centroblasts generate a pool of rapidly proliferating, densely packed cells forming the dark zone of the GC.

The rapidly expanding centroblast pool is used as substrate for diversification of antigen receptor by somatic mutation of the immunoglobulin variable regions. This process is termed somatic hypermutation (SHM) and relies on the enzyme activation-induced deaminase (AID). AID can transform cytidine (C) into uracil (U) within both ribonucleic acid (RNA) and DNA (Petersen-Mahrt et al., 2002). The resulting U:G lesion in the sequence of the variable region of the immunoglobulin gene is processed by one of several pathways and the pathway chosen will determine the type of base substitution generated (Di Noia and Neuberger, 2007). If DNA replication across the U:G lesion occurs, this will result in C to T (thymine) and G (guanine) to A (adenine) transition substitutions. Alternatively, the U:G lesion can be processed by the base excision repair (BER) or mismatch repair (MMR) machinery, both generating a gap lesion. In both cases, the resulting lesion will be filled in by error-prone DNA polymerases, which will potentially create transition (A:T) as well as transversion (C:G) mutations (Di Noia and Neuberger, 2007). In order to tolerate the high degree of DNA damage generated by SHM, centroblasts have

a suppressed DNA damage response (Phan and Dalla-Favera, 2004). This high tolerance of centroblasts towards DNA damage is counterbalanced by their increased sensitivity to undergoing apoptosis. This sensitivity is the result of the expression of several pro-apoptotic genes such as p53, Fas, Bax and c-Myc (cellular myelocytomatosis) (Martinez-Valdez et al., 1996). Moreover, centroblasts lack expression of common anti-apoptotic factors such as Bcl-2 and its family members (Liu et al., 1991a). The pro-apoptotic nature of centroblasts imposes a strong selective pressure on the cell population to diversify and improve their antigen receptors. After diversification of the BCR, centroblasts move to the light zone of the GC.

1.2.3 Centrocytes

Centroblasts that have ceased to proliferate rapidly and have moved to the GC light zone are termed centrocytes. Centrocytes test their freshly diversified and mutated antigen receptor for improved antigen binding, likely through interaction with antigen presented on the membrane of dendritic cells (Meyer-Hermann et al., 2006). Failure to establish a strong interaction with antigen and helper T cells induces Fas-mediated cell death (Choe et al., 1996, Koopman et al., 1997). Because the majority of random somatic mutations in the BCR are deleterious to binding of antigen, most centrocytes will undergo apoptosis in the light zone of the GC. If SHM has improved the antigen binding affinity, a strong interaction of the BCR with antigen in conjunction with CD40 signalling (T cell help) will allow the centrocyte to activate anti-apoptotic factors such as Bcl-2 and Bcl-X_L (Smith et al., 2000, Takahashi et al., 1999). CD40 signalling also induces the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which is viewed as an important contributor that keeps the pro-apoptotic tendency of centrocytes at bay. Moreover, CD40 signalling upregulates the interferon-regulatory factor 4 (IRF4), which seems to upregulate AID expression and thereby initiates class switch recombination (CSR) (Klein et al., 2006, Sciammas et al., 2006).

1.2.4 Class switch recombination

CSR allows centrocytes to diversify their antibody's effector functions without altering its antigen-binding affinity (Stavnezer, 1996). Prior to CSR, activated B cells express immunoglobulin (Ig) M and D (IgM and IgD) isotypes on their surface, which are generated by alternative splicing of a primary transcript (Early et al., 1980). Certain cytokines stimulate the initiation of CSR. In a first step transcription of so-called switch (S) regions leads to the generation of DNA-RNA hybrid structures termed R-loops. These R-loop structures are a preferred substrate for AID and are subjected to deamination as described for SHM (in section 1.2.2 p. 23) (Chaudhuri and Alt, 2004). As for SHM, the mismatch is then processed by BER or MMR thus generating a nick in the DNA strand. If two nicks are generated in close proximity on both DNA strands, this will create a double strand break in the S region which is repaired by the NHEJ machinery (Wuerffel et al., 1997). In this process the V(D)J segment is ligated to an alternative downstream constant heavy chain region, deleting all intervening DNA sequence. This somatic gene rearrangement confers a distinct biological function to the antibody without altering its affinity for antigen recognition.

1.2.5 Exit from the GC reaction

The GC reaction is terminated once testing of the antigen receptor and CSR is completed in the GC light zone, leading to terminal differentiation of GC B cells into either plasma cells or memory B cells. Plasma cells are non-dividing, terminally differentiated Ig-secreting cells which reside in the bone marrow (Shapiro-Shelef and Calame, 2005). Plasma cells can either differentiate directly from GC cells or memory B cells. Memory B cells are quiescent antigen-experienced B cells (Tarlinton, 2006) which can respond to secondary antigen encounter much more rapidly than naïve B cells (Tangye et al., 2003). Although it is currently unclear what causes the decision between differentiation into a plasma or memory B cell of a centrocyte at the end of the GC reaction, it is generally accepted that plasma cell differentiation is preceded by

downregulation of the transcriptional suppressor Bcl6 (see section 1.3 below for more detail). The factors required for memory B cell differentiation are less clear. It has been suggested that activation of the signal transducer and activator of transcription (STAT)-5 (Scheeren et al., 2005) and continued CD40 signalling (Arpin et al., 1995) can drive GC cells towards the memory B cell fate *in vitro*.

1.3 Regulation of normal B cell development by Bcl6

Bcl6 is a master transcription factor playing a crucial role at the affinity maturation stage of B cell development. Bcl6 protein is expressed in germinal centre cells but not in most other B cells within human lymphoid tissues (Cattoretti et al., 1995, Flenghi et al., 1995, Onizuka et al., 1995). At the GC stage of B cell differentiation, Bcl6 collaborates with other key transcription factors and thereby regulates late stages of B cell development (Figure 1.3).

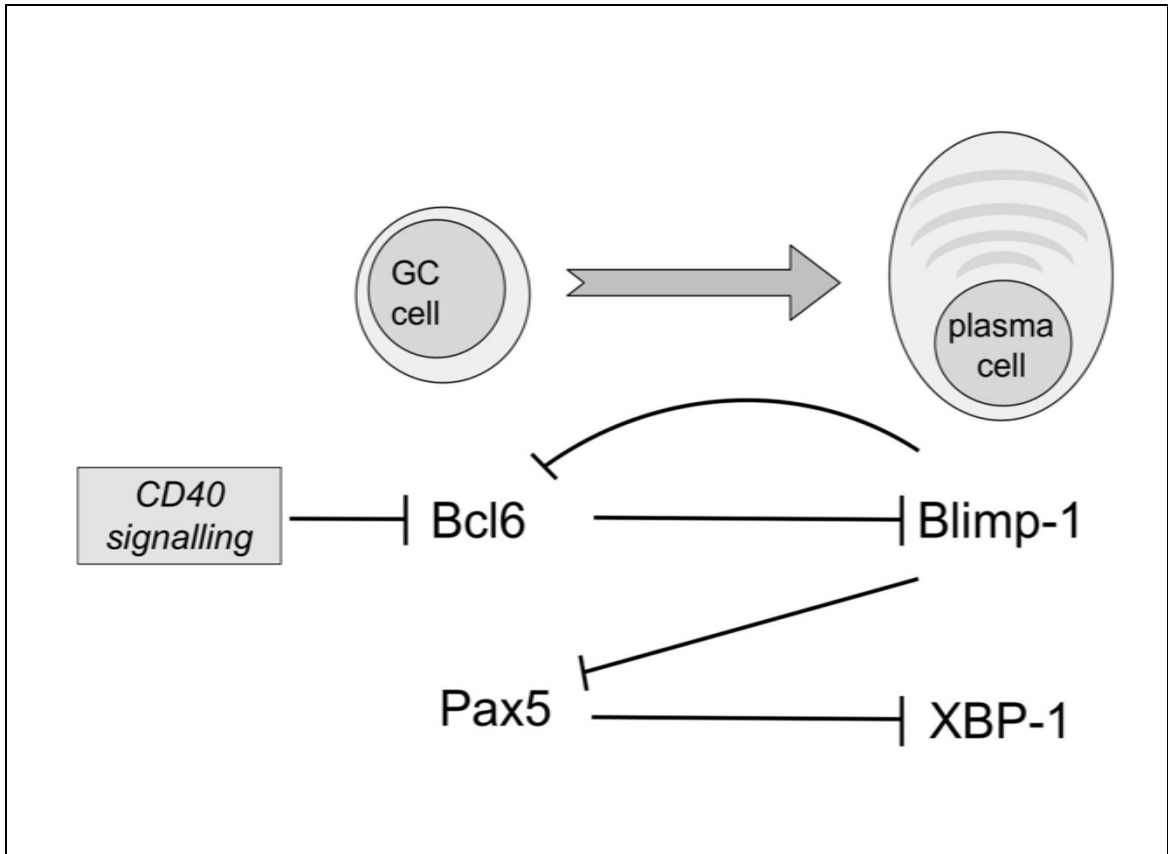


Figure 1.3: Role of Bcl6 in terminal B cell differentiation

In germinal centre (GC) cells, Bcl6 inhibits plasma cell differentiation through repression of the *PRDM1* gene (PR domain containing) encoding Blimp-1 (B lymphocyte-induced maturation protein-1). When Bcl6 is downregulated in response to CD40 signalling, *PRDM1* repression is released, Blimp-1 further downregulates *BCL6* transcription and induces terminal plasma cell differentiation. Repression of the GC transcriptional repressor gene *PAX5* (paired box protein) by Blimp-1 activates transcription of *XBP-1* (X-box binding protein-1), another key transcription factor in plasma cell terminal differentiation.

Bcl6 plays two important roles in the GC reaction. Firstly, Bcl6 secures proliferation of GC cells through suppression of genes involved in negative regulation of the cell cycle. Secondly, Bcl6 inhibits terminal plasma cell differentiation by repressing transcription of the master transcription factor of plasma cell differentiation i.e. B lymphocyte-induced maturation protein-1 (Blimp-1) which is encoded by the *PRDM1* (PR domain containing) gene (Shaffer et al., 2000). In order to terminally differentiate, a GC cell must downregulate Bcl6 activity and a T cell signal is thought to trigger this process.

When the CD40 ligand (CD40L) on a T cell engages the B cell CD40 receptor, Bcl6 activity is downregulated via two different routes. Firstly, CD40 signalling activates the NF- κ B pathway, which can disrupt the interaction of Bcl6 with the nuclear hormone receptor co-repressor (NCoR) (Espinosa et al., 2003, Espinosa et al., 2002) and relieves repression of some of the Bcl6 target genes such as ATR (ataxia telangiectasia and Rad3 related) (Ranuncolo et al., 2007). Secondly, strong CD40 signalling activates transcription of IRF4. IRF4 in turn binds to the *BCL6* promoter and downregulates its transcription (Saito et al., 2007). Bcl6 downregulation allows for Blimp-1 expression, which in turn downregulates a broad range of genes involved in cell growth and proliferation (Shaffer et al., 2002) and thereby guides GC cells towards the plasma cell fate.

Moreover, Blimp-1 activates a second key transcription factor in B cell terminal differentiation, Xbp-1 (X-box binding protein-1) (Reimold et al., 2001), through transcriptional repression of the GC transcription factor Pax5 (paired box protein 5) (Lin et al., 2002). Together, Blimp-1 and Xbp-1 drive GC B cells towards the plasma cell fate. Blimp-1 is thought to mostly contribute to suppression of proliferation (Shaffer et al., 2002), while Xbp-1 plays a vital role in the regulation of immunoglobulin secretion (Liou et al., 1990).

1.4 Bcl6 expression outside of B cell development

Bcl6 protein is expressed at the GC stage of B cell development and hence most of the research has focused on this cell type, however, there is a constantly expanding literature exploring additional functions of Bcl6 in different cell types (Albagli-Curiel, 2003). It is interesting to note that Bcl6 mRNA is expressed in almost all developing tissues of postnatal mice (Bajalica-Lagercrantz et al., 1998), emphasising a bigger role for Bcl6.

Pro-survival function of Bcl6 outside of B cell development

One of the main functions of Bcl6 in the GC reaction is that of a pro-survival factor and it is perhaps not surprising that Bcl6 can fulfil the same role in other tissues. It has been demonstrated in various cell lines and *in vivo* that Bcl6 can protect cells from certain stress stimuli, which would, under normal circumstances trigger an apoptotic response. When Bcl6 was knocked down in mouse skeletal myocytes by RNA interference (RNAi), cells no longer terminally differentiated but died in response to serum starvation (Kumagai et al., 1999). In accordance with this finding, cardiac myocytes of *BCL6* knockout mice are very prone to degeneration after 4 weeks of age (Yoshida et al., 1999) and spermatocytes from *BCL6* knockout mice are more sensitive to apoptosis induced by hyperthermia in comparison to their wild-type counterparts (Kojima et al., 2001).

Regulation of differentiation by Bcl6 outside of B cell development

Bcl6 blocks terminal differentiation in the B cell lineage and also seems to play a role in stabilising terminal differentiation of other cell types (Albagli-Curiel, 2003). Evidence for a role of Bcl6 in differentiation of other cell types has been demonstrated in monocytic cells (Yamochi et al., 1997), memory T cells (Ichii et al., 2002), keratinocytes (Yoshida et al., 1996), skeletal myocytes (Albagli-Curiel et al., 1998) and male germ cells (Yoshida 1996).

Pro-apoptotic function of Bcl6 outside of B cell development

In contrast to the well-established role of Bcl6 as a pro-survival factor, Bcl6 also appears to function as a pro-apoptotic factor under certain circumstances. Earlier studies demonstrated that enforced Bcl6 expression causes apoptosis of cell lines that do not naturally express Bcl6 (Albagli et al., 1999, Yamochi et al., 1999). These cell line-based observations were later supported by the finding that macrophages isolated from *BCL6* knockout mice display a better survival *in vitro* when compared to macrophages derived from wildtype mice (Tang et al., 2002). Taken together, these observations led to the suggestion that Bcl6 expression can potentially kill all cells that do not normally express Bcl6 (Dent et al., 2002).

1.5 Regulation of Bcl6 expression

1.5.1 Regulation of Bcl6 transcription

Bcl6 mRNA is abundant in resting B and T cells and also in GC B cells (Allman et al., 1996), however, only little is known about the factors involved in activating Bcl6 transcription. It has been suggested that certain STATs (Scheeren et al., 2005, Zhou and Ono, 2005) and the activator protein 1 (AP-1) (Arguni et al., 2006) have a potential function in this process. It remains to be demonstrated, however, how these pathways perform and interact *in vivo*.

Although not entirely clear, the transcriptional downregulation of Bcl6 is slightly better understood. It is now generally accepted, that negative autoregulation plays an important part. The observation that *BCL6* knockout mice expressed numerous truncated Bcl6 transcripts led to the suggestion that the Bcl6 protein negatively regulates transcription of its own gene, and in fact, two Bcl6 binding sites were discovered in the *BCL6* promoter region (Wang et al., 2002). It was later shown that the C-terminal (carboxy-terminal) binding protein 1 (CtBP1) functions as an important co-repressor in this process (Mendez et al., 2008).

The Bcl6 binding sites in the *BCL6* promoter region were often found mutated in patients suffering from diffuse large B cell lymphoma (DLBCL) (see section 1.9 below for more information on Bcl6-expressing B cell lymphomas), emphasising the biological significance of these sites for regulation of Bcl6 expression (Pasqualucci et al., 2003). Another factor that can downregulate *BCL6* transcription is CD40 stimulation as already described in section 1.3 (p.26).

1.5.2 Regulation of Bcl6 protein stability

Although Bcl6 mRNA is abundant in resting B and T cells and in GC B cells, only GC cells express high amounts of Bcl6 protein (Allman et al., 1996). This observation led to the conclusion that Bcl6 protein expression is strongly controlled by post-translational mechanisms. Bcl6 protein is detected by Western blotting as a broad band of an apparent molecular weight of 92-98 kilo Dalton (kDa) due to heavy phosphorylation (Onizuka et al., 1995) (predicted molecular weight 79 kDa (Kerckaert et al., 1993)). The site of this weighty phosphorylation maps to a sequence of the Bcl6 protein that is rich in proline, glutamic acid, serine and threonine (PEST domain) (see also section 1.6.2) and that contains several phosphorylation sites for mitogen-activated protein kinases (MAPKs) (Moriyama et al., 1997). Phosphorylation of Bcl6 at these residues in response to BCR signalling leads on to degradation of Bcl6 by the ubiquitin-proteasome pathway (Niu et al., 1998). As BCR signalling is also active upon initiation of the GC reaction, Bcl6 degradation must be suppressed in centroblasts. Three mechanisms have been proposed to aid Bcl6 protein stabilisation (Niu et al., 1998), the first two of which modulate the intensity of the BCR signal. Reducing BCR signalling will at the same time reduce MAPK activity and thereby protect Bcl6 from phosphorylation, subsequent ubiquitination and degradation. Firstly, downregulation of BCR expression itself at the centroblast stage has been observed in rats and might also function to reduce the amplitude of BCR signalling in humans (Liu et al., 1991b, MacLennan, 1994). Secondly, BCR signalling can be modulated by signals received from CD22 or Fc (fragment, crystallisable) γ receptor (Cambier, 1997,

Tedder et al., 1997). Thirdly, Bcl6 that has been targeted by ubiquitination in response to BCR signalling can be de-ubiquitinated by deubiquitinases (DUB) specifically expressed in GCs (Zhu et al., 1996).

1.6 Bcl6 functional domains and interaction sites

Bcl6 is a transcriptional repressor with three main functional protein domains. DNA binding is mediated via its C-terminal DNA binding domain consisting of six zinc finger (ZF) motifs. Transcriptional repression of Bcl6 is achieved mainly through recruitment of various co-repressors, most of which interact with Bcl6's most studied domain, i.e. its amino-terminal (N-terminal) BTB/POZ (broad complex, tramtrack and bric-à-brac / poxviruses and zinc finger) domain. It seems generally accepted that Bcl6 co-repressor availability varies between cell types and differentiation stages, and that this greatly modulates the target gene programmes repressed by Bcl6 in a given situation (Cimmino et al., 2008). The middle portion of Bcl6 has a central role in regulating Bcl6 protein degradation. As described above (section 1.5.2), the PEST domains in the central portion of the Bcl6 molecule are subjected to phosphorylation and consequent ubiquitination, which targets Bcl6 for proteosomal degradation. Figure 1.4 summarises various physical contacts that the Bcl6 protein makes with its interaction partners.

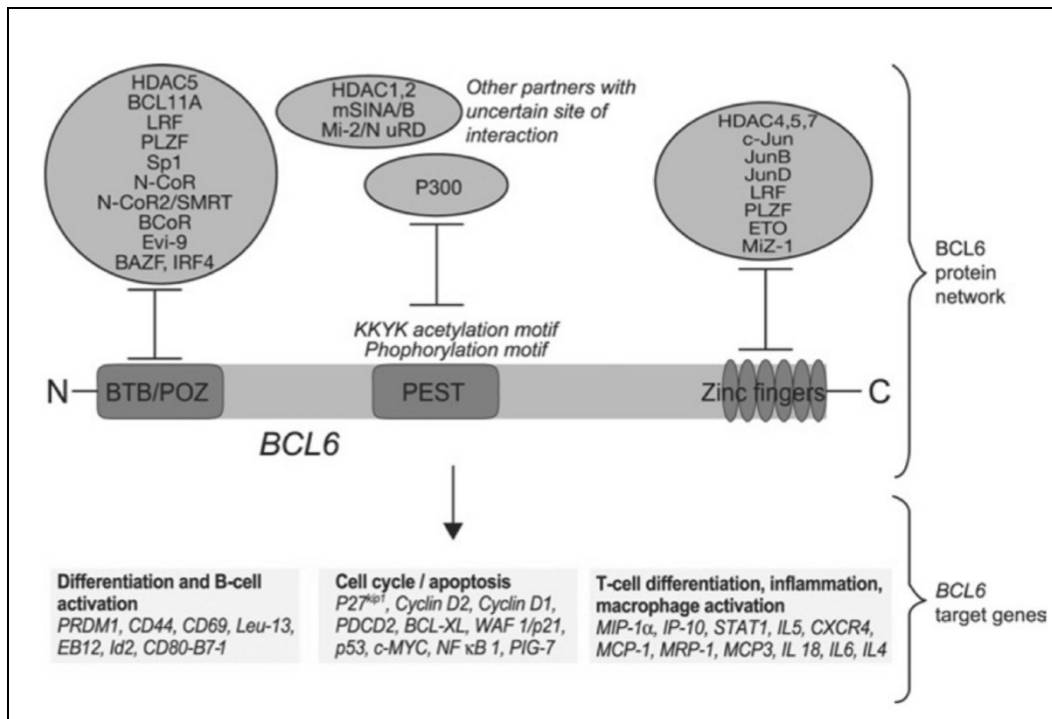


Figure 1.4: Bcl6-interacting proteins and target genes (Jardin et al., 2007)

This diagram shows the three functional domains of Bcl6 (middle), proteins that interact with these domains (top) and selected Bcl6 target genes summarised in three groups (bottom). Bcl6 has three functional domains, an N-terminal BTB/POZ domain mainly responsible for protein interactions, a central PEST domain that regulates Bcl6 half-life and some protein interactions, as well as six C-terminal zinc fingers, which are capable of interacting with DNA and various proteins. The zinc fingers bind to consensus sequences in promoter regions of Bcl6 target genes, thereby mediating repression.

1.6.1 Bcl6 N-terminal POZ domain

Bcl6 has an N-terminal BTB/POZ domain (amino acid (aa) residues 1-121) (Dhordain et al., 1995). BTB/POZ domains are found in many zinc finger transcription factors and can facilitate gene regulation by modifying local chromosome conformation (Ahmad et al., 2003). The Bcl6 BTB/POZ domain mediates homodimerisation with itself and heterodimerisation with other POZ domain containing proteins, such as the promyelocytic leukaemia zinc finger protein (PLZF) (Dhordain et al., 2000). Known partners that interact with the POZ domain of Bcl6 are the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and NCoR (Dhordain et al., 1997, Dhordain et al., 1998), Bcl6-interacting co-repressor (BCoR) (Ghetu et al., 2008, Huynh et al., 2000), CtBP (Mendez et al., 2008), Bcl6-associated zinc finger protein (BAZF) (Okabe et al., 1998), Myc-interacting zinc finger protein-1 (Miz-1) (Phan et al., 2005) and another zinc finger protein named ecotropic viral integration site 9 (Evi9) (Nakamura et al., 2000) (Figure 1.4).

The interaction of Bcl6 with SMRT, NCoR and BCoR depends on a charged pocket in the Bcl6 POZ domain, also referred to as lateral groove involving aa residues 25-54. This interaction site is thought to be the most crucial for Bcl6 function, as inhibitors interfering with this part of the Bcl6 molecule seem to completely abolish its function (see section 1.10 for below for details). The interaction of Bcl6 with CtBP seems to play a role in negative autoregulation of Bcl6 (Mendez et al., 2008) (see section 1.5.1 p.30).

Although many of the proteins that interact with the Bcl6 POZ domain have been identified and validated using experimental approaches such as mammalian two-hybrid, co-localisation, co-immunoprecipitation and reporter assays (Ahmad et al., 2003), the functional significance of the various interactions remains poorly understood. This area of Bcl6 research deserves

special attention, as it will provide a solid base for the development of Bcl6-targeting therapy (see section 1.10 below for details).

1.6.2 Central Bcl6 domain

The central part of the Bcl6 protein functions in transcriptional repression and regulation of protein stability. Although transcriptional repression is mostly attributed to the Bcl6's N-terminal POZ domain, the central portion of Bcl6 contributes to this function. Residues 191-383 of Bcl6 interact with the mammalian orthologue of the yeast SIN3 co-repressor (mSIN3A, component of a histone deacetylase (HDAC) complex) (Dhordain et al., 1998). Additionally, residues 370-386 of Bcl6 appear to be of functional significance in transcriptional repression too, although not through interaction with mSIN3A (Zhang et al., 2001). Furthermore, the central Bcl6 domain mediates the interaction with the metastasis-associated protein 3 (MTA3), a component of the Mi2-NuRD (HDAC) co-repressor complex (Fujita et al., 2004). The interaction of MTA3 with Bcl6 can be impaired by acetylation of the KKYK (lysine (K), tyrosine (Y)) motif of Bcl6 (aa 376-379) by the histone acetyl transferase (HAT) p300/CBP (CREB-binding protein) (Bereshchenko et al., 2002). Acetylation of this motif leads to upregulation of Bcl6 target genes involved in B cell terminal differentiation (Fujita et al., 2004, Jaye et al., 2007).

In addition to its role in co-repressor interactions, the central Bcl6 domain regulates protein stability. The PEST motifs in the central region of the Bcl6 molecule (aa 336-430) become phosphorylated by MAPK, inducing degradation of Bcl6 via the ubiquitin proteasome pathway (Moriyama et al., 1997, Niu et al., 1998) (see also section 1.5.2 p.31 for more information on the regulation of Bcl6 protein stability).

1.6.3 Bcl6 C-terminal zinc finger domain

The C-terminal portion of Bcl6 (aa 520-681) contains six zinc fingers. In addition to DNA binding, these zinc fingers interact with other proteins such as eighty-two-one (ETO) (Chevallier et al., 2004), Evi9 (Nakamura et al., 2000) and members of class II HDACs (Lemerrier et al., 2002).

Interestingly, a mutagenesis study demonstrated, that only the four C-terminal zinc fingers of Bcl6 participate in DNA binding and transcriptional repression, whereas the function of the two N-terminal zinc fingers remains to be determined (Masclé et al., 2003). Following this study, another group detected a new Bcl6 transcript variant that lacks exon 7 of Bcl6 and with it its two N-terminal zinc fingers. This small protein isoform (Bcl6S) appeared to have no difficulties in homo- or heterodimerisation, DNA binding, or transcriptional repression when compared to full length Bcl6 (Shen et al., 2008).

1.7 Mechanism of transcriptional repression by Bcl6

Bcl6 potentially regulates 485 target genes (Polo et al., 2007). Transcriptional repression is brought about by two different mechanisms, firstly Bcl6 directly binds to target gene promoters and secondly, Bcl6 associates with other DNA binding proteins. The Bcl6 zinc fingers recognise a 9 base pair (bp) consensus DNA sequence TTCCT(A/C)GAA that is found in the promoter region of many Bcl6 target genes (Chang et al., 1996, Seyfert et al., 1996). This consensus sequence may be located upstream or downstream of transcriptional start sites and transcriptional repression appears to be effective even over long distances (Albagli et al., 1996). Additionally, the Bcl6DNA recognition sequence is highly similar to that of STAT transcription factors (Seyfert et al., 1996). Consequently, Bcl6 is also capable of inhibiting STAT6-dependent gene transcription by blocking these binding sites (Chang et al., 1996, Kawamata et al., 1994b).

Furthermore, Bcl6 can also achieve transcriptional repression of genes that do not contain a Bcl6 binding site in their promoters through interaction with other transcription factors such as Jun proteins and Miz-1. Through interaction with Jun proteins (c-Jun, JunB and JunD), Bcl6 reduces their transcriptional activation function and represses transcription of *PRDM1* (Blimp-1) and *CDKN1A* (p21cip1/waf1) (Phan et al., 2005, Vasanwala et al., 2002). Through interaction with the transcriptional activator Miz-1, Bcl6 represses transcription of Miz-1 target genes such as *CDKN1A* (p21cip1/waf1) (Phan et al., 2005). Once bound to the regulatory areas within a target gene, Bcl6 interacts with class I and class II HDACs (Dent et al., 2002, Lemerrier et al., 2002) through its various protein interaction domains (see also section 1.6 p.32). Consequently, the recruited co-repressors make the chromatin structure inaccessible to the transcriptional machinery and other activating factors.

1.8 Bcl6 in malignant transformation

In normal B cell development, Bcl6 transcription is active at the GC stage and needs to be downregulated to allow B cells to terminally differentiate (see sections 1.3 p.26 and section 1.5.1 p.30). Supporting evidence for the oncogenic potential of Bcl6 due to misregulated gene expression is provided by two important observations; firstly, downregulation of Bcl6 transcription in response to CD40 stimulation is found to be impaired in many cell lines derived from GC-derived lymphomas (Allman et al., 1996, Gupta et al., 1999). Secondly, the *BCL6* gene is often translocated in B cell lymphoma (see also section 1.9 below). The *BCL6* promoter and the region immediately downstream form a major translocation cluster (MTC) at chromosome region 3q27 (Kerckaert et al., 1993, Otsuki et al., 1995, Ye et al., 1995). At least 20 translocation partner loci in which the *BCL6* gene is positioned under the influence of a heterologous promoter have to date been reported (Albagli-Curiel, 2003). Such promoter substitutions render affected cells resistant to the mechanisms that

downregulate Bcl6 expression under physiological conditions, thereby resulting in persistent Bcl6 expression.

The underlying mechanisms of the resistance of Bcl6 to transcriptional downregulation are the loss of the negative autoregulation of Bcl6 and to a lesser extent mutations in its transcribed sequence. During negative autoregulation, the Bcl6 protein binds to its own gene, thereby repressing its own transcription. A Bcl6 binding site required for negative autoregulation is located in non-coding exon 1 of *BCL6* and can be separated from its coding region as a result of translocations, or can be inactivated by point mutations. Point mutations in the *BCL6* locus are found in normal, as well as malignant GC B cells and seem to be resulting from aberrant targeting of the SHM machinery (Pasqualucci et al., 1998, Shen et al., 1998). However, because spreading of SHM to genes other than immunoglobulin genes is also observed in normal GC B cells, this process is unlikely to contribute to malignant transformation of GC B cells. There is, nevertheless, the interesting possibility that SHM and associated DNA damage within the *BCL6* gene, destabilises the genomic locus and thereby renders it more susceptible to translocation events (Dent et al., 2002).

1.9 Bcl6-expressing B cell lymphomas

Bcl6 protein is expressed in all GC-derived B cell malignancies such as follicular lymphoma (FL), Burkitt's lymphoma (BL) and DLBCL (Cattoretti et al., 1995). Follicular lymphomas almost always carry a Bcl-2 translocation (Tsujimoto et al., 1985). Bcl-2 inhibits apoptosis via the mitochondrial pathway, however, overexpression of Bcl-2 resulting from its translocation, appears to not be sufficient for malignant transformation as such translocations are also detected in normal B cells (Limpens et al., 1995). For this reason, secondary alterations in key cell cycle regulators such as p53, p16INK4a and c-Myc are

commonly observed in FL (Sanchez-Beato et al., 2003). In addition, *BCL6* translocations occur in approximately 6-14% of FLs (Bastard et al., 1994).

By definition, Burkitt's lymphoma is characterised by a translocation of the c-Myc proto-oncogene (Taub et al., 1982) and BL is commonly associated with infection by Epstein-Barr virus (EBV). The transcription factor c-Myc regulates at least 10-15% of all cellular genes (Knoepfler, 2007) thereby controlling cell proliferation, differentiation and apoptosis (Dang et al., 2006). Overexpression of c-Myc alone triggers p53-mediated cell cycle arrest and apoptosis and is hence not sufficient for malignant transformation (Zindy et al., 1998). Accordingly, c-Myc translocations are almost always accompanied by additional defects in the p53-mediated apoptotic pathway (Eischen et al., 1999). The contribution of Bcl6 to malignant transformation of BL is currently poorly understood. Importantly, however, the target gene programs of c-Myc and Bcl6 are highly interconnected (Figure 1.5). Interestingly, c-Myc induces genes involved in apoptosis, while Bcl6 can protect cells from an apoptotic response.

The *BCL6* locus is found translocated in 20-40% of DLBCL (Lo Coco et al., 1994). This mature B cell malignancy is further divided into two disease subsets according to their 'cell-of-origin' gene expression profile (Alizadeh et al., 2000). The GC-like subgroup has a gene expression profile that resembles that of normal centroblasts and expresses high levels of Bcl6, while the second subgroup, the activated B cell-like type, shows a post-GC gene expression profile and does not express Bcl6 (Alizadeh et al., 2000). Section 1.10 below provides further information on the role of Bcl6 in DLBCL and the associated potential for Bcl6 targeting therapy in this context.

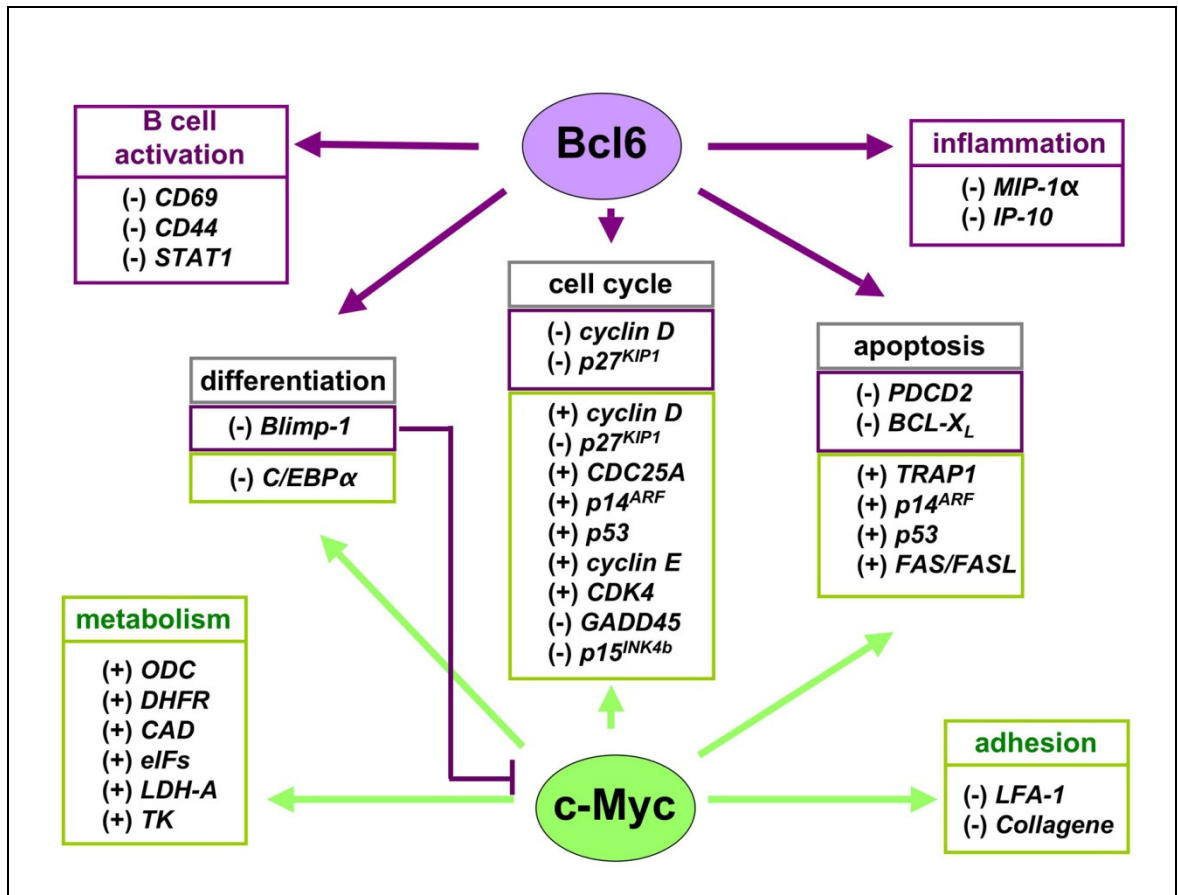


Figure 1.5: Interconnection of Bcl6 and c-Myc target gene programs

Bcl6 and c-Myc target genes are colour-coded in purple (Bcl6) and green (c-Myc) boxes respectively. Induction (+) or repression (-) of target genes is indicated in brackets. Adapted from (Sanchez-Beato et al., 2003).

1.10 Bcl6 as a therapeutic target

Bcl6 is a novel drug target currently being validated for therapy of DLBCL. However, although promising inhibitors have already been developed and tested *in vivo*, many issues in drug target validation of Bcl6 have not been addressed to date. In this section, I am going to discuss the current views of the potential of Bcl6 as a drug target in DLBCL and the associated challenges.

From a general point of view, a good drug target in a hematologic malignancy needs to fulfil two important requirements. Firstly, the drug target must be involved in a crucial biological pathway, so that therapeutic targeting can assert a useful effect. At the same time, however, the effects of interference with the candidate target must be exclusive to the malignant cell population and spare healthy tissue. Secondly, a good drug target needs to be 'well validated'. For drug target validation, it is crucial to understand the function and interactions of the target protein in healthy and malignant conditions as thoroughly as possible.

When examining the features of Bcl6 against these fundamental drug target requirements, there is exciting potential along with tricky challenges. Bcl6 seems to be essential for survival of DLBCL cells and inhibition of its function does not harm healthy tissue (Cerchietti et al., 2010, Cerchietti et al., 2009). The general acceptance that GC B cells and their malignant counterparts depend on Bcl6 results from numerous *in vitro* and *in vivo* studies. Firstly, *BCL6* knockout mice are unable to form GCs (Dent et al., 1997), while overexpression of Bcl6 in mice causes increased GC formation and the development of lymphomas that closely resemble a human DLBCL phenotype (Cattoretti et al., 2005). Secondly, when Shaffer and co-workers expressed a dominant-negative version of Bcl6 in a Burkitt's lymphoma cell line (Raji), increased apoptosis was observed (Shaffer et al., 2000). Thirdly, peptides interfering with the Bcl6 BTB/POZ domain cause growth suppression and apoptosis in DLBCL and BL cell lines (see section 1.9 p.38 for details on Bcl6-expressing lymphomas) and

human DLBCL xenografts (Cerchiatti et al., 2010, Cerchiatti et al., 2009, Chattopadhyay et al., 2006, Polo et al., 2004).

However, although many Bcl6 target genes and co-repressors have been identified, the role of specific protein-protein interactions remains elusive. As a result, it has not been established which part of the Bcl6 molecule and which interaction site would be most suitable for therapeutic targeting. One reason for this is the lack of good model systems to study the significance of Bcl6 interactions (see also section 1.11 below). To date, Bcl6 inhibitors have only targeted the POZ domain. Interference with the BTB/POZ domain appears to completely abolish Bcl6 function (Polo et al., 2007). Complete destruction of Bcl6 function, however, could be accompanied by too high toxicity in clinical trials.

To date, considerations of inhibiting sites of the Bcl6 molecule other than the POZ domain have been extremely limited, only targeting of the Bcl6-MTA3 interaction has been discussed in the literature. The Bcl6-MTA3 interaction is impaired by acetylation of Bcl6 protein by p300 and can thus be interfered with through inhibition of HDACs (Fujita et al., 2004). In fact, treatment of DLBCL with HDAC inhibitors caused the accumulation of acetylated Bcl6 that was unable to interact with MTA3 and enforced a differentiated phenotype (Bereshchenko et al., 2002). Although treatments that cause malignant cells to differentiate rather than kill them might appear to be too mild at first sight, such treatments may be of great benefit clinically because they are likely to show lower toxicity than approaches that trigger radical cell death. A successful example of this concept is the current treatment of acute promyelocytic leukaemia (APML). In APML, treatment with ATRA (all-trans retinoic acid) plus chemotherapy drives malignant cells towards differentiation into mature granulocytes and greatly improves patient survival (Melnick and Licht, 1999).

Although Bcl6 is a promising new drug target, the targeting potential of its multiple protein interaction sites has not been fully explored to date. Only two of its interaction sites have been considered for therapeutic targeting and the understanding of these interactions sites is nevertheless limited. In order to aid the development of Bcl6-targeting therapy, there is hence the need for developing novel model systems to study Bcl6 function and protein interactions.

1.11 Bcl6 model systems

As outlined in the previous section, it is essential to collect as much information on Bcl6 function as possible in order to optimise the development of Bcl6-targeted therapy. The following sections provide an overview of the model systems currently available to study Bcl6 function and their individual strengths and weaknesses.

1.11.1 Murine Bcl6 model systems

Loss-of-function studies in mice enable investigation of gene function within the complexity of a whole organism. *BCL6* knockout mice have been engineered by two independent groups and have generated valuable insight into the biological function of Bcl6 (Dent et al., 1997, Fukuda et al., 1997, Ye et al., 1997). *BCL6* knockout mice are born with normal lymphoid organs and no obvious defects in B and T cell development. Soon after birth, however, *BCL6*^{-/-} mice display growth retardation, severe inflammatory disease and die early. When challenged by pathogens, defects in T cell-dependent immune responses become apparent. *BCL6*^{-/-} B cells can differentiate into short-lived antibody secreting plasma cells (Tunyaplin et al., 2004, Ye et al., 1997), but not into long-lived plasma cells (Toyama et al., 2002). Consistent with this finding, GC formation in *BCL6*^{-/-} mice is absent, resulting in impaired affinity maturation and severe infections. Moreover, mice that lack Bcl6 display an inflammatory phenotype in many organs (Toney et al., 2000).

Despite the great contribution of these *in vivo* studies towards our understanding of Bcl6 function, *BCL6* knockout mice are very ill, die after a few weeks and can hence not be studied very well. Moreover, it has to be taken into account that although immunological responses are well conserved between humans and mice, the observed phenotypes must be interpreted with caution, as significant differences exist between the two species. An important observation for example is, that the inflammatory response is regulated differently in mice and humans (Del Prete et al., 1993).

1.11.2 Cell culture-based Bcl6 model systems

Cell line work is attractive for some purposes i.e. analysis of gene regulation, and avoids the use of animals, which is the direction of research that scientists are encouraged to follow, but cannot be used to show effects on the immune system as a whole. A beautiful study that provided exciting insight in the target gene program that Bcl6 regulates was published by Shaffer and co-workers (Shaffer et al., 2000). The authors established cell line-based gain-of-function and loss-of-function models and used these to screen for possible Bcl6 target genes by microarray analysis. In the gain-of-function approach, the authors expressed Bcl6 in cell lines that usually do not express Bcl6 (K562, WI-L2, SUDHL5). The loss-of-function model was based on a dominant-negative approach in which only the zinc finger portion of Bcl6 was expressed in BL cell lines that express endogenous Bcl6 (Raji and BJAB). Microarray analysis of the effects on gene expression of these cell line-based gain-of-function and loss-of-function models identified a large number of potential Bcl6 target genes involved in B cell activation, differentiation, inflammation and cell cycle regulation (Shaffer et al., 2000). Moreover, the authors of this study demonstrated that of two particular genes are direct Bcl6 target genes (cyclin D2 and MIP-1 α), by showing that Bcl6 transcriptional repression is dependent on Bcl6 binding site in their promoter region (Shaffer et al., 2000).

Despite of the intriguing insight into the broad transcriptional regulation capacity of Bcl6 gained from this study, it must not be neglected that the models used to generate the data are far from ideal. Firstly, the gain-of-function model involved expression of Bcl6 in a cell line that usually does not express Bcl6 and it has been demonstrated in other studies that this is likely to be toxic to the cells (Albagli et al., 1999, Yamochi et al., 1999). Furthermore, such cell lines may not express the same group of Bcl6 co-repressors as GC cells. Secondly, one cannot be certain that the effect of expression of only the ZF domain of Bcl6 in the loss-of-function model is only attributed to abrogation of Bcl6 function. In fact, the DNA sequence recognised and bound by the ZF domain of Bcl6 closely resembles that of STAT binding sites (Seyfert et al., 1996), hence the effect of abrogating Bcl6 function, might result from interfering with STAT transcriptional regulation. Nevertheless, this study revealed rich insights into the role of Bcl6 in the immune response and the development of lymphoma and serves as an excellent starting point for the development of more advanced system for studying Bcl6 function.

1.11.3 Bcl6 knockdown model systems

Alternatively to the dominant-negative concept chosen by Shaffer and co-workers, cell line-based loss-of-function can be also achieved by knockout (gene targeting) or knockdown (small interfering RNA (siRNA)) approaches. Both these strategies aim to eliminate any target protein expression, either by disruption of the genomic locus or by degradation of mRNA transcripts. siRNA has been previously used to downregulate Bcl6 expression in a human BL cell line (BJAB), however, only a 57 % depletion of Bcl6 protein was achieved (Baron et al., 2007). Although others have also reduced Bcl6 protein by siRNA in cell lines derived from BL, chronic myeloid leukaemia (CML) and gastric cancer (Fernandez de Mattos et al., 2004, Hirata et al., 2009, Kinugasa et al., 2007, Phan et al., 2005), no complete depletion was achieved and off-target effects of siRNAs are very common (Jackson and Linsley, 2010).

Alone, none of the available models for studying Bcl6 function is able to answer all open questions on the complex network surrounding Bcl6. I therefore decided to create a cell line-based model system in which complete Bcl6 protein depletion can be achieved. To accomplish this, I used gene targeting to disrupt the *BCL6* gene of a Bcl6-expressing, GC-derived B cell line. Due to the fact, however, that Bcl6 seems to be essential for the survival of GC cells, it was expected that a traditional knockout could be lethal. To overcome this potential problem, I decided to pursue a conditional knockout strategy as outlined in the following section.

1.12 Experimental outline

In order to overcome some of the limitations of the current models available for studying Bcl6 function, I have pursued a cell line-based conditional knockout strategy. My conditional *BCL6* knockout model was developed using the well-characterised BL cell line DG75. DG75 carries a c-Myc translocation and is deficient in p53-mediated apoptosis. Table 1 summarises some of the key characteristics of the parental cell line.

The conditional *BCL6* knockout approach involves gene targeting of the first allele of *BCL6*, while the product of the second *BCL6* allele ensures survival and proliferation of the cell. Before the second allele is targeted, an inducible *BCL6* copy is introduced, which rescues the cells when the second allele is targeted. Once both endogenous alleles are disrupted, the inducible copy can be switched off and the phenotype can be studied. Such conditional knockout systems have been successfully used in other cell lines, both in our lab (Carpenter and Porter, 2004, Itzhaki et al., 1997) and in others (Sonoda et al., 1998, Takagaki and Manley, 1998) to study gene function. The information generated from this model will greatly support the development of Bcl6 inhibitors and hopefully translate to clinical benefit for patients suffering from DLBCL.

Table 1: Summary of characteristics of the DG75 cell line

characteristic	description
EBV status	♦negative (Ben-Bassat et al., 1977)
translocation	♦t(8;14)(24;q32), MYC immunoglobulin heavy chain (IgH) (Basso et al., 1999)
Bcl6 status	♦no translocation (Yang et al., 2006) ♦one <i>BCL6</i> allele carries a SNP (Zhang et al., 2005) ♦no mutations in the coding region (as tested by sequencing)
markers	♦surface IgM kappa on its surface (Ben-Bassat et al., 1977)
apoptosis-resistance	♦p53 mutation impairs nuclear-cytoplasmic shuttling (Crook et al., 1998) ♦Bax is non-functional; frameshift mutation, premature stop codon (Brimmell et al., 1998) ♦resistance to cisplatin (Crook et al., 1998), ionomycin and serum starvation (Brimmell et al., 1998)
miscellaneous	♦AID is expressed at low levels (Scheller et al., 2010) ♦SHM not active (Scheller et al., 2010) ♦mismatch repair deficiency (Msh2-) (Brimmell et al., 1998)

CHAPTER 2 - Methodology

2.1 Bacterial culture

Escherichia coli (*E.coli*) strains TOP10 (Invitrogen), NEB-10-beta (New England Biolabs (NEB)) or XL2-Blue (Stratagene) were used for standard cloning procedures. Transformation was carried out according to the manufacturer's instructions. Bacterial cultures were grown in Luria Bertani (LB) medium or on LB agar plates (see section 2.9 p.69 for commonly used solutions) supplemented with 75 µg/ml ampicillin (amp) (Sigma). For *zeocin* (*zeo*) selection, bacteria were plated on low salt LB plates containing 25 µg/ml *zeocin* (Invitrogen). For long term storage, bacterial clones were kept in 20% glycerol volume per volume (v/v) (Sigma) at -80 °C.

