A Role for *Bik* in B Lymphocyte Apoptosis and its Regulation by Epstein-Barr Virus

A dissertation submitted for the Ph.D.

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of...........**Ph.D.**................ (insert title of degree for which registered) is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Dedication

This thesis is dedicated to my parents, Mr Masoud Hakimjavadi and Mrs Fatemeh Samandi, who have raised me to be the person I am today. Thank you for all the unconditional love, guidance, and support that you have always given me!

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Foremost, I would like to express my sincere gratitude to my advisor Dr Dermot Walls for the continuous support of my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. This thesis would not have been possible without the help, unequivocal support and encouragement throughout of my dear parents, my brother, Mr Amir Hakimjavadi and my sister, Ms Farzaneh Hakimjavadi for which my mere expression of thanks likewise does not suffice. I would like to thank Dr Eva Campion and Ms Joanne Lahouni for their friendship, support, knowledge and encouragement during the course of my Ph.D.

Abstract

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Roya Hakimjavadi

Inhibition of host cell apoptosis is central to the strategy of the B-lymphotrophic Epstein-Barr virus (EBV) and also plays an important role in the development of EBV-related malignant disease and immune disorders. EBV promotes the survival of its host B cell during transit through the Germinal Centre (GC) of lymphoid follicles. The cellular Bcl-2 family (BH3-only protein) Nbk/Bik is a potent inducer of apoptosis and loss of Bik has been implicated in the development of cancers and the resistance of tumour cells to various therapeutic agents.

Here, evidence is presented to show that the EBV growth programme down-regulates *bik* and that this host-virus interaction contributes to the survival of the infected B cell. *Bik* was transcriptionally repressed in the presence of the EBV nuclear antigen-2 (EBNA-2) when the latter was expressed as sole viral protein. Furthermore, ectopic EBNA-2 led to increased cell survival when B cell apoptosis was induced by B cell receptor (BCR) cross-linking with anti-IgM antibody and TGFβ1–induced signalling, both of which are key pathways of relevance to B cell survival. Transient *bik* knockdown by anti-*bik* siRNA was seen to inhibit apoptosis induced by TGFβ1 whereas this was not sufficient to protect cells from apoptosis triggered through the BCR.

An increased rate of apoptosis due to TGFβ1 was observed to coincide with elevated Smad3/4 recruitment to the *bik* promoter in EBV-negative cell lines and this effect was reversed in cells proliferating due to the EBV growth programme or in EBV-negative cells expressing ectopic EBNA-2. The latter coincided with a decreased level of total Smad3 present in cells proliferating due to EBV or expressing ectopic EBNA-2.

Furthermore for the first time *bik trans*-activation was observed to coincide with apoptosis induced by $1\alpha,25\text{-}(OH)_2D_3$, the active metabolite of vitamin D. *Bik* is therefore potentially a key common target for the major intrinsic apoptotic signalling pathways in the GC reaction and is a negative target of EBV. In summary, the EBV-*bik* interaction is likely to make an important contribution to the survival of EBV-infected B cells during EBV infection and in some EBV-associated malignant diseases.

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Abbreviations

A	Absorbance
A_{260}/A_{280}	Absorbance at 260nm/ Absorbance at 280nm
aa	Amino acid
AAD	Amino-actinomycin D
ABI	Applied Biosystems
ACT	Activation Domain
AIDS	Acquired immuno-deficiency syndrome
AML	Acute Myeloid Leukaemia
AP	Alkaline phosphatase
Apaf	Apoptotic protease activating factor 1
APS	Ammonium persulphate
ArgGly	Arginine–glycine
ATP	Adenosine tri-phosphate
BART	*Bam*HI A rightward transcript
BATF	B cell specific transcription factor
BCA	Bicinchoninic acid
B-CLL	B-cell chronic lymphocytic leukaemia
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BCR	B cell receptor
BD	Becton Dickinson Biosciences
BH	Bcl-2 homology
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
BTM	Basal transcription machinery
C	Cytosine
Ca^{2+}	Calcium
CAD	Caspase Activated DNase
CBF	C promoter binding factor