2.2 Nucleic acid isolation

2.2.1 Isolation of plasmid DNA

Bacterial plasmid DNA was isolated by alkaline lysis followed by high salt precipitation and plasmid DNA purification using mini (Omega bio-tek) or HiLink Pure maxi preparation kits (Invitrogen).

2.2.2 Isolation of mammalian RNA

Confluent cells were washed in phosphate buffered saline (PBS) (Lonza), 10^7 cells were pelleted and total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. This system relies on a guanidine-isothiocyanate lysis followed by silica-column based purification of total RNA.

2.2.3 Isolation of mammalian genomic DNA

Confluent DG75 cells were washed in PBS and 10^7 cells were pelleted. For cloning procedures, genomic DNA (gDNA) was extracted using the Gentra Puregene kit (Qiagen), for Southern blotting (see section 2.6), gDNA was extracted using a simplified DNA isolation procedure (Laird et al., 1991).

2.2.4 Quantification of nucleic acids

Nucleic acids were quantified using a BioPhotometer (Eppendorf) or a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). DNA and RNA concentrations were determined by measuring the optical density (OD) at 260 nm, where 1 OD unit equals 50 µg/ml of DNA or 40 µg/ml of RNA, respectively.

2.3 Mammalian cell culture

2.3.1 Culturing

All mammalian cells were grown in a humidified incubator (Napco 5410, Precision Scientific) at 37 °C with 5 % CO₂. Sterile handling of cells was carried out in a flow hood (Faster BHG2004, Jencons-PLS). All cell lines used in this project (see Table 2) were cultured in RPMI 1640 medium (Lonza), supplemented with 10 % fetal bovine serum (FBS) (Lonza) and 1 x Penicillin-Streptomycin-Glutamine (Invitrogen). For tetracycline (tet)-regulated transgene expression, 1 µg/ml tetracycline (tet) or doxycycline (dox) (both from Sigma) were added to the culture medium.

All cell lines were passaged at 80 % confluency or less. For passaging, adherent cells were washed in PBS, trypsinised (Gibco) and diluted in fresh medium. Suspension cells were passaged by directly diluting the culture in fresh medium. For long term storage, 10^7 mammalian cells were resuspended in

1ml freezing medium (see section 2.9), transferred into cryovials (CryoTube, Nunc), placed in a cryo-freezing container filled with isopropanol (at room temperature) and transferred to a -80 °C freezer. After 24 hours (hrs) at -80 °C, cells were transferred to liquid nitrogen. To thaw cells, cryovials were placed in a 37 °C water bath until the cell suspension started to thaw. Cells were transferred into pre-warmed medium, collected by centrifugation for 5 minutes (mins) at 700 x g and resuspended in complete medium, at high density, for recovery.

Table 2: Mammalian cell lines used throughout this project

cell line	description
DG75	humanBL (Ben-Bassat et al., 1977)
DG75-354	DG75-derivative that stably expresses the tetracycline transactivator (supplied by Dr B. Henglein, Institut Curie, Paris)
293T	human embryonic kidney cell line (Pear et al., 1993)
K-562	human chronic myeloid leukaemia cell line (Lozzio and Lozzio, 1975)
Jurkat	human acute T cell leukaemia (Schneider et al., 1977)
U266	human myeloma cell line (Nilsson et al., 1970)
JJN3	human myeloma cell line (Jackson et al., 1989)

2.3.2 Growth curve analysis

For growth curve analyses, trypan blue-excluding, i.e. viable cells were counted and cultures were split regularly so concentrations did not exceed or fall below the preferred cell density of $1-9 \times 10^5$ cells/ml. The doubling time (T_d) between time point 1 (t_1) and time point 2 (t_2) (in hours) was calculated by inserting cell concentrations at time point t_1 (c_1) and time point t_2 (c_2) into the following formula:

$$T_d = (t_2 - t_1) \times \frac{\ln(2)}{\ln(c_2/c_1)}$$

2.3.3 Electron microscopy

Dr Shaun Willimott (University of Leicester, Leicester) performed cell fixation for electron microscopy. Briefly, cells were fixed in 2% glutaraldehyde in 0.1 M

sodium cacodylate buffer (pH 7.4) at 4° C overnight. Dr David Dinsdale (MRC Toxicology Unit, Leicester) processed samples further and performed electron microscopy. For postfixing, cells were incubated in 1% osmium tetroxide/1% potassium ferrocyanide for 1 hr at room temperature. After fixation, cells were stained with 5% aqueous uranyl acetate overnight at room temperature, were dehydrated and embedded in Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK). Ultrathin sections were stained with lead citrate and recorded using a Megaview 3 digital camera and iTEM software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) in a Jeol 100-CXII electron microscope (Jeol UK Ltd., Welwyn Garden City, UK).

2.3.4 Transient transfections

Transient transfection of DG75 cells was carried out by nucleofection (Amaxa) using the V-kit in combination with nucleofection program 'O-006'. For each reaction, 3 million cells were nucleofected with 3 µg plasmid DNA. Transient transfection of 293T cells was achieved by lipofection with lipofectamineTM2000 (Invitrogen), according to the manufacturer's instructions. Transgene expression was monitored by flow cytometry or immunoblotting.

2.3.5 Stable transfections

Stable transfection was achieved by electroporation as described previously (Feederle et al., 2004). Briefly, 3 million cells were washed in medium without supplements and resuspended in 250 µl of the same medium and 3 µg of plasmid DNA. The suspension was transferred into pre-chilled, 4 mm-gap electroporation cuvettes (BTX Harvard Apparatus) and placed on ice. Electroporation was achieved with a Gene-Pulser (BioRad) at 240 volt (V) and capacitance 960 µF. Cells were immediately transferred into 3 ml pre-warmed medium including all supplements and left to recover for 24-48 hrs. After recovery, cells were plated in 96-well plates (VWR) at 10³ cells per well, together with the corresponding selective antibiotic. Geneticin (G418) (Invitrogen) was

used at 550 µg/ml, puromycin (Sigma) at 2 µg/ml and zeo (Invivogen or Invitrogen) at 100 µg/ml. The medium was exchanged every 4-7 days. Depending on the selective agent used, clones could be expanded after 14-20 days.

2.3.6 Flow cytometry

Unless otherwise indicated, 10,000 events were acquired with a FACSCalibur (BD). Acquisition and analysis of data were accomplished with CellQuest Pro software (BD) and the Flowjo software package (Treestar).

Cell surface staining

Anti-IgM-fluorescein isothiocyanate (FITC), anti-CD69-FITC and isotype control antibodies were purchased from BD Bioscience. Approximately 5×10^5 cells were collected by centrifugation, washed in PBS, resuspended in 1 ml PBS with 2 % (v/v) FBS and 3 µl of corresponding antibody and incubated on ice for 20 mins. Prior to fluorescent activated cell sorting (FACS) analysis, cells were washed twice in PBS with 2 % (v/v) FBS.

Annexin V assay

Apoptosis was measured using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). Briefly, 5×10^5 cells were washed in PBS, resuspended in 100 µl Annexin V binding buffer and incubated with 4 µl of each FITC Annexin V and propidium iodide (PI) staining solution for 15 minutes at room temperature in the dark. 400 µl Annexin V binding buffer were added to the samples prior to analysis by flow cytometry.

CFSE assay

Proliferation analysis was carried out by intracellular staining with carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described

(Quah et al., 2007). Briefly, 7×10^6 cells were collected by centrifugation, resuspended in 1 ml PBS with 5% v/v FBS and transferred to a fresh 15 ml tube. The tube was positioned horizontally and 110 μ l of PBS were added to a dry part at the top of the tube wall. 1.1 μ l of 5 mM CFSE (Sigma) were added to the PBS droplet, the tube was capped, quickly inverted and vortexed, protected from light and incubated at room temperature for 5 minutes. Subsequently, 10 ml of PBS with 5% v/v FBS were added and cells were collected by centrifugation. After two more washes, stained cells were distributed between two 10 ml cultures (with or without doxycycline) and analysed daily by flow cytometry.

Cell cycle / DNA content analysis

For cell cycle analysis, 10^6 cells were collected by centrifugation, washed in PBS and resuspended in 300 μ l PBS. 700 μ l ice-cold ethanol were added to the cell suspension whilst vortexing. Fixation was completed by incubation on ice for 1 hr or by storing samples at -20 °C over night. Fixed cells were collected by centrifugation, washed once in PBS and resuspended in 500 μ l PBS containing 0.2 mg/ml RNase A (Qiagen). Subsequent to digestion of RNA at 37 °C for 1 hr, PI (Sigma) was added to a final concentration of 40 μ g/ml and cells were analysed by flow cytometry.

2.4 Manipulation of nucleic acids

2.4.1 Restriction enzyme digestion of plasmid DNA

Restriction enzymes and protocols were from NEB. Typically, 1 μ g of plasmid DNA was digested with 3 units of enzyme for 1 hr at the incubation temperature recommended.

2.4.2 Restriction enzyme digestion of genomic DNA

Genomic DNA was heated up to 55 °C and gently resuspended by pipetting with a cut 1000 µl tip. Genomic DNA from 2×10^6 cells was typically digested at 37 °C over night, in a total volume of 200 µl, supplemented with 160 units *SphI* and 5 mM spermidine (Sigma). A fraction of the gDNA digest was visualised by agarose gel electrophoresis (section 2.4.4) to evaluate completion of the reaction. Digestion of human gDNA with *SphI* is expected to result in an average fragment size of 5,000 bp (calculated from public databases by NEB). Occasionally, more restriction enzyme had to be added and the reaction had to be incubated longer, in order to achieve complete digestion.

2.4.3 Phenol/chloroform extraction and ethanol precipitation

Phenol/chloroform extraction and ethanol precipitation, or ethanol precipitation alone were performed to purify DNA. For phenol/chloroform extraction, the DNA-containing sample was supplemented with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) equilibrated with 10 mM Tris, pH 8.0 (Sigma), vortexed for 1 minute and centrifuged at $>10k \times g$ for 1 min. DNA in the upper organic phase was subsequently purified by ethanol precipitation.

For ethanol precipitation, the DNA-containing sample was mixed with 1/10 volume of 3M sodium acetate and 2.5-3 volumes of ethanol (Fisher Scientific) were added. The sample was chilled at -20 °C up to several hrs and DNA was collected by centrifugation $>15k \times g$ at 4 °C for 30 mins. The DNA pellet was washed in 70% ethanol (v/v) in order to remove residual salt and phenol, dried and resuspended in tris-ethylene diamine tetraacetic acid (TE) (see section 2.9).

2.4.4 Agarose gel electrophoresis

Gels were casted with 0.5-1.5 % agarose weight per volume (w/v) (BDH or Bioline) in tris-acetate EDTA (TAE, see section 2.9) supplemented with Sybr Safe (Invitrogen). Agarose gels were run in a horizontal electrophoresis unit (Midi, Fisher Scientific) with TAE as running buffer at 35-125 V. DNA samples were mixed with DNA loading dye prior to gel electrophoresis and loaded alongside DNA ladders with known size standards such as 1kb ladder (NEB or Invitrogen), λ Hind marker (NEB), or 100 bp ladder (NEB). DNA was visualised with a blue light transilluminator (Invitrogen) and pictures were acquired with GrabIt software (UVP).

2.4.5 Purification of DNA from agarose gels

DNA-containing gel slices were excised from agarose gels using a razor blade and processed with the QIAquick gel extraction kit (Qiagen) as directed by the manufacturer.

2.4.6 Ligation

Ligations were carried out with T4 DNA ligase (NEB) according to the manufacturer's instructions. Typically, 50 ng of digested vector were incubated with a 3-fold molar excess of insert, 400 units T4 ligase and 1x T4 ligase reaction buffer (NEB), in a total volume of 15 μ l. Cohesive ends were ligated at 16 °C for 16 hrs, while blunt ends were ligated at room temperature for 2 hrs.

2.4.7 End-filling

For end-filling of 5' overhangs, 1 μ g of DNA was incubated in NEB buffer 2, supplemented with 33 μ M each deoxyribonucleotide triphosphate (dNTP) and 1 unit of Klenow enzyme (NEB) for 15 minutes, at room temperature, in a total volume of 20 μ l. The reaction was terminated by addition of ethylene diamine

tetra-acetic acid (EDTA, section 2.9) to a final concentration of 10 mM and the enzyme was heat inactivated for 20 mins at 75 °C.

2.4.8 Radiolabelling of DNA for Southern blotting

Marker labelling

Labelling of 1 µg DNA ladder (1kb, Invitrogen) was carried out in 1x restriction enzyme buffer 2 (NEB), supplemented with dNTPs (dATP, dTTP and dGTP (NEB, 50 µM final concentration each), 0.5 µl of α -³²P-dCTP (10 mCi/ml, Perkin Elmer) and 5 U of Klenow fragment (3' to 5' exo⁻) in 20 µl, for 30 minutes at 37 °C.

Probe labelling

25 ng of DNA probe were mixed with 250 ng of random hexamers (Promega), boiled for 2 minutes and quickly placed on ice before the remaining components were added. The labelling reaction was carried out in 20 µl as for the marker, however, 3 µl of α -³²P-dCTP were added and the reaction was incubated for 1 hour.

For purification of the labelling reaction, Illustra™ ProbeQuant™ G50 micro columns (GE Healthcare) were prespun for 1 minute at 735 x g, labelling reactions were made up to 100 µl with TE, applied to the column and spun for 2 mins at 735 x g. The percentage of radioisotope incorporation into the DNA probe was determined by comparing Geiger counts of the labelled probe before and after column purification. Percentages were around 60-70 %.

2.4.9 Phosphorylation of PCR primers

For site-directed mutagenesis primers were phosphorylated using T4 polynucleotide kinase (PNK). Briefly, 3 µl of primer (100 µM), 5 µl ATP (10 mM, NEB), 5 µl of 10x T4 PNK buffer (NEB) and 1 µl T4 PNK (10U/µl) were

incubated in 50 μ l at 37 °C for 30 mins. PNK was then heat inactivated for 20 mins at 65 °C and 2.4 μ l of the reaction were used per 30 μ l PCR reaction.

2.5 Cloning and PCR analysis

2.5.1 General PCR conditions

All polymerase chain reactions (PCR) were carried out in a Peltier Thermal Gradient Cyclor 200 (MJ Research).

2.5.2 Molecular cloning

All molecular cloning was performed with Phusion™ High-Fidelity DNA Polymerase (Finnzymes). Each reaction consisted of Phusion™ DNA polymerase (0.02 U/ μ l), 1x High Fidelity Buffer (Finnzymes), primers (0.5 μ M, Invitrogen), dNTPs (200 μ M each, NEB), 3 % dimethyl sulfoxide (DMSO) (NEB) and variable amounts of template DNA, depending on the source. Typically, 300 ng of gDNA, 10 ng of plasmid DNA or 200 ng complementary DNA (cDNA) were used as template. A classical PCR cycling profile for Phusion™ polymerase is shown below:

step	temperature	time
initial denaturation	98 °C	0:30
denaturation	98 °C	0:10
annealing	50-72 °C	0:30
extension	72 °C	15-30 seconds (sec) / kilobase-pair (kb)
final extension	72 °C	10:00

25-35 cycles

Cloning of the regions of homology for the targeting construct

Regions of homology for the targeting construct were amplified by PCR from DG75 gDNA using the following primers:

<u>KpnI</u> -homology-F	GAT <u>GGT ACC ATC CCA ACC GTC TCT TTC AC</u>
<u>SacII</u> -homology-R	CAT <u>CCC CGC GGC ACA CCT CTA ATA GGC TCA G</u>

The annealing temperature of this primer set was 68 °C for 5 cycles, followed by 72 °C for 30 cycles. The extension time of the 10,007 bp PCR product was 6 mins.

Cloning of the *zeocin* resistance cassette for the targeting construct

The *zeo* cassette contains the *zeo* coding region, as well as its eukaryotic and prokaryotic promoter and was cloned from plasmid pCMV/*Zeo* (Invitrogen) using the following primers:

<u>SbfI</u> - LoxP - <i>Zeo</i> -F	CAT CGA CCT GCA GGA CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATC GTT ACA TAA CTT ACG GTA A
<u>SbfI</u> - <u>SphI</u> - LoxP - <i>Zeo</i> -R	GTG CAT CCT GCA GGG CAT GCATAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT GCA GTG AAA AAA ATG CTT TA

The annealing temperature for this primer pair was 50 °C for 5 cycles, followed by 72 °C for 30 cycles. The extension time for the 1,161 bp product was 30 seconds.

Cloning tetracycline-regulated Bcl6

Total DG75 RNA was extracted with the RNeasy kit (Qiagen) and transcribed into cDNA using the Omniscript kit (Qiagen) according to the manufacturer's recommendations. 2 µl of cDNA were used as template for the PCR reaction. Human Bcl6 was hemagglutinin (HA)-tagged at the C-terminus using the following primers:

<u>Bam</u> HI-Bcl6-F	CAG GAT CCA TGG CCT CGC CGG CTG ACA G
<u>Hind</u> III-Bcl6- HA -R	CAG AAG CTT TCA AGCGTAATCTGGAACATCATAAGGGTA GCA GGC TTT GGG

The annealing temperature of this primer pair was 72 °C and the corresponding extension time for the 2,165 bp product was 1:30 mins for 30 cycles. The Bcl6 open reading frame (ORF) was sequenced by automated fluorescent sequencing using ABI3730xl and ABI3130xl (Applied Biosystems) using the following primers:

pTRE-tight-1F	CTC GTT TAG TGA ACC GTC AG
pTRE-tight-4R	CCT CTA CAA ATG TGG TAT GG
Bcl6-1R	CAG GTT GTT CTC CAC CAC
Bcl6-2F	GTG AGA GCA GAG CCT TTG
Bcl6-2R	CAG GCA TTC TTA CTG CTG C
Bcl6-3F	CAT CCT CCA GGC TTC TGG
Bcl6-3R	GTG TGC CTC TTG AGT GAG
Bcl6-4F	CAG ACC CAC AGT GAC AAA C

Cloning of complementation constructs

The Bcl6 ORF of all complementation constructs was sequenced using primers pcDNA3.1-F, pcDNA3.1-R and primers Bcl6-1R to Bcl6-4F (above).

pcDNA3.1-F	CTAGAGAACCCACTGCTTAC
pcDNA3.1-R	GAGGGGCAAACAACAGAT

Cloning of pcBcl6

Full length Bcl6 was amplified from DG75 cDNA using primers Bcl6-F and Bcl6-R (see below) (annealing temperature 72 °C, extension time 1:30, 35 cycles). The 2,121 bp product was inserted into the *PmeI* sites of pcDNA3.1/Hygro/lacZ (Invitrogen) and correct orientation of the insert was verified by restriction enzyme digestion.

Bcl6-F	ATGGCCTCGCCGGCTGACAG
Bcl6-R	TCAGCAGGCTTTGGGGAGCT

Cloning of pcBcl6-FLAG

To generate C-terminally-FLAG-tagged Bcl6 (pcBcl6-FLAG), site-directed mutagenesis was performed on pcBcl6 using phosphorylated primers Bcl6-FLAG-F and Bcl6-FLAG-R. The PCR reaction was performed using 1 ng of template, an annealing temperature of 72°C, an extension time of 4 mins and 25 cycles. Five µl of PCR product incubated with 1 µl of 10x T4 ligase buffer (NEB) and 1 µl of T4 ligase (20 U/µl, NEB) in a total volume of 10 µl over night at room temperature, prior to transformation into competent bacteria.

Bcl6-FLAG-F	GGTGGAGACTACAAAGACGATGACGATAAATGAAAACCCGCTGAT
	CAGCCTCGACTGTGC
Bcl6-FLAG-R	GCAGGCTTTGGGAGCTCCGGAGG

Cloning of pcBcl6^{N21K}

Site-directed mutagenesis was performed on pcBcl6 using primers N21K-F and N21K-R below. For the PCR reaction, an annealing temperature of 62 °C, an extension time of 2:30 mins and 18 cycles were used. Following amplification, 1 µl of *DpnI* (1 U/µl, NEB) was added to the reaction (total volume 50 µl) which was subsequently incubated for 1hr at 37°C before *DpnI* was heat inactivated for 20 mins at 80°C and the reaction was transformed into competent bacteria.

Bcl6-N21K-F	GTTCTTCTCCAAGCTTAATCGTC
Bcl6-N21K-R	GACGATTAAGCTTGAGAAGAAC

Cloning of pcBcl6^{N21K}-FLAG

pcBcl6^{N21K}-FLAG was derived from pcBcl6^{N21K} using the site-directed mutagenesis procedure described for construction of pcBcl6-FLAG above.

Cloning of pcBcl6^{ΔPOZ}-FLAG, pcBcl6^{D33A}-FLAG, pcBcl6^{R44A}-FLAG, pcBcl6^{D33R/R44D}-FLAG, pcBcl6^{QQYQ}-FLAG, pcBcl6^{ΔETO}-FLAG

pcBcl6^{ΔPOZ}-FLAG, pcBcl6^{D33A}-FLAG, pcBcl6^{R44A}-FLAG, pcBcl6^{D33R/R44D}-FLAG, pcBcl6^{QQYQ}-FLAG, pcBcl6^{ΔETO}-FLAG were derived from pcBcl6-FLAG using the site-directed mutagenesis procedure described for construction of pcBcl6-FLAG above. Using the following primers:

ΔPOZ-F	CGGAAGTTTATTAAGGCCAGTGAAGCAGAG
ΔPOZ -R	CATAAACGCTAGCCAGCTTGGGTCTCCC
D33A-F	CGAGACATCTTGACTGCTGTTGTCATTGTTGTG
D33A-R	ACTCCAGAGACGATTAAGGTTGAGAAGAAC
R44A-F	ACGCGTGAGCAGTTTGACAGCCATAAAACGGTC
R44A-R	CACAACAATGACAACATCAGTCAAGATGTC
D33R/R44D-F	AGCCGTGAGCAGTTTGATGCCCATAAAACGGTC
D33R/R44D-R	CACAACAATGACAACACGAGTCAAGATGTCTCG
QQYQ-F	CCCAAAGCCTGCAACTGGCAGCAATACCAGTTCATCGTGCTCAACAGC
QQYQ-R	GTCAGTGGGGCTCTTGGCTGGAGGGGAGCC
ΔETO-F	ACTGGTGAGAAGCCCTATCCCTGTGAAATCTG
ΔETO-R	GGGCTTCTCTCCAGAGTGAATTCGAGTGTG

2.5.3 PCR assays

Analysis of genetic manipulations in DG75-354 was carried out by PCR analyses. PCR assays for the following modifications were developed:

Targeting of the *BCL6* locus

Two PCR assays were used to screen for targeted disruption of the *BCL6* locus, a 'right homology PCR screen' flanks the right arm of homology of pTarg-*BCL6* (see CHAPTER 3 for details) and a 'left homology PCR screen' flanks the left arm of homology of pTarg-*BCL6* (see CHAPTER 3 and CHAPTER 5).

Cre-recombination of the resistance cassette

Two PCR assays were used to confirm excision of the *zeo* resistance cassette by Cre-mediated recombination at the first and second *BCL6* allele targeted, respectively (see sections 3.2.6 p.85 and 5.2.3 p.109). An additional PCR assay was designed to detect the circular Cre-recombination product (circular pop-out intermediate, see sections 5.2.3 and Figure 5.6).

Taq polymerase-based (Qiagen) assays were performed on whole cell lysates. Cells were collected by centrifugation and resuspended in 25 µl colony PCR lysis buffer supplemented with 30 µg/ml proteinase K (Qiagen). Reactions were incubated at 55 °C for 1 hr. Proteinase K was then heat inactivated (15 mins at 98°C) and the remaining PCR components were added. The PCR components (25 µl) were: Taq DNA polymerase (0.1-0.2 U/µl), 1 x PCR Buffer (Qiagen), primers (1 µM, Invitrogen or Sigma) and dNTPs (200 µM each, NEB). Primer sequences and PCR conditions are listed in Table 3, a typical cycling program was:

step	temperature	time
initial denaturation	94 °C	3:00
denaturation	94 °C	0:10
annealing	55-63 °C	0:30
extension	68-72 °C	1:00-6:00
final extension	68-72 °C	10:00

40-45 cycles

Table 3: PCR assay list, primer specifications and PCR conditions

PCR assay name	primer name and sequence (5'-3')	(1) annealing (2) extension (3) cycle number (4) product size
Gene targeting: Right-Homology Screen	Right-homology-assay-F (RHAF)	(1) 55 °C
	CTC GTT TAG TGA ACC GTC AG	(2) 5 mins at 68 °C
	Right-homology-assay-R (RHAR)	(3) 45
	CAC ACC TCT AAT AGG CTC AG	(4) 5293 bp
Gene targeting: Left-Homology Screen	Left-homology-assay-F (LHAF)	(1) 63 °C
	ATC CCA ACC GTC TCT TTC AC	(2) 6 mins at 68 °C
	Left-homology-assay-R (LHAR)	(3) 40
	CTG ACG GTT CAC TAA ACG AG	(4) 5940 bp
Cre recombination: 1 st <i>BCL6</i> allele	Cre-assay-F	(1) 55 °C
	ACG GTG TCT CTC CAT TG	(2) 2 mins at 72 °C
	Cre-assay-R	(3) 40
	CTA TGC CCA CAG AAC GTG	(4) 1967 bp
Cre recombination: 2 nd <i>BCL6</i> allele	2 nd -Cre-F	(1) 55 °C
	CTC GTT TAG TGA ACC GTC AG	(2) 1 min at 72 °C
	2 nd -Cre-R	(3) 40
	CTA TGC CCA CAG AAC GTG	(4) 925 bp
Cre recombination: (Detection of circular recombination product)	Cre-Circular-F	(1) 67.5 °C
	TTC GTG GCC GAG GAG CAG GA	(2) 1 min at 72 °C
	Cre-Circular-R	(3) 40
	GAG GCT GGA TCG GTC CCG GT	(4) 745 bp

2.5.4 Semiquantitative RT-PCR analysis

Total RNA was extracted as described in section 2.2.2 (p.48) and reverse transcribed using the ProtoScript® M-MuLV first strand cDNA synthesis kit (NEB). Serial dilutions of 1, 1:5, 1:25, 1:125 were prepared from cDNA generated from 1 µg total RNA in 50 µl. One µl of each dilution was used as template in a 25 µl PCR reaction. PCR reactions were set up with TopTaq™ PCR master mix (Qiagen) and a final primer concentration of 0.2 µM. Primers

used for characterisation of *BCL6*^{-/-}*tetBCL6-HA* cells in CHAPTER 6 (Table 4 below) and for validation of microarray data (Table 7, p. 68) are listed. A typical cycling program was:

step	temperature	time	
initial denaturation	94 °C	3:00	
denaturation	94 °C	0:30	
annealing	50-60 °C	0:30	30 cycles
extension	72 °C	1:00	
final extension	72 °C	5:00	

Table 4: Sq RT-PCR primers used in CHAPTER 6

gene	Primer sequence (5'-3')	(1) reference (2) annealing (3) product size
<i>PRDM1</i> (Blimp-1)	TGGACATGGAGGATGCGGATATG AGGTCCTTTCCCTTTGGACGGGTTG	(1) (Teng et al., 2007) (2) 60 °C (3) 762 bp
<i>PAX5</i>	AGCGTCAGTTCCATCAACAG GCCAGCGAGGCCATGGCTGA	(1) (Teng et al., 2007) (2) 60 °C (3) 422 bp
<i>B2M</i>	AGATATGCCTGCCGTGTG AATCCAAATGCGGCATCT	(1) (Ullmannova et al., 2003) (2) 55 °C (3) 110 bp
<i>MKI67</i> (Ki-67)	GGAGGCAATATTACATAATTTCA CAGGGTCAGAAGAGAAGCTA	(1) (Ullmannova et al., 2003) (2) 60 °C (3) 233 bp
<i>CCND3</i> (cyclin D3)	CCAGGAAATCATGTGCAATC CTGGCCATGAACTACCTGGA	(1) (Ullmannova et al., 2003) (2) 50 °C (3) 247 bp
<i>CDKN1A</i> (p21 ^{cip1} /waf1) <i>CDKN1B</i> (p27 ^{kip1})	CCTCTTCGGCCCGGTGGAC (F) CCGTTTTTCGACCCTGAGAG (p21R) TCTGCTCCACAGAACCGGC (p27R)	(1) (Ullmannova et al., 2003) (2) 57 °C (3) 367 bp (p21), 467 bp (p27)
<i>MYC</i>	TACCCTCTCAACGACAGCAG TCTTGACATTCTCCTCGGTG	(1) (Liao et al., 2007) (2) 55 °C (3) 478 bp

2.6 Southern blotting

SphI-digested gDNA (3 µg per 1 cm comb width) was separated on a 0.8 % agarose gel for 16 hours at 35-40 V, alongside 0.5 µl of radiolabelled marker mixed with 1 µg of cold marker (see also section 2.4.8 p. 56). The gel was subsequently rocked in denaturation solution 3 times for 15 mins, neutralised

for the same time and rinsed in water prior to transfer (solutions are listed in section 2.9). The transfer stack was assembled as follows: 3mm paper (Whatman) was placed on a plate with two opposing 3mm paper-ends reaching into a reservoir filled with 10x SSC (sodium chloride sodium citrate). The gel was placed on top, followed by a positively charged nylon membrane (HybondTM-N⁺, Amersham), two 3mm paper sheets, a stack of paper towels (of approximately 20 cm in height) and a weight of 500 g. The transfer was left for at least 16 hrs before the membrane was washed in 2x SSC and backed for 2 hours at 80 °C. The membrane was pre-hybridised with 15 ml Church mix at 65 °C in a glass bottle for 2 hrs. Subsequently, the radiolabelled probe was denatured for 5 minutes at 100 °C and added to the membrane in Church mix. After, hybridisation for 16-18 hrs at 65 °C, the probe was discarded and the bottle was rinsed with 50 ml of 2x SSC. The membrane was washed in 2x SSC and in 1x SSC for 15 minutes each at 65 °C, drained, wrapped in Saran wrap (Dow) and exposed to Hyperfilm MP (GE Healthcare) between two intensifying screens, at -80 °C for 48 hours to 7 days.

2.7 Protein analysis

2.7.1 Sample preparation and quantification

Cells were washed in PBS and pellets were lysed in 40-100 µl radioimmunoprecipitation (RIPA) buffer with 2% (v/v) non-specific protease inhibitors (Sigma). The lysate was incubated on ice for 30 mins and freed of debris by centrifugation at 4 °C for 20 mins at 15k x g. Protein concentrations were then determined by bicinchoninic acid assay (Sigma), according to the manufacturer's instructions.

2.7.2 Immunoblotting

After boiling in protein loading buffer, 20-100 µg of total protein was size fractionated on 7.5-15 % acrylamide gels at 100 V, using the Mini-Protean electrophoresis cell (BioRad). Molecular weight values were estimated using

prestained protein markers (Full Range Rainbow Marker (GE Healthcare) or ColorPlus Prestained Protein Ladder (NEB)). Transfer of proteins onto Immobilon-P polyvinylidene difluoride membrane (Sigma) was performed using a Trans-Blot SD semi-dry transfer cell (BioRad) according to the manufacturer's instructions. Membranes were incubated in blocking solution (5 % w/v marvel in tris-buffered saline/Tween20 (TBS/T)) for 1 hr at room temperature with shaking, washed in TBS/T and incubated in primary antibody in blocking solution (5 % bovine serum albumin (BSA, Sigma) was used instead of marvel where indicated) for 16 hours at 4 °C. After three washes of 10 minutes in TBS/T, blots were incubated with horseradish peroxidase (HRP)-coupled secondary antibodies with shaking, in blocking solution for 1 hr at room temperature. Table 5 and Table 6 list all antibodies and corresponding dilutions used in this project. Membranes were washed (3x 10 mins) and detected with ECL Blotting reagent (GE Healthcare) and X-OMAT LS-1 film (Kodak). As loading control, membranes were reprobbed with anti-GAPDH antibody or stained in amido black. Briefly, membranes were incubated in amido black for 15 mins, rinsed with water and destained in amido destain for 30 minutes.

Table 5: Primary antibodies used throughout this project

target	MW (kDa)	company	reference number	species	dilution
Bcl6 (aa 3-484)	87-98	Santa Cruz	I2607	rabbit	1:250
Bcl6 (C-terminus)	87-98	Cell Signaling	4242	rabbit	1:1k (5% BSA)
Blimp-1	95-100	Cell Signaling	9115	rabbit	1:1k (5% BSA)
Caspase-3	17/19/35	Cell Signaling	9662	rabbit	1:1k
CDK4	30	Cell Signaling	2906	mouse	1:1k
CDK6	36	Cell Signaling	3136	mouse	1:1k
c-Myc	57-70	Cell Signaling	9402	rabbit	1:1k (5% BSA)
Cyclin D3	31	Cell Signaling	2936	mouse	1:2k
Cyclin E	42/50	Santa Cruz	247	mouse	1:200
FLAG-tag	-	Cell Signaling	2368	rabbit	1:1k
GAPDH	37	Cell Signaling	14C10	rabbit	1:5k
HA tag	-	Cell Signaling	6E2	mouse	1:1k
LC3-B	16/18	Sigma	L7543	rabbit	1:1k
p15 INK4B	15	Cell Signaling	4822	rabbit	1:1k (5% BSA)
p21 Waf1/Cip1	21	Cell Signaling	2946	mouse	1:1k
p27kip1	27	Cell Signaling	2552	rabbit	1:1k (5% BSA)

Table 6: Secondary antibodies used throughout this project

target	company	reference number	species	dilution
mouse	Dako	P0447	goat (HRP-coupled)	1:1k
mouse	Cell Signaling	7076	goat (HRP-coupled)	1:2k
rabbit	Cell Signaling	7075	goat (HRP-coupled)	1:2k

2.8 cDNA microarray analysis

Total RNA was extracted using RNeasy minipreps (Qiagen) (see section 2.2.2 above) and concentration and quality were checked on the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) and the RNA Nano 6000 kit (Agilent Technologies) on a 2100 Bioanalyzer (Agilent Technologies). One hundred ng of total RNA was processed with the GeneChip® Eukaryotic Whole Transcript Sense Target Labelling Assay kit (Affymetrix) according to the manufacturer's details (see Figure 7.1 p.153 for a schematic representation of the steps involved). Hybridisation and scanning of GeneChips® was performed by Nathalie Lambie (CSC/IC Microarray Centre, MRC Clinical Sciences Centre Imperial College London). Data analysis was performed by Geraint Barton (Bioinformatics Support Service, Imperial College London). Briefly, pre-processing of data was performed using GeneSpring GX 10.0.2 software (Agilent Technologies) which applied the 'Exon RMA16' algorithm to the data set. Exon RMA16 performs background correction, quantile normalisation, median polish summarisation and variance stabilisation of 16.

In background correction, intensity values of each individual array are corrected for non-specific binding by subtracting the average signal intensity of the area between spots from each probe set. Normalisation is required so multiple chips can be compared to each other. Quantile normalisation adjusts the distribution of probe intensity of each array analysed and so that the distribution of probe intensities for each array in a set of arrays is the same (Bolstad et al., 2003). Probe summarisation refers to the conversion of probe level values (there

are approximately 26 probes per gene on each GeneChip®) to a single probe set expression value (Irizarry et al., 2003). Variance stabilisation of 16 refers to the addition of the value 16 to the expression values. By increasing the expression value, the variance of the data set is reduced and the distribution (defined by its mean and its variance) is stabilised (Huber et al., 2002).

Microarray data were validated by sq RT-PCR of 23 genes, as described in section 2.5.4 (p.62), using the primer set listed in Table 7 below and the following cycling program:

step	temperature	time	
initial denaturation	94 °C	3:00	
denaturation	94 °C	0:30	
annealing	60 °C	0:30	30 cycles
extension	72 °C	1:00	
final extension	72 °C	5:00	

Table 7: Primers used for validation of microarray results (CHAPTER 7)

Primer3-designed primers were located in different exons (Rozen and Skaletsky, 2000).

gene	primer sequence (5' to 3')	product size in bp
ARHGAP15	TGCCTGAGCCGCTCTTCCCT AGGCATCTGTCGCTTGAAAGGCA	455
BLNK	ACAGCTACGAGCCGCCTCCA CCTCGGTGGCGTTCAGCAGG	499 568
CAMK4	CCCCAGGGTACTGCGCACCT CCATGGCTGCTGCTGGCACT	430
CDC6	GGGTGAAGGCTGCGGGTTCC CCCTTTCCCTGGCAGGCAGC	578
CDKN1B	TCGGACAGCCAGACGGGGTT CCCTGCCCTCCCTTCCCCAA	495
CYSLTR1	TGTGCCCTGCTCTCTCCCCT GGTGCTGAGGCGGCACAAGA	658
E2F8	GTGCGCTCAGCTGGGACCTG GCTGTCGGTGTCCACGGCTC	350
EIF2AK3	CTGCGGCCGAGGTGACTGTG CCACTGCGAGGTCCGACAGC	562
FOXP1	CGGGCGGCAGCAACCACTTA GGCTGCCCGGGCTGAATTGT	577/578
GPRIN3	GATGGGTACACCCCGGGCCT GGGAGGGGAGCGCAGTCAGA	415
IFI44	GCGGCCTGTGCAGGGATGAC ACAGGGTCCAGCTCCCACTCA	424
IFI44L	TGGCCAAGCCGTAGTGGGGT CCCGCAGCATCTGCCTCAGTG	605
IKZF1	AAAGCATGGGCCTTCCGGGC TAGGCGCGGTGCTCCTCCTT	622
IRF9	AGAGATCAGCCGCCAGCCA GCAACATCCATGCGGCCCT	390
LYAR	GGCGCTGGCTGAGAGGCAAT CCCCTTGCTGTTCCACGGCG	669
PIK3IP1	TCTGGGCTACGTGCTGGGCA CTCAGCCACAGGGCCACCT	456
PLEK	TGACCCTGCTGGGGCAGAAGA ACCCCTGGGCTGTTTCCCGT	325
RB1	CCGGAGGACCTGCCTCTCGT CCTCGCCTGGGTGTTTCGAGG	653
SESN1	TGCTGCTTTGGGCCGTTTGGA CGCAAGGGACCAGCTGTGCT	367
STAT1	ACCAATGGCAGTCTGGCGGC GGGCACGCTCTCGCTCCTTG	583
STAT2	TCTGCCGGGACATTCAGCCCT GCGTTGCGTAGGTCCACCCC	637
SYK	CGGCTGGAGAGCGAGGAGGA GGCCCAGTCTTGGGCTGCAC	462
TAP1	TGCTGGAAGTGCAAGGTGCGG CGAGGGGTGCGGTCCAGGTA	409

2.9 Commonly used solutions

All chemicals were from BDH unless otherwise stated.

Standard solutions

50 x TAE	2 M Tris, 50 mM EDTA, 57.1 % (v/v) glacial acetic acid
DNA loading dye (10x)	0.5 % (w/v) bromophenol blue, 0.5 % (w/v) Orange G (Sigma), 60 % glycerol
TE	10 mM Tris-HCl, 10 mM EDTA, pH 7-8

Solutions for bacteria work

LB medium	10 % (w/v) bacto-tryptone, 5 % (w/v) bacto-yeast extract, 170 mM NaCl, pH 7.0
LB agar	15 % (w/v) agarose in LB medium
low salt LB agar	15 % (w/v) agarose in low salt LB medium
low salt LB medium	10 % (w/v) bacto-tryptone, 5 % (w/v) bacto-yeast extract, 85 mM NaCl, pH 7.0
SOC medium	20 % (w/v) bacto-tryptone, 5 % bacto-yeast extract, 9 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 20 mM glucose, pH 7.0

Solutions for cell culture

freezing medium	10 % DMSO (v/v) in FBS
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Solutions for Southern blotting

Church mix	0.4 M Na ₂ HPO ₄ , 0.1 M NaH ₂ PO ₄ , 7 % (w/v) sodium dodecyl sulfate (SDS), 1 % (w/v) BSA
denaturation solution	1.5 M NaCl, 0.5 M NaOH
neutralisation solution	1 M Tris-HCl, 1.5 M NaCl, pH 7.4
SSC (20 x)	3 M NaCl, 300 mM sodium citrate, pH 7.0

Solutions for protein analysis

20 x tris-buffered saline (TBS)	400 mM Tris-HCl, 2.76 M NaCl, pH 7.4
3 x protein loading buffer	34 mM Tris-HCl pH 6.8, 5.7 % (w/v) SDS, 15 % (v/v) β -mercaptoethanol, 28 % (v/v) glycerol, 0.06 % (w/v) bromophenol blue
5 x running buffer	250 mM Tris-base, 2M glycine, 17 mM SDS
amido destain	25 % Isopropanol, 10 % acetic acid
amido staining	1 % amido black (Merck) in H ₂ O
RIPA	150 mM NaCl, 1 % NP-40, 0.5 % deoxycholic acid, 0.1 % SDS, 50 mM Tris-HCl pH 8
separating gel (7.5 %)	375 mM Tris-HCl pH 8.8, 7.5 % (v/v) acrylamide, 3.5 mM SDS, 0.33 % (v/v) TEMED, 2.2 mM ammonium persulfate
stacking gel	375 mM Tris-HCl pH 6.8, 4.5 % (v/v) acrylamide, 3.5 mM SDS, 0.4 % (v/v) TEMED, 2.6 mM ammonium persulfate
TBS/T	0.1 % (v/v) Tween20 in 1xTBS
transfer buffer	200 mM glycine, 25 mM Tris, 20 % (v/v) methanol

Other solutions

colony PCR lysis buffer	0.045 % NP40, 0.045 % Tween20, 320 μ g/ml proteinase K (Qiagen) in 1x PCR buffer (Taq, Qiagen)
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CHAPTER 3 – Targeting of the first *BCL6* allele

3.1 Introduction

Homologous recombination is a naturally occurring process, which has been adapted by scientists to artificially modify genes in cell lines as well as whole organisms (Capecchi, 1989). Gene targeting by homologous recombination has led to ground-breaking discoveries in Biology and Medicine and a Nobel Prize was awarded to Mario R. Capecchi, Martin J. Evans and Oliver Smithies in 2007 for their outstanding contributions to this field (Mak, 2007).

The human GC B cell line DG75-354 cell line was chosen as the parental cell line for the conditional *BCL6* knockout. DG75-354 is a DG75-derivative, which stably expresses the tet-transactivator and was kindly supplied by Dr B. Henglein (Institut Curie, Paris) (see also section 4.2.1 p.93). DG75 is a Burkitt's lymphoma cell line (Ben-Bassat et al., 1977) that has previously been successfully used for targeted gene disruption (Feederle et al., 2004, Maier et al., 2005) and it was also observed in our lab that the ratio of targeted over random integration is remarkably high in these cells (R. Vangala and A. Porter, unpublished results).

3.2 Results

3.2.1 Design and cloning of the *BCL6* targeting construct

The *BCL6* gene has 10 exons, spread over a 26 kb genomic locus on chromosome 3 (band 3q27) with the start codon located in exon 3 (Kerckaert et al., 1993, Miki et al., 1994). The *BCL6* gene encodes at least three transcripts: one full length transcript, one that excludes exon 1b (Kawamata et al., 1994a) and one that encodes a smaller Bcl6 isoform, in which the first two zinc fingers (exon 7) are absent (Shen et al., 2008). I planned to disrupt exon 3, which contains the translation start site of *BCL6*. Figure 3.1 shows part of the *BCL6* locus and the gene targeting strategy designed. The targeting construct contains a *zeocin* (*zeo*) resistance cassette that is 'floxed', i.e. flanked, by *loxP* (locus of crossover) sites that serve as recognition sites for Cre (causes recombination) recombinase. This *floxed* cassette is followed by a downstream *SphI* restriction site and is flanked by arms of homology that target the construct to the *BCL6* gene, for disruption of exon 3. Furthermore, the targeting construct contains a single *KpnI* restriction site which facilitates linearisation prior to gene targeting. Following transformation of DG75-354 cells with the targeting construct and subsequent selection in *zeo*; targeted integration events are detected by PCR screening and confirmed by Southern blotting (see sections 3.2.2, 3.2.3, 3.2.4 and 3.2.5 below).

Gene disruption is targeted to the 11,763 bp genomic *SphI* fragment, which flanks exon 1b and finishes in exon 6 of *BCL6*. The large *SphI* fragment was analysed with the repeat masking program 'Censor' (Kohany et al., 2006) and it was found that ~11 % of the locus were made up of repetitive sequences (data not shown). Many of these repetitive elements interfered with primer design for amplification of the arms of homology and it was therefore decided to amplify and clone a larger genomic fragment, for which primer-design was easier, due to the absence of repetitive elements.

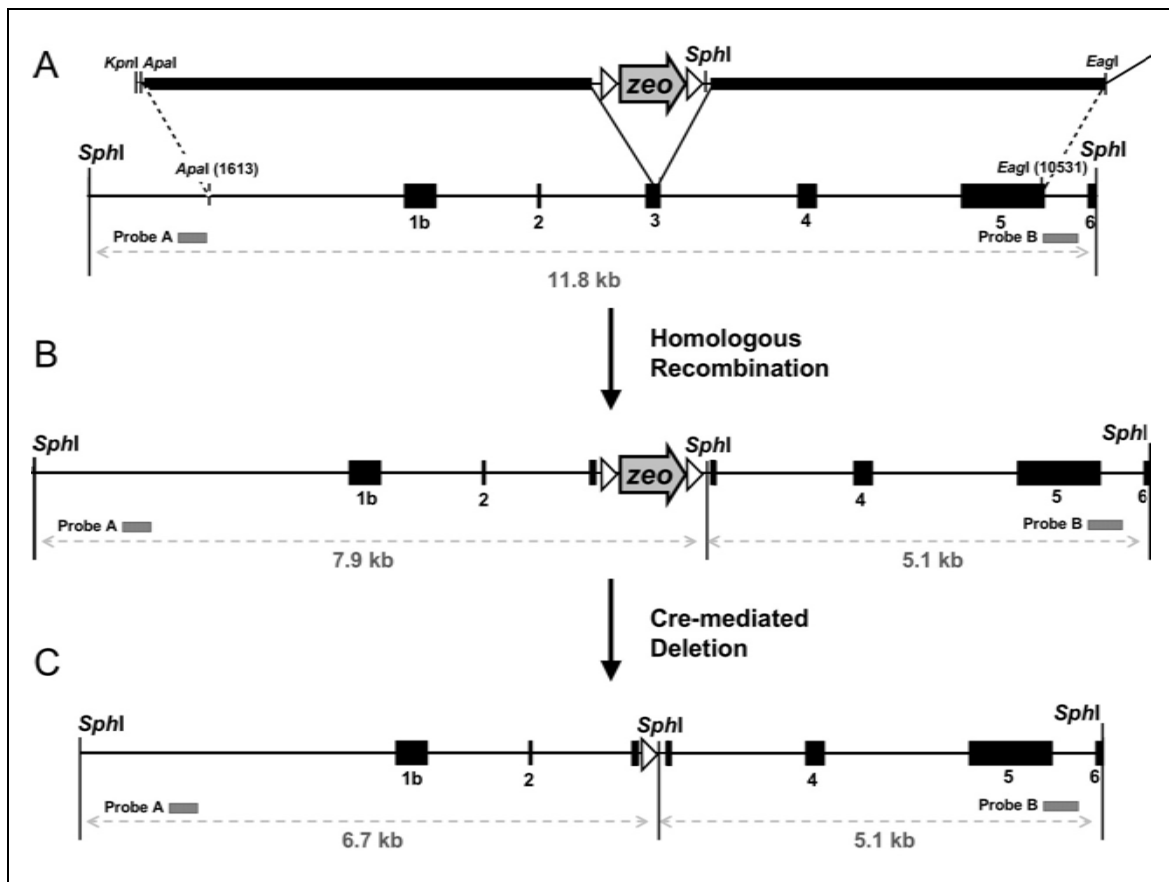


Figure 3.1: *BCL6* gene targeting strategy

A) The horizontal thin line represents part of the *BCL6* locus and selected restriction enzyme sites (*SphI*, *KpnI*, *Apal*, and *EagI*) are highlighted. Black boxes represent exons 1b-6. The targeting construct is shown above the *BCL6* locus and it contains a *zeocin* (*zeo*) resistance cassette flanked by *loxP* sites (triangles) and arms of homology (thick black line). A *SphI* site is located downstream of the second *loxP* site. Black dotted lines show the alignment of the targeting construct with homologous regions in the *BCL6* locus. Probe A and probe B represent sites outside the region of homology that can be used as probes for Southern blotting. Both probes (A+B) detect an 11.9 kb *SphI*-fragment (grey dotted line) at the native *BCL6* locus. B) After homologous recombination of the *BCL6* locus with the targeting construct, the *zeo* resistance cassette has disrupted exon 3. Probe A will detect a 7.9 kb *SphI*-fragment and probe B a 5.1 kb *SphI*-fragment by southern blotting. C) Cre recombination excises the *zeo* cassette and allows the targeting construct to be reused for targeting of the second *BCL6* allele. After Cre-mediated recombination, the fragment detected by Southern blotting with probe A will decrease from 7.9 kb to 6.7 kb.

The arms of homology for the targeting construct were subcloned as a smaller fragment derived from this initial big construct. The larger (~10 kb) region was amplified from genomic DNA (see section 2.5.2 p.57). This 9,992 bp *KpnI/SacI* fragment was ligated into pBluescript KS+ (Stratagene), resulting in vector p10kb-homology. The 8,918 bp *Apal/EagI* fragment of p10kb-homology was subcloned from p10kb-homology into pBluescript KS+, generating vector p8.9kb-homology. For disruption of the *BCL6* allele, a *zeo* cassette was inserted into the *SbfI* site of p8.9kb-homology (end of exon 3). This 1,245 bp *zeo* cassette (including its own promoter) was amplified from pCMV/*Zeo* using primers that added *loxP* sites to both ends of the *zeo* cassette, an *SphI* restriction site to its 3' end and *SbfI* restriction sites on both ends for subcloning (see section 2.5.2 p.58). The *SbfI*-digested PCR product was cloned into the *SbfI* site of p8.9kb-homology to generate the final targeting construct pTarg-*BCL6*. The orientation of the *zeo* cassette was verified by restriction enzyme digestion and the region surrounding the 5' *loxP* site was sequenced. Figure 3.2 shows the disruption of the *BCL6* locus at the nucleotide level before and after homologous recombination of the *BCL6* locus with pTarg-*BCL6*. After targeting of the first *BCL6* allele, cells can be transiently transfected with Cre-recombinase (pMC-Cre kindly supplied by H. Gu, University of Köln), which will excise the region between the *loxP* sites. The premature stop codon generated by targeted *BCL6* gene disruption is likely to result in the mRNA being degraded by nonsense-mediated decay (NMD) (Byers, 2002). The potential translation of the message would result in a 55 aa truncated protein which is expected to be non-functional and quickly degraded. Once *zeo* resistance is lost, the same targeting construct will be used to knockout the second *BCL6* allele.

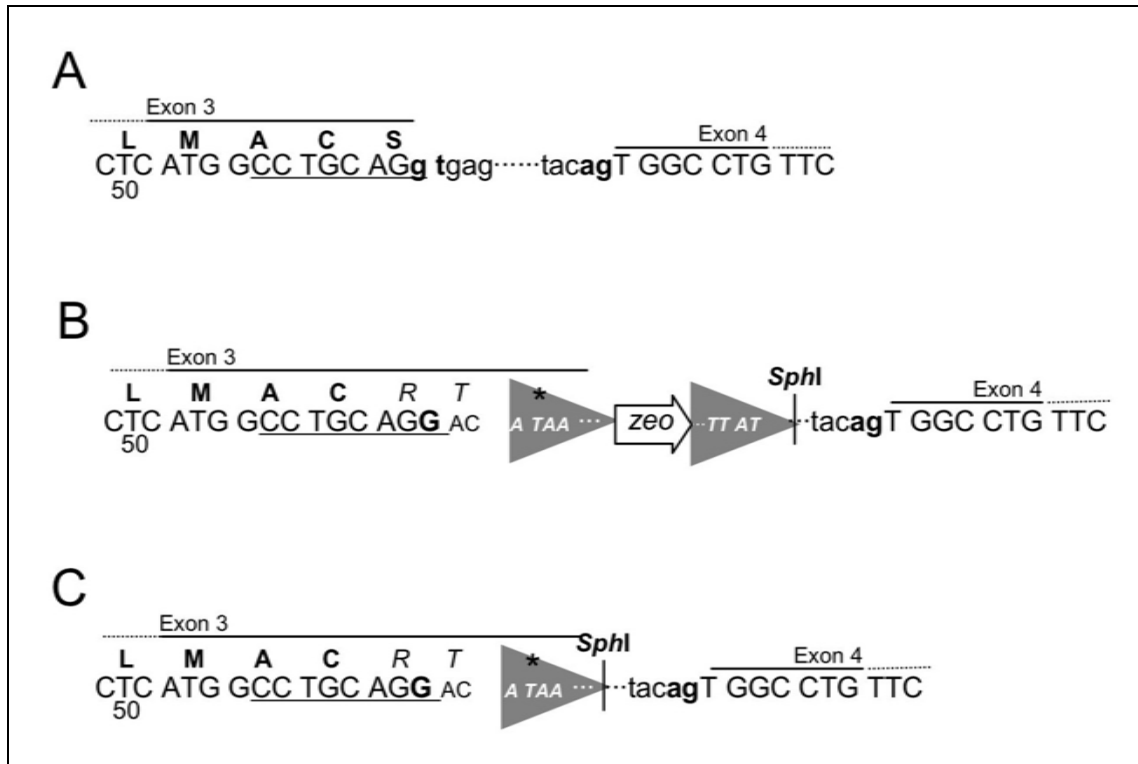


Figure 3.2: Disruption of the *BCL6* gene and marker-recycling at the nucleotide level

A) Exon 3 of wildtype *BCL6* codes for 54 amino acids. Underlined is a genomic *SbfI* restriction site used for insertion of the resistance cassette. Exons are shown in upper case, intronic sequences in lower case. Splice donor (gt) and splice acceptor sites (ag) are depicted in bold letters. B) Successful targeting of the *BCL6* locus inserts the *zeo* cassette into the genomic *SbfI* site. The disruption cassette contains 2 additional nucleotides (AC) followed by a 'floxed' (grey triangle) *zeo* expression cassette and an *SphI* restriction site. Integration of the cassette into the locus destroys the splice donor signal (gt, bold), the additional two nucleotides (AC) disrupt the *BCL6* open reading frame and induce a premature stop codon (*TAA) encoded by the *loxP* site. C) After marker recycling, i.e. deletion of the *zeo* resistance cassette by Cre-mediated recombination, the splice signal is still disrupted and the premature stop codon still present.

3.2.2 Design of PCR assay ‘Right-Homology’ to screen for successful targeting of the *BCL6* locus

When the targeting construct pTarg-*BCL6* integrates into the genome of DG75 it can either do so by homologous recombination, leading to knockout of *BCL6*, or by random integration. In both situations, the resulting clones are resistant to *zeo* because the *zeo* cassette contains its own promoter. Hence, a PCR assay was established to discriminate targeted, from random integrations (see section 2.5.3 p.61). The first PCR primer, Right-Homology-Assay-F (RHAF), anneals to the *zeo* cassette and the second primer Right-Homology-Assay-R (RHAR) anneals in the *BCL6* locus, outside the region of homology of pTarg-*BCL6* (Figure 3.3A). In the event of targeted integration a 5.3 kb PCR product can be amplified. If the targeting construct integrates into the genome randomly, no PCR product can be synthesised because one primer site (RHAF) is located at the site of random integration of the targeting construct (in the *zeo* cassette), not within reach of the second primer annealing site (RHAR, anneals in *BCL6* locus) which was lost in the subcloning step, from p10kb-homology to p8.9kb-homology (Figure 3.3B).

A positive control was designed to optimise PCR assay conditions and to ensure that a rare targeted event will not be missed due to insufficient sensitivity. The positive control construct mimicking successful targeting of *BCL6* was derived from p10kb-homology (parental vector of p8.9kb-homology). A unique *EcoRI* restriction site in vector p10kb-homology (3' of the genomic *SbfI* site, therefore located in the right arm of homology after insertion of the *zeo* cassette) was deleted by digestion with *EcoRI*, end-filling and re-ligation. The resulting construct (p10kb-homology^{-EcoRI}) was modified by insertion of the *zeo* cassette into its *SbfI* site, as for pTarg-*BCL6*, resulting in p10kb-homology^{-EcoRI}/*Zeo*. This construct mimics targeted gene disruption and serves as a positive control in the PCR assay because it contains both primer annealing sites. Both, construct p10kb-homology^{-EcoRI}/*Zeo* and a *BCL6* allele disrupted by pTarg-*BCL6*, can give rise to a 5.3 kb PCR product amplified by primer set

RHAF and RHAR, but only the PCR product of the latter can be cut by *EcoRI* (Figure 3.3A+C). This is important because p10kb-homology^{-*EcoRI*}/*Zeo* can potentially contaminate other samples during PCR screening and give rise to false positive PCR signals.

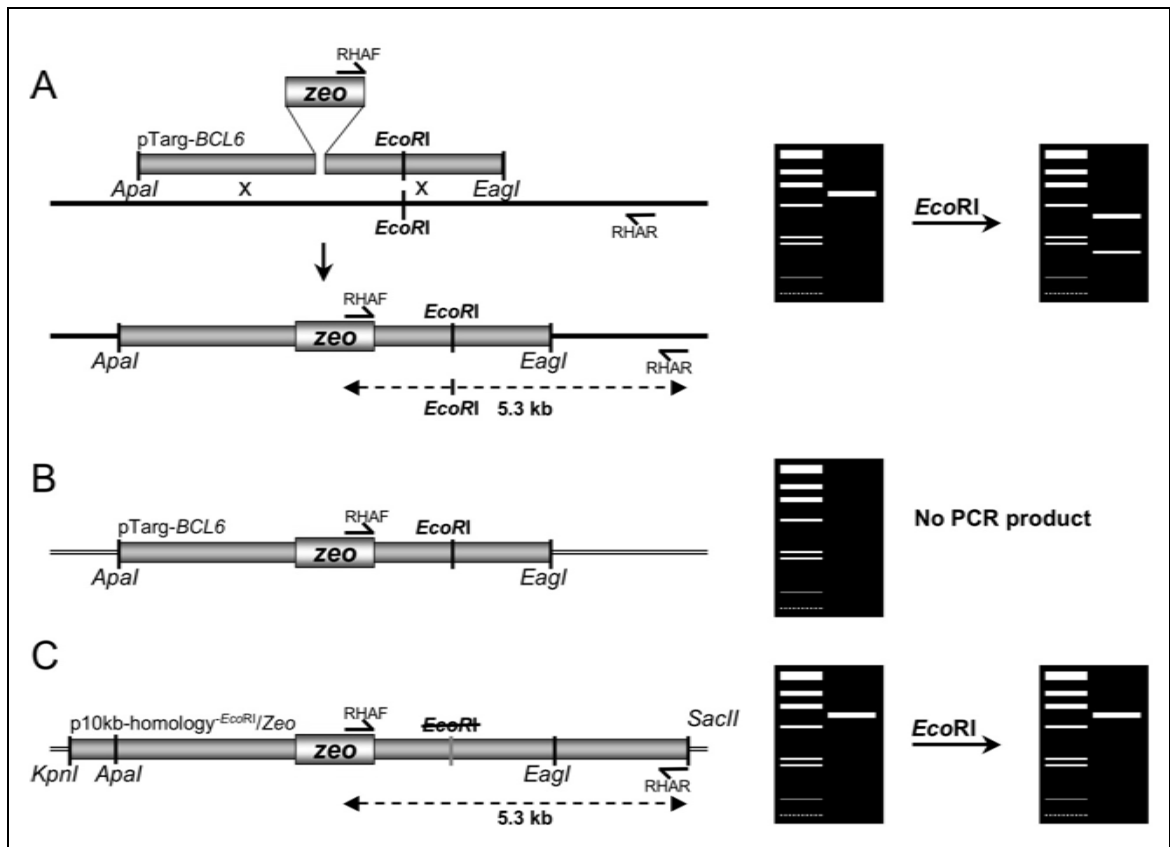


Figure 3.3: PCR Assay 'Right Homology' screens for targeted disruption of a *BCL6* allele

A) Targeting of the *BCL6* locus (black line) brings the two annealing sites of the primer set 'Right-Homology-Assay' (RHAF and RHAR) together and a 5.3 kb PCR product can be amplified. The resulting PCR product is cleavable by *EcoRI*. B) No PCR product can be synthesised if the targeting construct (pTarg-*BCL6*) integrates into a random genomic locus (black double line). pTarg-*BCL6* does not include the annealing site for the second primer (RHAR). C) Vector p10kb-homology^{-EcoRI}/Zeo includes annealing sites for both primers (RHAF+RHAR) and can give rise to a 5.3 kb PCR product. However, this PCR product is resistant to cleavage by *EcoRI*.

3.2.3 Optimisation of PCR assay ‘Right-Homology’ and screening for successful targeting of the *BCL6* locus

The sensitivity of PCR assay ‘Right-Homology’ was determined in order to screen a large number of clones for targeted disruption of *BCL6*. For this purpose DG75-354 cells were stably transfected with p10kb-homology^{-EcoRI}/*Zeo* and a single clone was isolated by plating transfected cells at limiting dilution. It was found that a robust PCR signal could be detected when 10⁴ cells were used as a template (data not shown) and this number was subsequently used to screen pTarg-*BCL6*-transfected,*zeo* resistant colonies (see also section 2.5.3 p.61).

Five *zeo* resistant clones isolated after transfection of DG75-354 with *KpnI*-linearised pTarg-*BCL6* were screened with the RHA, alongside positive (DG75-354 cells stably transfected with p10kb-homology^{-EcoRI}/*Zeo*) and negative (DG75-354 cells) controls. One clone was found to be positive and showed the expected 5.3 kb PCR product (Figure 3.4A). PCR products of the positive control and clone BA11 were gel extracted and digested with *EcoRI* and the digested products were visualised alongside the PCR products (Figure 3.4B). Only the PCR product derived from clone BA11 could be digested into the expected 3.6 kb and 1.7 kb fragments (see section 3.2.2 p.76 and Figure 3.4B). This confirmed successful disruption of the first *BCL6* allele in clone BA11 and ruled out contamination with the positive control. It was planned to test more clones in order to estimate the relative gene targeting frequency for the *BCL6* locus in DG75; however, the PCR assay suddenly stopped working and could not be optimised (see discussion in section 3.3.1 below). A new PCR assay was therefore designed to screen more clones (see section 3.2.4).

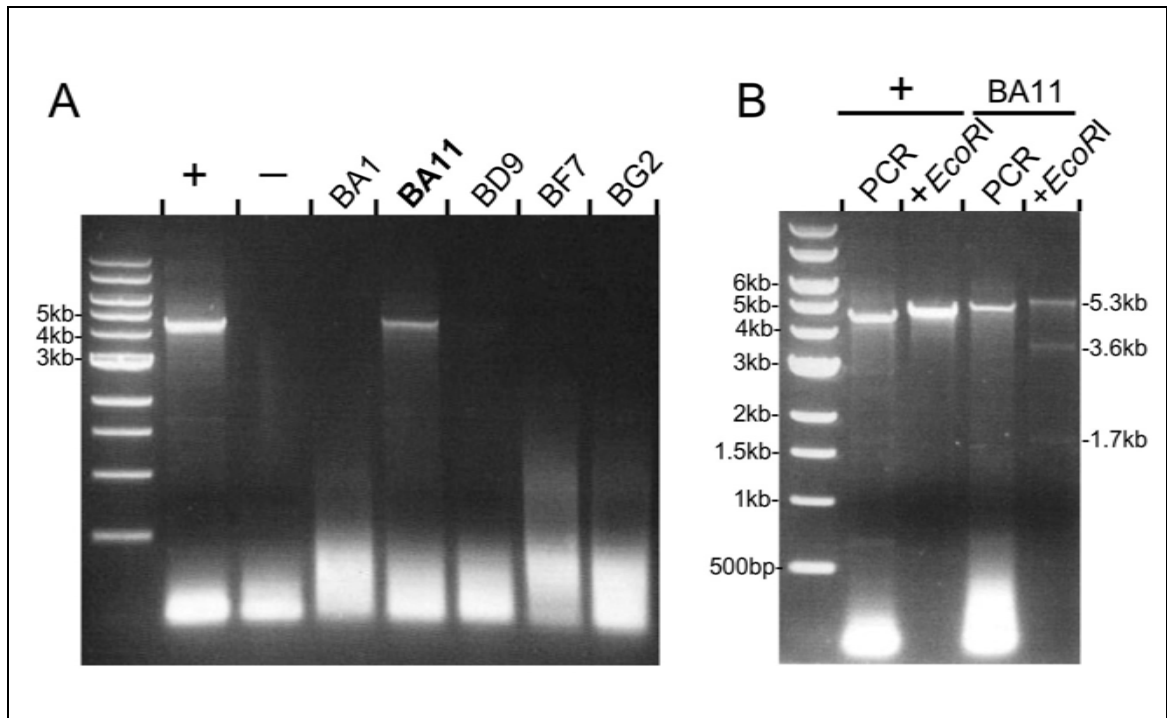


Figure 3.4: Screening of pTarg-*BCL6*-transfected clones with the 'Right Homology' PCR assay

A) Five pTarg-*BCL6*-transfected and *zeocin* resistant clones were screened by PCR assay (RHA). Cells stably transfected with p10kb-homology^{-EcoRI}/*Zeo* served as positive, non-transfected DG75-354 cells served as negative control. B) PCR products from A were gel extracted, subjected to digestion with *EcoRI* and visualised alongside undigested PCR products (see also Figure 3.3 p.78).

3.2.4 Optimisation of PCR assay ‘Left-Homology’ and screening for successful targeting of the *BCL6* locus

A new PCR assay was designed to screen more clones for successful targeting of the *BCL6* gene and to determine the targeting frequency that can be detected at this locus. The design of the new Left-Homology-Assay (LHA) is similar to the initial assay (RHA). The first primer, Left-Homology-Assay-R (LHAR) anneals to the *zeo* cassette and the second primer, Left-Homology-Assay-F (LHAF), anneals outside the region of homology of pTarg-*BCL6* but within the left arm of homology of constructs derived from p10kb-homology (Figure 3.5). Consequently, vector p10kb-homology^{-EcoRI}/*Zeo* could again be used as positive control. I decided, however, not to use this construct as positive control because there was no way to differentiate between PCR products derived from p10kb-homology^{-EcoRI}/*Zeo* and a successfully targeted clone (high risk of false positive PCR signals due to cross contamination). Instead, I chose to use successfully targeted clone BA11 (*BCL6*^{+/-}(*zeo*)) (Figure 3.4) as positive control.

The sensitivity of the LHA was determined with decreasing concentrations of BA11 cells and it was found that 10⁴ cells generated a robust signal (data not shown). Thirteen pTarg-*BCL6*-transfected and *zeo* resistant clones were screened alongside positive and negative controls and five showed the expected 5.9 kb PCR product indicative for successful targeting of the *BCL6* locus (Figure 3.5C). Another 26 clones were screened with this assay out of which 7 were found to be positive (data not shown). In summary, out of 44 *zeo*-resistant clones screened by PCR assays (sections 3.2.3 and 3.2.4), 13 had knocked out one of their *BCL6* alleles. This translated to a relative gene targeting frequency of 30 % (percentage of targeted over random integration).

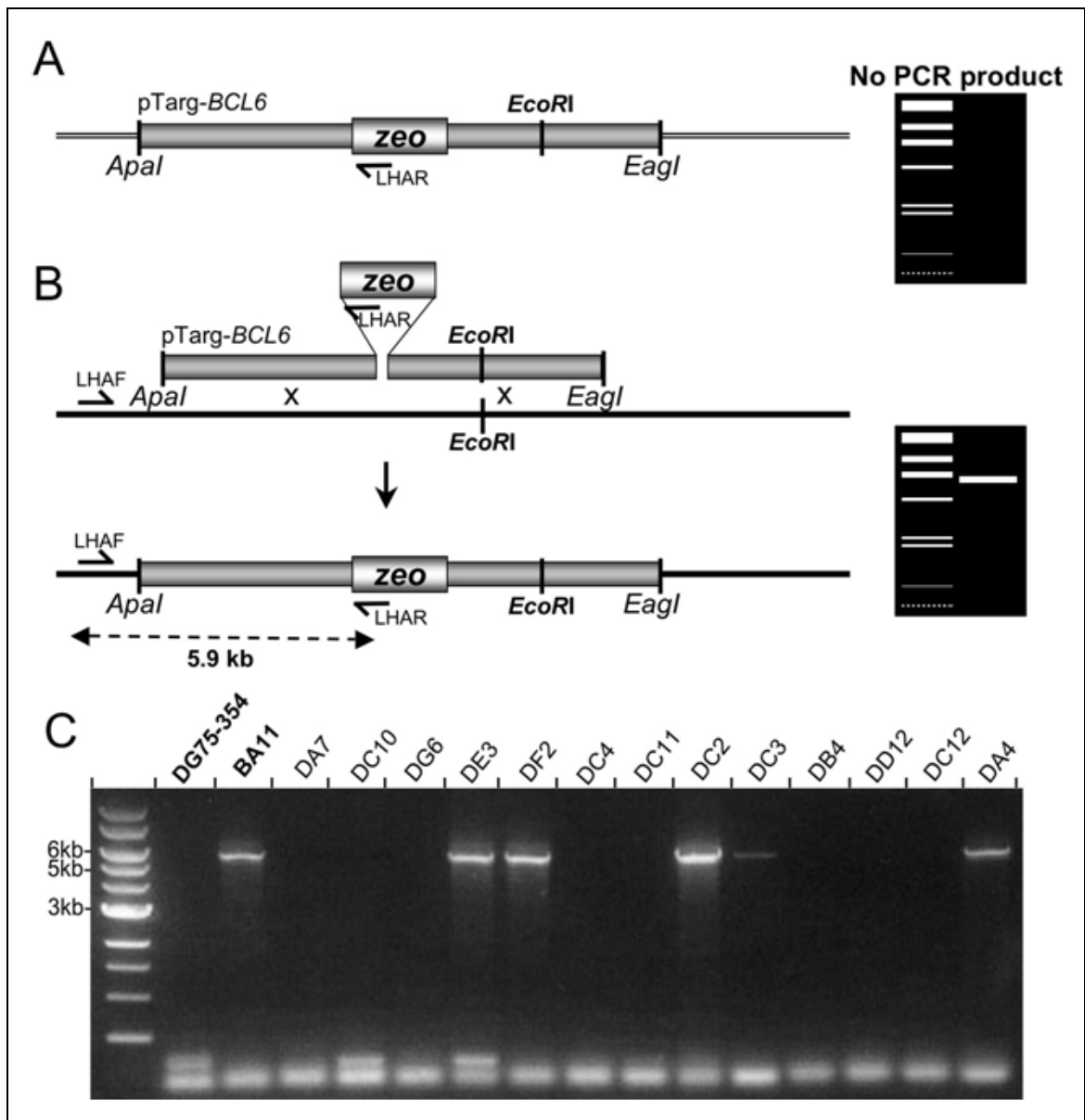


Figure 3.5: PCR Assay 'Left Homology' screens for targeted disruption of a *BCL6* allele

A) No PCR product can be synthesised if pTarg-*BCL6* integrates into a random genomic locus (black double line) because pTarg-*BCL6* does not contain the LHAF primer-annealing site. B) Successful targeting of the *BCL6* locus (black line) brings together the two annealing sites of primers LHAF and LHAR and the 5.9 kb PCR product is amplified. C) Thirteen pTarg-*BCL6*-transfected and *zeo* resistant clones were screened by PCR assay. BA11 cells (*BCL6*^{+/-}(*zeo*)) served as positive control, non-transfected DG75-354 cells served as negative control.

3.2.5 Screening for targeting of the *BCL6* locus by Southern blotting

After targeting of the first *BCL6* allele was confirmed for 13 out of 44 pTarg-*BCL6*-transfected and *zeo* resistant clones by PCR assays, 7 out of these 13 clones were expanded and tested for disruption of the *BCL6* locus by Southern blotting (see Figure A p.208 for an overview of all important clones generated in this project). Six out of 7 clones analysed by Southern blotting had successfully disrupted one of their *BCL6* alleles. Figure 3.6 shows the Southern blotting results for the targeted clone BA11 (*BCL6*^{+/-}(*zeo*)).

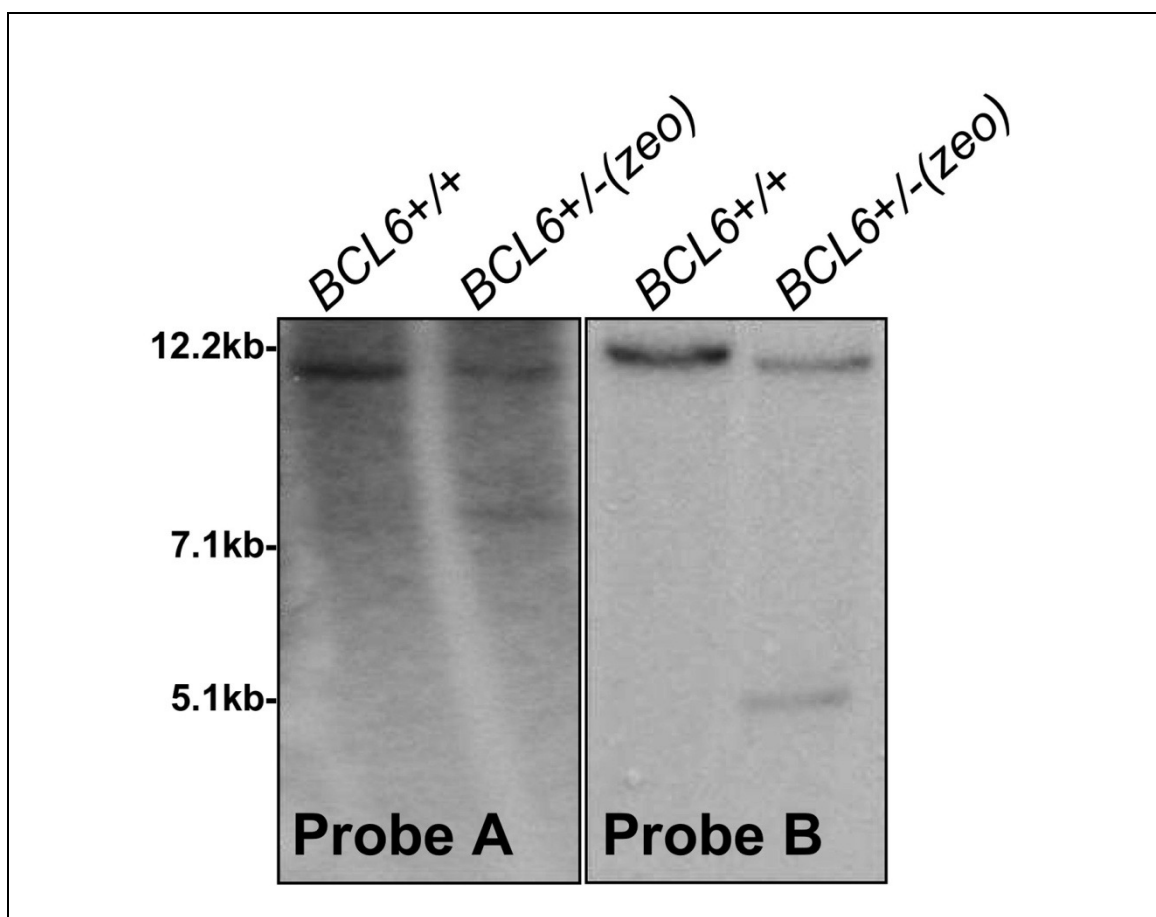


Figure 3.6: Analysis of the *BCL6* locus structure after gene targeting

SphI-digested genomic DNA of DG75-354 (*BCL6*^{+/+}) and BA11 (*BCL6*^{+/-}(*zeo*)) was probed with 2 probes. Both probes were excised from vector p10kb-homology (see section 3.2.1); probe A is a 345 bp *KpnI*/*Apal*-fragment and probe B a 730 bp *EagI*/*SacII*-fragment. The *BCL6* wildtype allele yields an 11.8 kb *SphI* fragment when detected with either probe A or B. DG75-354 has two wildtype *BCL6* alleles (*BCL6*^{+/+}). A targeted allele is detected as a 7.9 kb band when detected with probe A and a 5.1 kb band when detected with probe B. Clone BA11 carries a wildtype *BCL6* allele detected as an 11.8 kb fragment by probes A and B, as well as a targeted *BCL6* allele detected as a 7.9 kb band with probe A and a 5.1 kb band with probe B (*BCL6*^{+/-}(*zeo*)). See Figure 3.1 (p.73) for a graphic representation of this assay design and section 2.6 (p.63) for methodology.

3.2.6 Cre-mediated deletion of the *zeocin* resistance cassette

Cre-recombination after targeting of the first *BCL6* allele leaves the first *BCL6* allele disrupted and allows the targeting vector pTarg-*BCL6* to be reused for targeting of the second *BCL6* allele. Cre recombination will be detectable by Southern blotting and clones will have lost their *zeo*-resistance. In addition, a quick PCR assay was designed to screen for this event (Figure 3.7). In this PCR assay, primers flanking the *zeo* cassette can amplify three differently-sized PCR products. The wildtype *BCL6* allele gives rise to a 722 bp product that is resistant to cleavage by *SphI* (Figure 3.7A), while a targeted *BCL6* allele (that contains the *zeo* cassette) will result in a 1967 bp product (Figure 3.7B). A *BCL6* allele that was targeted by homologous recombination and modified by Cre-recombinase will produce a 773 bp PCR product that can be cleaved into two fragments of 543 and 230 bp by *SphI* (Figure 3.7C). After transient transfection of clone BA11 (*BCL6*^{+/-(zeo)}) with pMC-Cre (Gu et al., 1993) (see section 2.3.4 p.51), cells were plated at limiting dilution and 53 clones were tested for deletion of the *zeo* resistance cassette by PCR assay (Figure 3.7A-D). Two out of these 53 clones were found to have deleted the *zeo* cassette and died in *zeocin* (clones 4G12 and 7D4, *BCL6*^{+/(-)}, see also Figure A p.208). Figure 3.7D shows the PCR assay screen of clone 4G12.

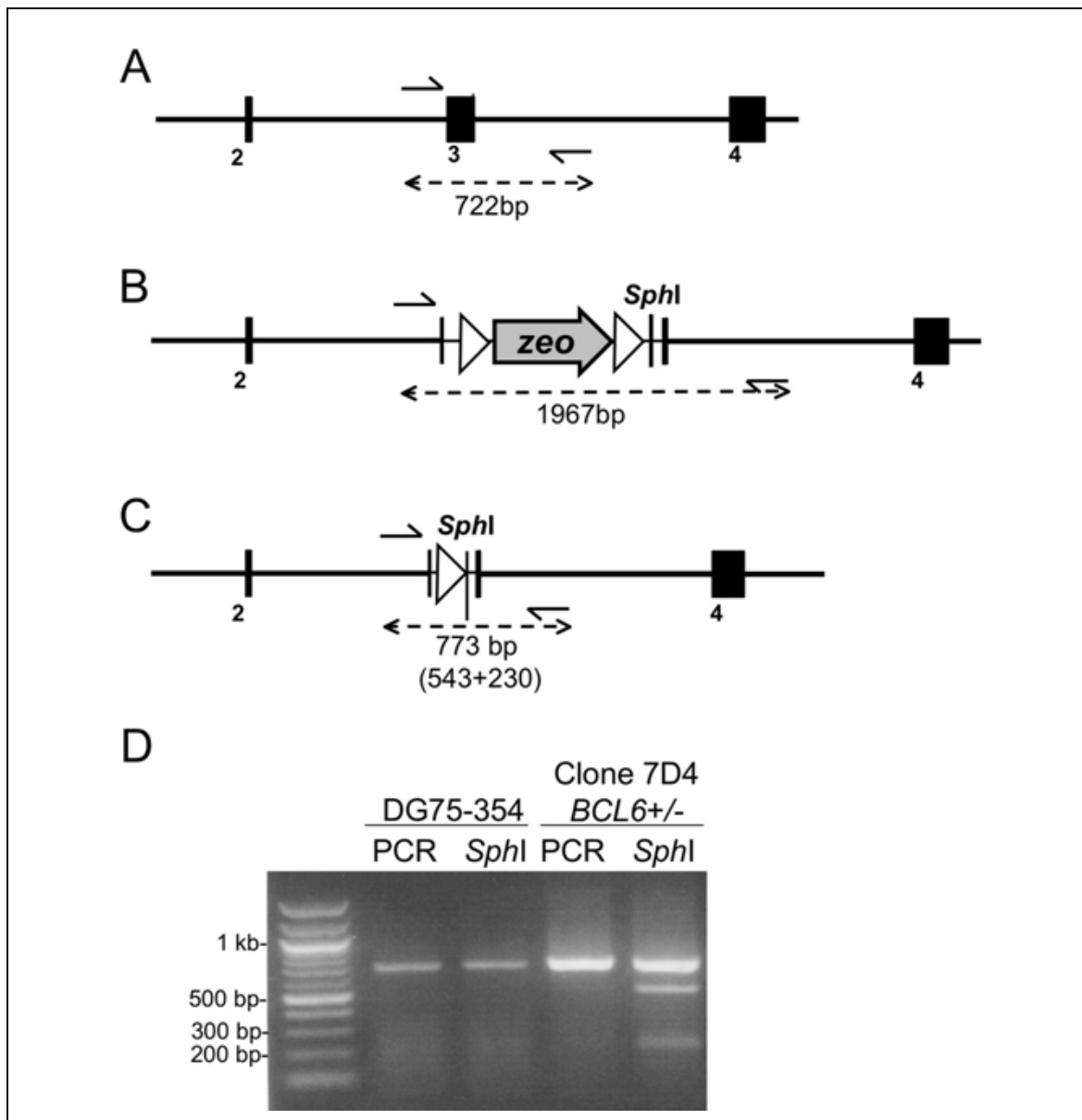


Figure 3.7: PCR assay 'Cre-recombination' screens for marker recycling

A) Cre-assay primers (see section 2.5.3 p.61) flank exon 3 of *BCL6* and amplify a 722 bp PCR product from a wildtype allele. B) A 1,967 bp product is amplified from a targeted *BCL6* allele. C) A 773 bp PCR product is amplified from a targeted *BCL6* allele that has undergone Cre-mediated recombination. This PCR product is cleavable into two fragments by *SphI* (543+230 bp). D) BA11 cells (*BCL6*^{+/-}(*zeo*)) were nucleofected with pMC-Cre and a clone (7D4) was isolated by plating at limiting dilution and tested for Cre-recombination with the PCR assay. The *BCL6* wildtype allele gives rise to a 722 bp PCR product, resistant to *SphI* digestion following gel extraction (DG75-354; *BCL6*^{+/+}). A targeted *BCL6* allele that has undergone Cre-recombination yields a 773 bp PCR product cleavable into 543 and 230 bp by *SphI* (7D4, *BCL6*^{+/-}).

3.3 Discussion

3.3.1 Disruption of the first *BCL6* allele

Gene targeting frequencies by homologous recombination are generally extremely low in mammalian cells, therefore, PCR-based methods are often used to screen for this event. For such a PCR assay, one primer is chosen that anneals to a region unique to the targeting vector and a second primer that anneals to the genomic DNA sequence of the target locus (Kim and Smithies, 1988). In theory, this primer pair will only generate a PCR product, if the targeting vector has integrated in the desired genomic region. In the event of random integration of the targeting construct, no PCR product should be amplified. In practice, however, PCRs are very susceptible to contamination (especially when positive controls are used) and false positives can also arise due to non-specific primer annealing or artefacts (Frohman and Martin, 1990). For these reasons, gene targeting of clones identified by PCR screens should be confirmed by Southern blotting.

PCR assay RHA (Figure 3.3) detected one targeted clone in 5 (Figure 3.4A) and it was confirmed by restriction enzyme digestion that the generated PCR product did not arise from the positive control vector used (Figure 3.4B). After the initial PCR screen, the assay stopped working and several attempts to reproduce the results, failed. PCR reactions produced smeary products between 5 kb and 500 bp (data not shown). All reagents were exchanged and the procedure was closely monitored for variations in sample processing. It was found that the smeary background appeared at high cycle numbers (>30) but the cycle number could not be reduced because the signal could not be detected at lower cycle numbers. The problem also occurred without any template, pointing towards a primer problem. I aimed to design new primers, however, this proved difficult as the locus contains many repetitive sequences (see also section 3.2.1). In order to design alternative primers I would have had to amplify a much larger PCR product (greater than 7 kb) which I did not

consider sensible. The RHA PCR assay required 40 PCR cycles at an extension time of 6 minutes greatly challenging Taq DNA polymerase; therefore adapting the extension time to an even larger PCR product was no favourable option. I tried to add fresh PCR reagents after 25 cycles but this too did not work. However, a new PCR assay designed (LHA) did not show any complications at high cycle numbers and was subsequently used to screen a larger number of clones. Overall, 44 clones were screened for integration of pTarg-*BCL6* into the *BCL6* locus by PCR assays out of which 13 were found positive (see also Figure A p.208).

Seven clones found positive for gene targeting by PCR assays were further analysed by Southern blotting and six were found to contain the expected genomic modifications in the *BCL6* locus (data only shown for clone BA11 in Figure 3.6). This points towards an unusually high gene targeting frequency in DG75 which is in accordance with the literature, with frequencies up to 69% being reported (Feederle et al., 2004). Gene targeting frequencies are, nevertheless, strongly locus-dependent, as frequencies as low as 5% were observed for a different locus in DG75 (Maier et al., 2005). Overall, however, frequencies reported for DG75 are extraordinarily high as frequencies in other B cell lines only reach 1-2% (Faili et al., 2002, Grawunder et al., 1998) or 11 % in the special case of Nalm-6 (Adachi et al., 2006).

As already mentioned by others (Feederle et al., 2004), DG75 is in many ways comparable to the chicken B cell line DT40, which been used extensively for knockout studies of both lineage-specific and non-lineage specific genes (Buerstedde and Takeda, 1991). DG75 and DT40 cell lines are similar because they both are of B cell lineage, support efficient homologous recombination and have a c-Myc translocation (Chang and Delany, 2004). DG75, however, has many advantages over DT40 regarding the functional analysis of human genes. Firstly, DT40 is an avian cell line and chickens have a different immune system to that of human. Chickens do not diversify their antibody repertoire by SHM but

gene conversion (Reynaud et al., 1994). Moreover, while human antibody maturation takes place in GCs, in chickens, antibody maturation takes place in the Bursa of Fabricius, a specialised organ located on the dorsal wall of the cloaca. Secondly, DG75 is karyotypically very stable, while DT40 has many cytogenetic abnormalities. The karyotypes of DT40 cell lines from different labs often vary due to its genetic instability. In addition to its c-Myc translocation t(8;14), a trisomy of chromosome 2, monosomy of chromosome 4 and a variant 4p have been reported for DT40 (Riley and Gordon, 1992). Although DG75 has a mismatch repair deficiency (Msh2-) (Brimmell et al., 1998), its karyotype is very stable, even after long periods in culture. The DG75 derivative DG75-354 used in this project has an additional t(1;3)(p35;q21) translocation (as tested by Valeria AS De Melo and Dr Alistair G Reid, Cytogenetics Laboratory, Hammersmith Hospital, London).

3.3.2 Cre-mediated deletion of the *zeocin* resistance cassette

The Cre/*Lox* recombination system is a very powerful tool in genetic modifications *in vitro* and *in vivo*. Cre recombinase interacts with specific *loxP* binding sites and deletes the sequence between them (Sternberg and Hamilton, 1981). *LoxP* sites consist of 13 bp inverted repeat sequences and an 8 bp asymmetric core which ensures directionality of recombination (Hoess and Abremski, 1985). By flanking a selectable marker of a targeting construct with *loxP* sites, the marker can be 'recycled' and the targeting construct can be reused for the knockout of the second allele. The concept of marker recycling has been proposed previously (Abuin and Bradley, 1996) and is an elegant way that simplifies gene targeting designs. After targeting of the first *BCL6* allele (see CHAPTER 3), clone BA11 (*BCL6*^{+/-}(*zeo*)) was transiently transfected with pMC-Cre (Gu et al., 1993) and clones were isolated by plating at limiting dilution. Out of 53 clones tested, only 2 were found to have lost their *zeo* resistance and it was confirmed by PCR assay that Cre-recombination at the targeted *BCL6* locus had taken place (resulting in *BCL6*^{+/-}) (Figure 3.7D) (see also Figure A p.208). The Cre-recombination efficiency observed in this project

seems rather low, as the efficiency of Cre-recombination between two *loxP* sites reaches almost 100% *in vivo* (Hennet et al., 1995, Kuhn et al., 1995), therefore efficiencies in cell culture systems should depend mostly on transfection efficiency. In DG75-354, typically 60-80 % of the transfected and viable population express the transgene (Figure 4.2C); therefore the low Cre recombination efficiency observed after nucleofection with pMC-Cre is likely to be caused by other reasons. This finding will be discussed in more detail in section 5.3.3 below.

CHAPTER 4 - Conditional Bcl6 expression in DG75-354

4.1 Introduction

Before the second endogenous *BCL6* allele could be disrupted, a tetracycline (tet)-regulatable Bcl6 expression had to be introduced into *BCL6*^{+/-} cells. This conditional Bcl6 expression was established with the Tet-Off system (Gossen and Bujard, 2002) which has been previously used in our laboratory (Carpenter and Porter, 2004, Sullivan et al., 2001). The Tet-Off system employs the tetracycline transactivator (tTA), which, in the absence of tetracycline, binds to a tet-response element (TRE) in the promoter region of the gene of interest and thereby promotes transcription by RNA polymerase II. In the presence of tetracycline (or doxycycline (dox)), the tTA is released from the promoter region and transcription ceases (Figure 4.1).

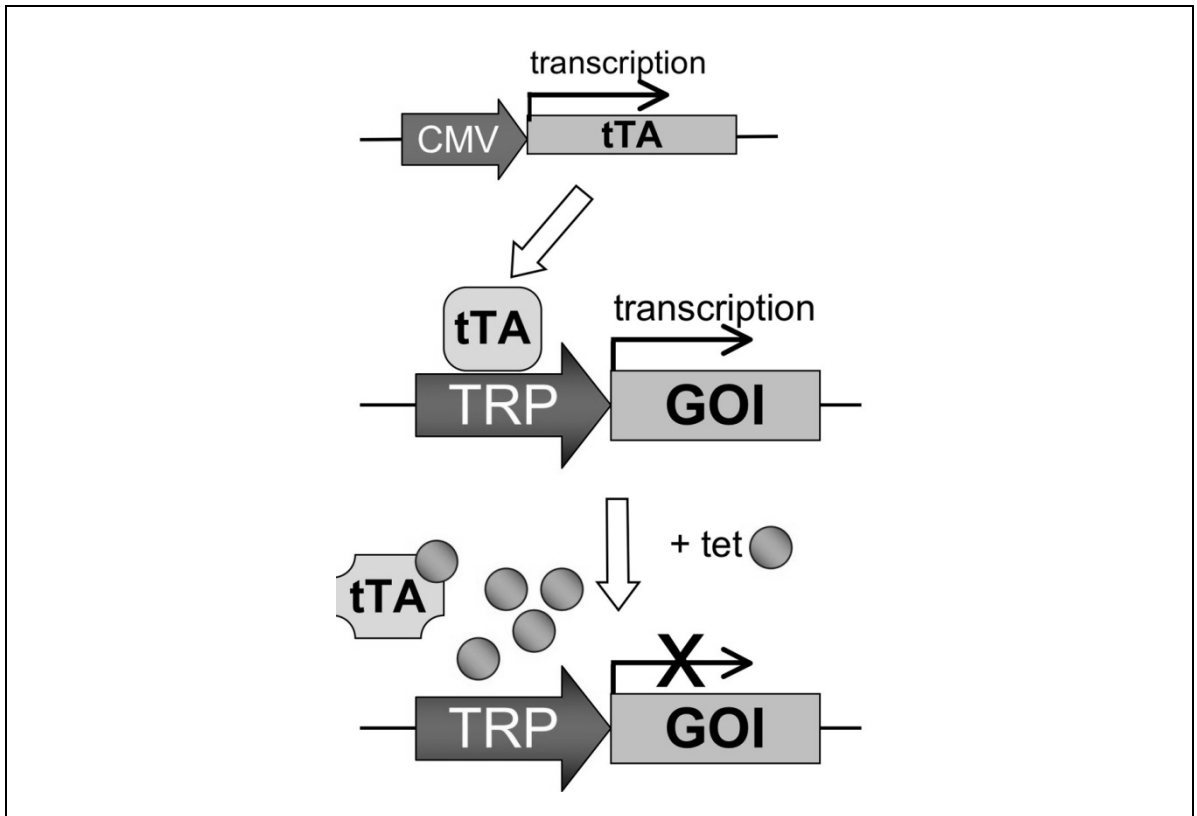


Figure 4.1: The Tet-Off system

The tetracycline transactivator (tTA) is expressed from a constitutive promoter and induces transcription of the gene of interest (GOI) by binding to the tetracycline responsive promoter (TRP). Binding of tetracycline (tet) to the tTA induces a conformational change of tTA, causes its dissociation from the TRP and transcription ceases.