CBP	CREB Binding Protein
cdk	Cyclin dependent kinase
CDKI	Cyclin dependent kinase Inhibitor
cDNA	Complementary DNA
c-IAP	Cellular inhibitor of apoptosis
CIP	Calf intestinal phosphatase
CIR	Co-repressor of CBF1
CKII	Casein kinase II
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
Cp	C promoter
CpG	Cytosine and Guanine
CR	Conserved region
CSL	CBF1/Su(H)/Lag-1/RBP-Jκ/KBF2
CST	Complementary-strand transcript
C_T	Threshold Cycle
CTAR	C-terminal activating region
CTL	Cytotoxic T Lymphocyte
C-terminal	Carboxy terminal
DD	Death domain
DEAE	Diethyl aminoethyl
DEPC	Diethylpyro-carbonate
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA Methyltransferase
dNTP	Deoxyribonucleotide
DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,Ndimethyl-1-propanaminium trifluoroacetate
DRP	Dynamin-related protein

DS-DNA	Double stranded DNA
DSL	Delta, Serrate and LAG-2
E. coli	*Escherichia coli*
EA	Early antigen
EA-D	Diffuse early antigen
EA-R	Restricted early antigen
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen-leader protein
EBV	Epstein-Barr virus
EBV-1/A	EBV Type I/A
EBV-2/B	EBV Type II/B
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
ERE	EBNA2-responsive element
ER/EBNA2	Estrogen-responisve EBNA2
EtBr	Ethidium bromide
ETOH	Ethanol
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FAM	6-carboxyfluorescein
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FSC	Forward light scatter
G	Growth Phase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Germinal centre
GFP	Green fluorescent protein
Gly	Glycine
gp	Glycoprotein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase

HDAC i	Histone deacetylase Inhibitor
HL	Hodgkin's lymphoma
HP1α	Heterochromatin protein 1
HRP	Horseradish peroxidase
H/RS	Hodgkin/Reed-Sternberg
HRT	Hairy-related transcription factor
HSV	Herpes simplex virus
IAP	Inhibitor of Apoptosis Protein
IC	Intracellular
Ig	Immunoglobulin
IgK	Immunoglobulin K
IgG	Immunoglobulin G
IgH	Immunoglobulin H
IgL	Immunoglobulin L
IgM	Immunoglobulin M
IκB	Inhibitor of κB
IKK	IκB kinase complex
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal repeat
JAK	Janus kinase
JNK	c-Jun NH_2 terminal kinase
Lat I	Latency I
Lat III	Latency III
LB	Luria-Bertani broth
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
M	Mitosis
M/MW	Molecular weight marker
MA	Membrane antigen
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase

MCS	Multiple cloning site
MEF	Mouse Embryonic Fibroblast
MHC	Major histocompatability complex
M-MLV RT	Moloney Murine leukemia virus reverse transcriptase
MMP	Mitochondrial Membrane Potential
MOMP	Mitochondrial Outer Membrane Potential
mRNA	messenger RNA
MZ	Mantle Zone
NEB	New England Biolabs
NF-κB	Nuclear factor κB
NHL	Non-Hodgkin lymphomas
NK	Natural killer cell
NLS	Nuclear localisation signal
NotchIC	Notch intracellular domain
NPC	Nasopharyngeal carcinoma
Nt	Nucleotide
N-terminal	Amino terminal
O.D.	Optical density
OHL	Oral Hairy leukoplakia
OMM	Outer Mitochondrial Membrane
ORF	Open reading frame
oriP	Origin of replication
p53	Protein 53
p38/MAPK	p38/mitogen activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP ribose) polymerase
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PE	Phycoerythrin
pH	Power of the hydrogen ion
PI	Propidium Iodide

PI3-K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethanesulphonylfluoride
pRb	Retinoblastoma protein
PS	Phosphatidyl serine
PTLD	Post transplant lymphoproliferative disorder
Qp	Q promoter
QPCR	Quantitative PCR
R	Region
RbCl$_2$	Rubidium chloride
RBP-Jκ	Jκ-recombinant-binding protein
RCC	Renal Cell Carcinoma
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROX	6-carboxyl-X-rhodamine
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
S	Synthetic Phase
SAP30	SIN3-associated protein 30
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
sIgG	Surface immunoglobulin G
SKIP	Ski-interacting protein
Smad	Small mother against decapentaplegic
SSC	Side light scatter
STAT	Signal transducers and activators of transcription
SV40	Simian virus 40
TAD	Trans-activation domain
TAE	Tris acetate ethylenediamine tetraacetic acid
TAMRA	Tetramethyl-6-carboxyrhodamine
tBid	Truncated Bid