4.2 Results

4.2.1 Validation of DG75-354 as a suitable system for conditional gene regulation

The Tet-Off system relies on a tTA, which in the absence of tetracycline (or doxycycline), binds to the tetracycline responsive promoter of the gene of interest and activates its transcription. DG75-354 is a DG75-derivative, which stably expresses the tet- transactivator and was kindly supplied by Dr B. Henglein (Institut Curie, Paris). To generate DG75-354, DG75 was stably transfected with a derivative of pUHD15-1 (Gossen and Bujard, 1992), which expresses the tTA, as well as a puromycin resistance gene. In order to test the potency of tet-regulation in this cell line, DG75-354 was nucleofected with a tet-regulated green fluorescent protein (GFP) construct, pTARG4 (Brough et al., 2007) and subsequently cultured with or without tetracycline. Figure 4.2 shows that good tet-regulation can be achieved in this cell line. In the absence of tetracycline, over 70 % of pTARG4-transfected DG75-354 cells express GFP (Figure 4.2C). However, 18-48 hours after transfection with pTARG4 and addition of tetracycline, GFP expression is remarkably reduced to less than 3 % (Figure 4.2B).

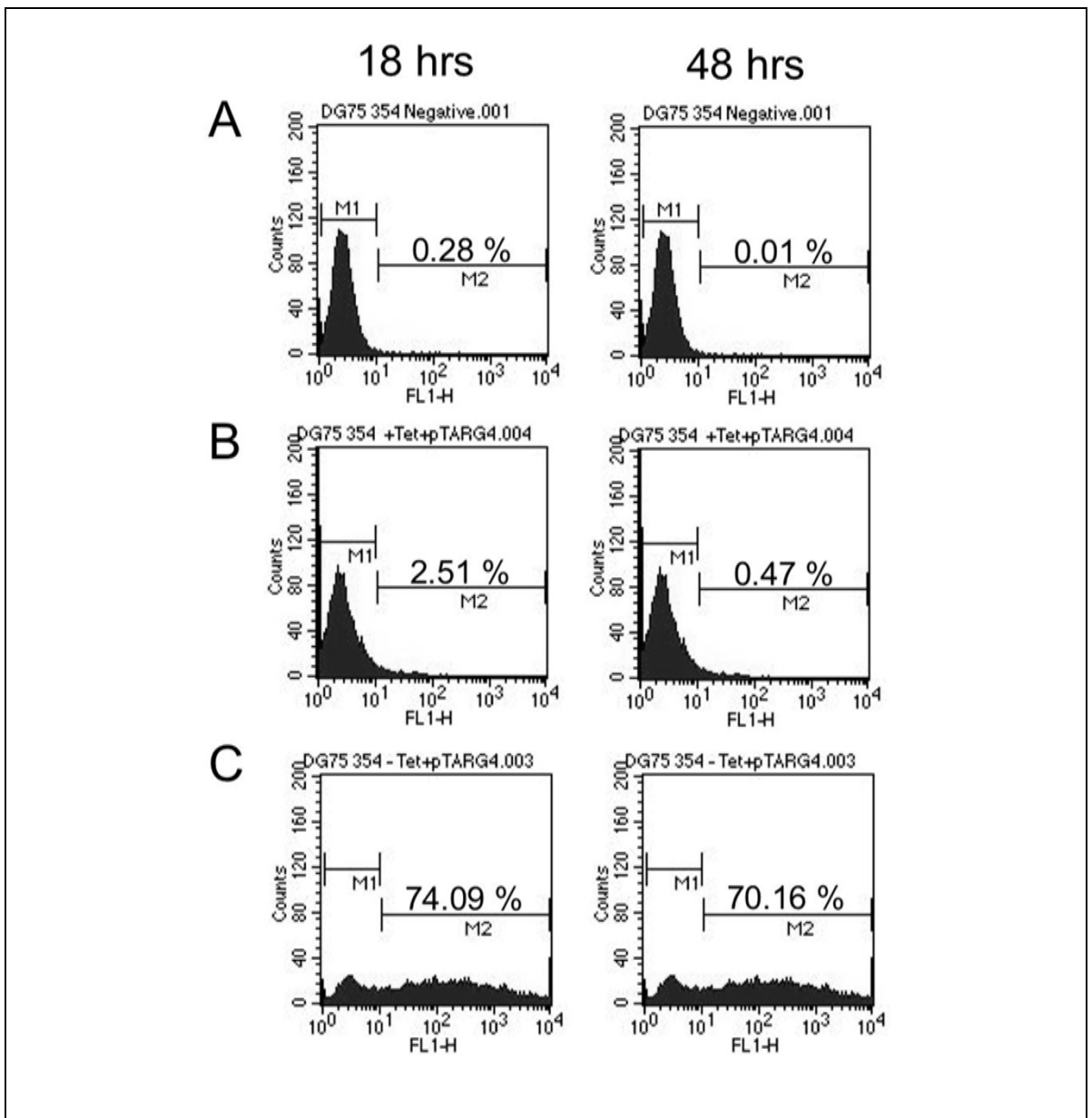


Figure 4.2: Transient tet-regulated GFP expression in DG75-354

DG75-354 cells were grown up for transfection in the absence of tetracycline and screened by flow cytometry 18 and 48 hrs after transfection. GFP expression was detected in the FL1-H channel and percentages represent gated populations of viable cells. See section 2.3.6 p.52 for details on flow cytometry. A) Negative control cells were nucleofected without DNA. B) Cells were nucleofected with tet-regulated GFP (pTARG4 (Brough et al., 2007)) and promptly incubated in medium supplemented with tetracycline (1 μ g/ml). C) Cells were nucleofected with tet-regulated GFP (pTARG4 (Brough et al., 2007)) and incubated in the absence of tetracycline.

4.2.2 Establishing stable tet-regulated Bcl6 expression in *BCL6*^{+/-} cells

Tetracycline-regulated Bcl6 was amplified and cloned from DG75 cDNA and tagged with the hemagglutinin (HA) peptide sequence YPYDVPDYA (see section 2.5.2 p.57). The 2,154 bp Bcl6-HA insert was introduced into the *Bam*HI and *Hind*III sites of pTRE-tight (Clontech) and the resulting construct pTRE-Bcl6-HA was sequenced with 4 sets of primers covering both DNA strands in order to validate that no mutations are present.

A heterozygous *BCL6* knockout *BCL6*^{+/-}-clone (7D4) was co-electroporated with pTRE-Bcl6-HA and pSV2-neo (Southern and Berg, 1982) (molar ration 10:1). The resulting G418-resistant clones were isolated by plating at limiting dilution and tested for tet-regulatable Bcl6-HA expression by Western blotting. Figure 4.3 shows three clones that stably expressed Bcl6-HA from the pTRE-Bcl6-HA construct and this expression was stringently regulatable by tetracycline. No more protein was detected 24 hrs after the addition of tetracycline. Having established conditional Bcl6 expression in the heterozygous knockout clone (*BCL6*^{+/-};tet*BCL6*-HA), I moved on to target the second *BCL6* allele.

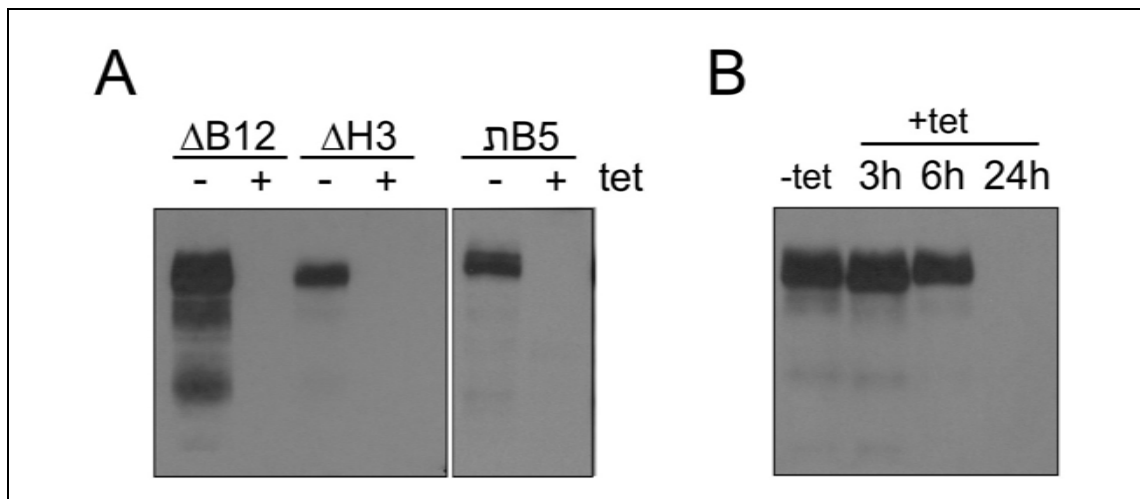


Figure 4.3: Stable and tightly regulated Bcl6 expression in *BCL6*^{+/-} cells

A) A number of clones were screened for tet-regulatable Bcl6 expression. The expression pattern of three typical clones in the absence and presence (72h) is shown. B) Clone $\Delta B12$ (*BCL6*^{+/-};*tetBCL6-HA*) was cultured without tet or with tet for 3, 6 and 24 hrs respectively. An anti-hemagglutinin antibody was used for detection (see section 2.7 p.64).

4.3 Discussion

4.3.1 Validation of DG75-354 as a suitable system for conditional gene regulation

Conditional gene expression is popular in molecular biology and many systems are now available to achieve robust regulation and tet-dependent gene regulation is a widely used system (Gossen and Bujard, 2002). For the conditional *BCL6* knockout project, it is important that gene regulation is tightly controlled and that complete depletion of Bcl6 protein is achieved. Figure 4.2 shows that stringent transgene regulation is feasible in DG75-354 with less than 1% of cells expressing GFP protein 48 hrs after transfection and addition of tet. It is likely that even tighter regulation can be achieved, when a clonal population is monitored for transgene regulation as it has been reported that tetracycline regulation occurs homogeneously on the single cell level of a clonal population (Fukagawa et al., 1999).

4.3.2 Establishing stable tet-regulated Bcl6 expression in *BCL6*^{+/-} cells

A hemagglutinin (HA) tag was attached to the Bcl6 protein in order to be able to differentiate it from endogenous Bcl6 protein (Ledoux et al., 1996). Epitope tags are widely used in molecular biology studies because high affinity antibodies are available for their detection (Jarvik and Telmer, 1998). However, one has to take into account that the addition of such a tag may abolish or interfere with the function of the protein of interest. For this project, the influenza-hemagglutinin peptide has been chosen because it has been extensively used in recombinant expression systems (Field et al., 1988). It is hoped that it does not interfere with Bcl6 function due to its small size.

The characteristics of expression achieved in *BCL6*^{+/-};*tetBCL6-HA* clones are in accordance to expectations. The stringency of tet-regulation is known to be very tight for the system used and has already been confirmed in DG75-354 by

flow cytometry (Figure 4.2). The time it takes for all tet-regulated protein to disappear strongly depends on the specific half-life of the protein of interest which has been reported to be around 4-6 hrs in the case of Bcl6 (Niu et al., 1998). It is therefore not surprising that complete Bcl6 protein depletion is observed 24 hrs after addition of tet.

CHAPTER 5 – Targeting of the second *BCL6* allele

5.1 Introduction

I was hopeful that successful targeting of the second *BCL6* allele could be achieved because it is generally believed that gene targeting efficiency is dependent on the genomic locus targeted and the quality of the targeting construct used. As the same genomic locus had been successfully targeted before and the same targeting construct was to be used (see CHAPTER 3 above), similar gene targeting frequencies were expected.

5.2 Results

5.2.1 Targeting of the second *BCL6* allele

For targeting of the second *BCL6* allele, *BCL6*^{-/+};*tetBCL6-HA* cells (clone ΔH3) were cultured without tetracycline and transfected by electroporation with *KpnI*-linearised pTarg-*BCL6*. Seventeen *zeocin*-resistant clones were isolated and screened by PCR assay LHA (see Figure 3.5 p.82), in which one primer is located in the resistance cassette of pTarg-*BCL6* and the second in the *BCL6* locus. The 5.9 kb PCR product can hence only be amplified if the targeting construct has integrated into the *BCL6* locus. A typical result of such a screen is shown in Figure 5.1A. Out of 17 clones screened, 4 were found to have integrated the targeting vector into the *BCL6* locus (approximately 24%) (see also Figure A p.208). Due to the fact that I used a marker recycling gene targeting approach, such clones could have either re-targeted the already targeted *BCL6* allele, or have disrupted the second allele. I therefore performed an additional PCR assay to distinguish between these two events (Figure 5.1B) and a typical result of the screen is shown in Figure 5.1B. It was found that three out of four clones tested had targeted the second *BCL6* allele (clones 2D7, 5D7, 6D1), and only one (clone 5D6) had re-targeted the first allele. Following this initial PCR screen, the results obtained were confirmed by Southern blotting.

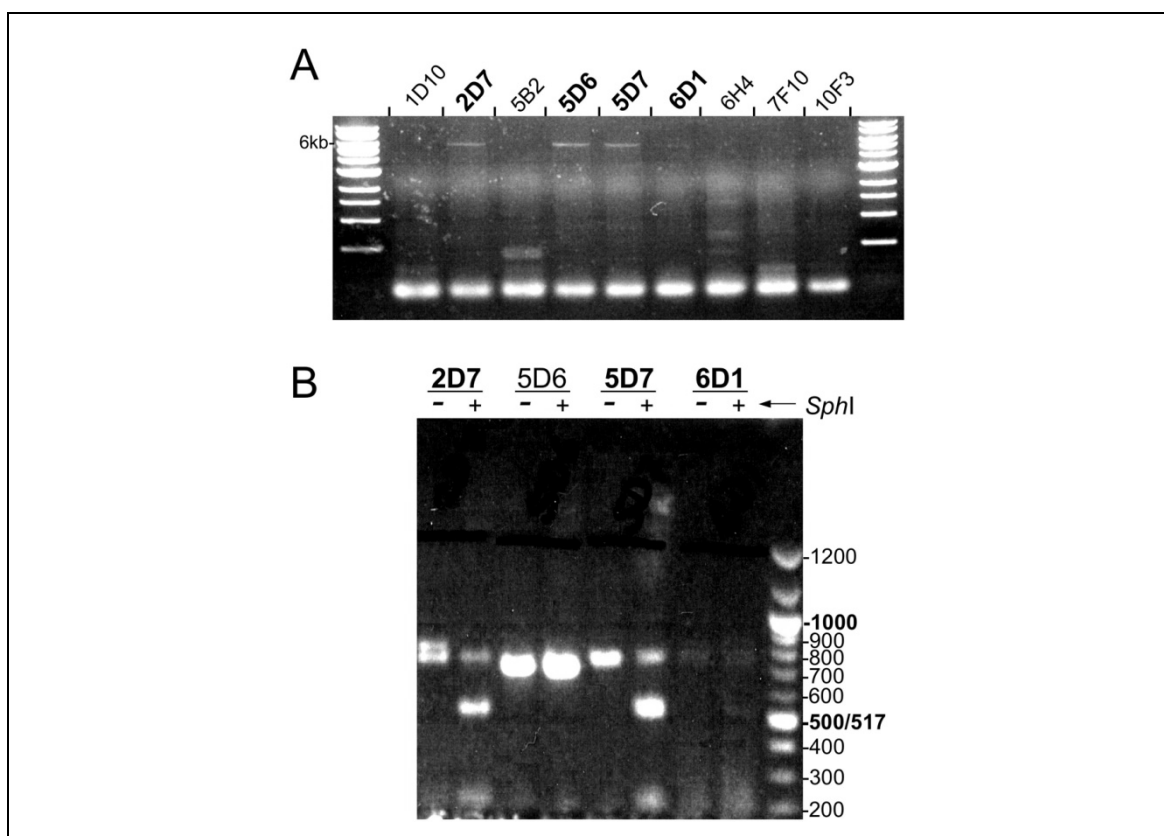


Figure 5.1: PCR screens detect targeting of the second *BCL6* allele

A) In PCR assay 'Left Homology' one primer anneals within the *zeocin* disruption cassette of the targeting construct and one primer is located in the *BCL6* locus - a 5.9 kb PCR product indicates targeted disruption of the *BCL6* allele (Figure 3.5 p.82). *BCL6*^{+/-};tet*BCL6*-HA cells (clone ΔH3) were transfected with pTarg-*BCL6* and screened by LHA PCR assay. An example of such a PCR screen is shown, clones 2D7, 5D6, 5D7 and 6D1 (very weak signal) were identified to have disrupted a *BCL6* allele. B) The 'Cre-recombination' PCR assay can differentiate between re-targeting of the first *BCL6* allele and gene targeting of the second allele (Figure 3.7 p.86). The primers used for this screen amplify a 722 bp product for a wildtype *BCL6* allele, a 1967 bp product for a targeted *BCL6* allele and a 773 bp product that is cleavable into two fragments (543 + 230 bp) by *SphI* for a targeted *BCL6* allele that has undergone Cre recombination. The existence of the 543 bp band after cleavage of the PCR product with *SphI* indicates that the first *BCL6* allele was not re-targeted. 'Cre recombination' PCR assay results for clones found positive in A). Clones 2D7, 5D7 and 6D1 (very weak) show the desired 543 bp band after digestion of the PCR product with *SphI*. Hence three clones targeted the second *BCL6* allele, while clone 5D6 re-targeted the first *BCL6* allele.

Southern blotting was performed to confirm that only a single integration of the targeting vector had occurred, and that this integration had occurred at the *BCL6* locus. In order to demonstrate that only one integration of the construct had occurred, I chose a hybridisation probe specific for the targeting vector. The 1218 bp probe used for this purpose is the *zeocin* resistance gene, which was excised from vector pCMV/*Zeo* using restriction enzymes *Xho*I and *Eco*RI. Figure 5.2A shows the resulting Southern blot. As expected; only the clones that contain the *zeocin* disruption cassette in one of their *BCL6* alleles show the predicted 7.9 kb *Sph*I fragment recognised by the *zeocin* probe (Figure 3.1 p.73). The hybridisation site of the *zeocin* probe is located on a 6.3 kb *Sph*I-*Kpn*I fragment; *Kpn*I was used for linearisation of the targeting vector and *Sph*I was used for digestion of genomic DNA prior to Southern blotting. Therefore any additional integration of the targeting construct elsewhere in the genome would be detected as an additional band recognised by the *zeocin* probe that is equal to or greater than 6.3 kb.

In addition to confirming that only one integration of the cassette had occurred in each targeted clone, I also intended to demonstrate that the targeting construct had disrupted the correct i.e. the *BCL6* locus. In order to show this, I re-probed the same blot (Figure 5.2A) with a probe that hybridises to the *BCL6* locus but is not part of the targeting vector (probe B see also Figure 3.1 p.73). Figure 5.2B shows the resulting Southern blot confirming that all three clones tested (2D7, 5D7 and 6D1) successfully disrupted both endogenous *BCL6* alleles and also that only a single copy of the tet-regulated Bcl6-expression had integrated into their genome.

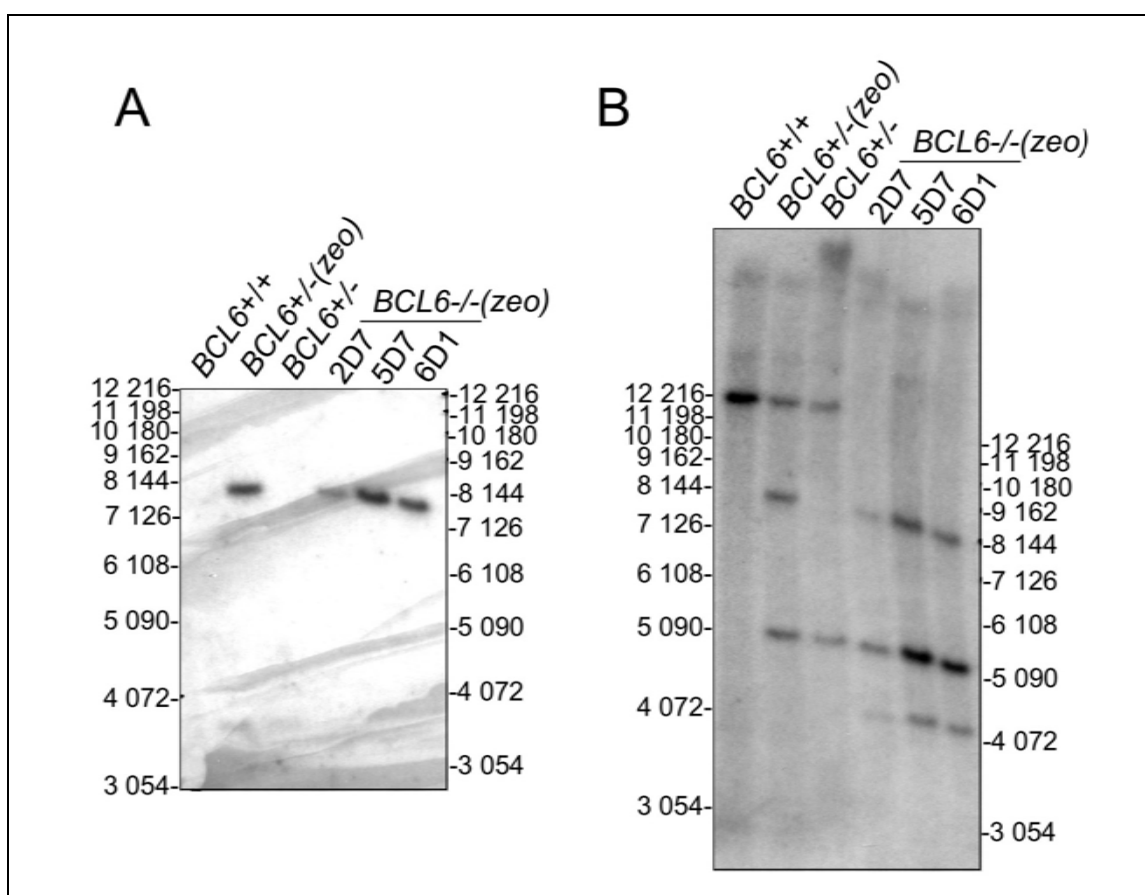


Figure 5.2: Southern blotting confirms single copy integration of pTarg-*BCL6*-transfected cells and targeting of the second *BCL6* allele

A) *SphI*-digested genomic DNA of control and knockout clones was probed with a *zeocin* probe. Targeting of a *BCL6* allele is expected to detect a 7.9 kb fragment (same as probe A, see Figure 3.1 p.73). A second random integration of the targeting vector would result in an additional band detected that is greater than or equal to 6.3 kb (see text). B) The same blot as in A) was re-probed with probe B which detects an 11.8 kb *SphI*-fragment for a wild-type allele and a 5.1 kb fragment for a targeted *BCL6* allele. 7.9 kb bands result from remaining activity of the *zeocin* probe (as in A). Probe B hybridises to exon 5 of *BCL6*, which is part of the protein-coding region of Bcl6 and thus part of the vector pTRE-Bcl6-HA stably transfected into the heterozygous knockout clone (Figure 4.3 p.96). Therefore an additional 4.1 kb band is detected in clones 2D7, 5D7 and 6D1. Because only 623 bp of the 730 bp probe B can hybridise to the tet-regulatable Bcl6 construct. The signal detected for this 4.1 kb band is slightly weaker than that detected for the bands to which the probe hybridises fully (5.1 and 11.8 kb). All three clones were derived from the same tet-regulated Bcl6-expressing clone (Figure 4.3, clone ΔH3), which explains why the band is the same in all clones in which it is found. Moreover, this finding confirms that only one copy of the tet-regulatable *BCL6* construct has integrated.

5.2.2 Detection of an aberrant Bcl6 protein variant in *BCL6*^{+/-}(zeo) and *BCL6*^{-/-}(zeo);*tetBCL6-HA* cells

In addition to confirming the disruption of both *BCL6* alleles by Southern blotting, Western blot analysis was performed to verify that complete Bcl6 protein depletion has been achieved in *BCL6*^{-/-}(zeo);*tetBCL6-HA* clones. Unexpectedly, an antibody raised against the N-terminal two thirds of Bcl6 (Santa Cruz, see Table 5 p.65) detects an additional protein of approximately 80-85 kDa in *BCL6*^{-/-}(zeo);*tetBCL6-HA* clones which is absent in parental cells (DG75-354; *BCL6*^{+/+}) (Figure 5.3A). It is likely that the protein originated from the endogenous *BCL6* allele rather than the exogenous Bcl6 expression, as it is neither tet-regulatable (Figure 5.3A) nor detected with an antibody recognising the HA tag (Figure 4.3 p.96). In order to determine the origin of the additional Bcl6 protein variant, all clones generated during the construction of the conditional knockout clone were probed with a Bcl6 antibody raised against a peptide located at the carboxy terminus of Bcl6 (Cell Signalling, see Table 5 p.65). This antibody also detects a Bcl6 variant protein of approximately 80-85 kDa in all clones carrying a *zeocin* cassette in their *BCL6* locus (Figure 5.3B).

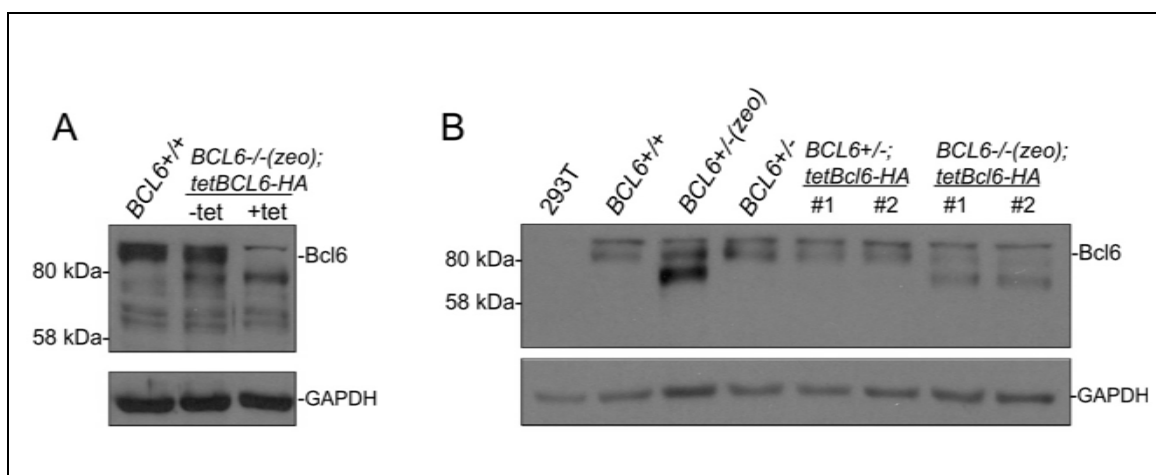


Figure 5.3: Bcl6 protein expression of targeted clones

A) Bcl6 protein (MW 92-98 kDa) is detected in parental cells (DG75-354; *BCL6*^{+/+}) and conditional knockout cells (*BCL6*^{-/-}(zeo);*tetBcl6-HA*; clone 2D7) when grown in the absence of tetracycline. Bcl6 is switched off after culturing conditional knockout cells in tetracycline (1 µg/ml) for 24 hours. A Santa Cruz anti-Bcl6 antibody recognises aa 3-484 of Bcl6 also detects an additional protein of approximately 80-85 kDa in *BCL6*^{-/-}(zeo);*tetBCL6-HA* cells. B) A Cell Signaling anti-Bcl6 antibody that recognises the C-terminus of Bcl6, detects an additional protein of 80-85 kDa for the heterozygous *BCL6* knockout clone before Cre recombination (*BCL6*^{+/-}(zeo); clone BA11) and homozygous *BCL6* knockout clones (*BCL6*^{-/-}(zeo);*tetBcl6-HA*; clone 2D7 (#1) and 5D7 (#2)). No such Bcl6 variant protein is detected in parental cells (DG75-354; *BCL6*^{+/+}) or heterozygous knockout clones in which the *zeocin* cassette has been deleted (*BCL6*^{+/-}; *tetBCL6-HA*, clones ΔB12 (#1) and ΔH3 (#2)).

There is the possibility that the cytomegalovirus (CMV) promoter or an enhancer element in the *zeocin* cassette activates transcription of the *BCL6* locus, downstream of the site disrupted by gene targeting. As the *BCL6* allele was disrupted at the end of exon 3 (see Figure 3.1 p.73), such an aberrant transcript would lack the first coding exon of Bcl6 (exon 3) but translation could potentially be initiated from an alternative downstream start codon within a subsequent exon, thus generating an N-terminally truncated Bcl6 variant protein.

With the aim of exploring the possibility that an N-terminally truncated Bcl6 version is generated, the Bcl6 protein coding nucleotide sequence was screened for alternative start codons. It transpired that the Bcl6 coding sequence contains 38 ATG codons including 18 alternative in-frame start codons which could potentially generate N-terminally truncated Bcl6 proteins. Figure 5.4A shows the Bcl6 amino acid sequence with all in-frame start codons highlighted, the potential truncated Bcl6 proteins and their predicted molecular weights are listed in Figure 5.4B.

In order to speculate which of these in-frame start codons is likely being used by cells that carry the *zeocin* cassette in one of their *BCL6* alleles, it has to be taken into account that the Bcl6 wildtype protein does not run at its predicted molecular weight when analysed on Tris-Glycine acrylamide gels (Onizuka et al., 1995). The predicted molecular weight of Bcl6 is 79 kDa but its apparent molecular weight is 92-98 kDa. This difference is attributed to its high degree of phosphorylation and its amino acid composition. When dephosphorylated, Bcl6 protein runs as a single band of 87 kDa, suggesting that differential phosphorylation accounts for a difference of 5-11 kDa (Onizuka et al., 1995). The remaining difference of 8 kDa (87 kDa dephosphorylated Bcl6 versus 79 kDa predicted MW), is attributed to the high proline content of Bcl6, affecting its electrophoretic mobility (Onizuka et al., 1995). Taking this into account, an N-terminally truncated version of Bcl6 with an apparent molecular weight of 80-85

kDa would correspond to a predicted molecular weight of around 66/67 kDa. The first alternative start codon is located in exon 3 (codon position 51, Figure 5.4), however, translation from this site would terminate after only 5 amino acids due to a premature stop codon generated by the targeted disruption of the *BCL6* gene (see Figure 3.2 p.75). Therefore an alternative start codon downstream of exon 3 would have to be used. The first alternative start codon in exon 4 lacks the first 89 amino acids of Bcl6 and thus lacks a major part of the Bcl6 POZ domain (aa 1-121) and an important charged pocket which is viewed as being essential for co-repressor interaction (Melnick et al., 2002). Moreover, a mutagenesis study revealed that the deletion of part of the POZ domain of PLZF (corresponding to Bcl6 residues 1-54) disabled PLZF dimerisation and the protein almost completely lost its ability to repress transcription (Melnick et al., 2000). Because the POZ domains of PLZF and Bcl6 are highly similar, a Bcl6 protein that lacked the first 89 amino acids would most likely be non-functional. However, in order to rule out that any residual function of the truncated Bcl6 variant interfered with the conditional knockout phenotype, I aimed to delete the *zeocin* cassette from the second targeted *BCL6* allele by Cre recombination.

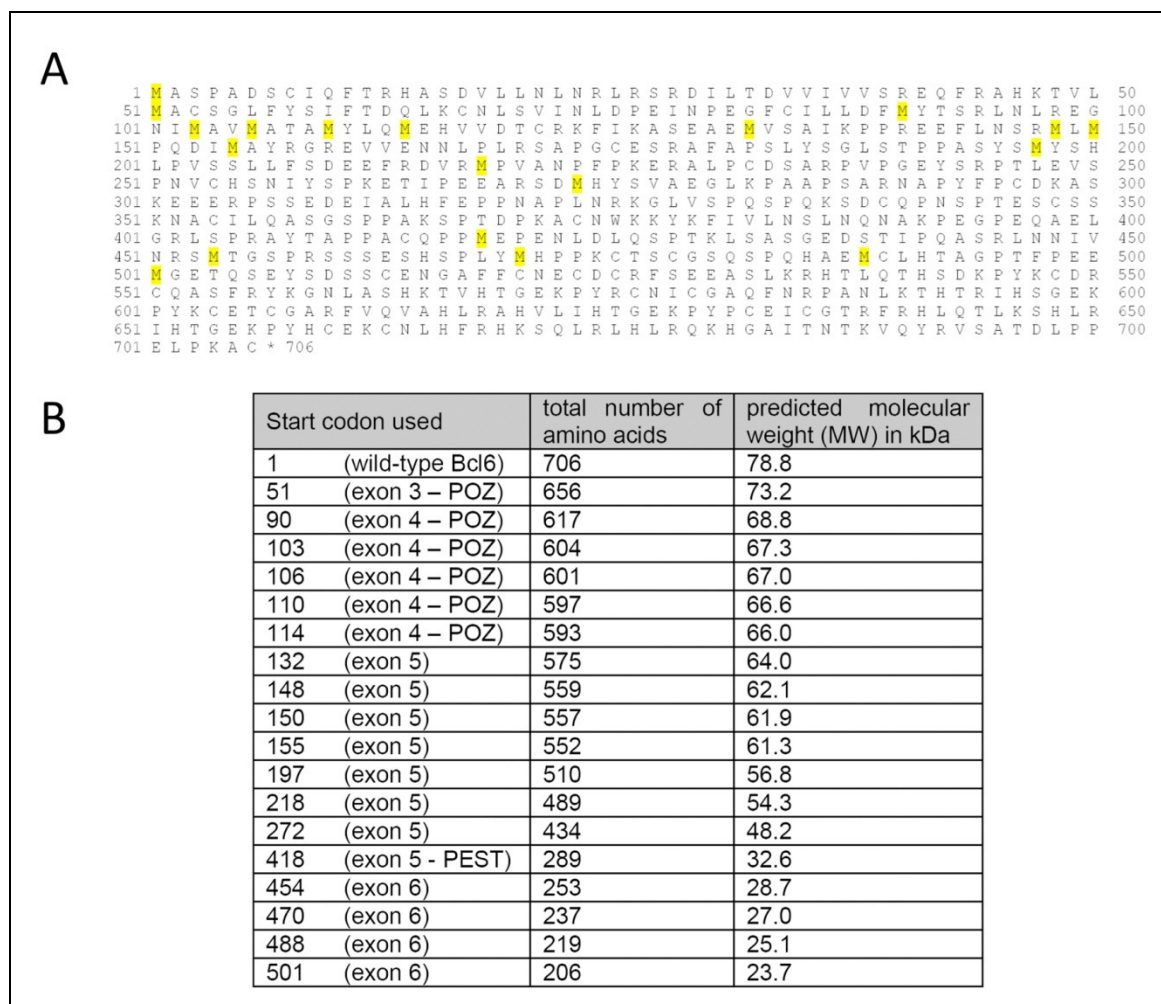


Figure 5.4: Alternative start codons in the Bcl6 coding sequence and potential Bcl6 truncations

A) The open reading frame of Bcl6 codes 706 amino acids. Alternative in-frame translation start sites (methionine (M)) are highlighted in yellow. B) List of potential N-terminally truncated Bcl6 variants translated from alternative in-frame start codons highlighted in A) and predicted molecular weights.

5.2.3 Cre-mediated deletion of the *zeocin* cassette in *BCL6*^{-/-} (*zeo*);*tetBCL6-HA* cells

Given that the truncated Bcl6 protein appears to be expressed only when the *zeocin* cassette is present in exon 3 of *BCL6* (Figure 5.3B), it was attempted to remove the cassette by Cre recombination in *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells. The efficiency of Cre recombination was expected to be low (see section 3.3.2 p.89), as only 2 out of 53 clones were found to have removed the cassette when it was attempted to excise it from the first *BCL6* allele targeted. The PCR screen that tests for Cre recombination at the second *BCL6* allele used a forward primer specific for the *zeo* cassette and a reverse primer that anneals in the *BCL6* locus further downstream. These primers amplify a 925 bp region of a *BCL6* allele that has been disrupted by the *zeo* resistance cassette (Figure 5.5A) but cannot generate a product once successful Cre recombination has taken place (Figure 5.5B).

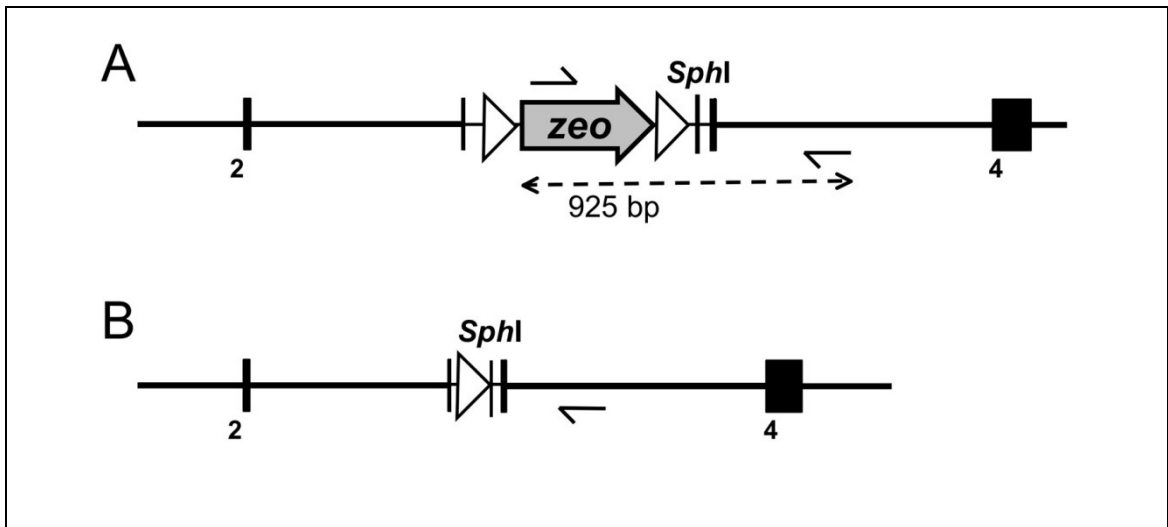


Figure 5.5: PCR assay screen to identify Cre recombination of the second *BCL6* allele

A) A *BCL6* allele that is disrupted by the *zeo* resistance cassette in exon 3 gives rise to amplification of a 925 bp PCR product. The forward primer (Right-Homology-F see sections 3.2.2 and 3.2.3) anneals to the *zeo* cassette and the reverse primer (Cre-R section 3.2.6) anneals to the intronic region downstream of exon 3 of *BCL6*. B) PCR primers cannot amplify a PCR product from a targeted *BCL6* allele that has been subjected to Cre recombination.

Using this PCR assay (Figure 5.5), I screened 410 clones isolated after nucleofection of *BCL6*^{-/-}(*zeo*);*tetBCL6*-HA cells with pMC-Cre in the absence of tetracycline. These 410 clones were derived from 3 independent nucleofections of clones 2D7 and 5D7 and I could not detect a single clone that had successfully deleted the *zeo* resistance cassette. In order to account for the possibility that deletion of the *zeo* cassette, and associated loss of the Bcl6 variant protein, conferred a disadvantage to cell proliferation, I paid special attention to clones that took longer than average to grow up. Because PCR screening for loss of a PCR signal is particularly susceptible to cross contamination, I also tested clones for their ability to survive in *zeocin* following screening by PCR, however, without success. This led to the idea that the desired clones may not be viable because they depend on the truncated protein for survival and that its loss may not be adequately compensated for by the tet-regulated Bcl6 copy introduced earlier (see section 4.2.2 p.95). In order to test this idea and rule out any technical problem associated with Cre recombination, I aimed to demonstrate that Cre recombination does take place at the *BCL6* locus shortly after nucleofection with the Cre-expression construct (pMC-Cre).

In order to demonstrate that Cre recombination does occur at the *BCL6* locus, I developed a PCR assay that can detect the Cre-recombination product shortly after nucleofection with Cre recombinase. In this assay, two primers anneal to the *zeo* cassette but face away from each other and can therefore only amplify a product after circularisation of the *zeo* cassette by Cre-recombination (Figure 5.6). Using this assay, successful Cre recombination of the *BCL6* allele can be detected shortly after transfection, even though the clones may not be viable enough to proliferate and grow up after the genetic manipulation. The circular *zeo* cassette (pop-out intermediate) could be detected when whole cell lysates of clones containing the floxed *zeo* resistance cassette were incubated with Cre recombinase *in vitro*, proving that the locus is capable of undergoing efficient Cre recombination. However, no PCR signal could be detected after transient nucleofection of *BCL6*^{-/-}(*zeo*);*tetBCL6*-HA cells with pMC-Cre (data not shown).

I concluded that the event either did not occur at all, or that it occurred at a too low frequency to be detected by the PCR assay. As other lab members had successfully used the same pMC-Cre construct and plasmid preparation for Cre recombination in different cell lines, a defect in the plasmid itself was considered unlikely. This left the possibility that transient expression of Cre-recombinase from this vector was too inefficient in my cell line. Dr Timothy Ley and his colleagues (Washington University, St Louis, USA) made the observation that Cre recombination after transient transfection of embryonic stem cells with pMC-Cre is very inefficient (1-2%). Consequently, the group cloned a Cre-expression vector, which was capable of increasing the efficiency of Cre recombination to 50 % in mouse embryonic stem cells. The improved vector is derived from pCAGGS (Niwa et al., 1991) in which the gene of interest is expressed from a chicken β -actin promoter supported by an upstream CMV enhancer. The group inserted a Cre recombinase modified with an N-terminal NLS into pCAGGS, resulting in vector pTurbo-Cre (Westervelt et al., 2003). When nucleofection of the *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* clone was repeated with this improved Cre expression vector, the circular pop-out intermediate could be detected 18 hrs after transfection (Figure 5.6B).

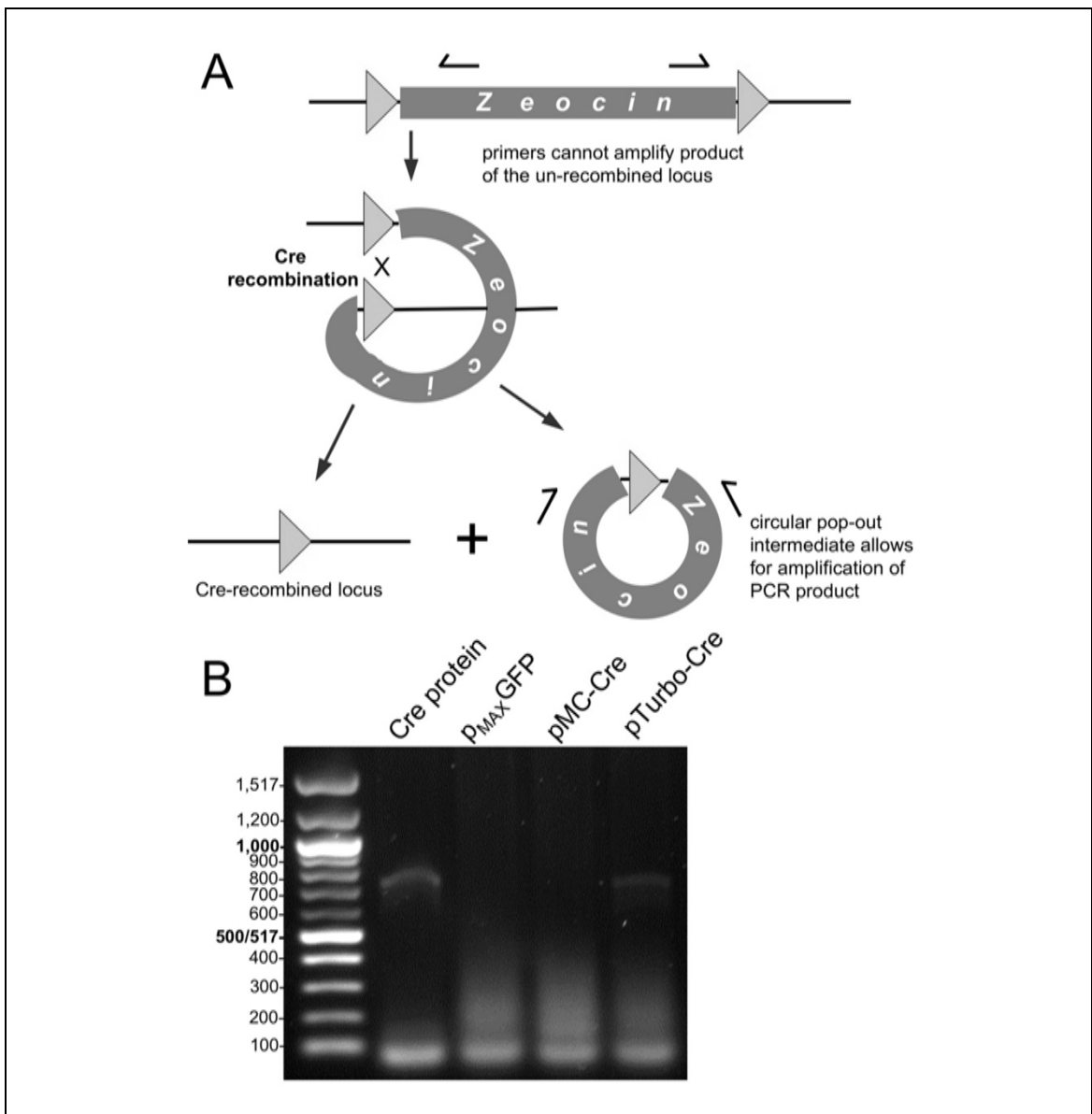


Figure 5.6: PCR assay for monitoring Cre recombination of the *zeocin* cassette at the *BCL6* locus

A) During Cre recombination, *loxP* sites (triangles) align and crossover recombination leaves a single *loxP* site in the recombined locus and a circular pop-out intermediate (Nagy, 2000). Primers cannot amplify a PCR product from the un-recombined *BCL6* locus because they are pointing in opposite directions. The circular pop-out intermediate, however, allows for amplification of a 745 bp PCR product. B) Detection of the circular pop-out intermediate by PCR was performed on whole cell lysate of clone 5D7 (*BCL6*^{-/-}(*zeo*);*tetBCL6*-HA) incubated with recombinant Cre recombinase protein or on lysates of the same clone 18 hrs after nucleofection with vector p_{MAX}GFP, pMC-Cre or pTurbo-Cre (see section 2.5.3 p.61 for technical details).

Having established that deletion of the *zeo* cassette from exon 3 of the *BCL6* locus is possible when the improved Cre-expression construct pTurbo-Cre is used (Figure 5.6B), I next isolated clones from *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells (clone 5D7) nucleofected with pTurbo-Cre in the absence of tetracycline. Out of 84 clones screened, 3 were found to have successfully deleted the *zeo* cassette. Figure 5.7A shows an example PCR screen of nine clones, amongst them are the 3 clones that have deleted the resistance cassette: AB7, CD9 and FA8 (see also Figure A p.208). Moreover, all 3 clones died when cultured in the presence of *zeocin*.

Following deletion of the *zeo* cassette, it remained to be shown that the truncated Bcl6 protein was no longer expressed in the *BCL6*^{-/-}; *tetBCL6-HA* clones. Western blotting confirms that all 3 isolated clones completely switch off Bcl6 protein expression when cultured in doxycycline and that the truncated Bcl6 protein is almost completely absent (Figure 5.7B). Furthermore, Bcl6 expression is still inducible in *BCL6*^{-/-}; *tetBcl6-HA* cells (Figure 5.7C). The faint residual expression of the variant Bcl6 protein will be discussed further in section 5.3.3 below.

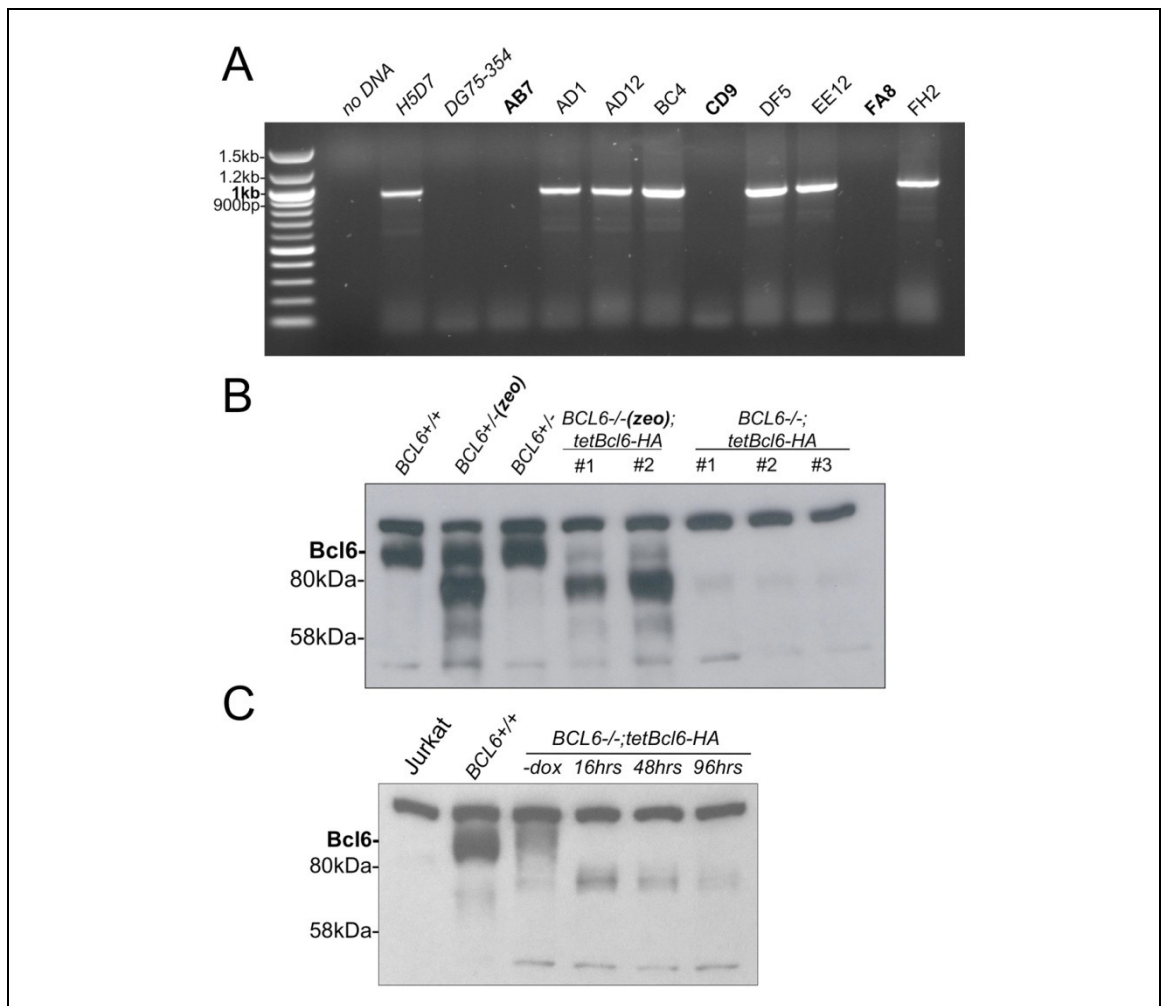


Figure 5.7: PCR screening for *BCL6*^{-/-};*tetBCL6-HA* clones and validation of loss of Bcl6 protein by Western blotting

A) *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells (clone 5D7) were transfected with pTurbo-Cre, single clones were isolated by plating at limiting dilution and screened for loss of the *zeo* cassette in the second *BCL6* allele by PCR assay (Figure 5.5 p.110). Negative controls: lane 1 (no DNA) and 3 (DG75-354, *BCL6*^{+/+}); positive control: lane 2 (*BCL6*^{-/-}(*zeo*);*tetBCL6-HA*, clone 5D7), clones screened: lanes 9 to 12. B) Detection of Bcl6 protein in *BCL6* knockout clones. *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells (clone 2D7 (#1) and clone 5D7 (#2)) were cultured in the absence of doxycycline (express Bcl6-HA). *BCL6*^{-/-};*tetBCL6-HA* clones (clones AB7 (#1), CD9 (#2), and FA8 (#3)) were cultured in doxycycline (1 µg/ml) for 3 days (no Bcl6-HA expression). The truncated Bcl6 protein detected in *BCL6*^{+/-}(*zeo*)(clone BA11) and *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* clones (Figure 5.3) is almost completely absent in *BCL6*^{-/-};*tetBCL6-HA* cells. C) Bcl6 protein is still inducible in conditional *BCL6* knockout cells. *BCL6*^{-/-};*tetBcl6-HA* cells (clone AB7) were cultured without doxycycline (-dox) or with doxycycline for 16, 48, or 96 hrs. Jurkat cells serve as a negative control for Bcl6 expression, DG75-354 cells (*BCL6*^{+/+}) allow for comparison of Bcl6 protein expression between parental and conditional *BCL6*^{-/-} cells.

5.3 Discussion

5.3.1 Targeting of the second *BCL6* allele

Following successful targeting of the first *BCL6* allele in CHAPTER 3, knockout of the second *BCL6* allele was expected to be equally efficient. In fact, 4 out of 17 *zeo*-resistant clones isolated were found to have undergone gene targeting integration after transfection with the *BCL6*-targeting construct. This relative gene targeting frequency of ~24% is similar to that observed for targeting of the first *BCL6* allele (see section 3.3.1). However, an additional screen to determine which allele has been disrupted by the targeting vector was necessary because the same construct was used for targeting of the first and second *BCL6* allele, and hence no selection against re-targeting of the first *BCL6* allele exists. In theory, chances of re-targeting of the first *BCL6* allele and targeting of the second *BCL6* allele are expected to be equal. In fact, out of four clones tested, three had successfully targeted the second *BCL6* allele as confirmed by PCR screening and Southern blotting (Figure 5.1 and Figure 5.2) (see also Figure A p.208).

5.3.2 Detection of an aberrant Bcl6 variant in *BCL6*^{+/-}(*zeo*) and *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells

An aberrant Bcl6 protein variant was detected in *BCL6*^{+/-}(*zeo*) and *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells by Western blotting (Figure 5.3) which coincided with the integration of the *zeo* resistance cassette into either of the two *BCL6* alleles targeted. The occurrence of this aberrant protein was unexpected, as I predicted that any mRNA transcripts generated from a targeted *BCL6* allele would be degraded by nonsense-mediated decay (NMD). NMD is a cellular control mechanism that prevents the expression of truncated or erroneous proteins through detection of premature termination codons (PTCs) in mRNAs. During mRNA splicing in the nucleus, a protein complex is deposited 20-24 nucleotides upstream of exon-exon junctions (Le Hir et al., 2001). These exon junction complexes (EJCs) are displaced by the ribosome during translation

until a stop codon is reached. In aberrant transcripts, however, the ribosome reaches a PTC before all downstream EJC have been displaced and this will trigger NMD i.e. degradation of the non-functional mRNA (Chang et al., 2007). As the *BCL6* gene was disrupted at the third out of ten exons, the PTC at the end of exon 3 (see Figure 3.2 p.75) should activate the NMD machinery and degradation of the non-functional *BCL6* mRNA transcript. There is, however, the possibility that exon 3 is spliced out of the transcript or that a transcript starts downstream of exon 3, thereby bypassing recognition by the NMD pathway. In fact, the occurrence of the aberrant protein coincided with the presence of the *zeo* cassette in one of the *BCL6* alleles (Figure 5.3B). It is therefore highly likely that the enhancer element of the *zeo* cassette activates a cryptic promoter downstream of exon 3 of *BCL6*. Such a cryptic promoter might express a Bcl6 transcript that starts downstream of the PTC engineered to disrupt the reading frame at the end of exon 3 and an alternative start codon in the Bcl6 ORF could be used to start translation (Figure 5.4). To identify such a transcript, 5' RACE (rapid amplification of cDNA ends) could be performed. In 5' RACE analysis, the 5'-untranslated region of a messenger RNA is amplified by PCR and subsequently sequenced (Scotto-Lavino et al., 2006). Once the 5'UTR of such a message is known, this may help to predict the translational start site used to generate the truncated Bcl6 protein expressed. Although such analysis would be interesting, I decided to focus on the deletion of the *zeocin* from the *BCL6* locus in order to eliminate the aberrant protein.

5.3.3 Cre-mediated deletion of the *zeocin* cassette in *BCL6*^{-/-}(*zeo*);*tetBCL6*-HA cells

Cre-mediated deletion of the *zeo* cassette in *BCL6*^{-/-}(*zeo*);*tetBCL6*-HA cells was expected to be difficult because only 2 out of 53 clones could be isolated when the same genetic modification was performed on the first *BCL6* allele targeted (see sections 3.2.6 and 3.3.2). When Cre-recombination was attempted on the second *BCL6* allele targeted, 410 clones derived from 3 independent transfections with pMC-Cre were screened for Cre recombination

and not one clone could be identified to have deleted the resistance cassette. Although other lab members had successfully used the same Cre-expression construct and plasmid preparation, there is the possibility that Cre-expression from pMC-Cre is less efficient in DG75. When reviewing the literature, I found an alternative Cre expression construct pTurbo-Cre (Westervelt et al., 2003). In this construct, Cre-recombinase is equipped with a nuclear localisation signal (NLS) and a CAG promoter drives its expression. In fact, an older study (Araki et al., 1997) compared Cre-recombination efficiency in mouse embryonic fibroblast cells using 4 different promoters. Amongst those promoters tested were the polyoma enhancer/herpes simplex virus thymidine kinase promoter (MC promoter; as in pMC-Cre) and the CAG promoter (as in pTurbo-Cre). The study reported a Cre-recombination frequency less than 0.3 % for the MC promoter and a frequency of up to 25 % for the CAG promoter (Araki et al., 1997). When I used the CAG-driven Cre-expression construct I managed to isolate 3 out of 84 clones (Figure 5.7A) and although this frequency of ~4 % is far lower than what has been reported in the literature, it is sufficient when working with an immortalised cell line and potent screening assays are available.

The deletion of the *zeo* resistance cassette generated a conditional *BCL6* cell line (Figure 5.7C). Levels of Bcl6 protein expressed in parental cells are higher than in *BCL6*^{-/-};*tetBcl6-HA* cells cultured without doxycycline. Options to more closely match conditional Bcl6 protein levels to that of parental cells are discussed in section 6.3.3 below. Deletion of the *zeo* resistance cassette in this cell line almost completely eliminated the truncated Bcl6 protein variant (Figure 5.7B) and any potential residual function of it that could interfere with the conditional *BCL6* knockout phenotype. It is not entirely clear why a weak band is still detected. The only difference between a wildtype and the knockout *BCL6* allele is the presence of a *loxP* site at the end of exon 3. The possibility remains that the *loxP* site interferes with mRNA splicing thereby expressing a low

amount of the Bcl6 variant. However, as discussed in sections 5.2.2 and 5.3.2, such a protein is likely to be non-functional.

Characterisation of the resulting conditional *BCL6* knockout cell line (*BCL6*^{-/-}; *tetBcl6-HA*) was subsequently performed and the results are presented in the following chapters.

CHAPTER 6 - Characterisation of *BCL6*^{-/-};*tetBCL6-HA* cells

6.1 Introduction

On the grounds of the general acceptance that Bcl6 target genes are especially involved in cell cycle regulation and B cell differentiation (Shaffer et al., 2000), I focused particularly on these processes in the course of the characterisation of the *BCL6*^{-/-};*tetBCL6-HA* cell line. Nonetheless, for the reason that the conditional knockout cell line was derived from a cancer cell line, one has to bear in mind and carefully account for known pre-existing genetic abnormalities when interpreting the phenotype.

Like many cancer cell lines, DG75 has accumulated genetic alterations in the cell cycle machinery, an overview of which is illustrated in Figure 6.1. Three aberrations of cell cycle regulators have to date been reported in DG75. Firstly, p53 is inactivated due to a mutation causing its exclusion from the nucleus and preventing induction of p53-dependent apoptosis (Brimmell et al., 1998, Crook et al., 1998). Secondly, DG75 has inactivated the cyclin-dependent kinase (CDK) inhibitor p16ink4a. p16ink4a inhibits the kinase activity of cyclin D-CDK4/6 and can thereby induce cell cycle arrest in G₁ (Serrano et al., 1993). p16ink4a is found to be inactivated by point mutations in the protein coding region, or by hypermethylation of its promoter in most BLs (Klangby et al., 1998, Lindstrom and Wiman, 2002). In fact, treatment of DG75 with a demethylating agent revealed that the locus encoding p16ink4a is hypermethylated in DG75 (Poirier et al., 2001). Thirdly, DG75 cells have an altered cyclin D3 / p27kip1 expression pattern. Cyclin D1 and D2 are not expressed in GC B cells and cyclin D3 is essential for GC formation (Peled et al., 2010). Normal GC-B cells do not express any detectable p27kip1 protein, consistent with their high proliferative index. However, like many aggressive B cell lymphomas, DG75 expresses an unusually high amount of cyclin D3 along

with detectable p27^{kip1} protein (Barnouin et al., 1999). p27^{kip1} in these cells, however, does not seem to restrict cell cycle progression, as p27^{kip1} is found to be almost exclusively bound and sequestered by overexpressed cyclin D3 (Sanchez-Beato et al., 1999).

One of the key characteristics of GC B cells is their extremely short doubling time in conjunction with the absence of detectable CDK inhibitor p27^{kip1} protein (Quintanilla-Martinez et al., 1998, Sanchez-Beato et al., 1999). In fact, Bcl6 contributes to this phenotype by promoting proliferation of GC-B cells through suppression of transcription of the *CDKN1B* gene (encoding p27^{kip1} protein). At the G₁ to S phase transition, p27^{kip1} is a potent inhibitor of CDK2 which, when in complex with cyclin E, promotes entry into S phase. p27^{kip1} mRNA is downregulated in response to Bcl6 expression in cells without endogenous Bcl6, however, a direct regulation could not be demonstrated (Shaffer et al., 2000). A further cell cycle inhibitor gene known to be repressed by Bcl6 is *CDKN1A* (encoding p21^{cip1}/waf1 protein). p21^{cip1}/waf1 inhibits CDK4-cyclin D activity at the G₁ to S phase transition and its transcription is induced upon p53 activation (Sherr and Roberts, 1999). Bcl6 represses transcription of *CDKN1A* through interaction with Miz-1 and this enables GC cells to sustain the elevated degree of DNA damage resulting from SHM and CSR (Phan et al., 2005).

Besides inhibition of the negative cell cycle regulators p27^{kip1} and p21^{cip1}/waf1, Bcl6 also appears to repress transcription of the positive cell cycle regulator cyclin D2. A Bcl6 binding site was identified in the *CCND2* promoter (encoding cyclin D2) and the functional interaction was confirmed by electrophoretic mobility shift assays as well as reporter gene assays (Shaffer et al., 2000). It is not clear why Bcl6 represses a gene that promotes cell cycle progression but an interesting suggestion is, that another D-type cyclin may be better suited for the rapid cell cycle progression of germinal centre cells (Dent et al., 2002).

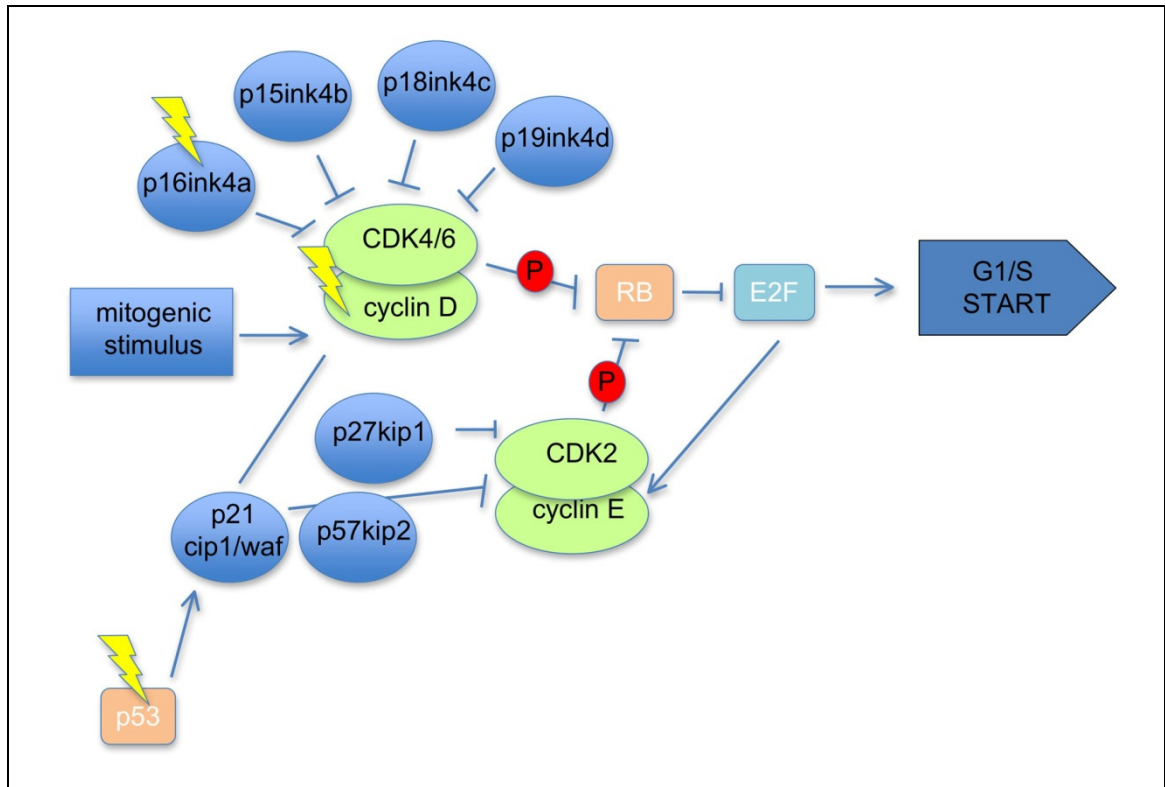


Figure 6.1: Alterations the G₁/S transition in DG75

Mitogenic signalling increases expression of cyclin D, which complexes with CDK4 and CDK6. Cyclin D-CDK4/6 is bound by cip/kip inhibitors (p27kip, p21cip1/waf1, p57kip2) but it seems that this does not impair its kinase activity. Cyclin D alone can bind and thereby sequester p27kip1. Cyclin D-CDK4/6 complexes phosphorylate the retinoblastoma (Rb) protein, thereby leading to degradation of Rb and activation of transcription of E2F target genes. Amongst the E2F target genes are regulators of S phase progression and *CCNE* (encoding cyclin E protein). Cyclin E complexes with CDK2, the kinase activity of which is greatly inhibited by p27kip1. Only once cyclin D and D-CDK4/6 complexes have sequestered enough p27kip1 away from E-CDK2, can E-CDK2 phosphorylate remaining Rb and mediate entry into S phase of the cell cycle. Three aberrations of the cell cycle machinery in DG75 have been documented in the literature and are marked in the above scheme by yellow bolts.

As outlined above, an activated B cell that enters into the GC reaction must apply important changes to its cell cycle regulation to adapt the GC B cell phenotype and also repress lineage inappropriate genes, i.e. B cell activation genes. Bcl6 contributes to this development through repression of the B cell activation genes *CD69* and *CD44* (Shaffer et al., 2000). *CD69* and *CD44* are early activation markers involved in migration of both B and T lymphocytes (Aruffo et al., 1990, Sanchez-Mateos et al., 1989). A Bcl6-binding site was identified in the promoter region of *CD69* (Shaffer et al., 2000), its functional significance, however, has yet to be demonstrated. With regard to stabilisation of the GC-B cell phenotype, Bcl6 also prevents terminal plasma cell differentiation through suppression of *PRDM1* gene that encodes Blimp-1, the master transcription factor regulating plasma cell differentiation (see section 1.3 p.26) (Shaffer et al., 2002, Shaffer et al., 2000). The *PRDM1* gene contains two Bcl6-binding sites, the functional importance of which has been demonstrated by chromatin immunoprecipitation (Parekh et al., 2007, Tunyaplin et al., 2004). When Bcl6 is downregulated towards the end of the GC reaction, suppression of *PRDM1* released, so that Blimp-1 subsequently prompts plasma cell development.

With Bcl6's impact on cell cycle progression and differentiation in mind, I next asked how my *BCL6*^{-/-};*tetBCL6-HA* cells reacted, when depleted of Bcl6 protein.

6.2 Results

6.2.1 Bcl6-depletion causes morphological changes and reduced growth properties

The response of *BCL6*^{-/-}(*zeo*);*tetBCL6-HA* cells to Bcl6-depletion caused by the addition of doxycycline was followed for several days. Distinct morphological changes manifested approximately 3 days after switching off wildtype Bcl6 expression. Cells appeared slightly smaller, more granular and displayed irregular shapes and blebs (Figure 6.2).

In addition to the morphological changes observed, *BCL6*^{-/-};*tetBCL6-HA* cells required much less frequent splitting when cultured in doxycycline. Growth curve analysis revealed that, while the addition of doxycycline to the culture medium had almost no effect on cell growth of parental cells (*BCL6*^{+/+}, DG75-354), all three conditional *BCL6* knockout clones (*BCL6*^{-/-}(*zeo*);*tetBCL6-HA*; AB7, CD9 and FA8) showed dramatically reduced growth in response to Bcl6-depletion (Figure 6.3). Some evidence suggested that growth rates of Bcl6-depleted cells increased slightly following an initial crisis within the first week after addition of doxycycline. Although this effect was difficult to confirm and quantify, it could potentially result from the accumulation of mutations in response to high selective pressure and will be discussed further in section 6.3 below. I next tested whether the perceived overall reduction of cellular growth was caused by cell death (see section 6.2.2 below).

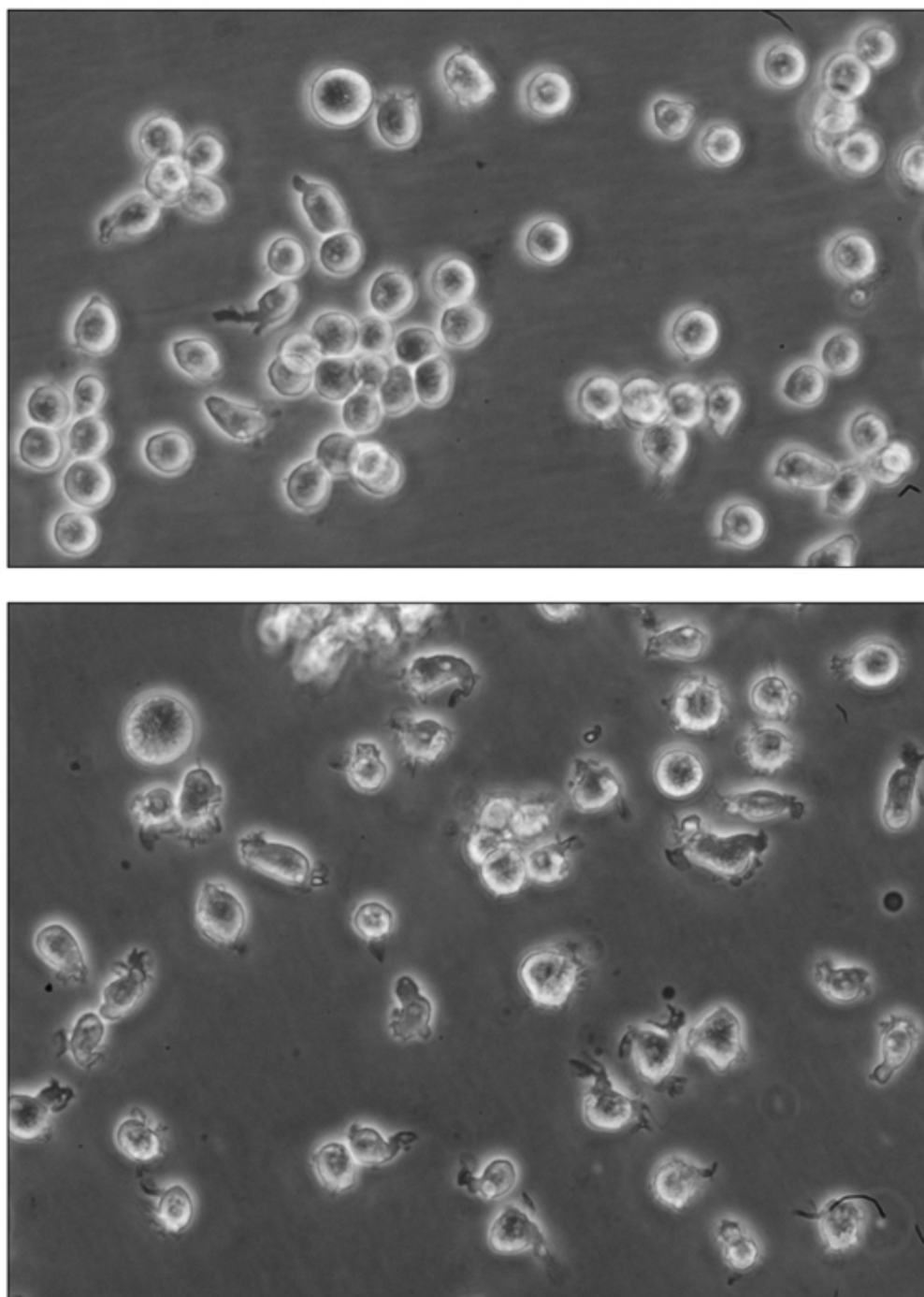


Figure 6.2: Phase contrast microscopy of *BCL6*^{-/-};*tetBCL6-HA* cells

BCL6^{-/-};*tetBCL6-HA* cells (clone FA8) were grown in the absence (top) or presence (bottom) of doxycycline (1 µg/ml) for 7 days.

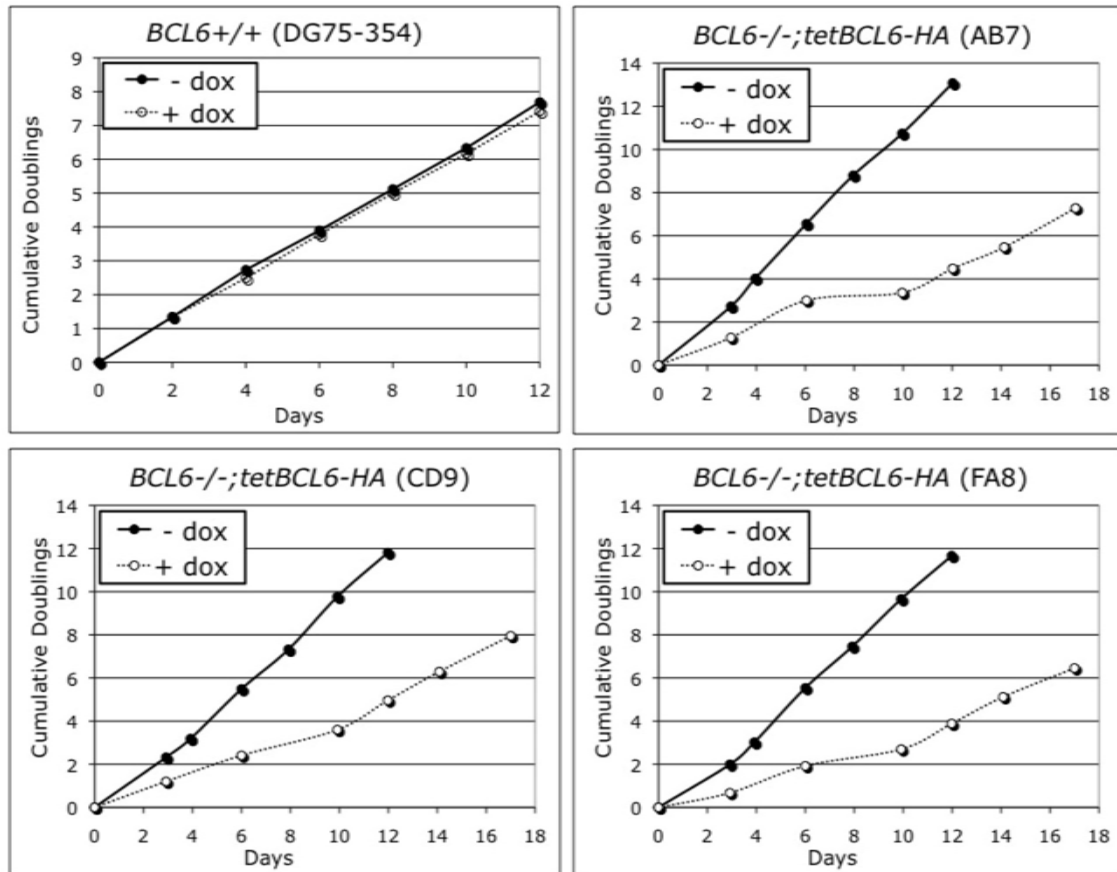


Figure 6.3: Growth characteristics of *BCL6*^{-/-};tet*BCL6*-HA cells

Cells were counted and subsequently passaged so that a preferred cell density of $1-9 \times 10^5$ cells/ml was maintained. Only viable i.e. trypan blue-excluding cells were included in the analysis (see section 2.3.2 p.50 for technical details).

6.2.2 Bcl6-depletion does not cause cell death

The reduced cell size, membrane irregularities, enhanced granularity observed by phase contrast microscopy (Figure 6.2) and the dramatically reduced growth properties (Figure 6.3) suggested, that cells were dying when depleted of Bcl6 protein. Annexin V / PI staining was performed in order to test for cell death; however, no increase in apoptotic or dead cells was detected (Figure 6.4). Moreover, no caspase 3 activation was detected by Western blotting in response to Bcl6 depletion (Figure 6.5). This is not entirely surprising as DG75 cells have a defect in the p53-mediated apoptotic response (see sections 1.12 p.46 and 6.1 above).

In addition to apoptosis and necrosis, cells can utilise a third mode of cell death i.e. autophagy (Hotchkiss et al., 2009). Similarly to apoptosis, the induction of autophagy is also mediated by p53 (Levine and Abrams, 2008), however, the autophagic response has been reported to be mediated by cytoplasmic p53 (Tasdemir et al., 2008) and should accordingly be functional in DG75. In order to investigate whether Bcl6-depletion activates autophagy in the conditional *BCL6* knockout cell line, we performed electron microscopic analysis of the cell line, however, no phagophores (cytosolic smooth, ribosome-free double membrane cisternae) or autophagosomes (single membrane enclosing cytoplasmic structures) indicative of autophagy (Klionsky et al., 2008, Larsen and Sulzer, 2002) could be detected (Figure 6.6). To confirm these results, I also tested *BCL6*^{-/-};*tetBCL6-HA* cells for post-translational modification of the autophagy marker light chain 3B (LC3B) (Wu et al., 2006). During autophagy the 18 kDa LC3B-I isoform becomes lipidated and converted into the 16 kDa LC3B-II isoform (Kabeya et al., 2004). Figure 6.5 shows a low level of lipidated LC3B-II isoform in Bcl6-expressing *BCL6*^{-/-};*tetBcl6-HA* cell which appears to be induced when doxycycline is added and Bcl6 protein is downregulated. Although this upregulation of lipidated LC3B-II isoform in response to Bcl6-depletion is not as strong as observed for the positive control (K562 cells), this

effect could potentially be studied further. After ruling out that the reduced growth rates observed (Figure 6.3) were caused by cell death, I concluded that Bcl6-depletion reduces the proliferative capacity and I performed DNA content analysis to monitor alterations in cell cycle progression.

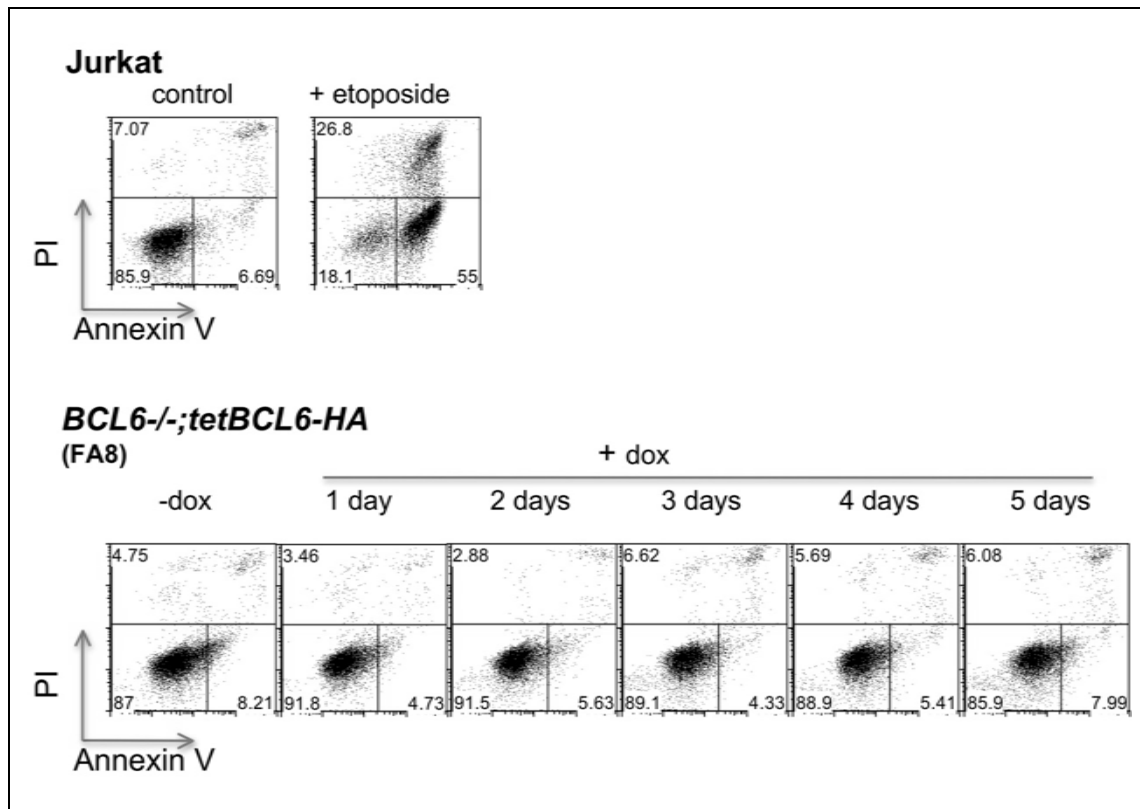


Figure 6.4: *BCL6*^{-/-};*tetBCL6-HA* cells do not apoptose in response to Bcl6-depletion

Annexin V / PI staining was performed on Jurkat cells grown in 10 μ M etoposide for 24 hrs and on *BCL6*^{-/-};*tetBCL6-HA* cells (clone FA8) cultured in the absence or presence of doxycycline (1 μ g/ml) over a time course of 5 days. Apoptotic cells stain positive for Annexin V and necrotic and dead cells stain positive for both Annexin V and PI. See section 2.3.6 p.52 for technical details.

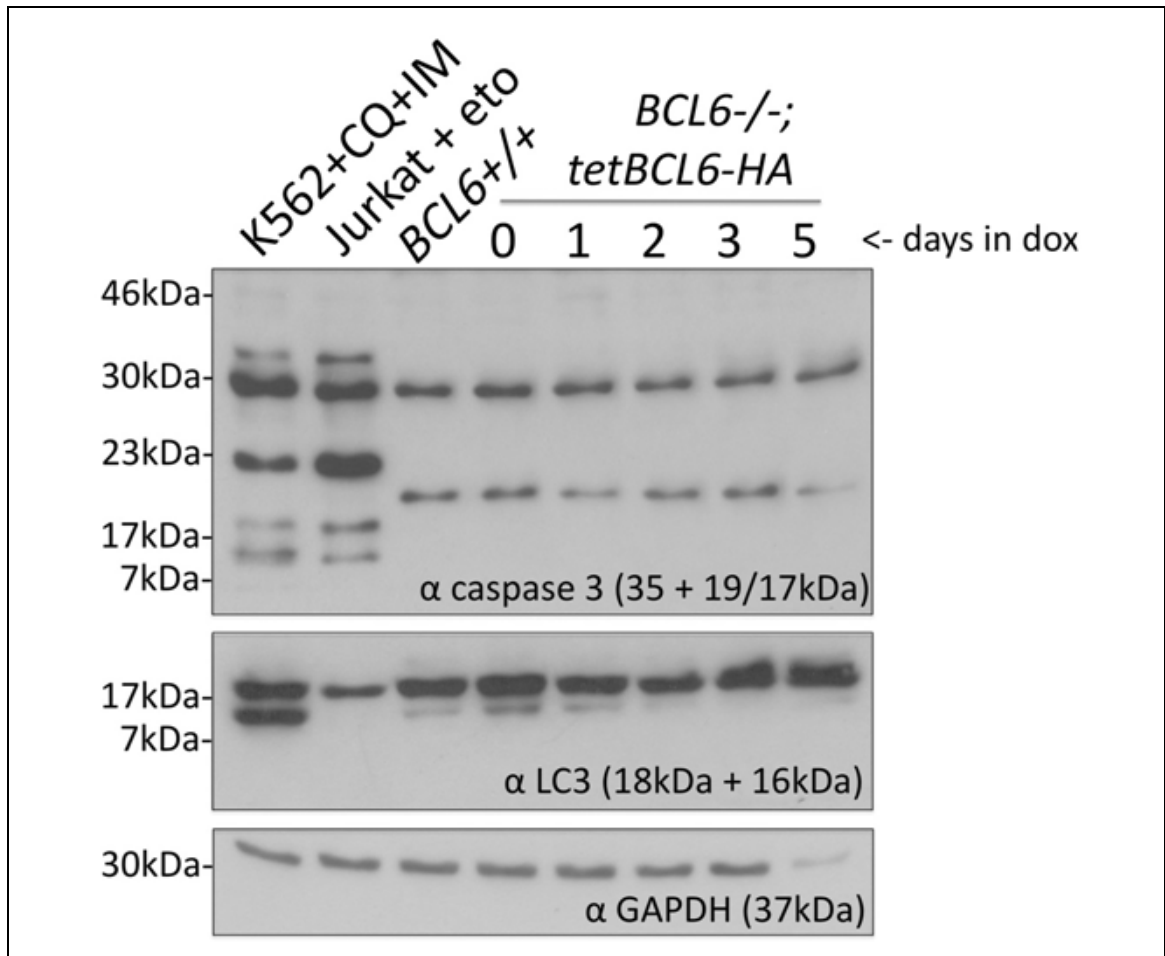


Figure 6.5: No apoptosis or autophagy is observed in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells

Detection of caspase 3 cleavage and LC3 lipidation in response to Bcl6 depletion of *BCL6*^{-/-};*tetBCL6-HA* cells (clone CD9). Jurkat cells were treated with 10 μ M etoposide (Sigma) for 24 hrs were used as a positive control for caspase 3 activation. The antibody used detects full-length caspase-3 (35k Da) and the large fragment (17/19 kDa) generated after activation and cleavage of caspase 3. K562 cells were treated with 25 μ M chloroquine (CQ) (Sigma) and 0.25 μ M imatinib (IM) for 48 hrs as a positive control for LC3B activation (Bellodi et al., 2009). DG75-354 cells (*BCL6*^{+/+}) served as negative control. *BCL6*^{-/-};*tetBCL6-HA* cells were cultured without (0) or with 1 μ g/ml doxycycline for 1, 2, 3 and 5 days.

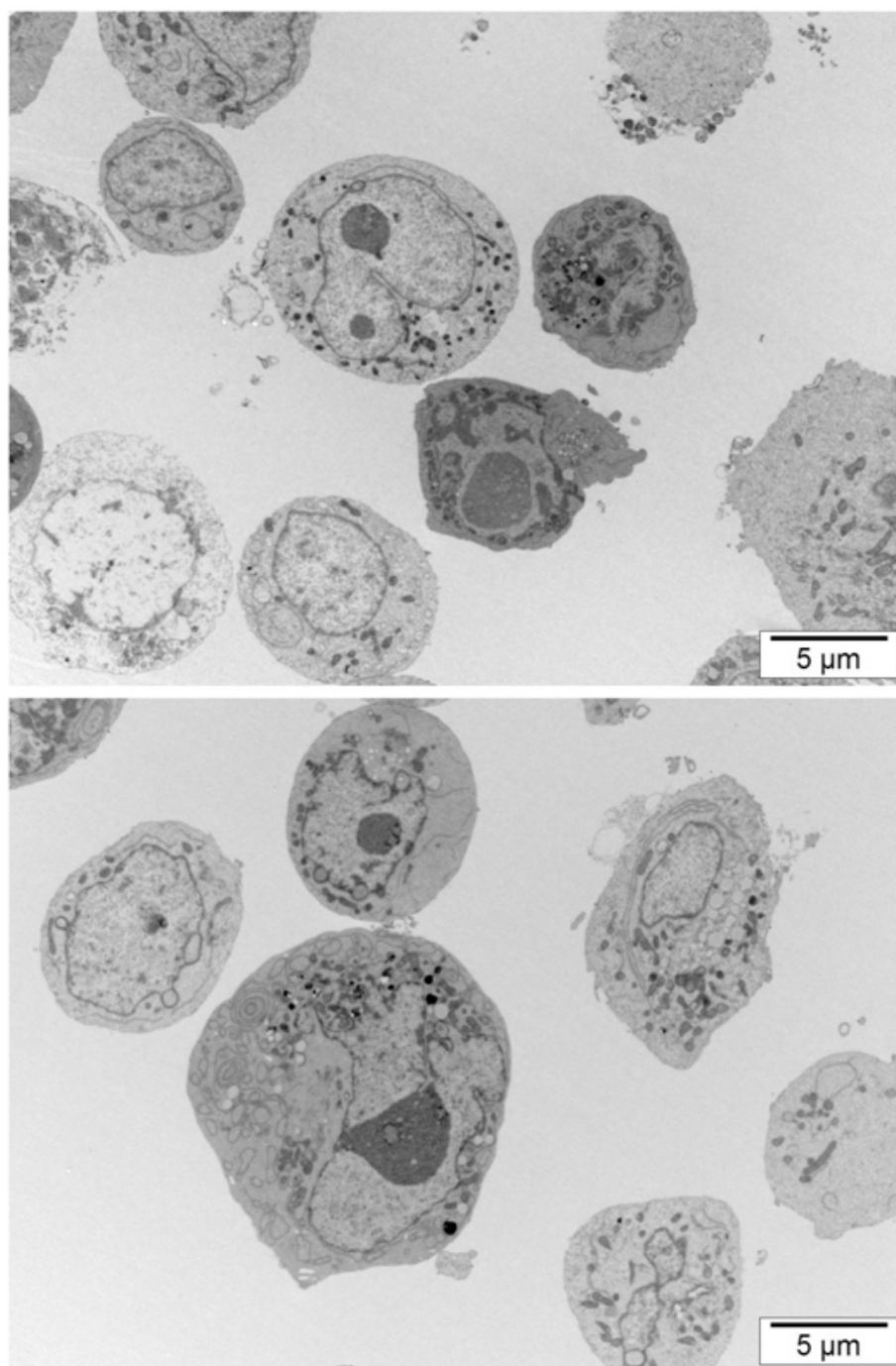


Figure 6.6: Electron microscopic analysis of *BCL6*^{-/-};*tetBCL6-HA* cells

BCL6^{-/-};*tetBCL6-HA* cells (clone AB7) were cultured without (top) or with 1 µg/ml doxycycline (bottom) for 5 days prior to electron microscopic analysis by Dr Shaun Willimott and Dr David Dinsdale (see section 2.3.3 p.50 for technical details).

6.2.3 Bcl6-depletion causes accumulation of cells in G₀/G₁-phase of the cell cycle

Aiming to elucidate the reason for reduced growth properties of Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells (see section 6.2.1 above), I conducted cell cycle analysis by flow cytometry. DNA content analysis revealed, that Bcl6-depletion increases the percentage of cells in the G₀/G₁-phase of the cell cycle (see Figure 6.7). This could either be due to some of the cells exiting the cell cycle while others continued to proliferate or due to homogeneous prolongation of G₀/G₁-phase of all cells in the population. In order to investigate which of the two possibilities was causing the apparent growth retardation, I labelled cells with CFSE prior to Bcl6-depletion and followed their divisions by flow cytometry. Figure 6.8 shows that all CFSE-labelled populations proliferate homogeneously and that no non-dividing cell population is left behind. Having established that a key phenotype of the *BCL6*^{-/-};*tetBCL6-HA* cell line lies in their reduced proliferative capacity, I next set out to examine changes in expression of key regulators of cell cycle progression and differentiation (see section 6.2.4 below).

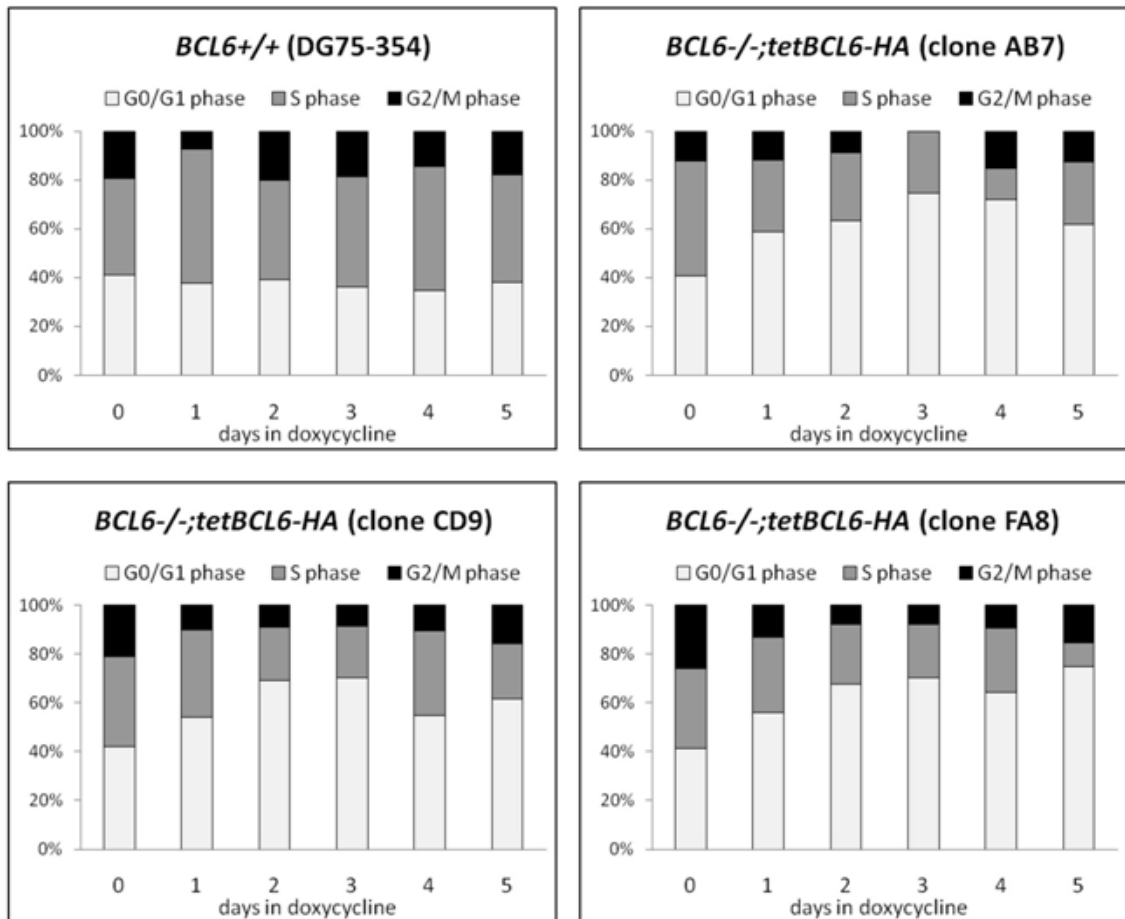


Figure 6.7: *Bcl6*-depleted *BCL6*^{-/-};*tetBCL6-HA* cells accumulate in G₀/G₁ phase of the cell cycle

BCL6^{+/+} (parental cells, DG75-354) or *BCL6*^{-/-};*tetBCL6-HA* clones (AB7, CD9, FA8) were incubated without doxycycline (0) or with 1 µg/ml doxycycline for 5 days. Cells were fixed, PI-stained and their DNA content was analysed by flow cytometry daily (see section 2.3.6 p.52 for further details).