TBS	Tris buffered saline
TBS-T	Tris buffered saline + Tween 20
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet	Tetracycline
TFB	Transformation buffer
TGF	Transforming growth factor
T_m	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TR	Terminal repeat
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factors
TRAIL	TNF-related apoptosis inducing ligand
upH$_2$0	Ultra-pure water
UV	Ultraviolet
UTR	Untranslated Region
UP	Undecylprodigiosin
VCA	Viral capsid antigen
v-IL	Viral Interleukin
v/v	Volume per volume
Wp	W promoter
wt	Wild type
w/v	Weight per volume
XIAP	X-linked mammalian inhibitor of apoptosis protein
zVAD-fmk	*N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone
$\Delta\Delta C_T$	Delta Delta C_T
+1	Transcriptional Start Site
3D	Three dimensional

Units

%	Percentage
˚C	Degrees Celsius
bp	Base pairs
cm	Centimetre
g	Grams
H	hours
Kb	Kilobase pairs
kDa	Kilo Dalton
Kg	Kilogram
L	Litres
M	Molar
mA	Milliamperes
mg	Milligrams
ml	Millilitres
mM	Millimolar
ng	Nanograms
nm	Nanometres
nt	Nucleotides
pmole	Picomoles
rpm	Revolutions per minute
U	Enzyme units
V	Volts
x g	Relative centrifugal force
W	Watts
μF	Micro Faraday
μg	Micrograms
μl	Microliter
μM	Micromolar
μm	Micrometre/micron

Publications

Manuscript

- Eva M. Campion[1*], **Roya Hakimjavadi[1*]**, Sinéad T. Loughran[1, 3], Susan Phelan[1, 4], Sinéad M. Smith[1, 4], Brendan N. D'Souza[1, 5], Paul A. Cahill[1, 2] and Dermot Walls[1#]. Downregulation of the pro-apoptotic cellular *bik/nbk* gene by the Epstein-Barr Virus growth programme. *Submitted for publication.* * Joint first authors.

- Kennedy E, Greene C, **Roya Hakimjavadi,** Fitzpatrick E, Guha S, Collins L, Mackle J, Morrow D, Redmond EM and Cahill PA (2013). Embryonic rat vascular smooth muscle cell A10 and A7r5 lines revisited - a model for neonatal, neointimal vSMC or differentiated vascular stem cells? *In preparation.*

- Mooney C, Kennedy E, **Roya Hakimjavadi,** Fitzpatrick E, Guha S, Collins L, Mackle J, Morrow D, Redmond EM and Cahill PA (2013). Vascular smooth muscle cells in culture are derived from resident multipotent vascular stem cells. *In preparation*

Presentations: Posters

- **Roya Hakimjavadi[1*],** Eva M. Campion[1*], Sinéad T. Loughran[1, 3], Susan Phelan[1, 4], and Dermot Walls[1]. A Role for *bik* in B Lymphocyte Apoptosis and its Regulation by EBV. BT Annual Research Day, DCU, Ireland, 25th January 2013.

- **Roya Hakimjavadi[1*],** Eva M. Campion[1*], Sinéad T. Loughran[1, 3], Susan Phelan[1, 4], and Dermot Walls[1]. Epstein-Barr Virus-mediated *bik* down-regulation enhances B cell resistance to TGFβ1 and BCR engagement. T3/BPS conference, DCU, Ireland, 11th January 2011.

- **Roya Hakimjavadi[1*],** Eva M. Campion[1*], Sinéad T. Loughran[1, 3], Susan Phelan[1, 4], and Dermot Walls[1]. Epstein-Barr Virus-mediated *bik* down-regulation enhances B cell resistance to TGFβ1 and BCR engagement. BT Annual Research Day, DCU, Ireland, 25th January 2013.

- **Roya Hakimjavadi**. Abstract title "Modulation of cytokine secretion and immunoglobulin production in specif B cell subsets" submitted for the Ruggero Ceppellini Advanced School of Immunology Course on: "The Role of B Cells in the Physiology and Pathology of the Immune System". Sorrento, Italy. 5-9th November 2009.

Presentations: Oral

- **Roya Hakimjavadi**. Investigation of A Role for *Bik* in B Lymphocyte Apoptosis and its Regulation by EBV. Transfer from M. Sc. to Ph. D presentation, 12th July 2012.

- **Roya Hakimjavadi[1*],** Eva M. Campion[1*], Sinéad T. Loughran[1, 3], Susan Phelan[1, 4], and Dermot Walls[1]. Epstein-Barr Virus-mediated *bik* down-regulation enhances B cell resistance to TGFβ1 and BCR engagement. T3/BPS conference, DCU, Ireland, 11th January 2011.