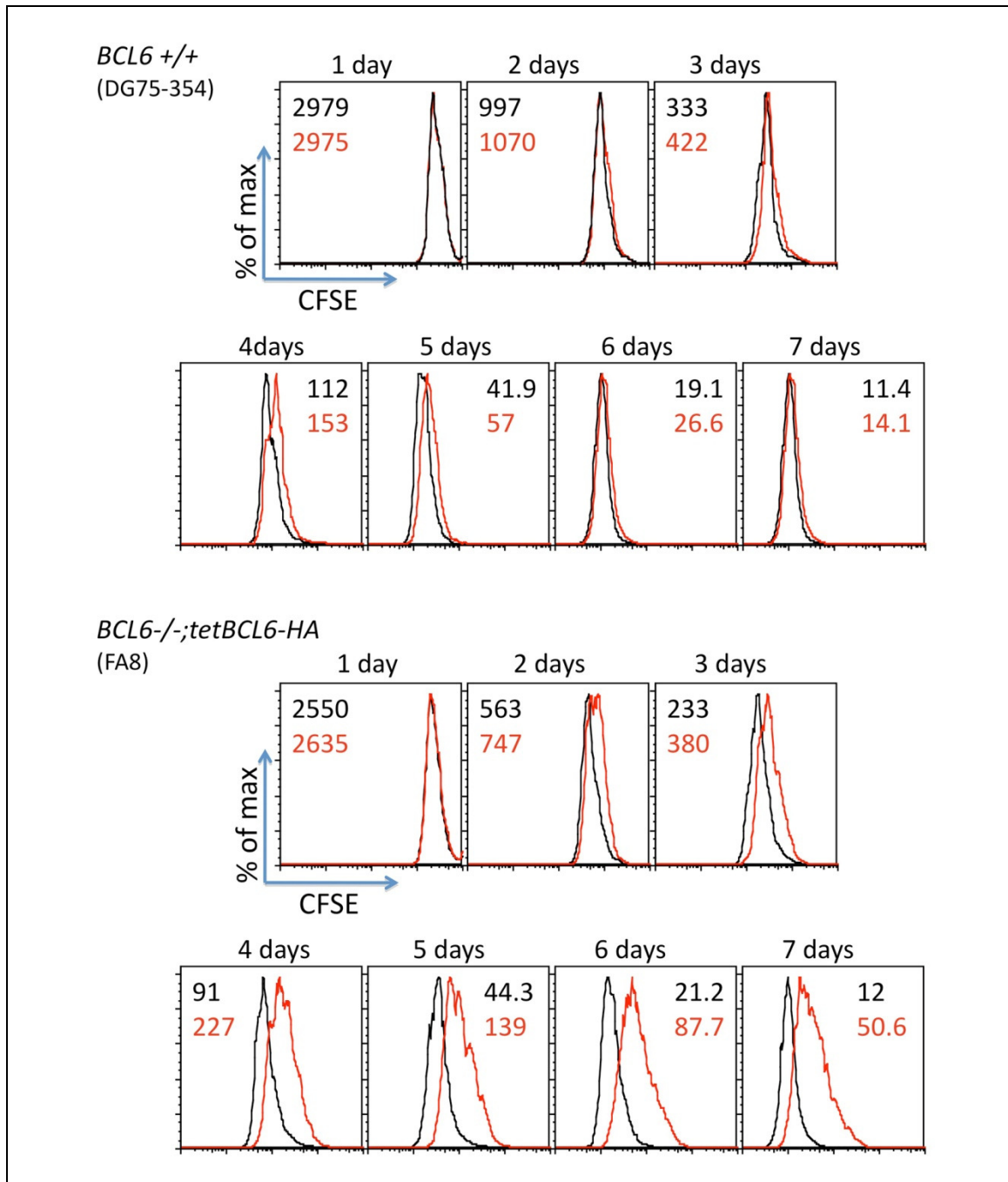


Figure 6.8: Cell proliferation assay of Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells

BCL6^{+/+} (parental cells, DG75-354) or *BCL6*^{-/-};*tetBCL6-HA* (clone FA8) were labelled with CFSE and subsequently incubated without doxycycline or with 1 µg/ml doxycycline for 7 days. Cells were analysed by flow cytometry daily and overlay histograms of cells grown without (black) or with doxycycline (red) were produced. Numbers in histograms show corresponding mean fluorescence intensities (see section 2.3.6 p.52 for further details).

6.2.4 Influence of Bcl6-depletion on Bcl6-target genes and regulators of proliferation and differentiation

Flow cytometry (Figure 6.9 and Figure 6.10), Western blotting (Figure 6.11 and Figure 6.12) and semiquantitative (sq) RT-PCR analysis (Figure 6.13) were applied to test for changes in expression of known Bcl6 target genes and key regulators of differentiation and cell cycle progression.

Bcl6 represses genes involved in B cell activation and a Bcl6 binding site has been detected in the promoter region of the *CD69* gene (Shaffer et al., 2000). CD69 is a cell surface marker and a change in expression can therefore be monitored by flow cytometry. Figure 6.9 shows that little if any CD69 expression is induced upon depletion of Bcl6 in response to addition of doxycycline. Further to inhibiting genes involved in earlier B cell development, Bcl6 also suppresses terminal B cell differentiation through inhibition of the *PRDM1* gene encoding Blimp-1 (Shaffer et al., 2000). However, neither induction of Blimp-1 mRNA nor protein could be detected in response to Bcl6-depletion (Figure 6.11 and Figure 6.13). I moreover observed an increase in Pax5 mRNA expression, the expression of which is expected to be downregulated once Blimp-1 expression is induced (Figure 6.13).

CSR of *BCL6*^{-/-};*tetBCL6-HA* cells would be detected as complete loss of IgM-expression of some or all cells of the population. However, this effect was not observed in response to Bcl6-depletion, indicating that no progression towards terminal B cell differentiation is taking place (Figure 6.10). There is some evidence, however, that IgM-expression is slightly decreased in response to Bcl6-depletion in *BCL6*^{-/-};*tetBCL6-HA* cells and this could potentially be studied further (Figure 6.10).

When I focused on gene expression changes of key regulators of cell cycle regulation in response to Bcl6-depletion, I found no changes in expression of c-

Myc (Western blotting and sq RT-PCR), p15^{ink4B} (Western blotting), CDK4/6 (Western blotting), cyclin E (Western blotting) and cyclin D3 (Western blotting and sq RT-PCR) between conditional *BCL6*^{-/-} cells cultured in the absence or presence of doxycycline (Figure 6.11-Figure 6.13).

Although no changes in p21^{cip1/waf1} mRNA expression were detected between conditional *BCL6*^{-/-} cells cultured in the absence or presence of doxycycline (Figure 6.13), p21^{cip1/waf1} protein levels changed with *BCL6* status and Bcl6 protein expression (Figure 6.12). Somewhat surprisingly, p21^{cip1/waf1} protein was barely detectable in parental DG75-354 cells (*BCL6*^{+/+}), however, much more strongly expressed in Bcl6-expressing *BCL6*^{-/-};tet*Bcl6*-HA cells. Furthermore, the significant level of p21^{cip1/waf1} protein expressed in *BCL6*^{-/-};tet*Bcl6*-HA cells is downregulated upon Bcl6 protein depletion to a level still exceeding that of parental DG75-354 (*BCL6*^{+/+}) cells (Figure 6.12). This finding will be further discussed in section 6.3 below. Encouragingly, I detected an upregulation of p27^{kip1} both by Western blotting and sq RT-PCR (Figure 6.12 and Figure 6.13).

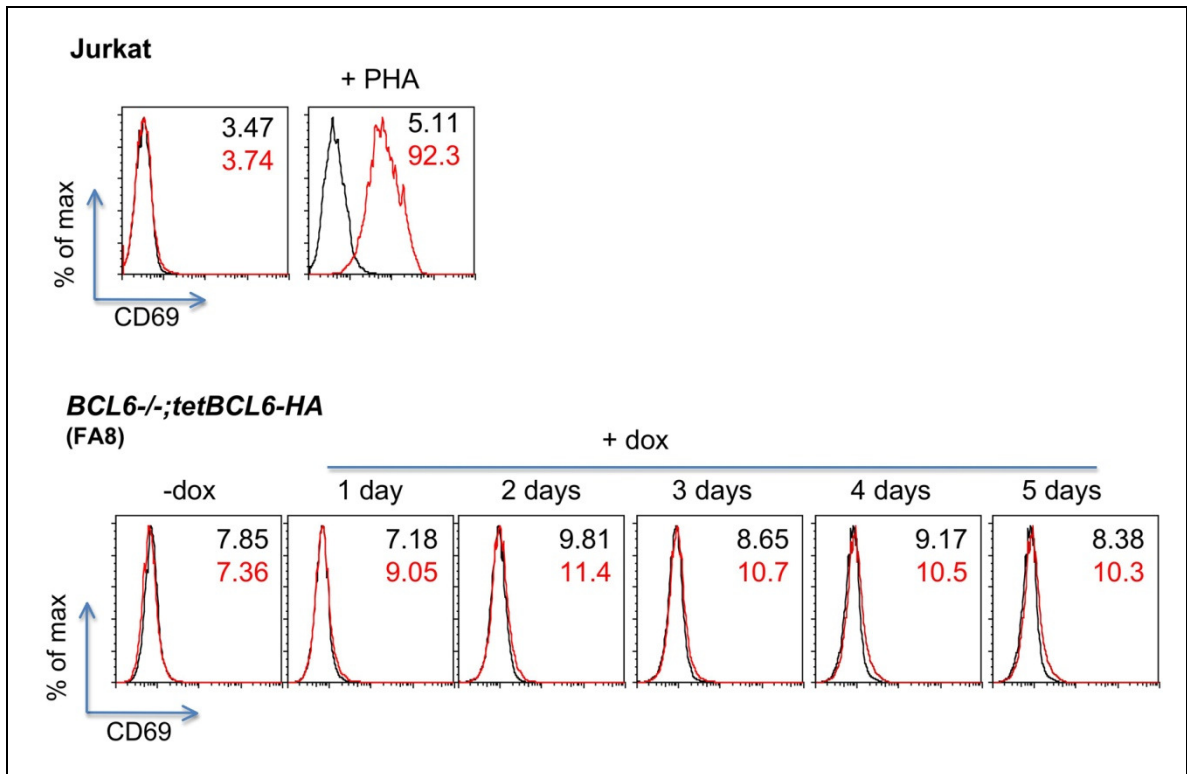


Figure 6.9: No CD69 expression is induced in *Bcl6*-depleted *BCL6*^{-/-};*tetBCL6-HA* cells

Jurkat cells were stimulated with 0.1 % (v/v) phytohemagglutinin (PHA; M form, Invitrogen) for 24 hrs. *BCL6*^{-/-};*tetBCL6-HA* cells (clone FA8) were cultured without or with 1 μ g/ml doxycycline and analysed daily. All graphs are overlays of α -CD69-FITC (clone FN50) (red line) and the mouse IgG₁ κ isotype control (clone MOPC-21)stained cells (BD Biosciences) (black line). Values in graphs denote mean fluorescence intensities of isotype controls (black) and α -CD69 (red) (see section 2.3.6 p.52 for further details).

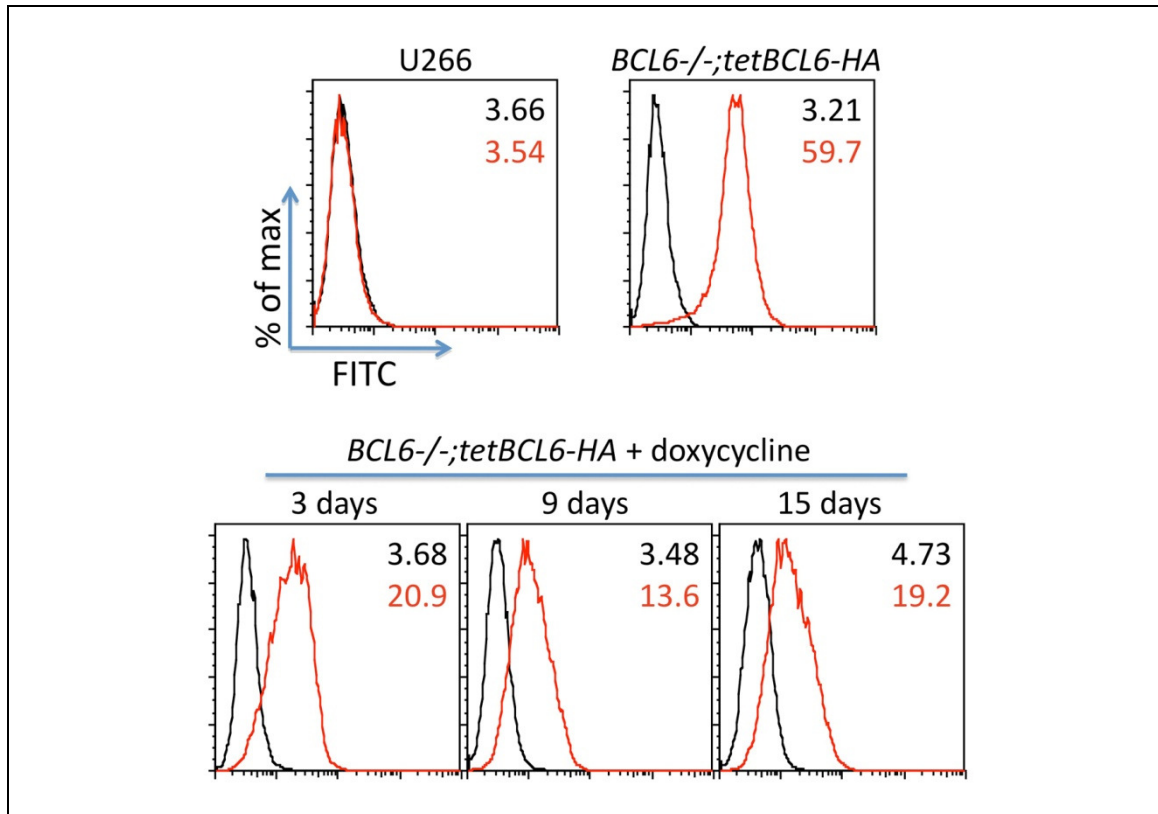


Figure 6.10: No class switch recombination is induced in *Bcl6*-depleted *BCL6*^{-/-};*tetBCL6-HA* cells

IgM-expressing *BCL6*^{-/-};*tetBCL6-HA* cells (clone FA8) were cultured without or with 1 µg/ml doxycycline for 15 days and analysed after 3, 9 and 15 days. IgE-expressing U266 cells served as negative control. All graphs are histogram overlays of α-IgM-FITC (clone G20-127) (red line) and mouse IgG₁κ isotype control (clone MOPC-21) stained cells (black line) (BD Biosciences). Values in graphs represent mean fluorescence intensities (see section 2.3.6 p.52 for further details).

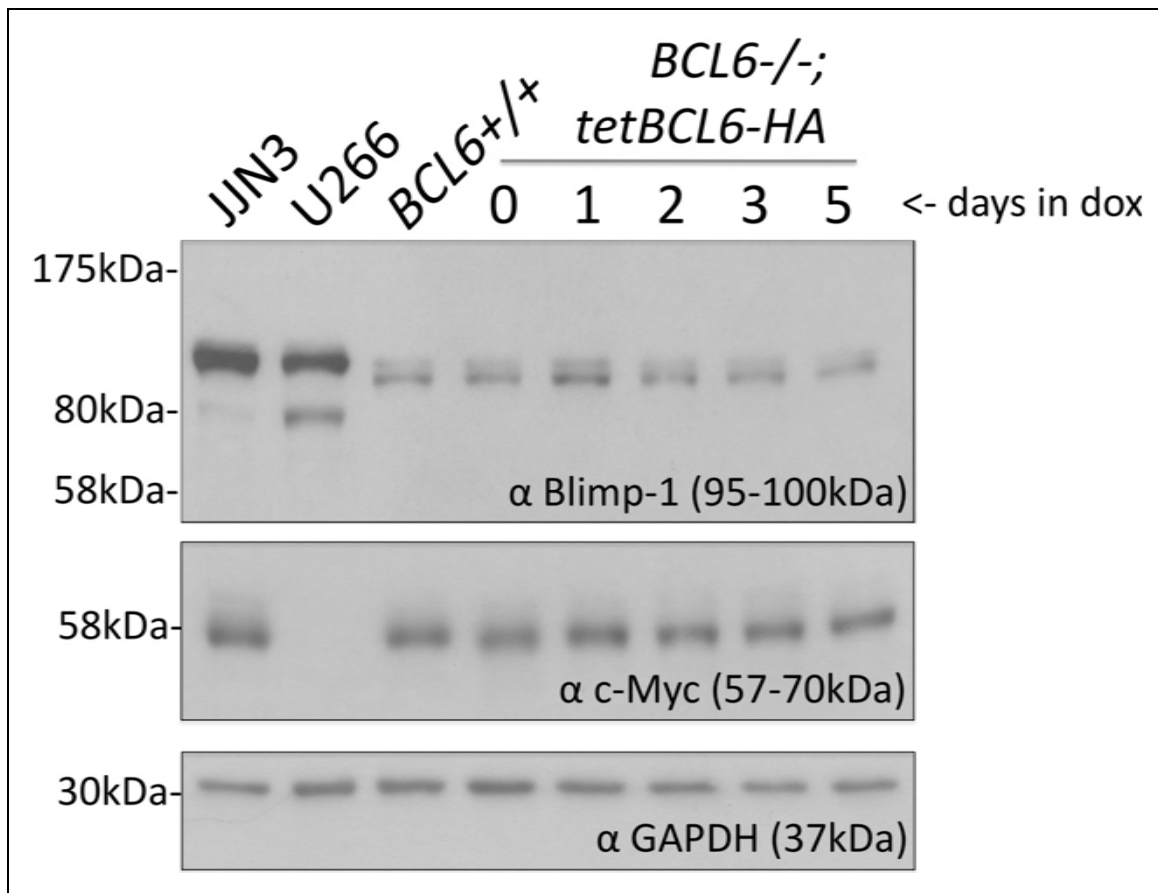


Figure 6.11: No changes in Blimp-1 and c-Myc protein expression are observed in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells

JJN3 and U266 are myeloma cells serving as positive controls for expression of Blimp-1. Parental cells (*BCL6*^{+/+}; DG75-354) served as negative control. *BCL6*^{-/-};*tetBCL6-HA* cells (clone CD9) were cultured without (0) or with 1 µg/ml doxycycline for 1, 2, 3 and 5 days.

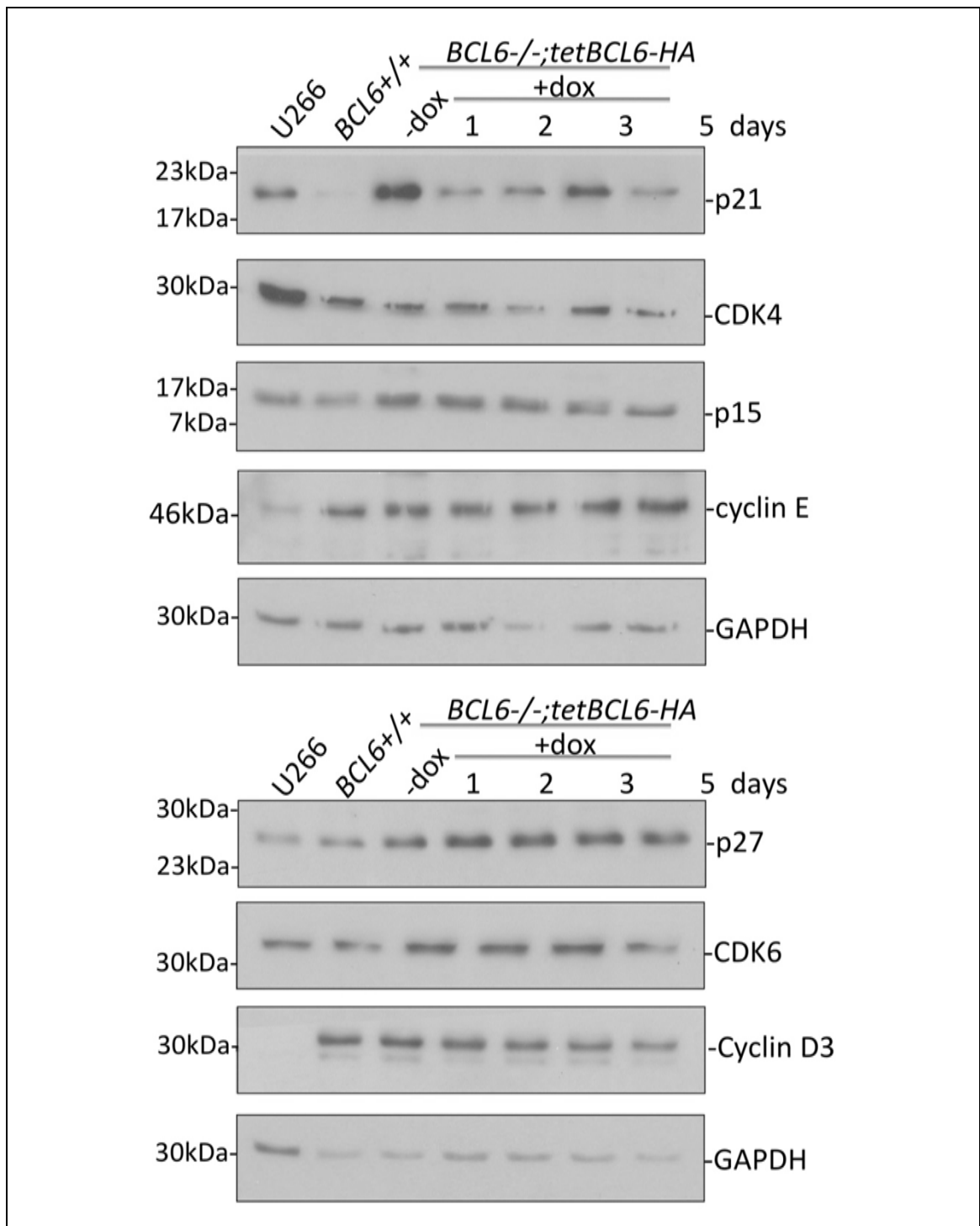


Figure 6.12: Effect of Bcl6-depletion in *BCL6*^{-/-};*tetBCL6-HA* cells on cell cycle regulatory proteins

U266 and parental cells (*BCL6*^{+/+}; DG75-354) served as controls. *BCL6*^{-/-};*tetBCL6-HA* cells (clone CD9) were cultured without (0) or with 1 µg/ml doxycycline for 1, 2, 3 and 5 days.

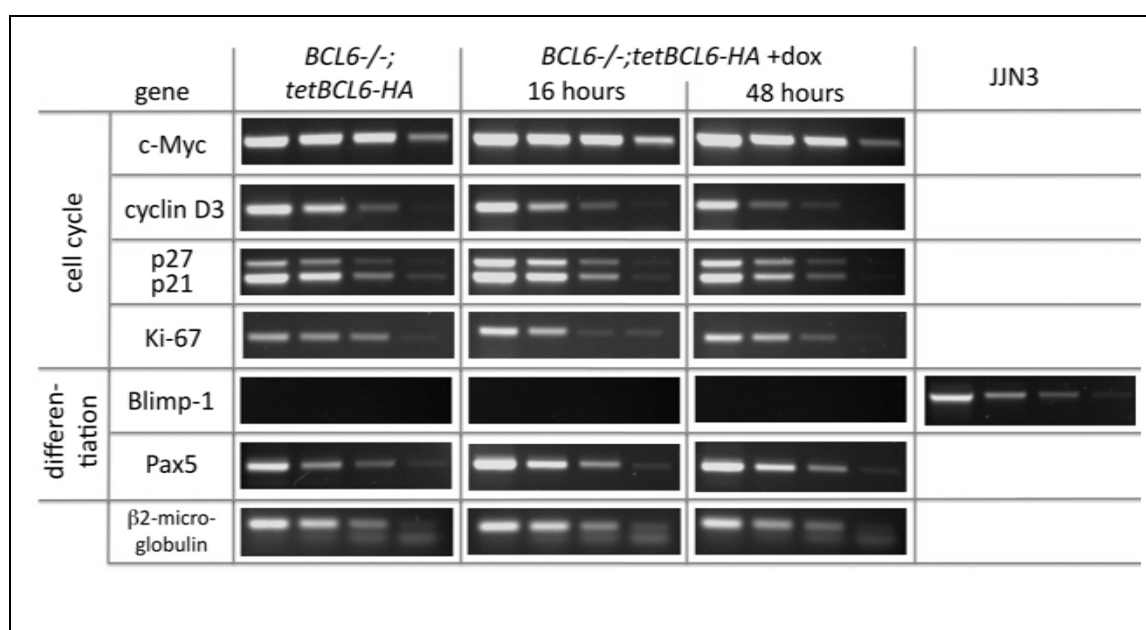


Figure 6.13: Sq RT-PCR analysis of selected mRNA transcripts involved in cell cycle regulation and differentiation in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells

Total RNA from JJN3 cells and *BCL6*^{-/-};*tetBCL6-HA* cells (clone AB7) cultured without or with doxycycline for 16 or 48 hours was extracted and reverse transcribed. Dilutions of 1, 1:5, 1:25 and 1:125 (left to right) of the resulting cDNAs were prepared and used as template for the following PCR reaction. See section (see section 2.5.4 p.62) for more details.

6.3 Discussion

6.3.1 Bcl6-depletion does not induce cell death in *BCL6*^{-/-};*tetBCL6-HA* cells

Analysis of the *BCL6*^{-/-};*tetBCL6-HA* phenotype by microscopy, flow cytometry, sq-RT-PCR and Western blotting revealed that Bcl6-depletion reduces the proliferative capacity of the cell line. Contrary to our expectations, Bcl6-depletion did not cause cell death as no apoptosis, necrosis or autophagy was detected by flow cytometry, electron microscopy and Western blotting (Figure 6.4, Figure 6.6 and Figure 6.5). As already reviewed in section 1.10 (p.41), it is believed that GC B cells and their malignant counterparts depend on Bcl6 expression for survival. This view was based on *BCL6* knockout mice studies (Dent et al., 1997), microarray analysis of a BL cell line (Raji) (Shaffer et al., 2000) and several studies in which the Bcl6 POZ domain was inhibited (Cerchietti et al., 2010, Cerchietti et al., 2009, Chattopadhyay et al., 2006, Polo et al., 2004). It is not entirely clear why Bcl6-depletion in our system does not confirm the absolute requirement of Bcl6 for survival but it should be considered that the DG75 cell line is severely compromised in its apoptotic pathway (see section 1.12) and that some of the models that had been used to establish that Bcl6-depletion in GC-cells is lethal, may not be optimal to draw such a conclusion.

Like all Burkitt lymphomas, DG75 carries the t(8;14) translocation, which places the c-Myc gene under the control of the immunoglobulin heavy chain promoter, resulting in overexpression of c-Myc. Typically, c-Myc induces p19ARF, in turn sequestering Mdm2, resulting in p53 activation and cell cycle arrest or apoptosis (Zindy et al., 1998). In order to overcome this tumour suppressor mechanism, most Burkitt lymphomas accumulate mutations in the p53-pathway. In fact, DG75 carries a heterozygous p53 mutation, which impairs p53 shuttling into the nucleus. The respective mutant p53 protein acts in a dominant-negative way, by homodimerising with and thereby sequestering

wildtype p53 in the cytoplasm (Crook et al., 1998). Moreover, DG75 cells lack expression of one of the pro-apoptotic Bcl2-family members. A frameshift mutation in the Bax coding region further desensitises DG75 cells to p53-dependent cell death (Brimmell et al., 1998). Taken together, these aberrations could possibly contribute to the absence of increased apoptosis observed in the *BCL6*^{-/-};*tetBCL6-HA* model system.

The apoptotic defect in DG75 cells is one explanation for the unexpected absence of cell death in response to Bcl6-depletion in my system; however, it must also be considered that the models that were used to demonstrate the apparent strong correlation between Bcl6-depletion and the apoptotic response may not be suitable to draw such a conclusion. While studies in *BCL6* knockout mice only demonstrated that Bcl6 is required for GC formation i.e. proliferation of GC B cells (Dent et al., 1997), cell death was only demonstrated when a dominant-negative Bcl6 variant was transiently expressed in the BL cell line Raji (Shaffer et al., 2000) and when Bcl6-expressing cells were treated with Bcl6 POZ domain inhibitors (Cerchiatti et al., 2010, Cerchiatti et al., 2009, Chattopadhyay et al., 2006, Polo et al., 2004). The dominant-negative approach involved transient overexpression of only the zinc finger domain of Bcl6 in a cell line and it cannot be ruled out that this artificial protein interferes with cellular functions unrelated to Bcl6. It is possible that conformational changes resulting from the deletion of the larger part of the Bcl6 protein alter the DNA binding affinity of the zinc finger domain. The Bcl6 zinc fingers have been shown to not only operate in DNA binding but also interact with other proteins such as ETO (Chevallier et al., 2004). Therefore, the interaction of the Bcl6 ZF portion with other proteins might be altered and overexpression of such a mutant protein could potentially sequester an unpredictable range of other cellular proteins, thereby triggering an apoptotic response unrelated to specific Bcl6-depletion.

Off-target side effects have to be considered for expression of the dominant-negative Bcl6 truncation and also for the utilisation of Bcl6 inhibitors. Although the authors of the corresponding studies have demonstrated that such inhibitors only cause apoptosis in cell lines expressing Bcl6 (Cerchietti et al., 2010, Cerchietti et al., 2009, Chattopadhyay et al., 2006, Polo et al., 2004), this does not prove that the apoptotic response is solely attributable to Bcl6 inhibition.

It could well be that off-target side effects of Bcl6 inhibitors differ between Bcl6-expressing and not Bcl6-expressing cell lines. Furthermore, it is possible that a combination of off-target side effects and Bcl6-depletion is required to cause cell death, and that either alone is insufficient. It would therefore be incredibly interesting to test whether the phenotype I observed for Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells can be reproduced when Bcl6 inhibitors are added to DG75 cells or *BCL6*^{-/-};*tetBCL6-HA* cells cultured without tet or dox (Bcl6-expression is on). If such an experiment reproduced the *BCL6*^{-/-} knockout phenotype I observed in this project, this would provide solid support for the specificity of Bcl6 inhibitors. However, should Bcl6 inhibitors produce a more severe phenotype or even cell death in DG75 cells or Bcl6-expressing *BCL6*^{-/-};*tetBCL6-HA* cells, this would argue strongly for a combinatorial effect of Bcl6 inhibition and off-target side effects and thereby challenge the current belief that Bcl6 is essential not only for proliferation but also survival of GC B cells.

6.3.2 Bcl6-depletion does not affect differentiation of *BCL6*^{-/-};*tetBCL6-HA* cells

Besides the unexpected absence of cell death in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells, my model system also did not confirm the absolute requirement of Bcl6 for maintaining the GC-B cell stage of B cell development.

I detected no upregulation of genes involved in earlier or later stages of B cell differentiation in response to Bcl6-depletion. CD69 is one of the earliest

expression markers of lymphocyte activation and is involved in migration and cytokine secretion and is regulated by Bcl6, probably to manifest the GC phenotype (Shaffer et al., 2000). *CD69* gene expression seems to be regulated differently in B and T cells and is only poorly understood to date (Vazquez et al., 2009). The biological significance of the Bcl6-binding site of the *CD69* promoter could not be demonstrated to date (Shaffer et al., 2000) but there is a potential alternative way, how Bcl6 could suppress *CD69* transcription. As mentioned in section 1.7 (p.36), Bcl6 interacts with Jun proteins that are components of the activator protein 1 (AP-1) complex and can thereby repress transcription of *PRDM1* (Blimp-1) (Vasanwala et al., 2002). Interestingly AP-1 is one of the activating transcription factors that regulate *CD69* expression (Castellanos et al., 1997). However, whether Bcl6 regulates *CD69* expression directly through binding to its binding site in the *CD69* promoter or indirectly via interaction with Jun proteins of the AP-1 complex, remains to be explored experimentally. I failed to detect any marked surface *CD69* expression in response to Bcl6-depletion in my conditional Bcl6 expression model. This is not entirely surprising as activation of *CD69* transcription appears to be activated by interplay of not only the AP-1 but also EGR and ATF/CREB families (Castellanos Mdel et al., 2002), which are likely being repressed by Bcl6-independent mechanisms in GC B cells.

Besides the lack of evidence for differentiation back to developmental stages earlier than the GC-stage of Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells, I have collected no unequivocal data suggesting a progression of cells towards terminal plasma cell differentiation. As overexpression of Blimp-1 in mature B cells causes plasma cell differentiation, Blimp-1 appears to be both necessary and sufficient for plasma cell differentiation (Shapiro-Shelef and Calame, 2005). *PRDM1* can be considered the best characterised Bcl6-target gene, somewhat surprisingly, however, no Blimp-1 upregulation was observed in response to Bcl6 depletion in my system - no Blimp-1 protein and more importantly no Blimp-1 mRNA could be detected. Although the *PRDM1* locus can be altered in

DLBCL cells (Pasqualucci et al., 2006) and I have not formally confirmed the integrity of the *PRDM1* locus in DG75, I provisionally concluded, that Bcl6 downregulation is required but not sufficient for Blimp-1 expression. Blimp-1 expression is assumed to be mainly regulated on the transcriptional level, as no experimental evidence has been reported to date that indicates posttranslational modifications. Moreover, Blimp-1 mRNA expression appears to correlate well with protein expression. Bcl6 is a well-established, but not the only repressor of *PRDM1* transcription. Apart from Bcl6, two additional transcriptional repressors of *PRDM1* are likely being expressed in DG75, i.e. Pax5 and Bach2 (Calame, 2008) (see Figure 6.14).

Pax5 is expressed from the pro-B cell stage throughout development until it is downregulated upon plasmacytic differentiation and functions in suppressing lineage-inappropriate genes. Pax5 represses *PRDM1* transcription through binding to its exon 1 (Mora-Lopez et al., 2007) and this repressive effect is likely to be active in *BCL6*^{-/-};*tetBCL6-HA* cells as parental DG75 cells express Pax5 protein (Renne et al., 2006) and an increase in mRNA abundance in response to Bcl6-depletion was detected by sq RT-PCR in my model system (Figure 6.13). Bach2, a further repressor of the *PRDM1* gene, is expressed from the pro-B cell stage, only silenced upon plasmacytic differentiation and therefore very likely to be expressed in DG75. *BACH2* knockout mice show a defect in GC formation and have elevated Blimp-1 mRNA levels without any alteration in Bcl6 expression (Muto et al., 2004). On a molecular basis, Bach2 alone can repress *PRDM1* expression in reporter gene assays, while Bcl6 had no effect in the system. However, maximal repression of Blimp-1 expression was only achieved when Bach2 and Bcl6 were co-expressed (Ochiai et al., 2008).

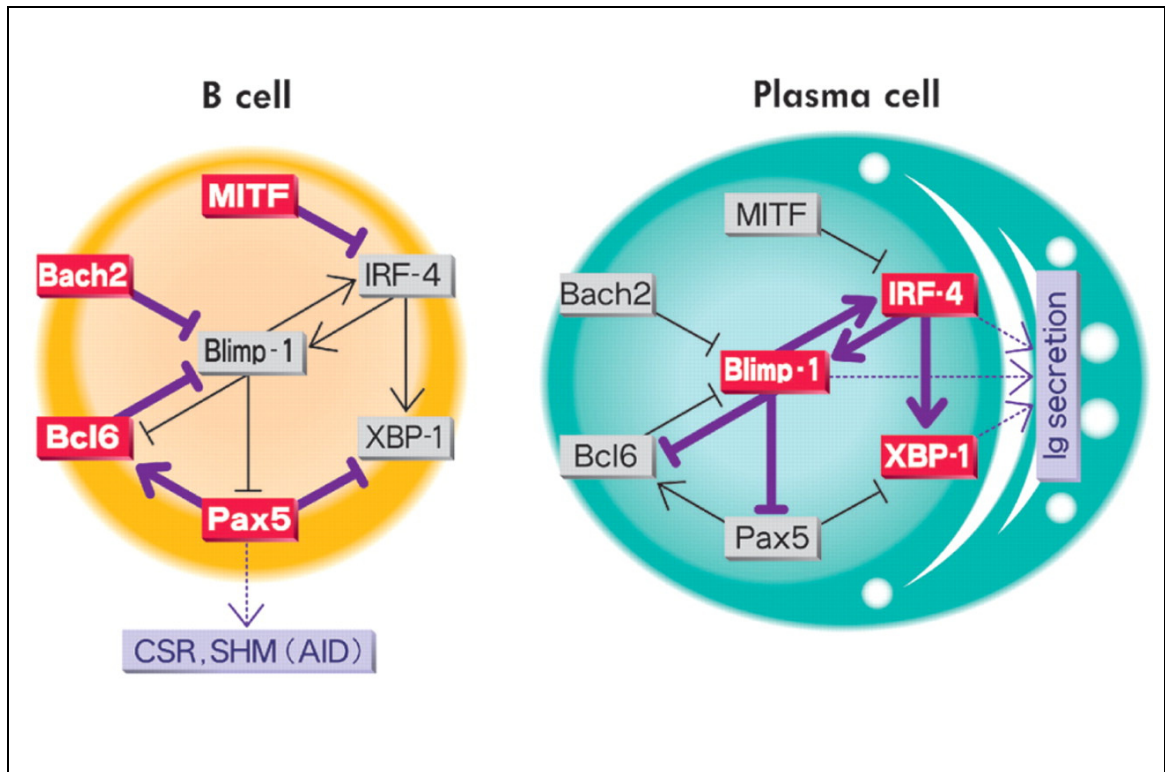


Figure 6.14: Transition of transcription factor network from GC B to plasma cells (Igarashi et al., 2007)

“In B cells, Bach2, Bcl6, MITF and Pax5 repress the plasmacytic transcription factors (left). Blimp-1, IRF-4 and Xbp-1 promote plasma cell differentiation (right). Red boxes indicate genes that are expressed. Gray boxes indicate genes that are not expressed. Bold lines and arrows indicate effective repression and effective activation, respectively. Dashed arrows represent biological outputs.” (Igarashi et al., 2007)

In addition to its regulation by transcriptional repressors, *PRDM1* expression is also controlled by the transcriptional activators IRF4, STAT3, STAT5, AP-1 and NF- κ B (Calame, 2008). IRF4 is expressed in immature B cells in the bone marrow, absent in centroblasts and re-expressed in some centrocytes and plasma cells (Pernis, 2002). Accordingly, the Blimp-1 activator IRF4 is not normally expressed in DG75 (Xu et al., 2008). Moreover, *PRDM1* expression is activated by STAT3 and STAT5 in response to cytokine stimulation. STAT3 is often found constitutively activated in EBV positive BLs, however, this is not true for DG75 (Kube et al., 2001, Weber-Nordt et al., 1996). It would certainly be interesting to test whether induced STAT3 activation in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells provides the necessary signal for *PRDM1* induction. Binding of the transcriptional activator AP-1 to the *PRDM1* promoter has also been reported, however, it seems that it is not essential for *PRDM1* expression (Ohkubo et al., 2005).

An upregulation of *PRDM1* expression was detected when the zinc finger domain of Bcl6 was transiently expressed in Raji (BL) cells (Shaffer et al., 2000) and Bcl6 was shown to bind to the *PRDM1* promoter in chromatin immunoprecipitation analysis of Ramos cells (BL) and WIL2 cells (lymphoblastoid B cell lymphoma) (Parekh et al., 2007, Tunyaplin et al., 2004), however, Blimp-1 was not induced in response to Bcl6-depletion in my system (Figure 6.11 and Figure 6.13). In order to discuss this finding, one has to take into account that the above mentioned study was based on a different cell line. Even though Raji and DG75 cell lines both share a BL phenotype, the accompanying genetic lesions in both cell lines are diverse. DG75 is EBV negative and cytogenetically very stable, while Raji is EBV positive and displays several cytogenetic abnormalities the functional consequences of which have not been studied (Karpova et al., 2005). In summary, various transcriptional repressors and activators regulate expression of *PRDM1* but it remains to be investigated which transcription factors impose the strongest impact on *PRDM1* transcription and how the removal of individual repressors

and induction of individual activators collaborate to induce *PRDM1* expression and plasma cell differentiation. The results obtained in my *BCL6*^{-/-};*tetBCL6-HA* model strongly suggest that although necessary, the selective removal of only Bcl6 from the centroblast gene expression program is not sufficient to induce *PRDM1* and terminal plasma cell differentiation.

6.3.3 Bcl6-depletion reduces proliferation of *BCL6*^{-/-};*tetBCL6-HA* cells

The influence of Bcl6 on repression of negative cell cycle regulators such as p27kip1 is well established (Shaffer et al., 2000) and it is hence perhaps not surprising to observe reduced growth rates and cell cycle progression in response to Bcl6 depletion in my *BCL6*^{-/-};*tetBCL6-HA* system (Figure 6.3 and Figure 6.7). Growth curve analysis revealed a severely reduced proliferation following Bcl6-depletion (Figure 6.3). Although not quantified, proliferation rates slightly increased again after an initial crisis that lasted approximately 7 days. This is likely due to the powerful selective pressure imposed on these cells upon withdrawal of Bcl6. One way cells could overcome this unfavourable condition, would be to accumulate mutations in the tetracycline transactivator. The tTA is required for Bcl6-HA expression in *BCL6*^{-/-};*tetBCL6-HA* cells and inactivated when bound to tetracycline or doxycycline. Mutations that impair binding of the transactivator to tet/dox could thereby reactivate Bcl6-HA expression, causing a weakening of the phenotype and a growth advantage. This could be tested by re-analysing Bcl6 protein depletion in *BCL6*^{-/-};*tetBCL6-HA* cells after long-term culture in doxycycline. For characterisation of the *BCL6*^{-/-};*tetBCL6-HA* cell line, it is therefore essential to focus on early rather than late phenotypic changes as there is a chance that late changes are being caused by directed evolution rather than Bcl6-depletion.

It is not clear why, in contrast to parental DG75-354 cells, *BCL6*^{-/-};*tetBCL6-HA* cells express p21cip1/waf1 protein (Figure 6.12). It has been previously shown by Northern blotting that p21cip1/waf1 mRNA abundance is very low in DG75

and that it cannot be induced by cisplatin treatment due to the inability of mutant p53 to translocate into the nucleus (Crook et al., 1998). This suggests that p21cip1/waf1 expression has been activated during the process of constructing the conditional *BCL6*^{-/-} cell line. It remains to be tested whether the remaining two *BCL6*^{-/-};*tetBCL6-HA* clones (AB7 and FA8) also express p21cip1/waf1. If this is the case, it needs to be investigated at which stage during the construction of the *BCL6*^{-/-};*tetBCL6-HA* cell line p21cip1/waf1 expression started. Once it is known at what point p21cip1/waf1 expression started, it could be considered to repeat some of the genetic modifications with an alternative clone or to investigate the mechanism by which p21cip1/waf1 expression has been activated. Although I have not formally analysed it, a possible explanation for this finding is that p21cip1/waf1 expression is caused by the reduced Bcl6-protein expression in *BCL6*^{-/-};*tetBCL6-HA* cells over parental cells (Figure 5.3B p.105 and Figure 5.7B p.115). p21cip1/waf1 transcription is not only activated by p53 in response to DNA damage, but also repressed by Bcl6 during the GC reaction via interaction with Miz-1 (Phan et al., 2005). It may well be that the reduced amounts of Bcl6 protein expressed in *BCL6*^{-/-};*tetBCL6-HA* cells activate p21cip1/waf1 transcription without the need for nuclear p53. To adjust the exogenous (tet-regulatable) Bcl6 expression in *BCL6*^{-/-};*tetBCL6-HA* cells to a level that closer resembles that of parental DG75-354 cells, it could be tried to target the second *BCL6* allele of a clone that expresses higher tet-regulatable Bcl6 protein levels (such as Δ B12 (*BCL6*^{+/-};*tetBCL6-HA*) in Figure 4.3 p.96), or one could alternatively introduce an additional tet-regulatable Bcl6 copy into *BCL6*^{-/-};*tetBCL6-HA* cells (clone AB7, CD9 or FA8).

However, the above mentioned possibility that lower than physiological Bcl6 protein levels in *BCL6*^{-/-};*tetBCL6-HA* cells could account for reactivation of p21cip1/waf1 expression, does not explain why p21cip1/waf1 protein expression is downregulated in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells (Figure 6.12). I am not sure why this is the case, but as the experiment was only

performed once it should best be repeated on different clones in order to confirm the finding.

The results obtained from growth curve analysis, proliferation assay, Western blotting and sq RT-PCR are consistently reflecting a reduced proliferative capacity of *BCL6*^{-/-};*tetBCL6-HA* cells in response to Bcl6-depletion (Figure 6.3, Figure 6.7, Figure 6.8, Figure 6.12 and Figure 6.13). In summary, however, the phenotypic changes observed for Bcl6-depleted *BCL6*^{-/-};*tet-BCL6-HA* cells, such as the altered morphology (Figure 6.2), cannot not be solely attributed to increased p27^{kip1} expression. In order to gain a wider view on molecular pathways likely altered in my conditional knockout cells and to identify future perspectives in the field of studying Bcl6 function, I decided to monitor the entire transcriptome of *BCL6*^{-/-};*tet-BCL6-HA* by cDNA microarray analysis (see CHAPTER 7 below).