Chapter 1 :
Introduction

1.1 Epstein-Barr virus Discovery

The Epstein - Barr virus (EBV) is a ubiquitous human gamma herpes virus that infects approximately 95% of the world's adult population. EBV was discovered in 1958, in Uganda by an Irish surgeon, Denis Burkitt, who observed what he described as a lymphosarcoma primarily affecting children in equatorial Africa. This malignancy would later be re-designated and named Burkitt's lymphoma (BL) (Harford 2012). Due to the onset of BL at a young age and the climatic and geographical distribution of the patients affected, Burkitt began to suspect that an external pathogen, such as a virus, may play a role in the development of this form of cancer. In order to further investigate and better understand of the cause of this endemic cancer, he sent tumour biopsies from Kampala to London in 1961. A few years later in 1964 Anthony Epstein, Bert Achong, and Yvonne Barr observed and described a virus particle in electron microscopic images of cultured Burkitt lymphoma cell lines that they named Epstein-Barr Virus (EBV) (Harford 2012).

High titers of anti-EBV antibody were found in BL patients compared to control patients (Henle, et al. 1969). In south China high antibody titers were also observed in a lymphoepithelial malignancy with a high incidence (Henle, et al. 1970). These observations together with further subsequent investigations led to the implication of EBV as the first candidate for a human oncogenic virus, not only in Burkitt's lymphoma but also in a number of other malignancies including undifferentiated nasopharyngeal carcinoma (NPC; Reviewed in (Bornkamm 2009)). Interestingly however, it soon became evident that EBV infection was widespread, indicating that EBV alone was not a causative agent for malignancy, and that other genetic and environmental factors were required for tumour development (Bornkamm 2009). EBV is now well known as the causative agent of infectious mononucleosis (IM; also known as glandular fever) and for its capacity to potently growth-transform its principal target cell, the B lymphocyte. In culture, these 'immortalised' cells are referred to as lymphoblastoid cell lines (LCLs). In later life Burkitt noted that his 'lymphoma belt' study carried out on BL was also a map of malaria-infested regions in Africa. The link between endemic BL occurrence and malarial transmission areas had been established, but to date no defined functional role for malaria in EBV-led BL has been elucidated (Reviewed in Moormann, Snider and Chelimo 2011 and Griffin,B., Rochford, R. 2005).

1.2 EBV Epidemiology

Infection with EBV is worldwide, affecting more than 95% of human population during the first two decades of their lives (Ng and Khoury 2009). In developing countries, primary infections occur mainly in young children and are usually asymptomatic. In developed countries, primary EBV infection can occur in 25% to 50% of children younger than five years of age. A second peak of primary infection occurs in adolescence, and 30% to 50% of cases will go on to develop the self-limiting condition known as glandular fever/infectious mononucleosis (IM) (Ng and Khoury 2009, Papesch and Watkins 2001). While the virus is normally a harmless passenger throughout one's life, EBV has been associated with several malignancies, as will be described below (see section 1.11). Co-factors that contribute to the development of clinical disease include immune status, genetic and dietary factors, age of the individual at the time of exposure and various physiological variables (reviewed in (Crowcroft, et al. 1998, Hocker, et al. 2013, Taylor and Blackbourn 2011, Dojcinov, et al. 2011)).

1.3 EBV Classification

The herpesviridae is a family of herpes viruses that infects birds, reptiles and mammals (Lacoste, et al. 2010). The herpes family of viruses can be separated into three sub-types according to their genome organisation, growth characteristics and cell tropism, namely α-herpes viruses, β-herpes viruses and γ-herpes viruses (Hardie 2010). The γ-herpes viruses are further divided into lymphocryptoviruses (LCV; also known as gamma1-herpes virus) and rhadinoviruses (RV; also known as gamma2-herpes virus) (Lacoste, et al. 2010). It is generally accepted that the LCV evolved from the RV due to the more divergent genomes that are found in RVs. EBV [also known as human herpes virus 4 (HHV4)], is a γ-herpes viruses of the genus lymphocryptovirus that targets B lymphocytes (Lacoste, et al. 2010, Hardie 2010, Barozzi, et al. 2007). EBV was the first herpes viral genome to be completely sequenced (Baer, et al. 1984). Typical γ-herpes viruses replicate in epithelial cells and then establish a lifelong latent infection in host lymphocytes (Hardie 2010, Kieff, E., Rickinson, A, B. 2007).

The EBV genome, like that of all herpes viruses, has a signature structure of conserved genes divided by defined regions of genetic variation (Lacoste, et al. 2010). EBV, has extensively co-evolved with its host, adapting its genetic code and incorporating genes of host origin to enable the latent virus to go undetected by the immune system (Hardie 2010).

1.4   Structure of EBV

Herpes viruses possess a toroid shaped DNA/protein associated core enclosed in a 162 capsomer nucleocapsid. This icosahedral nucleocapsid is surrounded by a protein tegument that is itself confined by an outer envelope. This outer envelope is coated by glycoprotein spikes (Kieff, E., Rickinson, A, B. 2007, Thompson and Kurzrock 2004) see (Figure 1-1).
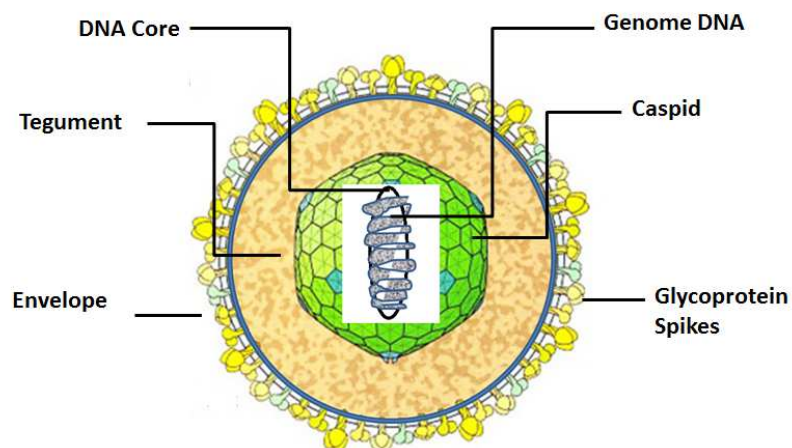
Figure 1-1: Schematic representation of herpes virus structure.
The EBV associated toroid protein core is illustrated within the icosahedral nucleocapsid. This capsid is itself covered by a protein tegument and enclosed in a glycoprotein covered envelope. Adapted from (van Kooten and Banchereau 1997)

The EBV genome is 170-184 kilobase pairs (kb) in length with a guanine/cytosine content of approximately 60%, encoding around 85 genes. The viral genome is wrapped around a toroid-shaped protein. This structure is covered with a nucleocapsid with 162 capsomers. The space between the nucleocapsid and the viral envelope is filled by a protein tegument and a range of glycoprotein spikes are seen on the outer envelope of the virus, consisting of the viral glycoproteins gp350, gp85, gp25, gp110, and gp84/11 (Thompson and Kurzrock 2004, Knipe, D.M, Howley, P.M. 2001). Due to the splicing potential of EBV RNA transcripts, it is likely that the number of possible proteins exceeds the number of genes present on the viral episome. The ends of the EBV genome contain a 500 base pair (bp) motif of variable number terminal repeats, this genome variation is further compounded by the presence of four internal tandem repeats that divide the genome into unique long and short fragments (Lacoste, et al. 2010). The internal repeats include one 3 kb tandem internal direct repeat (IR1) that defines the unique short region (U_S) from the unique long region (U_L) which contains another three short tandem internal direct repeats (IR2-4)(Lacoste, et al. 2010, Kieff, E., Rickinson, A, B. 2007) (see Figure 1-2). Upon entry into the host cell, the linear genome becomes circularised by the fusion of the two termini (Lindahl, et al. 1976). In latently infected cells the change in viral genome structure from linear to episomal occurs around 12-24 h after infection of B cells, therefore each latently infected cell contains about 10-20 circular viral episomes in its nucleus. Viral episomes divide as independent replicons once per cell cycle in early S phase using cellular DNA polymerase II and III (Knipe, D.M, Howley, P.M. 2001).
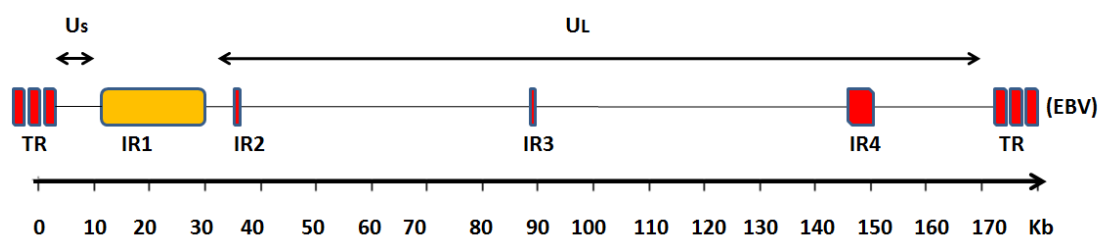
Figure 1-2: The EBV Genome.
A schematic drawing of the 172 kb EBV genome, including the terminal repeats (TR), internal repeats (IR) 1-4, the unique long arm (U_L) and the unique short arm (U_S) as indicated. Figure adapted from Lacoste *et al* 2010.