CHAPTER 7 - Gene expression profiling of *BCL6*^{-/-};tet*BCL6*-HA cells

7.1 Introduction

Following the examination of growth behaviour and gene/protein expression changes of a limited number of genes in Bcl6-depleted *BCL6*^{-/-};tet*BCL6*-HA cells (CHAPTER 6), I decided to perform microarray analysis. DNA microarray analysis can simultaneously measure gene expression of the entire cellular transcriptome and also provides hints towards cellular pathways altered under certain conditions. Microarray technology is therefore a powerful tool for the characterisation of a specific phenotype and has previously been used to identify putative Bcl6 target genes (Shaffer et al., 2000).

One important consideration in the design of a microarray experiment is whether two-colour or single-colour arrays are used. In two-colour analysis, two samples are labelled with different fluorophores and are hybridised to the same array, while in single-colour arrays each sample is labelled with the same fluorophore and hybridised to a separate array. While it appears more economical to only use one array for two samples, it has been shown that the labelling efficiency of different fluorophores varies and that such experiments require additional controls in which the dyes on the samples are swapped and hybridised to a second array (Dombkowski et al., 2004). Therefore, I used GeneChip® technology (Affymetrix) which relies on single channel detection (Figure 7.1). GeneChip® technology (Human GeneChip 1.0 ST Array, Affymetrix) allows for the detection of 28,869 well-annotated mRNA transcripts, each of which is represented by approximately 26 probes on the array. Figure 7.1 shows a schematic representation of the procedure.

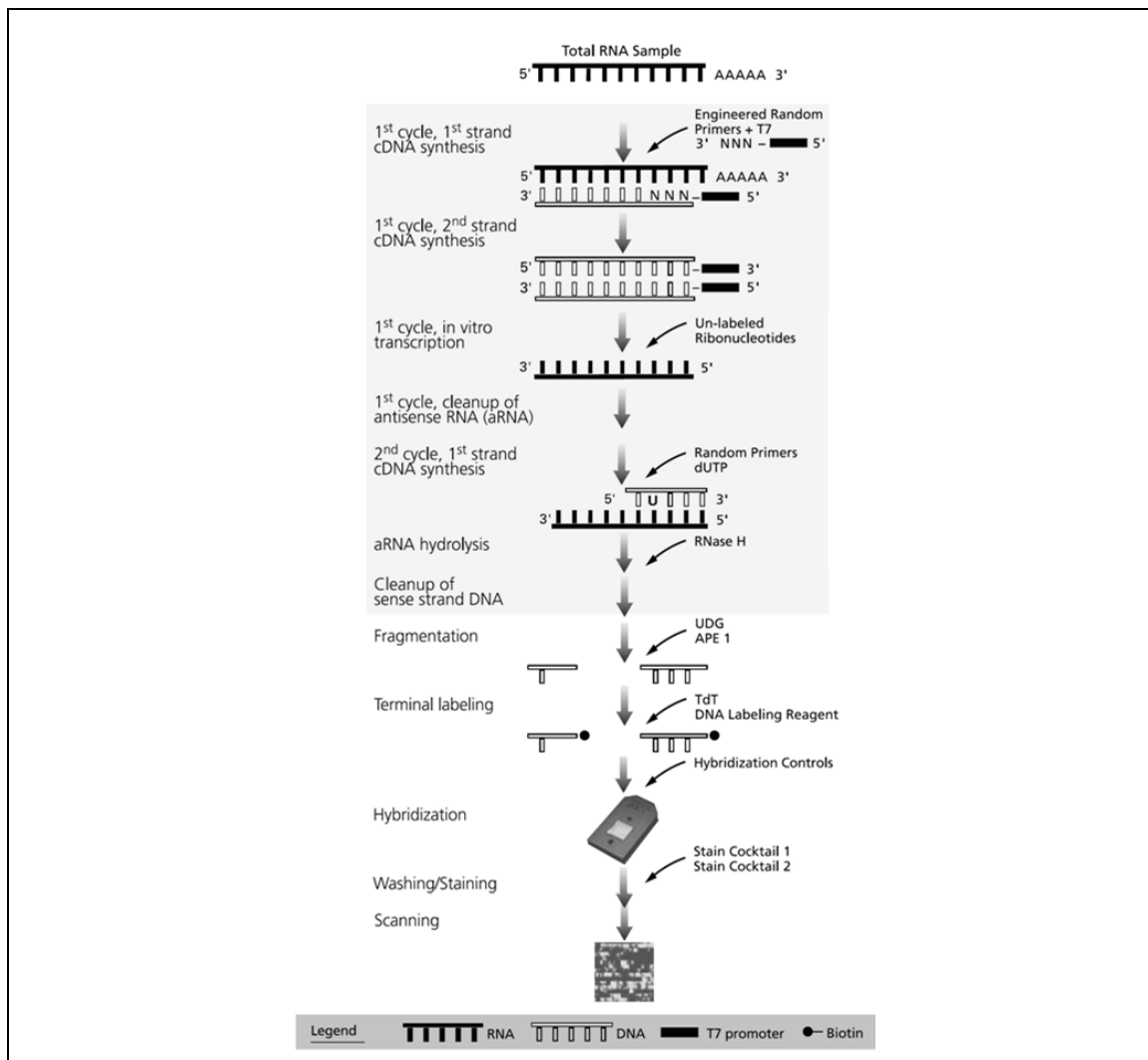


Figure 7.1: Microarray assay schematic

Double stranded cDNA is synthesised from total RNA using random hexamers that are tagged with a T7 promoter sequence. Linear amplification of the resulting cDNA by *in vitro* transcription by T7 RNA polymerase produces antisense cRNA, which is subsequently purified and used as template for a repeated round of cDNA synthesis. Synthesis of single-stranded cDNA in sense orientation uses random primers and a dUTP + dNTP mix. Following RNA hydrolysis, sense cDNA is fragmented. In this step uracil-DNA glycosylase (UDG) removes the uracil residues from the single stranded cDNA molecule and apurinic/apyrimidinic endonuclease 1 (APE 1) cleaves the phosphodiester backbone where the base is missing and thereby generates fragments of approximately 40-70 nucleotides. cDNA fragments are labelled by terminal deoxynucleotidyl transferase (TdT) with biotin allonamide triphosphate and are hybridised to the array. For final scanning, probes are visualised by staining with streptavidin-phycoerythrin (SAPE), biotin-anti-streptavidin and SAPE again. Figure taken from Affymetrix GeneChip® WT terminal labelling and hybridisation user manual.

7.2 Results

I had two choices in the design of the microarray experiment. (1) Analysing six arrays including three biological replicates of a control sample (-doxycycline) and three biological replicates from one time point after Bcl6-depletion or (2) analysing four arrays including the control sample and three different time points after Bcl6-depletion (excluding biological replicates). Ideally, I would have used three biological replicates of multiple time points. Because of economical reasons and the wish to follow gene expression changes over a couple of days, I decided to pursue the latter option. *Bcl6*^{-/-}*tetBCL6-HA* cells (clone AB7) were either grown without doxycycline (control) or with 1 µg/ml doxycycline for 16, 48 or 96 hours. Further information on methodology involved can be found in section 2.8, p.66. The quality of microarray data was subjectively evaluated by scatter plot analysis (Figure 7.2). Ideally, data should be distributed along a straight line, based on the assumption that expression of most genes does not change during the experiment. If microarray data presents itself 'banana-shaped' or 'boomerang-shaped' then the variation between the arrays more likely reflects obscuring variation (resulting from technical variation) rather than interesting variation (resulting from biological variation) (Bolstad et al., 2003).

Two hundred and five genes with a ≥ 2 -fold up-or downregulation in any sample cultured in doxycycline (16 hours, 48 hours or 96 hours) compared to the control (-doxycycline) were identified. Thirteen out of these 205 transcripts were excluded from further analysis because no complete coding sequence is known for them or their existence is only supported by expressed sequence tags or computational prediction. The remaining 192 genes corresponded to well-curated transcripts and these were selected for further analysis. Interestingly, out of these 192 genes, 163 genes (85 %) were upregulated in response to Bcl6-depletion, nicely correlating with the fact that Bcl6 is a transcriptional repressor. Table 8 lists these 192 genes in alphabetical order. For validation of

microarray data, 23 genes were selected from Table 8 and expression changes were confirmed by sq RT-PCR analysis (Figure 7.3). In the selection process of genes to be validated, genes with no alternative transcripts (ease of primer design) and those with bigger expression changes were favoured. Validation of these genes was not performed on the same clone on which microarray analysis was performed (clone AB7) but on mRNA isolated from a different clone (clone FA8). Furthermore, validation by sq RT-PCR was only performed once, the results of which are shown in Figure 7.3.

In sq RT-PCR, 14 out of 23 genes (61 %) fully supported the microarray data. These were *IFI44L*, *CYSLTR1*, *IFI44*, *SESN1*, *IKZF1*, *STAT1*, *SYK*, *PIK3IP1*, *FOXP1*, *IRF9*, *STAT2*, *E2F8*, *CDC6* and *LYAR*. Eight out of 23 genes (35 %) failed to support the changes detected by microarray analysis. These were *PLEK*, *CAMK4*, *ARHGAP15*, *RB1*, *BLNK*, *CDKN1B*, *EIF2AK3* and *GPRIN3*. The result for the *TAP1* gene showed a clear difference to the microarray data (Figure 7.3). However, taking the limits of sq RT-PCR analysis into account and bearing in mind that the experiment was only performed once on a clone different to the one that the microarray data was generated from, these results, together with quality control data (Figure 7.2) were of sufficient quality to perform pathway analysis.

In order to identify biological pathways altered in response to Bcl6-depletion, I submitted the genes listed in Table 8 to the publicly available ‘DAVID bioinformatics database’ (Dennis et al., 2003, Huang da et al., 2009) and performed pathway mapping. The integrated pathway mapping tool of DAVID, compares a submitted gene list to biological pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) and thereby identifies pathways that are altered under the experimental condition studied. Using this integrated pathway-mapping tool, I identified three cellular pathways affected by Bcl6. These are (1) cell cycle regulation, (2) BCR

signalling and (3) calcium signalling (Figure 7.4 - Figure 7.7). The influence of Bcl6 on these pathways will be discussed in section 7.3 below.

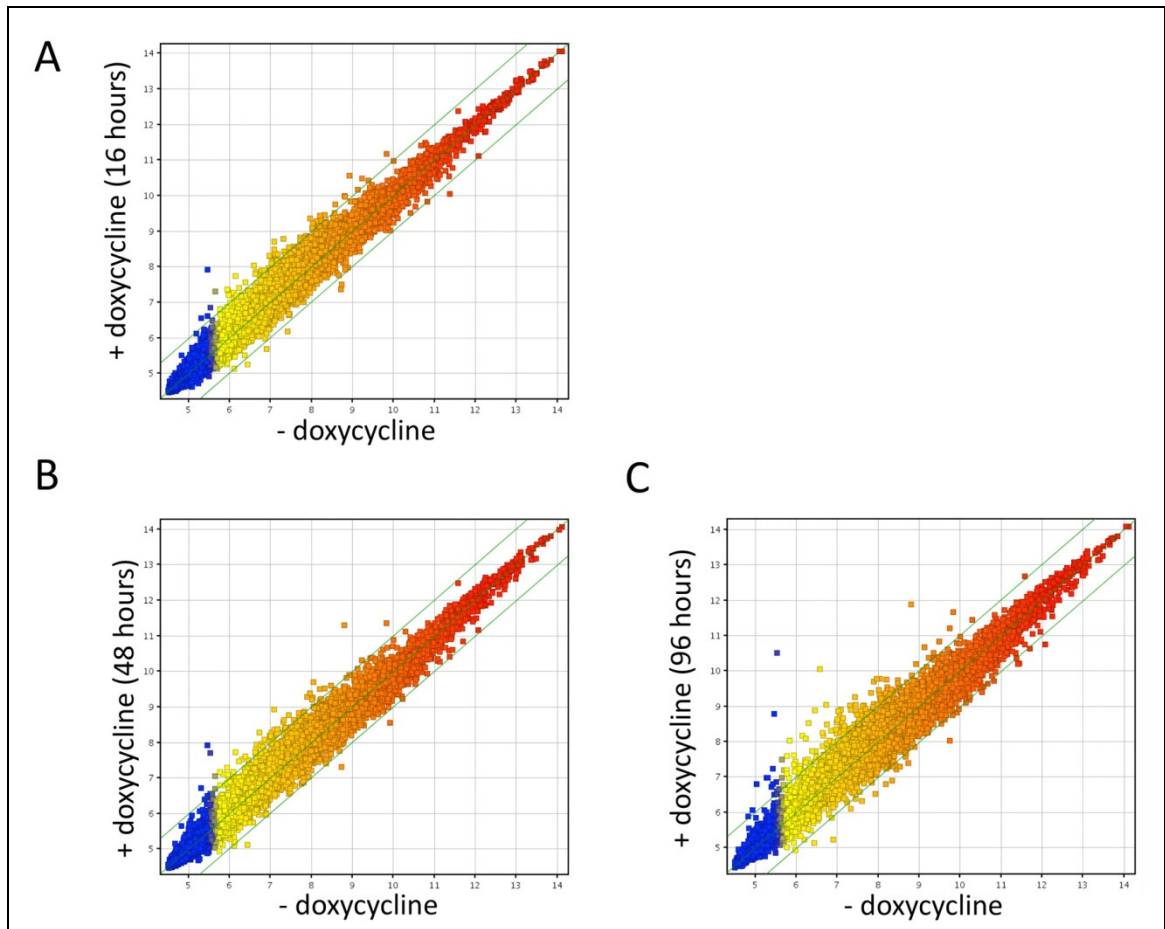


Figure 7.2: Quality control of microarray data by scatter plot analysis

Scatter plots compare normalised intensity values (base-2 logarithm) of gene expression between two arrays and thereby visualise data distribution. Data were generated from clone AB7 (*BCL6*^{-/-}*tetBCL6-HA*) cultured without doxycycline (x-axis) or with doxycycline (y-axis) for 16 hrs (A), 48 hrs (B) or 96 hrs (C). Genes represented by data points outside the region between the three diagonal lines have a greater than 2-fold change in expression between the 2 arrays compared. Colours encode signal intensities of the control sample (- doxycycline, x-axis), i.e. blue reflects low, yellow/orange reflect medium and red reflects high transcript expression.

Table 8: Genes displaying largest fold mRNA expression changes in response to Bcl6-depletion in *BCL6*^{-/-};tet*BCL6*-HA cells

Listing of gene expression changes in response to Bcl6-depletion as detected by microarray analysis. Genes listed were selected on the basis that at least one of the samples incubated in doxycycline (for 16, 48 or 96 hours) displayed an at least 2-fold change compared to the control to which no doxycycline was added. Genes are listed in alphabetical order. Last three columns list fold-changes, most of which are upregulated. Downregulations are highlighted in grey.

gene symbol	gene description	16	48	96
7A5	putative binding protein 7a5	2.28	2.32	2.42
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	1.79	1.97	2.04
ACSM3	acyl-CoA synthetase medium-chain family member 3	1.19	2.06	2.11
AIM1	absent in melanoma 1	2.06	1.86	2.16
ALDOC	aldolase C, fructose-bisphosphate	2.52	1.16	1.06
ANKRD44	ankyrin repeat domain 44	2.26	2.12	2.34
ARHGAP15	Rho GTPase activating protein 15	2.52	2.35	2.39
ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	1.79	2.50	3.04
ATM NPAT	ataxia telangiectasia mutated nuclear protein, ataxia-telangiectasia locus	1.75	1.83	2.26
ATP2A2	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2	1.33	1.57	2.02
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	2.06	2.12	2.34
BCL2A1	BCL2-related protein A1	2.42	2.68	3.22
BLNK	B-cell linker	1.57	1.92	2.36
BMI1	BMI1 polycomb ring finger oncogene	1.69	1.71	2.06
BST2	bone marrow stromal cell antigen 2	1.25	1.52	2.12
BTN3A1	butyrophilin, subfamily 3, member A1	1.84	1.95	2.03
BTN3A2	butyrophilin, subfamily 3, member A2	1.81	2.03	2.32
BTN3A3	butyrophilin, subfamily 3, member A3	1.63	1.77	2.31
C10orf64 WD FY4	chromosome 10 open reading frame 64 WDFY family member 4	1.37	2.18	2.42
C11orf53	chromosome 11 open reading frame 53	1.25	1.72	2.30
C13orf18 LO C728970	chromosome 13 open reading frame 18 hypothetical LOC728970	3.13	3.65	3.55
C18orf17	chromosome 18 open reading frame 17	1.95	1.93	3.25
C5orf13	chromosome 5 open reading frame 13	1.89	1.58	2.05
CAMK4	calcium/calmodulin-dependent protein kinase IV	2.30	2.37	2.48
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	1.59	1.78	2.28
CCL3	chemokine (C-C motif) ligand 3	1.65	2.09	2.74
CCL3L1	chemokine (C-C motif) ligand 3-like 1	1.76	2.63	3.51
CCPG1	cell cycle progression 1	1.02	1.49	2.31
CCR7	chemokine (C-C motif) receptor 7	1.02	1.71	2.77
CD180	CD180 antigen	1.96	1.73	2.31
CD48	CD48 antigen	1.39	1.79	2.25
CD72	CD72 receptor	1.16	1.49	2.25
CDC25A	cell division cycle 25 homolog A (<i>S. pombe</i>)	1.33	1.92	2.07
CDC2L6	cell division cycle 2-like 6 (CDK8-like)	2.05	1.82	1.87
CDC6	cell division cycle 6 homolog (<i>S. cerevisiae</i>)	1.43	1.99	2.03

CHAPTER 7 – Gene expression profiling of *BCL6*^{-/-tet}*BCL6*-HA cells

CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	2.19	1.74	2.08
CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)	1.08	1.52	2.60
CHML	choroideremia-like (Rab escort protein 2)	2.15	2.31	2.20
CNR2	cannabinoid receptor 2 (macrophage)	1.37	1.63	2.15
CTDSP2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2	1.96	1.76	2.10
CTH	cystathionase (cystathionine gamma-lyase)	1.05	1.79	2.68
CXorf21	chromosome X open reading frame 21	3.16	2.66	3.60
CYSLTR1	cysteinyl leukotriene receptor 1	5.51	5.60	10.2
DDIT4	DNA-damage-inducible transcript 4	2.30	2.05	3.40
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.50	1.66	2.04
DGKD	diacylglycerol kinase, delta 130kDa	1.50	1.73	2.11
DNMBP	dynamitin binding protein	1.87	2.47	2.87
DTX3L	deltex 3-like (Drosophila)	1.90	2.10	2.96
DZIP3	DAZ interacting protein 3, zinc finger	1.87	1.85	2.14
E2F8	E2F transcription factor 8	1.38	1.81	2.03
EBI2	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	1.41	1.76	3.33
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	1.56	1.50	2.08
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	2.10	2.00	2.03
ENPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3	1.32	1.51	2.29
ERAP2	endoplasmic reticulum aminopeptidase 2	1.62	1.87	2.06
EVI2A EVI2B	ecotropic viral integration site 2A ecotropic viral integration site 2B	3.03	3.02	4.08
FAIM3	Fas apoptotic inhibitory molecule 3	1.29	1.58	3.52
FLI1 EWSR1	Friend leukemia virus integration 1 Ewing sarcoma breakpoint region 1	2.84	2.45	3.34
FLJ31958	Hypothetical protein FLJ31958	1.23	2.02	1.93
FNBP1	formin binding protein 1	1.55	1.58	2.08
FOXP1	forkhead box P1	1.66	1.69	2.14
FRYL	FRY-like	2.01	1.62	1.77
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	2.24	2.16	2.70
GAB1	GRB2-associated binding protein 1	2.37	2.25	3.08
GCET2	germinal center expressed transcript 2	2.00	2.01	2.95
GPR174	G protein-coupled receptor 174	2.75	2.60	2.70
GPRIN3	GPRIN family member 3	2.75	1.67	1.58
HEG1	HEG homolog 1 (zebrafish)	1.83	1.65	2.02
HERC2P2 L	hect domain and RLD 2 pseudogene 2 hypothetical protein LOC283755 hect domain and RLD 2 pseudogene 3	1.62	1.70	2.02
OC283755 L				
OC440248 H				
ERC2P3	hect domain and RLD 2 pseudogene 4	1.61	1.85	2.07
HERC2P4				
HIST1H2BK	histone cluster 1, H2bk	1.45	2.29	3.11
HIVEP2	human immunodeficiency virus type I enhancer binding protein 2	2.57	2.11	2.65
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	2.48	1.92	1.99
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	1.91	1.85	2.47
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	3.13	1.98	2.42
IFI16	interferon, gamma-inducible protein 16	1.83	1.81	2.08
IFI27	interferon, alpha-inducible protein 27	1.18	1.66	11.4
IFI35	interferon-induced protein 35	1.45	1.87	2.16
IFI44	interferon-induced protein 44	2.06	2.96	5.05

CHAPTER 7 – Gene expression profiling of *BCL6*^{-/-}*tetBCL6-HA* cells

IFI44L	interferon-induced protein 44-like	2.54	4.58	31.8
IFI6	interferon, alpha-inducible protein 6	1.32	1.37	2.45
IFIH1	interferon induced with helicase C domain 1	1.67	1.64	2.28
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	1.54	2.04	2.56
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	1.25	1.93	2.58
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	1.23	1.67	2.85
IFITM1	interferon induced transmembrane protein 1 (9-27)	1.30	1.52	3.59
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3	1.76	1.85	2.19
IGF2R	insulin-like growth factor 2 receptor	1.63	1.89	2.27
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	1.76	1.88	2.16
IKZF1	IKAROS family zinc finger 1 (Ikaros)	2.39	2.47	2.96
IKZF2	IKAROS family zinc finger 2 (Helios)	2.45	1.74	2.49
IL10RA	interleukin 10 receptor, alpha	1.73	2.09	2.03
IRF9	interferon regulatory factor 9	1.31	1.57	2.07
ITPR1	inositol 1,4,5-triphosphate receptor, type 1	2.02	1.92	2.06
ITPR2	inositol 1,4,5-triphosphate receptor, type 2	1.61	1.90	2.37
KBTBD8	kelch repeat and BTB (POZ) domain containing 8	1.59	2.07	1.85
KIAA1618	Ring finger protein 213	2.00	2.33	2.91
KLRC4 KLRK1	killer cell lectin-like receptor subfamily C, member 4 killer cell lectin-like receptor subfamily K, member 1	2.13	2.26	2.36
KLRK1 KLRC4	killer cell lectin-like receptor subfamily K, member 1 killer cell lectin-like receptor subfamily C, member 4	1.66	1.89	2.58
LAPTM5	lysosomal associated multispinning membrane protein 5	2.33	5.76	8.59
LGALS9 LOC284194 LOC654346	lectin, galactoside-binding, soluble, 9 (galectin 9) galectin-9 like similar to galectin 9 short isoform	1.20	1.67	2.64
LGALS9 LOC654346 LOC284194	lectin, galactoside-binding, soluble, 9 (galectin 9) similar to galectin 9 short isoform galectin-9 like	1.30	1.73	2.67
LGMN	legumain	1.96	1.82	2.13
LMO4	LIM domain only 4	1.82	1.98	2.40
LOC389765	similar to KIF27C	2.29	2.16	1.68
LOC85389	RNA, small nucleolar	1.28	1.26	2.02
LOC85391	RNA, small nucleolar	1.29	1.08	2.61
LRRC33	leucine rich repeat containing 33	1.26	1.59	2.17
LRRN3	leucine rich repeat neuronal 3	2.68	2.24	2.34
LYAR	Ly1 antibody reactive homolog (mouse)	1.42	1.59	2.01
MAML3	mastermind-like 3 (Drosophila)	2.30	2.35	2.59
MAP1A	microtubule-associated protein 1A	1.17	1.31	2.08
MAPK10	mitogen-activated protein kinase 10	1.80	1.36	2.00
MCTP2	multiple C2 domains, transmembrane 2	1.78	1.82	2.06
MEF2C	myocyte enhancer factor 2C	2.41	2.12	2.23
MEIS2	Meis homeobox 2	2.04	2.11	2.48
MGAT5	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetylglucosaminyltransferase	1.76	1.86	2.17
MGC16169	TBC1 domain containing kinase	2.08	1.21	1.91
MLKL	mixed lineage kinase domain-like	1.59	2.09	2.53
MLLT3	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3	2.21	2.34	2.26
MRT04	mRNA turnover 4 homolog (S. cerevisiae)	1.56	1.60	2.12

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NEK7	NIMA (never in mitosis gene a)-related kinase 7	2.18	1.64	1.50
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	1.60	1.86	3.40
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1.87	2.10	5.41
OXR1	oxidation resistance 1	1.49	1.74	2.10
PARP9	poly (ADP-ribose) polymerase family, member 9	2.31	2.41	3.67
PCMTD1	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	1.79	1.65	2.10
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	2.58	2.91	3.62
PIK3IP1	phosphoinositide-3-kinase interacting protein 1	1.29	1.75	2.43
PLEK	pleckstrin	1.60	1.90	2.63
PLSCR1	phospholipid scramblase 1	1.88	1.55	2.01
PLXNC1	plexin C1	1.79	1.95	2.04
PMEPA1	prostate transmembrane protein, androgen induced 1	1.83	1.84	2.01
PML	promyelocytic leukemia	1.43	1.64	2.37
PNRC1	proline-rich nuclear receptor coactivator 1	1.38	1.70	2.29
PRDM15	PR domain containing 15	1.88	1.98	2.12
PSAT1	phosphoserine aminotransferase 1	1.08	1.40	2.28
PSCD1	pleckstrin homology, Sec7 and coiled-coil domains 1(cytohesin 1)	1.49	1.97	2.20
PSCDBP	pleckstrin homology, Sec7 and coiled-coil domains, binding protein	2.26	1.70	2.09
PTK2B	Pyk2	1.61	1.64	2.14
PTPN6	protein tyrosine phosphatase, non-receptor type 6	1.74	1.78	2.23
PTPRC	protein tyrosine phosphatase, receptor type, C	2.10	1.44	1.62
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	1.63	2.23	2.37
RASSF6	Ras association (RalGDS/AF-6) domain family member 6	2.64	2.42	1.96
RB1	retinoblastoma 1 (including osteosarcoma)	2.27	1.82	2.37
RCN2	reticulocalbin 2, EF-hand calcium binding domain	2.07	1.59	1.58
RCSD1	RCSD domain containing 1	2.05	1.69	2.09
REPIN1	replication initiator 1	2.12	2.31	2.73
RNF213	ring finger protein 213	1.98	2.52	3.03
RP5-1022P6.2	hypothetical protein KIAA1434	1.48	2.02	1.95
RPL27A	ribosomal protein L27a	1.83	1.51	2.11
RPS29	ribosomal protein S29	1.22	1.53	2.10
S100A10	S100 calcium binding protein A10	1.06	1.83	2.78
SAMD9	sterile alpha motif domain containing 9	1.82	1.68	2.22
SAMD9L	sterile alpha motif domain containing 9-like	2.10	2.71	3.60
SCN3A	sodium channel, voltage-gated, type III, alpha subunit	2.24	2.07	2.08
SERPINA9	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9	1.85	1.43	2.04
SESN1	sestrin 1	2.66	2.72	3.56
SESN3	sestrin 3	2.38	2.05	2.33
SFRS6	splicing factor, arginine/serine-rich 6	1.76	1.82	2.17
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.01	1.81	2.12
SLC30A4	solute carrier family 30 (zinc transporter), member 4	2.27	1.88	1.62
SLC44A5	solute carrier family 44, member 5	1.75	1.74	2.11
SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	1.16	1.83	3.26
SMAP2	stromal membrane-associated GTPase-activating protein 2	1.74	2.00	2.55

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SNORD50B	small nucleolar RNA, C/D box 50B	1.29	1.35	2.35
SNORD75	small nucleolar RNA, C/D box 75	1.71	1.51	2.07
SNTB1	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	1.98	2.09	2.06
SP110	SP110 nuclear body protein	2.14	2.42	3.64
SP140	SP140 nuclear body protein	1.55	2.47	4.67
ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltransferase 1	1.49	1.83	2.26
STAMBPL1	STAM binding protein-like 1	2.03	1.58	1.31
STAT1	signal transducer and activator of transcription 1, 91kDa	1.46	1.95	2.96
STAT2	signal transducer and activator of transcription 2, 113kDa	1.61	1.57	2.05
SYK	spleen tyrosine kinase	2.18	2.54	2.71
TAGAP	T-cell activation RhoGTPase activating protein	1.34	1.43	2.11
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	1.62	1.70	2.01
TBCEL	tubulin folding cofactor E-like	2.02	1.87	2.31
TFRC	transferrin receptor (p90, CD71)	1.56	1.74	2.31
TIPIN	TIMELESS interacting protein	1.43	1.73	2.04
TMEM154	transmembrane protein 154	2.06	2.17	2.07
TNFSF8	tumor necrosis factor (ligand) superfamily, member 8	1.30	1.41	2.64
TP53INP1	tumor protein p53 inducible nuclear protein 1	2.10	2.39	2.79
TP63	tumor protein p63	2.23	3.16	3.35
TRIM22	tripartite motif-containing 22	1.37	1.76	2.05
TUBB2C	tubulin, beta 2C	1.34	1.80	2.08
UGCG	UDP-glucose ceramide glucosyltransferase	1.87	1.73	2.45
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	1.01	1.42	3.45
UTRN	utrophin	1.86	2.04	2.54
VSTM3	V-set and transmembrane domain containing 3	1.24	1.72	2.10
WDFY4 C10 orf64	WDFY family member 4 chromosome 10 open reading frame 64	1.36	1.98	2.37
XAF1	XIAP associated factor 1	1.90	2.39	4.74

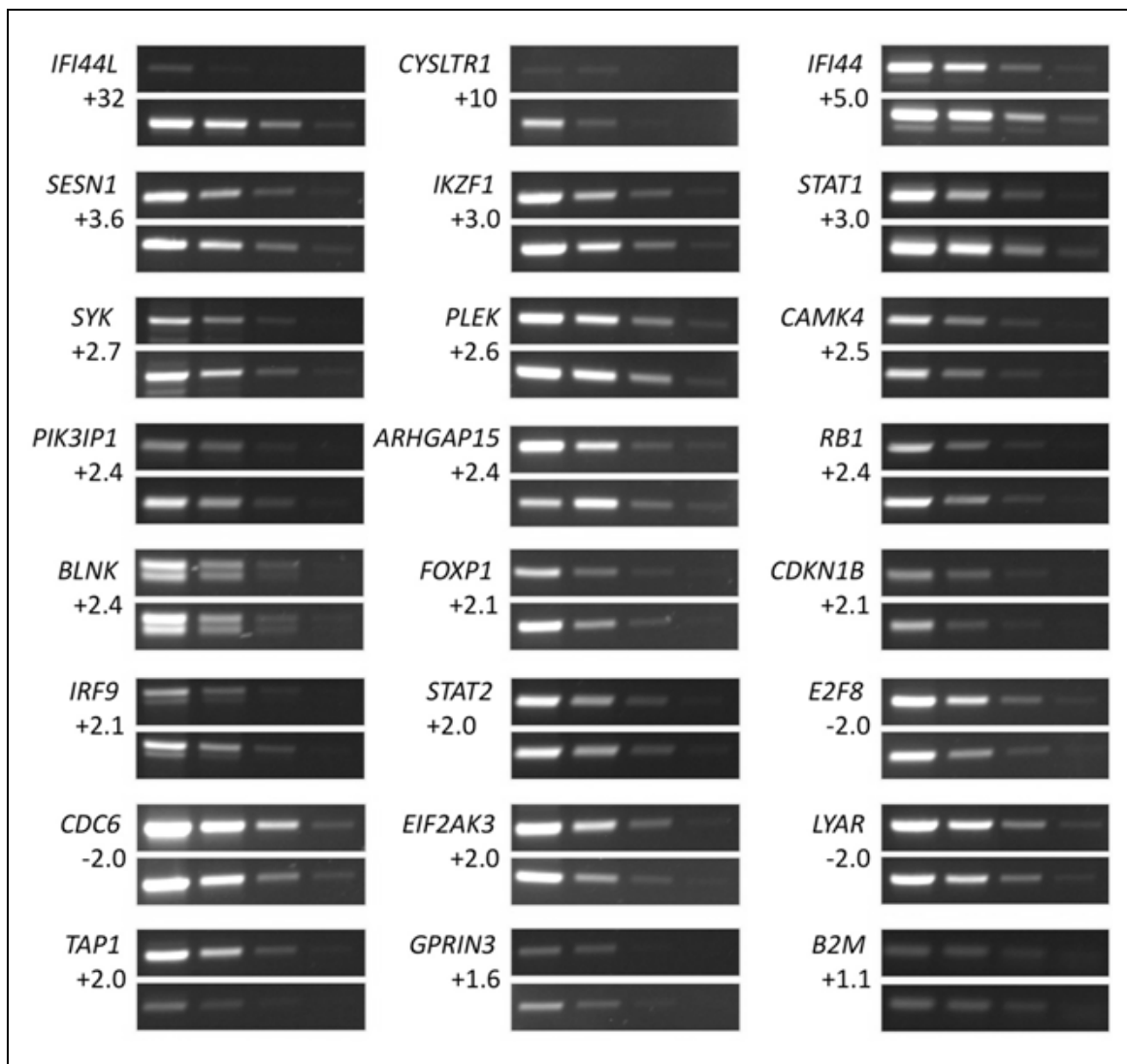


Figure 7.3: Validation of microarray data by sq RT-PCR

Microarray analysis was performed on *BCL6*^{-/-};tet*BCL6*-HA clone AB7. Validation of microarray results by sq RT-PCR was performed on *BCL6*^{-/-};tet*BCL6*-HA clone FA8. cDNA dilutions of 1, 1:5, 1:25 and 1:125 (left to right) were prepared and used as template for the PCR reaction. See section 2.5.4 and Table 7 p.68 for more details. For each gene, the upper gel picture represents the control (cells cultured without doxycycline) while the lower panel corresponds to PCR reactions based on cDNA generated from cells cultured in presence of doxycycline (1 µg/ml) for 96 hours. Below each gene symbol is the fold up (+) or down (-) regulation in gene expression expected based on microarray results obtained for *BCL6*^{-/-};tet*BCL6*-HA clone AB7 (Table 8). *B2M* (β-2 microglobulin) served as control.

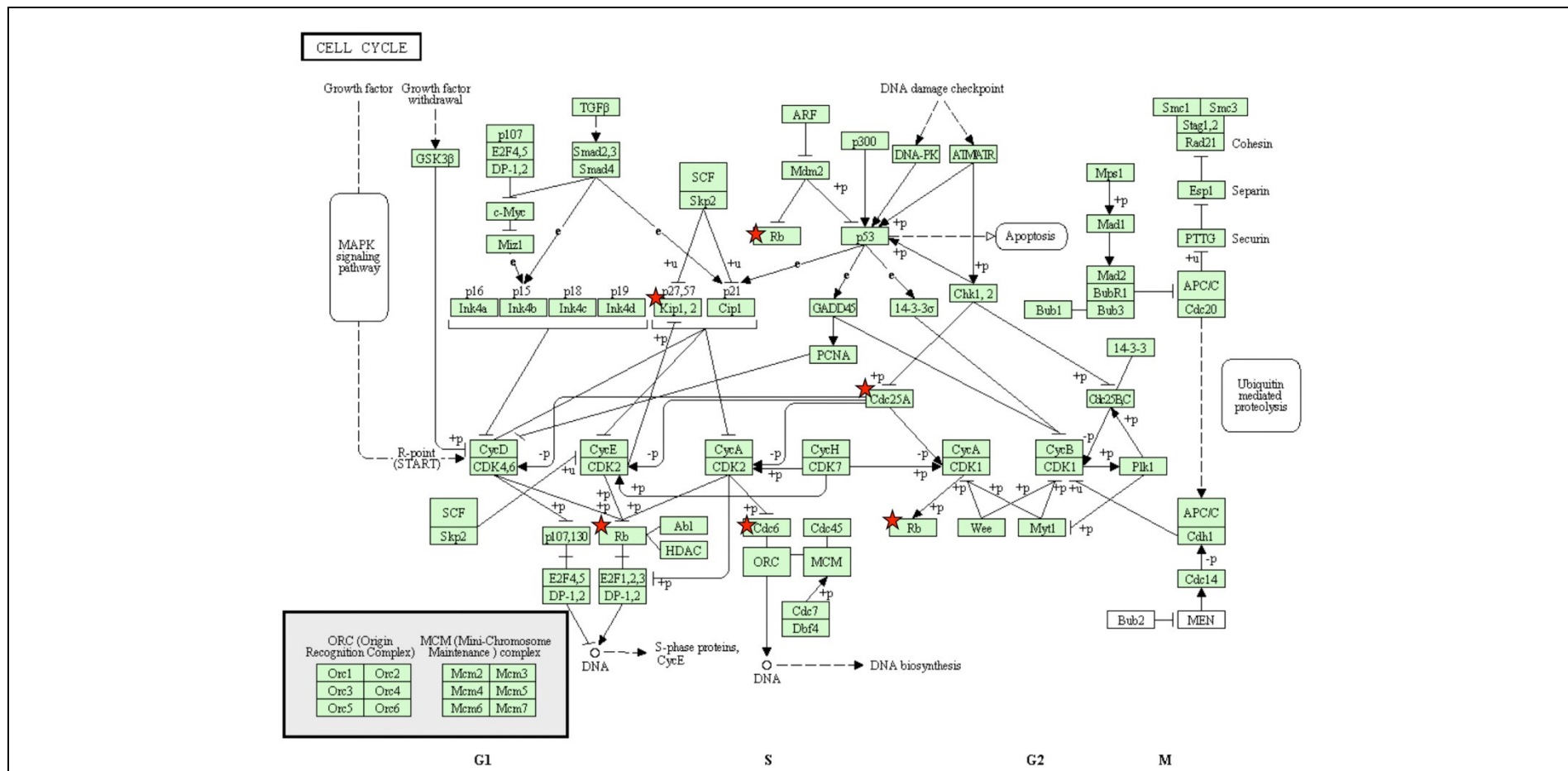


Figure 7.4: Bcl6 affects transcription of cell cycle regulators

Genes listed in Table 8 (192 genes) were submitted to the pathway mapping tool of the 'DAVID functional annotation program' (Dennis et al., 2003, Huang da et al., 2009) and four of these genes (*CDC6*, *CDC25A*, *CDKN1B* and *RB1*) were mapped to the cell signalling KEGG pathway (indicated by red stars, diagram was generated by pathway mapping tool) (Kanehisa and Goto, 2000).

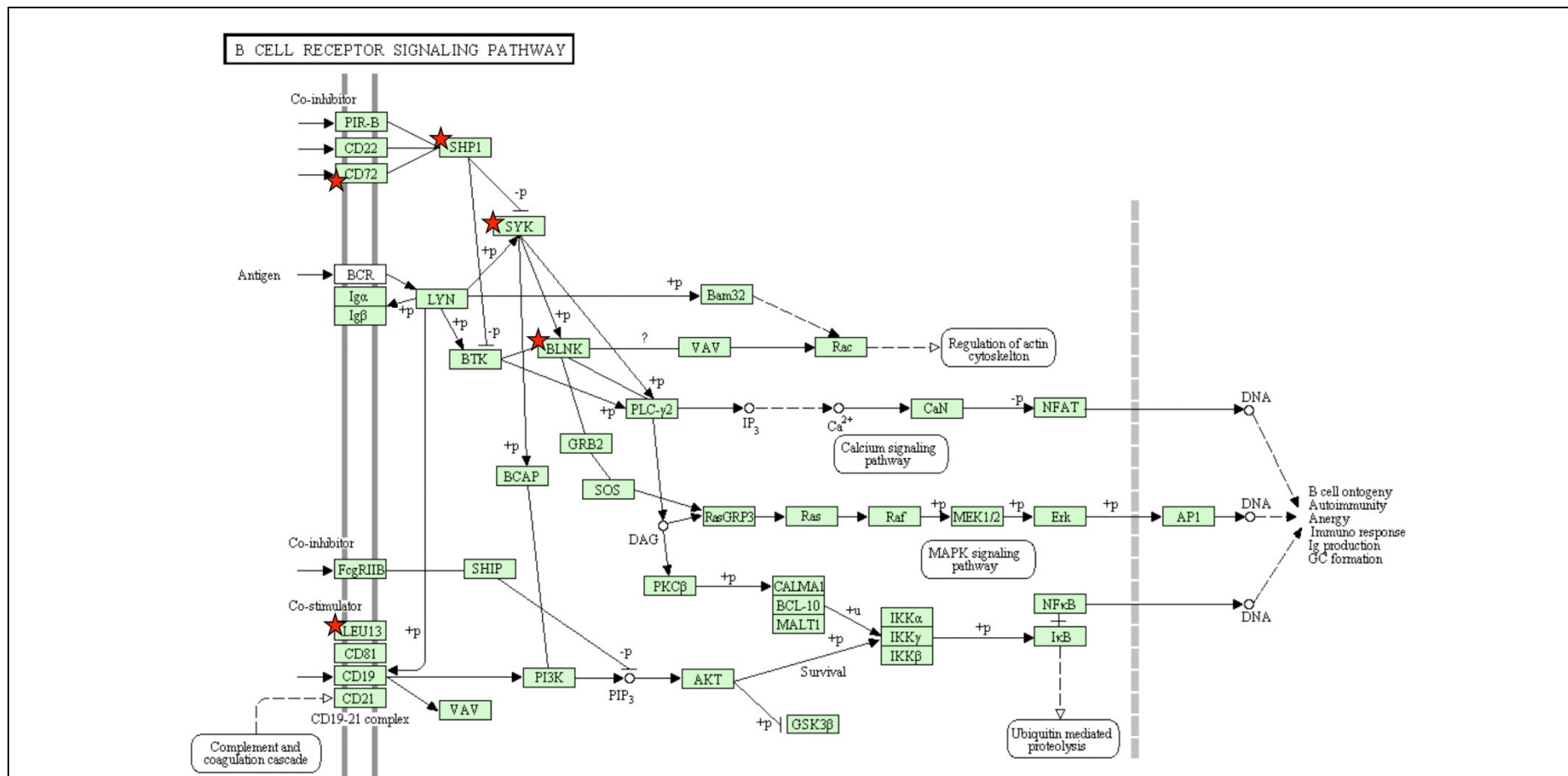


Figure 7.5: Bcl6 affects transcription of BCR signalling components

Genes listed in Table 8 (192 genes) were submitted to the pathway mapping tool of the 'DAVID functional annotation program' (Dennis et al., 2003, Huang da et al., 2009) and five of these genes (*BLNK*, *CD72*, *IFITM1/LEU13*, *PTPN6/SHP-1* and *SYK*) were mapped to the BCR signalling KEGG pathway (indicated by red stars, diagram was generated by pathway mapping tool) (Kanehisa and Goto, 2000).

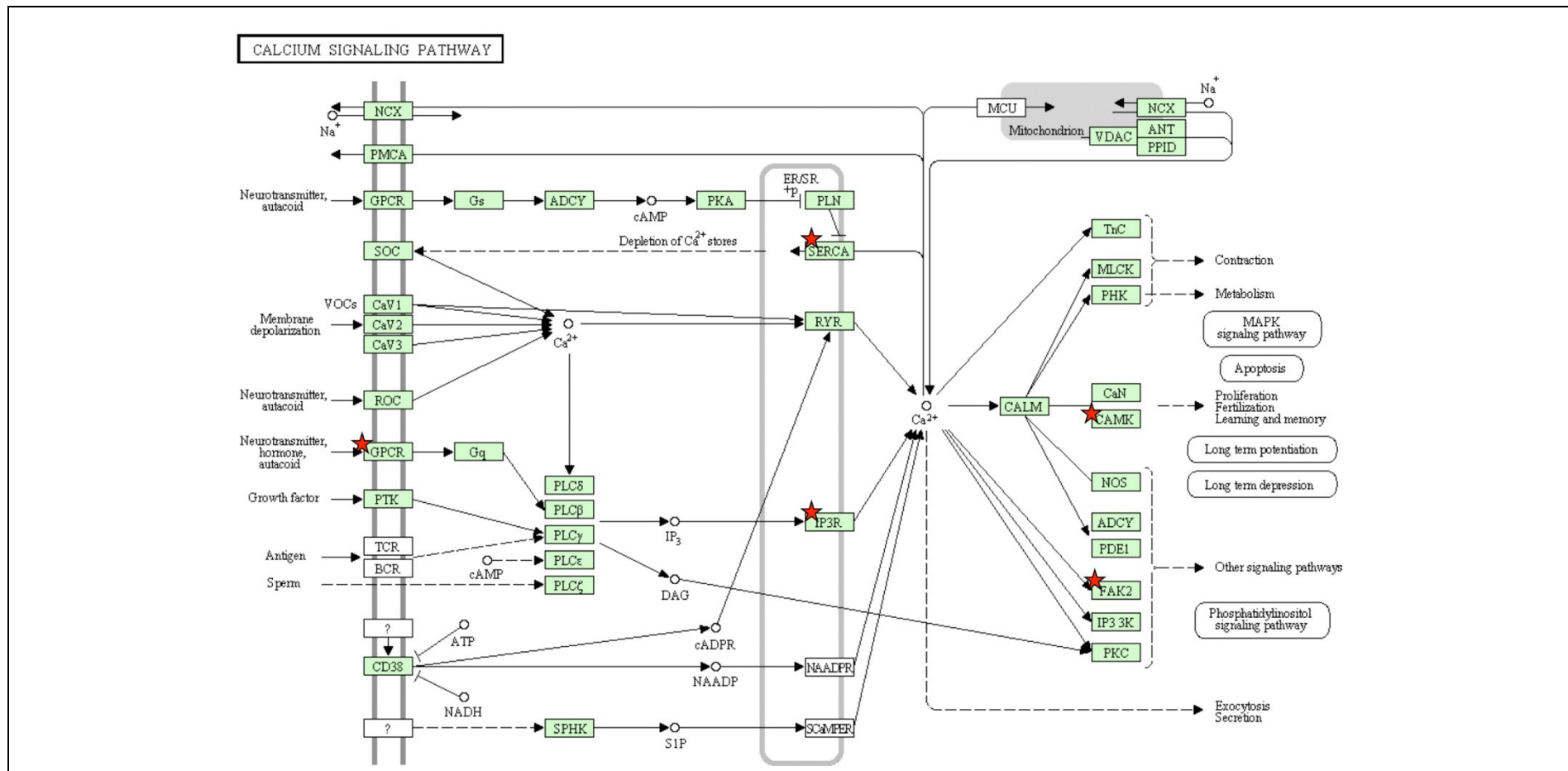


Figure 7.6: Bcl6 affects transcription of calcium signalling components

Genes listed in Table 8 (192 genes) were submitted to the pathway mapping tool of the 'DAVID functional annotation program' (Dennis et al., 2003, Huang da et al., 2009) and six of these genes (*ATP2A/SERCA*, *CAMK4*, *CYCLTR1/GPCR*, *ITPR1/2/IP3R* and *PTK2B/FAK2*) were mapped to the BCR signalling KEGG pathway (indicated by red stars, diagram was generated by pathway mapping tool) (Kanehisa and Goto, 2000).

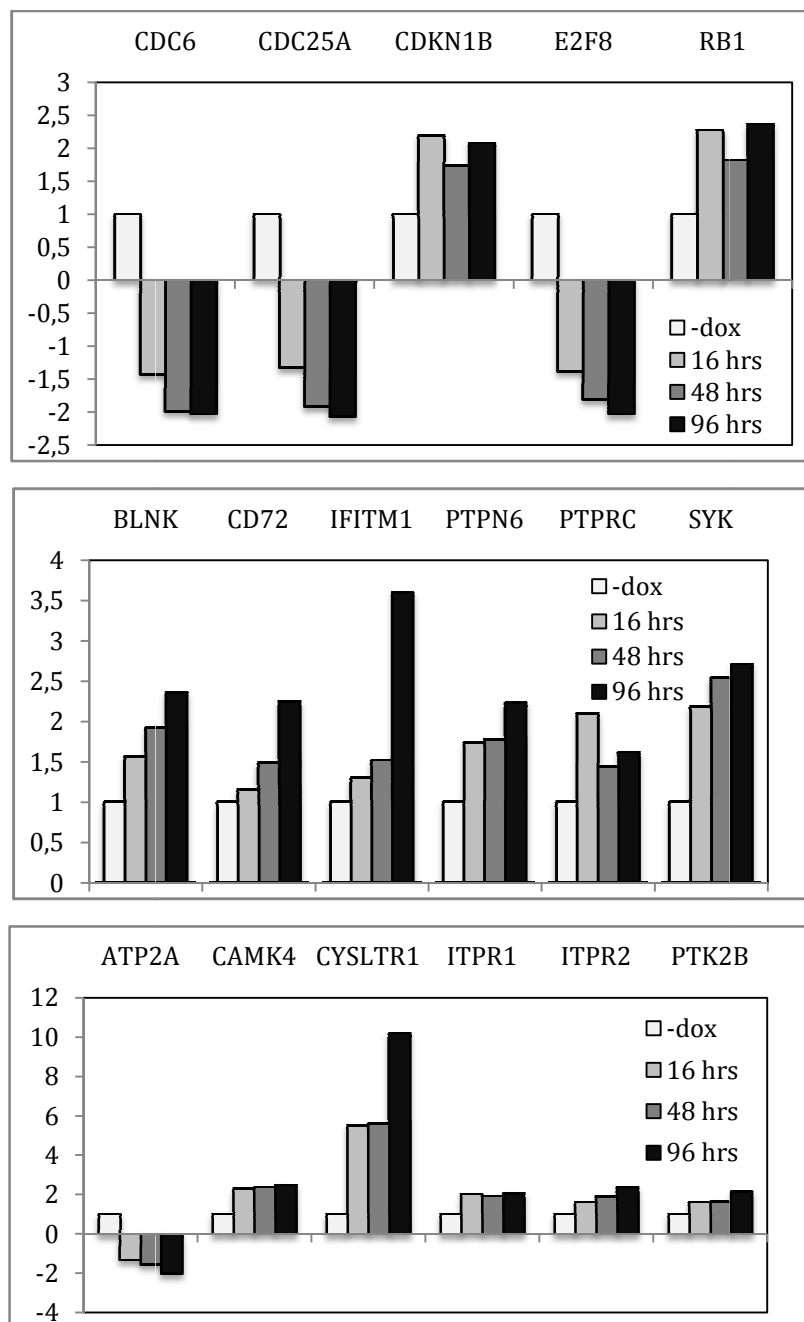


Figure 7.7: Graphic representation of fold gene expression changes of selected genes

Gene expression changes of genes highlighted by red stars in Figure 7.4 (and *E2F8*, cell cycle regulation, top), Figure 7.5 (and *PTPRC*, BCR signalling, middle) and Figure 7.6 (calcium signalling, bottom).

7.3 Discussion

7.3.1 Quality control of microarray data

Quality control of microarray data was performed by subjective inspection of scatter plots (Figure 7.2). However, statistical analysis of data was not performed, as no biological replicates were included in the analysis for economical reasons. In order to reduce the signal to noise ratio, it is generally advised to use three replicates for each sample and even more when working with very heterogeneous cell populations (such as tissue) or when only very few starting material is available for a sample. As the cell line *BCL6*^{-/-}*tetBCL6-HA* is a very homogeneous cell population that is derived from a single cell clone and availability of large cell numbers is no point of concern, the signal to noise ratio of samples was found to be very low. This was judged by the fact that expression of most genes analysed did not change when each of the data sets derived from Bcl6-depleted samples was compared to the control (-doxycycline) (Figure 7.2). Nevertheless, the experimental design could be improved by including biological replicates and hence the data generated must be carefully validated and can only serve as a guide as to which biological pathways are influenced by Bcl6 and warrant further study.

Microarray data are often validated by sq RT-PCR analysis (Chuaqui et al., 2002) and typically less than 10 genes are selected for validation (Al Moustafa et al., 2002, Porkka et al., 2002). I validated 23 genes by sq RT-PCR (Figure 7.3). For validation by sq RT-PCR, I chose a clone different to the one I performed microarray analysis on, in order to demonstrate a degree of universality of the microarray results (microarray analysis clone AB7, sq RT-PCR clone FA8) (Chuaqui et al., 2002). Supportive results were obtained for 14 out of 23 genes analysed, no supporting effect was observed for 8 out of 23 genes and 1 out of 23 (*TAP1*) showed a clear discrepancy to the microarray data (Figure 7.3). It is not clear why a discrepancy was detected for *TAP1*. In order to maximise accuracy of the sq RT-PCR experiment, all 96 PCR

reactions were performed on the same day. There is the possibility the clone chosen for validation (FA8) may behave slightly different to that used in the microarray experiment (AB7). In order to determine the exact cause of this discrepancy, validation for the *TAP1* gene would have to be repeated, ideally on both clones. Overall, however, quality control results (Figure 7.2 and Figure 7.3) are satisfactory and sufficient to obtain an overview of the biological pathways involved in the *BCL6* knockout phenotype of *BCL6*^{-/-}*tetBCL6-HA* cells.

7.3.2 Bcl6 and cell cycle regulation

Pathway mapping with the 'DAVID functional annotation program' of microarray data provided an interesting insight into the gene expression changes correlating with the reduced proliferative capacity of Bcl6-depleted *BCL6*^{-/-}*tetBCL6-HA* cells (Figure 7.4). I detected an upregulation of the demonstrated Bcl6 target gene *CDKN1B* (p27kip2) (Shaffer et al., 2000), which is in accordance with the upregulation of p27kip2 mRNA and protein demonstrated in CHAPTER 6 (Figure 6.12 p.140 and Figure 6.13 p.141). No change in transcript expression was observed for p21cip1/waf1 in microarray analysis although a decrease in protein abundance was observed in section 6.2.4 (Figure 6.12 p.140), while no difference was detected in sq RT-PCR analysis (Figure 6.13 p.141). Although p21cip1/waf1 protein analysis needs to be repeated in *BCL6*^{-/-}*tetBCL6-HA* cells (see discussion section 6.3 p.149), posttranscriptional regulation of p21cip1/waf1 is possible.

In addition to changes in *CDKN1B* (p27kip2), microarray analysis identified gene expression changes of further regulators of cell cycle progression namely *CDC25A*, *CDC6*, *E2F8* and *RB1*. Transcription of *CDC25A*, *CDC6*, and *E2F8* was downregulated in response to Bcl6-depletion; hence they cannot be direct Bcl6 target genes, *RB1* transcription was upregulated (Table 8). Cdc25A is a phosphatase that can remove inhibitory phosphorylations from CDKs involved in G₁/S as well as G₂/M phase transition (Aressy and Ducommun, 2008).

Following DNA damage, Cdc25A is rapidly degraded leading to cell cycle arrest (Zhao et al., 2002). Cdc6 is a component of the pre-replication complex and contributes to regulation of DNA replication in S phase (Yan et al., 1998). E2F8 is an E2F-like cell cycle regulator that inhibits transcription of E2F target genes by blocking their binding sites in target gene promoter regions (Christensen et al., 2005). Interestingly, *CDC25A*, *CDC6* and *E2F8* are all E2F target genes (Christensen et al., 2005, Vigo et al., 1999, Yan et al., 1998) and were downregulated in response to Bcl6-depletion. Because the E2F transcriptional activators are inactive when bound to retinoblastoma (Rb), upregulation of *RB1* in response to Bcl6-depletion (Table 8) provides a possible explanation for the downregulation of E2F target genes. Not much is known about how *RB1* gene expression is regulated. The *RB1* promoter closely resembles that of typical housekeeping genes, which is in agreement with its ubiquitous expression (Goodrich, 2003) and its regulation by posttranslational mechanisms. Phosphorylation of Rb by CDKs in G₁/S phase causes its degradation and activates transcription of E2F-dependent gene program (Giacinti and Giordano, 2006). According to my microarray data, *RB1* expression is upregulated in response to Bcl6-depletion, however, a link between Bcl6 and *RB1* has not been demonstrated to date. It would be interesting to perform reporter gene assays to elucidate whether *RB1* is a direct Bcl6 target gene or whether its gene expression is upregulated as a secondary event.

7.3.3 Bcl6 and B cell receptor signalling

Expression of six genes with key functions in B cell receptor signalling was upregulated in response to Bcl6-depletion; these genes were *SYK*, *BLNK*, *PTPRC* (CD45), *IFITM1* (LEU13), *CD72* and *PTPN6* (SHP-1). *SYK*, *BLNK*, *CD45* and *LEU13* are activators of BCR signalling, while *CD72* and *SHP-1* negatively modulate the BCR signal. A simplified overview of BCR signalling is shown in Figure 7.8.

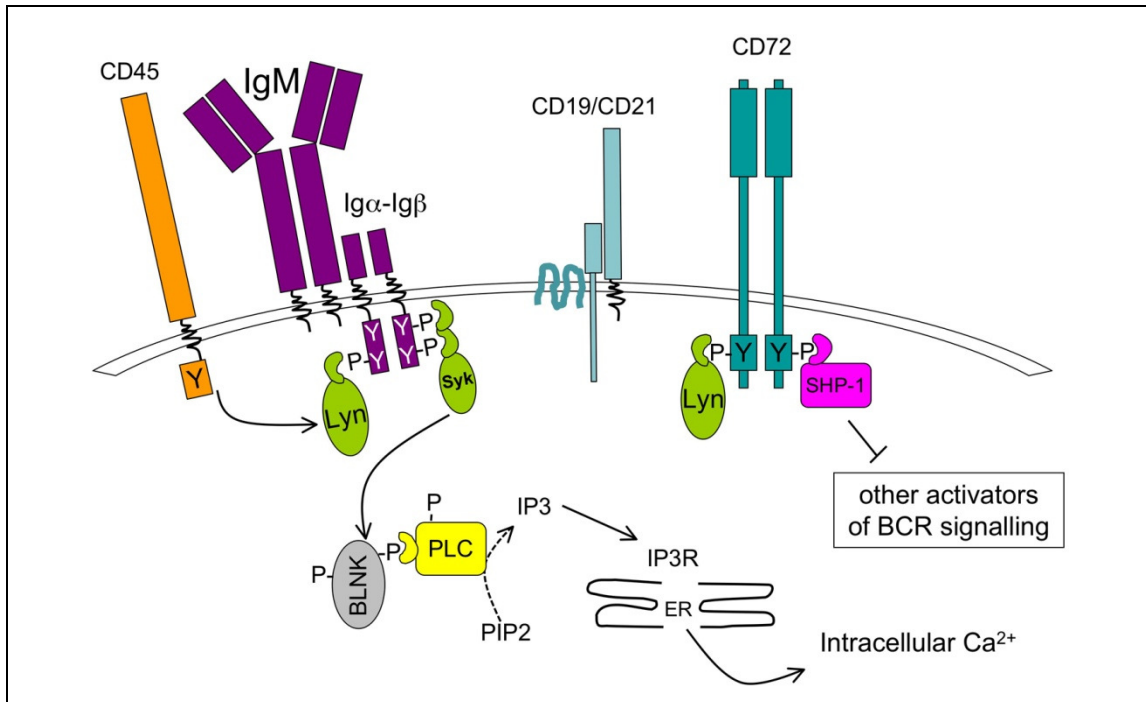


Figure 7.8: BCR and calcium signalling adapted from (Nitschke and Tsubata, 2004)

Upon ligation of the BCR (IgM), tyrosines (Y) of the intracellular BCR-associated Igα-Igβ heterodimer are phosphorylated by Lyn. This phosphorylation facilitates association and activation of SYK (spleen tyrosine kinase), which in turn phosphorylates and activates the B cell linker (BLNK). BLNK activation recruits and activates phospholipase C (PLC) which hydrolyses phosphatidyl 4,5-bisphosphate (PIP₂) and thereby produces the second messenger inositol 1,4,5-trisphosphate (IP₃). IP₃ in turn activates the IP₃ receptor (IP₃R) in the endoplasmic reticulum (ER), which in response releases calcium (Ca²⁺) into the cytoplasm. Rise of intracellular Ca²⁺ levels induces various cellular responses.

The inhibitory BCR co-receptor CD72 binds and activates SHP-1, which dephosphorylates and thereby inactivates intracellular signalling molecules that induce the BCR signal. The phosphatase CD45 is a positive co-regulator of BCR signalling that can remove inhibitory phosphorylations from LYN (Hermiston et al., 2003). Another BCR-activating effect is provided by the CD19/CD21 complex that consists of CD19, CD21, CD81 and Leu13 (Fearon and Carroll, 2000). The CD19/CD21 complex has been shown to prolong the signal generated from the BCR, possibly through direct interaction with the BCR (Cherukuri et al., 2001). CD72 is a negative regulator of BCR signalling (Li et al., 2006). In response to BCR engagement, LYN not only phosphorylates the BCR-associated Igα-Igβ heterodimer but also the intracellular portion of the inhibitory BCR co-receptor CD72 (Adachi et al., 1998). Phosphorylation of CD72 recruits and activates the phosphatase SHP-1, which moves on to inactivate intracellular signalling molecules functioning in BCR activation (Nitschke and Tsubata, 2004).

Bcl6-depletion upregulates mRNA transcripts of at least four activators of BCR signalling (SYK, BLNK, CD45 and LEU13). It cannot be evaluated whether these changes are a direct effect of Bcl6-depletion (i.e. that the upregulated genes are direct Bcl6 target genes) or whether they are secondary results of Bcl6-induced changes but this could be tested by reporter gene assays. Nothing is known about the transcriptional regulation of *PTPRC* (CD45) and *SYK*; however, some information is available for the remaining three candidates. *BLNK* transcription is activated by Pax5 (Schebesta et al., 2002b) and repressed by NF- κ B (Gupta et al., 2008). *IFITM1* (LEU13) has been suggested to be Bcl6 target gene. Gain-of function (overexpression of Bcl6 in cell lines that do not normally express Bcl6) and loss of function (expression of dominant-negative Bcl6 in BL cell line) models studied by Shaffer and co-workers provided evidence for transcriptional regulation of *IFITM1* by Bcl6 (see also section 1.11.2 p.44), although no information on putative Bcl6 binding sites in the *IFITM1* is available to date (Shaffer et al., 2000).

CD72 and SHP-1 function in negative regulation of BCR signalling and their expression was upregulated in response to Bcl6-depletion. It has been shown that Bcl6 can bind the *CD72* promoter through interaction with PU.1 in Ramos and BJAB cells, as demonstrated by chromatin immunoprecipitation (Wei et al., 2009). Although not demonstrated for *CD72*, downregulation of Bcl6 in BJAB cells by siRNA upregulated expression of genes that Bcl6 can interact with through PU.1 (Wei et al., 2009). Together with the results obtained from my *BCL6*^{-/-}*tetBCL6-HA* model system, it appears highly likely that *CD72* is a direct Bcl6 target gene in GC B cells. Less evidence, however, is available for *PTPN6* (encoding SHP-1). The *PTPN6* promoter contains a STAT1 binding site, the deletion of which was shown to reduce transcription in reporter gene assays performed in mouse embryonic fibroblasts (An et al., 2008). Interestingly, *STAT1* itself was also found to be upregulated in response to Bcl6-depletion (Table 8), which could potentially account for the upregulation of *PTPN6* expression. Interestingly, EBV-negative BL cell lines and primary centroblasts

have been previously shown to express reduced SHP-1 protein levels (Delibrias et al., 1997). This observation was proposed to account for a lower threshold of cytokine and BCR signalling at this particular stage of B cell development (Delibrias et al., 1997).

Overall, Bcl6 appears to regulate components involved in positive and negative regulation of BCR signalling in *BCL6*^{-/-}*tetBCL6-HA* cells. The influence of Bcl6 on BCR signalling has been previously reported. In DLBCL, Bcl6 is found to repress transcription of the SYK phosphatase PTPROt (Juszczynski et al., 2009). Reporter gene assays in HEK293T cells and modulation of Bcl6 expression in naive B cells and centroblasts validated the direct influence of Bcl6 on *PTPROt* gene expression (Juszczynski et al., 2009). By repressing the SYK inhibitor PTPROt, Bcl6 contributes to activation of BCR signalling, Bcl6-depletion would lead to reduced BCR signalling.

Although this project was carried out in a BL cell line, the findings generated from the *BCL6*^{-/-}*tetBCL6-HA* model may also have implications for the understanding of Bcl6 function in DLBCL. DLBCLs are classified by their gene expression profile. When Monti and co-workers performed microarray analysis on nodal biopsies obtained from 176 newly diagnosed DLBCL patients, they identified three DLBCL subsets based on the enrichment of gene expression of functional gene expression clusters (Monti et al., 2005). The ‘Ox/Phos’ subset expressed genes involved in oxidative phosphorylation, mitochondrial function and the electron transport chain. The second subgroup, termed ‘host response’, expressed markers of the T cell-mediated immune response, the classical complement pathway as well as inflammatory mediated and connective tissue components. The third DLBCL subset termed ‘BCR/proliferation’ expressed genes enriched in cell cycle regulation, DNA repair, BCR signalling as well as B cell-specific transcription factors (Monti et al., 2005). Interestingly, Bcl6 overexpression and translocations were

significantly associated with the BCR/proliferation subgroup of DLBCL (Monti et al., 2005), underscoring the importance of Bcl6 in this molecular pathway.

It is not entirely clear why Bcl6 represses genes involved in positive as well as negative regulation of BCR signalling. GC B cells are under great selective pressure to improve BCR binding for its antigen because they depend on BCR signals for survival (see sections 1.2.2 and 1.2.3). It is possible that both mechanisms exist *in vivo* to fine-tune BCR signalling at the GC stage of B cell development.

7.3.4 Bcl6 and calcium signalling

The concentration of intracellular calcium is an important regulator of cellular function and gene transcription. Intracellular calcium concentration is increased either by release of calcium from cellular stores such as the endoplasmic reticulum (ER) or it can enter the cytosol from the extracellular space via specialised ion channels and pumps (Cahalan et al., 2001). BCR signalling leads to phosphorylation and activation of the B cell linker (BLNK) which in turn recruits BTK and phospholipase C (PLC) (Scharenberg et al., 2007). When BTK and PLC are brought together by BLNK, BTK phosphorylates and thereby activates PLC (Kurosaki and Tsukada, 2000). Activated PLC hydrolyses the membrane phospholipid phosphatidyl 4,5-bisphosphate (PIP₂), thereby forming the second messenger molecule inositol 1,4,5-trisphosphate (IP₃) (Rhee, 2001). IP₃ then diffuses to the ER where it activates IP₃-dependent ion channels to release calcium from the ER into the cytosol (Patterson et al., 2004) (see also Figure 7.8). Elevated cytosolic calcium binds and activates calmodulin which in turn activates calmodulin/calcium-dependent kinases (CAMK). Once these effector kinases such as CAMK4 are activated, they phosphorylate and thereby activate downstream effectors, such as transcription factors or components involved in the remodelling of the cytoskeleton (Berridge et al., 2003).

Genes involved in the calcium signalling pathway that were altered in response to Bcl6-depletion are *CYSLTR1* (GPCR), *ATP2A* (SERCA1), *ITPR1/ITPR2* (IP₃R), *CAMK4* and *PTK2B* (FAK2, Pyk2) (Table 8). The *CYSLTR1* gene encodes a G-protein-coupled receptor (GPCR) located in the plasma membrane (Lynch et al., 1999) and its expression was upregulated in Bcl6-depleted *BCL6*^{-/-}*tetBCL6-HA* cells. Upon binding of its ligand leukotriene LTD₄, *CYSLTR1* undergoes a conformational change and activates downstream signalling molecules such as PLCβ, thereby inducing calcium signalling independently of the BCR (Capra et al., 2004). Most research on *CYSLTR1* is centered on its role in chronic inflammation in human asthma, where it is thought to play a role in chemotaxis of eosinophils (Mita et al., 2001), however, there are yet no data on its function or transcriptional regulation in B cells.

The calcium ATPase SERCA1 (encoded by *ATP2A*) was downregulated in Bcl6-depleted *BCL6*^{-/-}*tetBCL6-HA* cells, hence it is no direct transcriptional target of Bcl6 but is more likely downregulated by an unknown mechanism as a secondary effect resulting from Bcl6-depletion. SERCA1 resides in the ER membrane where it pumps cytosolic Ca²⁺ back into the lumen (MacLennan et al., 1997).

The *ITPR1* and *ITPR2* genes encode members of the IP₃R family that reside in the ER membrane, where they release calcium into the cytosol upon activation by the second messenger IP₃ (Coquil et al., 1999). Both *ITPR1* and *ITPR2* were upregulated in response to Bcl6-depletion, hence they could potentially be direct Bcl6 target genes, however, no data exist about their transcriptional regulation in cells of the immune system. While the transcription factors AP-1, AP-2, NF-κB were suggested to bind to the *ITPR1* promoter (Kirkwood et al., 1997), the promoter of murine *ITPR2* is suggested to behave like that of a housekeeping gene, in accordance with its ubiquitous expression pattern (Tamura et al., 2001).

Bcl6-depletion also upregulated transcription of *CAMK4*. Intracellular calcium complexes with calmodulin and binds to CAMK4, thereby activating it (Anderson and Kane, 1998). Activated CAMK4 then activates calcium-responsive transcription factors (Healy et al., 1997). No data on *CAMK4* transcriptional regulation in immunologic cells or connection to Bcl6 have been described to date.

Another gene involved in calcium signalling and upregulated in response to Bcl6-depletion is *PTK2B*. *PTK2B* encodes the protein tyrosine kinase Pyk2 which becomes phosphorylated and activated as a result of BCR signalling and plays a role in regulating B cell morphology (Tse et al., 2009). In monocytes, *PTK2B* is regulated by the MEK/ERK pathway through C/EBP (Park et al., 2008), however, no information is available for transcriptional regulation of *PTK2B* in the B cell lineage. Nevertheless, the upregulation of *PTK2B* mRNA in Bcl6-depleted *BCL6*^{-/-}*tetBCL6-HA* cells presents a possibly intriguing explanation for the morphological changes observed for Bcl6-depleted *BCL6*^{-/-}*tetBCL6-HA* cells in CHAPTER 6 (Figure 6.2 p.125). Pyk2 is a known regulator of macrophage motility (Okigaki et al., 2003) and Bcl6 is also expressed in macrophages (Yamochi et al., 1997). Macrophages from *BCL6*^{-/-} mice have an altered morphology; they display large numbers of small apical membrane protrusions, increased pseudopodia formation and migrate more slowly, probably due to loss of cell polarisation caused by increased RhoA activation (Pixley et al., 2005). Moreover, Pyk2 appears to be required for marginal zone B cells (Guinamard et al., 2000). *PTK2B*^{-/-} mice cannot produce marginal zone B cells, most likely due to defects in cell motility and responsiveness to chemokines resulting in a failure of B cells to localise to corresponding niche (Guinamard et al., 2000).

In summary, microarray analysis provided an exciting insight into the transcriptional changes resulting from Bcl6 protein depletion and the data generated supply much inspiration for future directions of study. The list of 163

genes upregulated in response to Bcl6-depletion likely contains many novel Bcl6 target genes that await validation. Implications of these results for future work and Bcl6-expressing lymphomas will be further discussed in CHAPTER 8.

CHAPTER 8 - Final discussion and future work

8.1 Rationale for developing the *BCL6*^{-/-};*tetBCL6-HA* cell line

Bcl6 has proven to be a promising drug target in DLBCL first *in vitro* and then in *in vivo* experiments in mice and it is tempting to hope that Bcl6-targeting therapy could follow on to a success story similar to that of Bcr-Abl-targeting therapy for treatment of chronic myeloid leukaemia (Goldman and Melo, 2008). This may just be possible because Bcl6, like Bcr-Abl, has been shown to be sufficient for development of malignant disease in mouse models (Cattoretti et al., 2005, Van Etten, 2001), and Bcl6 appears to be required for survival of primary DLBCL and DLBCL cell lines (Cerchiatti et al., 2009). However, unlike Bcr-Abl, which is uniquely expressed in leukaemic cells, Bcl6 is widely expressed.

In order to create the best possible potential for this to happen, it is essential to collect as much information as possible on Bcl6 function within its wide interaction network in B cells. The two most powerful systems for studying Bcl6 function to date are the knockout-mouse model and cell line-based gene expression analysis using gain-of-function and loss of function systems (Dent et al., 1997, Fukuda et al., 1997, Shaffer et al., 2000, Ye et al., 1997). Both models, however, have major limitations. The fact that *BCL6* knockout mice suffer from severe inflammatory disease and die early, underscores the importance of Bcl6 in the lymphoid lineage and also other tissues. At the same time, however, working with mice suffering from such a severe phenotype makes studying Bcl6 function difficult in this model.

The second source of information that has been very valuable to our understanding of the impact of Bcl6 on a wide range of regulatory networks was

based on microarray gene expression analysis (Shaffer et al., 2000). The two systems examined in this study were the gain-of function (i.e. Bcl6 overexpression in cells that do not express Bcl6) and a loss of function system (i.e. expression of dominant-negative Bcl6 in Bcl6-expressing BL cell lines) (see section 1.11.2 p.44). Both these systems, however, were based on transient transfections of cell lines. In such transfections expression of the construct of interest is temporary and uncontrolled and this makes results difficult to reproduce. I have therefore generated a novel model system that allows for precise Bcl6-protein depletion, by gene targeting of the *BCL6* locus.

8.2 Generation of the *BCL6*^{-/-};*tetBCL6-HA* cell line

The genetically tractable human B cell line *BCL6*^{-/-};*tetBCL6-HA* was derived from the cell line DG75. This Burkitt lymphoma cell line expresses high levels of Bcl6 protein and has been previously shown to support efficient somatic gene manipulation (Feederle et al., 2004, Maier et al., 2005). In the initial step towards generating my model system, I have disrupted the first *BCL6* allele by gene targeting (CHAPTER 3). The targeting vector I designed contains a 'floxed' *zeocin* resistance cassette, which allowed me to excise the selectable marker after successful targeting of the first *BCL6* allele so that I could reuse the same targeting construct to disrupt the second allele (CHAPTER 5). Before the second *BCL6* allele was targeted, I stably introduced a tetracycline-regulated *BCL6* gene into the cell line, which compensated for loss of endogenous Bcl6 expression when the second *BCL6* allele was targeted (CHAPTER 4). Unexpectedly, the presence of the *zeo* cassette and its strong CMV promoter in the *BCL6* locus generated a truncated Bcl6 protein variant. Despite technical difficulties, the *zeo* cassette was deleted from the second *BCL6* allele. After deletion of the *zeo* cassette from the second *BCL6* allele, expression of the truncated Bcl6 variant protein stopped, ruling out any falsification of the phenotype observed in *BCL6*^{-/-};*tetBCL6-HA* cells (CHAPTER 5).

8.3 Characterisation of the *BCL6*^{-/-};*tetBCL6-HA* cell line

I have carried out detailed analysis of the *BCL6*^{-/-};*tetBCL6-HA* cell line and have demonstrated the contribution of Bcl6 to cell cycle regulation in this model system (CHAPTER 6). Somewhat surprisingly, I detected no changes in gene expression of Bcl6 target genes involved in B cell activation and terminal plasma cell differentiation that were identified in previous studies (Schebesta et al., 2002a, Shaffer et al., 2000). Possible explanations for this are provided in the discussion of CHAPTER 6 (section 6.3). In the particular case of Blimp-1 (*PRDM1*), I concluded that the expression of other transcriptional repressors in addition to Bcl6 are responsible for the fact that no Blimp-1 expression can be induced in the DG75-based *BCL6*^{-/-};*tetBCL6-HA* system. An interesting experiment would therefore be to downregulate the additional transcriptional repressors of *PRDM1*, i.e. Pax5 and Bach2 and to induce expression of the *PRDM1* transcriptional activator IRF4. In this context, the *BCL6*^{-/-};*tetBCL6-HA* system would serve as a powerful model to test the precise requirements necessary for induction of *PRDM1* expression and thereby greatly contribute to our understanding of terminal B cell differentiation.

Microarray analysis revealed that Bcl6 protein-depletion in *BCL6*^{-/-};*tetBCL6-HA* cells affects not only genes involved in cell cycle regulation but also BCR and calcium signalling (CHAPTER 7). These data, together with a long list of putative Bcl6 target genes awaiting validation, provide exciting direction for future experiments.

8.4 Complementation analysis

Another exciting potential of the *BCL6*^{-/-};*tetBCL6-HA* cell line will be its use in complementation analyses. The cell line can be used as a platform to precisely dissect the interactions between Bcl6 and its various co-repressors. I have already designed, cloned and performed preliminary tests on eight Bcl6 complementation constructs that can be introduced into *BCL6*^{-/-};*tetBCL6-HA* cells (Figure 8.1). These complementation constructs express C-terminally FLAG-tagged (and mutated) Bcl6 from vector pcDNA3.1/Hygro/lacZ (Invitrogen) (see section 2.5.2 p.57).

(1) **pcBcl6-FLAG** encodes FLAG-tagged Bcl6 which is expected to be fully functional as it produces a similar band pattern to that of wildtype Bcl6 when transiently expressed in 293T cells (compare Figure 8.1 to Figure 5.7 p.115). When introduced into *BCL6*^{-/-};*tetBCL6-HA* cells, pcBcl6-FLAG is expected to completely rescue the phenotype observed when tet-regulated Bcl6 is switched off in *BCL6*^{-/-};*tetBCL6-HA* cells.

(2) **pcBcl6^{ΔPOZ}-FLAG** lacks the entire Bcl6 BTB/POZ domain (aa 1-121) of Bcl6. Most of the interactions between Bcl6 and its co-repressors are mediated through its POZ domain (see section 1.6 p.32), which is also the domain targeted by all Bcl6 inhibitors published to date because it is thought to be the most important site for Bcl6 function (Chattopadhyay et al., 2006, Polo et al., 2004, Cerchietti et al., 2008). It is not entirely clear whether the POZ domain truncated Bcl6 variant is properly folded, as expression was found to be low in 293T cells and the band pattern is not as diffuse as observed for full length Bcl6 (Figure 8.1). The diffuse banding pattern for Bcl6 is attributed to heavy phosphorylation of its PEST domains in the central portion of the protein (Onizuka et al., 1995), which is still present in the truncation. Nevertheless, this construct could become a valuable control for complementation studies that will be unable to, or only rescue very little of the *BCL6* knockout phenotype.

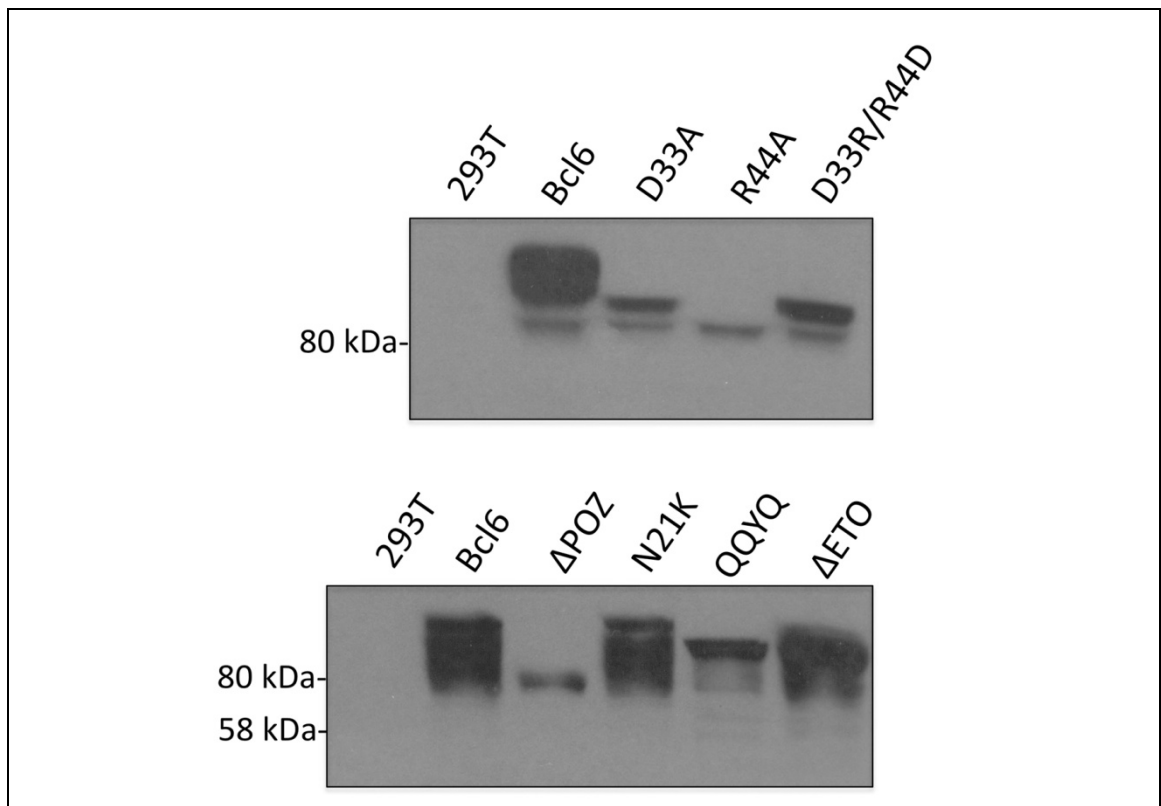


Figure 8.1: Testing Bcl6 complementation constructs in 293T cells

Cell lysates of 293T alone (293T) or 293T cells lipofected with pcBcl6-FLAG (Bcl6), pcBcl6^{D33A}-FLAG (D33A), pcBcl6^{R44A}-FLAG (R44A), pcBcl6^{D33R/R44D}-FLAG (D33R/R44D), pcBcl6^{ΔPOZ}-FLAG (ΔPOZ), pcBcl6^{N21K}-FLAG (N21K), pcBcl6^{QQYQ}-FLAG (QQYQ) or pcBcl6^{ΔETO}-FLAG (ΔETO) were analysed by Western blotting and detected with an αFLAG antibody (Cell Signaling) 24 hours after transfection.

(3) The Bcl6 variant expressed from **pcBcl6^{N21K}-FLAG** seems to be functionally folded, as its expression pattern in 293T cells closely resembles that of wildtype Bcl6 (Figure 8.1). The N21K mutation disables the interaction between Bcl6 and SMRT and NCoR (Ahmad et al., 2003). When Bcl6-expressing DLBCL cell lines were treated with a peptide targeting the SMRT binding site of Bcl6, this led to growth suppression and apoptosis (Polo et al., 2004). A new study from the same group reports a peptomimetic inhibitor of Bcl6 targeting the SMRT interaction site which suppressed the growth of human DLBCL xenografts in mice and killed primary human DLBCL cells, but had no effect on normal lymphoid tissue or other tumors (Cerchietti et al., 2010, Cerchietti et al., 2009). Therefore, the disruption of this major co-repressor interaction is expected to rescue, very little, if any of the phenotype of Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells.

(4)-(6) Constructs **pcBcl6^{D33A}-FLAG**, **pcBcl6^{R44A}-FLAG** and **pcBcl6^{D33R/R44D}-FLAG** were created to express Bcl6 variants resistant to inhibition by the peptide aptamer (Apt48), which was previously reported to encourage terminal differentiation of Bcl6-expressing Burkitt cell lines (Chattopadhyay et al., 2006). This resistance to inhibition by Apt48 is accomplished by charge switching at the interphase of the Apt48 – Bcl6 interaction through point mutations such as D33A, R44A and D33R/R44D (Ko Ferrigno, personal communication). These constructs could potentially be used to mimic therapeutic targeting of Bcl6 by Apt48 and would therefore be of great use in translational research. However, when tested in 293T cells it was found that all three mutated Bcl6 variants appeared very unstable (Figure 8.1). This finding is in agreement with an in-depth mutagenesis study of the POZ domain, in which it was found that alterations at this site of the Bcl6 molecule lead to severe protein folding defects that rendered Bcl6 non-functional (Melnick et al., 2000). These three mutant Bcl6 versions will therefore not be used for future complementation studies.

(7) **pcBcl6^{QQYQ}-FLAG** encodes a Bcl6 variant with an alteration in its central domain that is thought to regulate the interaction MTA3 complex (Fujita et al., 2004). Interaction of Bcl6 with MTA3 can be abolished by mutating the central acetylation motif KKYK to a motif mimicking constitutive acetylation (QQYQ) (Mendez et al., 2008). Robust amounts of Bcl6^{QQYQ}-FLAG can be expressed in 293T cells and hence this variant is believed to retain the remaining functions of the Bcl6 protein (Figure 8.1). The difference in banding pattern observed for Bcl6^{QQYQ}-FLAG (less diffuse) when compared to Bcl6-FLAG can be explained by the interference of the QQYQ mutation with the phosphorylation sites of Bcl6 (Figure 8.1). In plasma cell lines, overexpression of Bcl6 and MTA3, but not Bcl6 alone, downregulated Bcl6 target genes involved in plasma cell differentiation (such as Blimp-1) while B cell markers were upregulated (Fujita et al., 2004). Furthermore, it has been observed that depletion of MTA3 from lymphoma cells induces plasma cell differentiation in DLBCL cells (Parekh et al., 2007). It would be interesting to see to what extent complementation with this construct rescues the *BCL6*^{-/-} phenotype, as Blimp-1 induction was not observed in the *BCL6*^{-/-};tet*BCL6*-HA system. Should a residual phenotype remain, then this would argue strongly for a function of Bcl6 acetylation that extends transcriptional repression of *PRDM1* (Blimp-1).

(8) In **pcBcl6^{ΔETO}-FLAG**, the fourth zinc finger of Bcl6 (aa residues 602-624) is deleted which abolishes the interaction of Bcl6 with its co-repressor ETO (see section 1.6.3 p.36) (Chevallier et al., 2004). The 23 aa deletion causes a size decrease of approximately 3 kD and seems to not affect overall stability or phosphorylation of Bcl6, as a banding pattern similar to that of the control (Bcl6-FLAG) was observed by Western blotting (Figure 8.1). It would certainly be interesting to see which effect the disruption of the interaction between Bcl6 and ETO has on the *BCL6*^{-/-} phenotype.

In summary, 5 out of 8 complementation constructs appear to be functional when expressed in 293T cells (Figure 8.1) and are available for in-depth

analysis of the functional significance of some of the interaction sites between Bcl6 and its co-repressors.

8.5 Conclusion and clinical implications

This project and the conditional *BCL6*^{-/-};*tetBCL6-HA* cell line generated make an important contribution to the field of Bcl6 research and its future development. Through complementation analysis, the conditional *BCL6* cell line will facilitate the precise genetic dissection of the interactions that Bcl6 makes with its co-repressors. To date, we are able to disrupt the co-repressor interactions of Bcl6 with SMRT/NCoR, MTA3 and ETO. In addition, as more information on the sites within the Bcl6 molecule that function in co-repressor interactions becomes available, the panel of complementation constructs can be easily expanded. Such analyses will greatly contribute towards our understanding of the importance of these interactions and possibly identify alternative sites (other than the BTB/POZ domain) within the Bcl6 molecule that are worth targeting therapy against.

In addition to its use in complementation studies, the *BCL6*^{-/-};*tetBCL6-HA* cell line will serve as a valuable tool for translational research. Several Bcl6 inhibitors are currently being developed and vigorous testing of these small molecules has been performed in cell culture systems, primary patient cells and mouse models (see section 1.10 p.41). However, a pitfall of this technology is that specific effects mediated through inhibition of Bcl6 cannot be distinguished from off-target side effects. The data generated from my cell line represents a unique resource to be able to overcome this problem for two reasons. Firstly, a potential Bcl6 antagonist tested on DG75 cells or Bcl6-expressing *BCL6*^{-/-};*tetBCL6-HA* cells would be expected to reproduce the growth phenotype that I documented but not further stress or kill these cells. Secondly, I have identified a panel of genes that are strongly regulated by Bcl6 deficiency. These genes can be developed from a panel of PCR assays to give a semiquantitative

measure of how closely a potential Bcl6 antagonist mimics the effects of Bcl6 deficiency in the DG75 cell line.

I demonstrated that loss of Bcl6 protein is not sufficient to kill Bcl6-expressing BL cells (CHAPTER 6). This is contrary to the current belief that Bcl6 is necessary for survival of Bcl6-expressing GC representative B cell lines such as Raji (Polo et al., 2004), Ramos (Chattopadhyay et al., 2006), SUDHL4, SUDHL6 (Cerchietti et al., 2009), Farage, OCI-Ly1, OCI-Ly7 or OCI-Ly10 (Cerchietti et al., 2010). A possible explanation for this finding is that the Bcl6 inhibitors studied to date have off target side effects, which can now be easily tested in DG75 or Bcl6-expressing *BCL6*^{-/-};*tetBCL6-HA* cells. When adding Bcl6-inhibitors to these cells, if the inhibitors were specific, this should precisely reproduce the tet/dox-induced Bcl6-depletion phenotype. Furthermore, these inhibitors should have no effect on the already existing *BCL6*^{-/-}-phenotype in Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells. However, if Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells showed any phenotype alteration or died when treated with Bcl6 inhibitors, this would demonstrate that these inhibitors do not kill cells only through specific inhibition of Bcl6.

A similar approach has been followed in the development of inhibitors targeting pro-survival members of the Bcl-2-family. The Bcl-2 protein family regulates apoptosis and its pro-survival members are hence attractive targets in cancer therapy (Adams and Cory, 2007). Induction of apoptosis by Bcl-2 family proteins strictly depends on Bax/Bak (Zong et al., 2001) hence the inhibitors of Bcl-2 pro-survival proteins were expected to not kill *BAX/BAK* knockout cells. However, when *BAX/BAK* knockout cells were treated with potential inhibitors ('BH3 mimetics'), six out of seven compounds killed Bax/Bak-deficient cells due to off-target side effects, while only one proved specific (van Delft et al., 2006). Just as Bax/Bak deficient cells could screen for the most specific 'BH3 mimetic' drugs, Bcl6-depleted *BCL6*^{-/-};*tetBCL6-HA* cells can now be used to evaluate the specificity of Bcl6 inhibitors.

A further important implication of this project, other than studying the functional significance of the interactions between Bcl6 and its co-repressors and its use in the evaluation of Bcl6 inhibitors, is that the findings presented in this project may also translate into the clinical setting, i.e. the management of Burkitt's lymphoma. The current treatment regime of BL involves intensive chemotherapy and although the disease is curable, it is difficult to manage and associated with many toxicities (Levine, 2002). Treatment of BL patients with aggressive chemotherapy is associated with two major complications. Firstly, BL occurs in immunodeficient individuals (i.e. patients suffering from human immunodeficiency virus (HIV) or EBV infection), hence these patients may not be stable enough to receive intensive chemotherapy. Secondly, the extremely high proliferative index of BL (~ 100 %) puts patients at elevated risk of potentially fatal tumour lysis syndrome (TLS) in response to intensive chemotherapy (Schiffer, 2001). For these reasons, any intervention that allowed for reduction of the chemotherapeutic dose administered would be of great benefit for treatment of BL patients. Much attention has been paid to the importance of the c-Myc translocation in BL, however, as depletion of Bcl6 in *BCL6*^{-/-};*tetBCL6-HA* cells greatly reduces cell proliferation (CHAPTER 6), my results argue strongly for a considerable contribution of Bcl6 to the malignant phenotype of BL. Thus, the administration of Bcl6 inhibitors in combination with reduced intensity chemotherapy to BL patients comprises promising potential for the future.

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Appendix A

① Targeting of first *BCL6* allele ($BCL6^{+/+} \Rightarrow BCL6^{+/-}(zeo)$)

- transfection of DG75-354 ($BCL6^{+/+}$) with pTarg-*BCL6*, isolation of 44 *zeocin*-resistant clones
- 13 out of 44 clones were identified to have targeted the first *BCL6* allele by PCR assay
 - clones: **BA11**, BD9, DA4, **DC2**, DC3, **DE3**, DE8, **DF2**, **DF4**, DG3, **DG5**, **DG11**, FD2
- 7 out of 13 clones were analysed by Southern blotting:
 - clones: **BA11**, DC2, ~~DE3~~ (*BCL6* not targeted), DF2, DF4, DG5, DG11

② Cre-recombination ($BCL6^{+/-}(zeo) \Rightarrow BCL6^{+/-}$)

- nucleofection of clone BA11 ($BCL6^{+/-}(zeo)$) with pMC-Cre, isolation of 53 clones
- 2 out of 53 clones were identified to have deleted the *zeocin* cassette by PCR assay
 - clones: **7D4**, ~~4G12~~ (lost due to mycoplasma infection)

③ Introduction of tet-regulatable Bcl6 expression ($BCL6^{+/-} \Rightarrow BCL6^{+/-};tetBCL6-HA$)

- co-transfection of clone 7D4 ($BCL6^{+/-}$) with pTRE-Bcl6-HA and pSV2neo, isolation of 34 G418-resistant clones
- 7 out of 34 clones were tested positive for Bcl6-HA expression by Western blotting
 - clones: αB6, δG4, **ΔH3**, ΔB12, ηB5, *B10, *B10, ⊗G2

④ Targeting of second *BCL6* allele

($BCL6^{+/-};tetBCL6-HA \Rightarrow BCL6^{-/-}(zeo);tetBCL6-HA$)

- transfection of clone ΔH3 ($BCL6^{+/-};tetBCL6-HA$) with pTarg-*BCL6*, isolation of 17 *zeocin*-resistant clones
- 4 out of 17 clones were identified to have targeted a *BCL6* allele by PCR assay
 - clones 2D7, 5D6, 5D7, 6D1
- 3 out of 4 clones were identified to have targeted the second *BCL6* allele by PCR assay and Southern blotting
 - clones 2D7, **5D7**, 6D1

⑤ Cre recombination ($BCL6^{-/-}(zeo);tetBCL6-HA \Rightarrow BCL6^{-/-};tetBCL6-HA$)

- nucleofection of clone 5D7 ($BCL6^{-/-}(zeo);tetBCL6-HA$) with pTurbo-Cre, isolation of 84 clones
- 3 out of 84 clones were identified to have deleted the *zeocin* cassette by PCR assay
 - clones AB7, CD9, FA8

Figure A: Overview of clones generated and analysed in this study

Bold clones were further analysed or used in subsequent genetic modification steps.