1.5 Primary Infection and Lytic Replication

Upon initial infection following exposure to saliva carrying EBV, the virus infects the oropharyngeal epithelial cells where it replicates, and then infects naïve IgD+ B cells in the oropharynx ((Kieff, E., Rickinson, A, B. 2007, Bollard, Rooney and Heslop 2012) B cells have the most important role in the EBV infection process, as patients with X-Linked agammaglobulinemia disorder, who do not have mature B cells, do not show any evidence of infection with EBV during their life (Faulkner, et al. 1999).

EBV infection of B cells is instigated with the attachment of the gp 350/220 viral membrane glycoprotein to the CD21 (complement C3d component (CR2)) molecule on lymphocytes. It has recently been shown that CD21 is not the only cellular receptor for EBV glycoprotein, but also CD35, the human complement receptor type I, which serves as another attachment receptor for gp350/220 glycoprotein (Ogembo, et al. 2013). Other viral envelope proteins including gp42 (part of the trimolecular viral envelope glycoprotein complex gp42/gp85/gp25 (also known as gH/gL complex)), which binds to HLA class II molecules and gp110 which also initiates the interaction of virus and its target cells are also involved in mediating viral entry into B cells (Ogembo, et al. 2013). It has been shown that the infection rate of epithelial cells increases when they are in close contact with virus-producing B cells (Shannon-Lowe, et al. 2006). EBV binds via its envelope glycoprotein gH/gL complex to gHgL receptor (gHgLR) on epithelial cells. In addition to this interaction, higher infection efficiency is obtained when the EBV multi-spanning envelope glycoprotein BMRF2, binds to $\alpha5\beta1$ integrin on the surface of epithelial cells (Klein, Klein and Kashuba 2010, Odumade, Hogquist and Balfour 2011).

EB virions secreted from reactivating B cells (or plasmablasts) are low in gp42 and thus show a greater tropism for epithelial cells which are HLA-class II-negative cells. On the other hand viruses derived from epithelial cells possess higher levels of gp42 envelope glycoprotein, which increases viral affinity for B cells (HLA-class II positive cells) (Hutt-Fletcher 2005, Borza and Hutt-Fletcher 2002). This finding suggested that epithelial cell infection facilitates viral access to B cells and that this process is critical for virus cycle maintenance (Hutt-Fletcher 2005). Studies have shown that most of the EBV genome and gp350 remains at the surface of the B cells for several days following virus/cell contact (Shannon-Lowe, et al. 2006). In addition that study showed that a gp350 knockout-EBV cell free virus preparation was able to infect epithelial cells with high efficiency, but unable to bind and infect B cells, thus it was suggested that the

gp350 envelope glycoprotein was an inhibitor for direct infection of epithelial cells by EBV, and that interaction of gp350 with CD21 is required to remove this inhibition and facilitate the unmasking of other virus envelope components required for epithelial cell infection. In this proposed model of primary infection, EBV binds to the surface of resting B cells and uses them as a vehicle to infect epithelial cells rather than there being an initial round of virus replication in B cells prior to epithelial cell infection (Shannon-Lowe, et al. 2006). After antigen binding, EBV is endocytosed by CD21-derived cytoplasmic vesicles and viral capsids are released into the cytoplasm (Bollard, Rooney and Heslop 2012, Nemerow and Cooper 1984, Carel, et al. 1990). This initiates mRNA synthesis, B cell blast formation, homotypic cell adhesion, surface CD23 expression and interleukin (IL)-6 production (Thompson and Kurzrock 2004). The linear viral genome moves into the nucleus within 12 hours whereupon it becomes a circular episome (Reviewed in (Kieff, E., Rickinson, A, B. 2007)) with a variable number of terminal repeats (TR) (Figure 1-2) which gives rise to a DNA fingerprint for the original infected cell and any arising daughter cells. This is a valuable feature that can be exploited to determine the clonality of EBV-associated tumours (Kutok and Wang 2006, Raab-Traub and Flynn 1986). EBV does not encode its own RNA polymerase for transcription, it utilises the cellular RNA polymerase II (Kieff 1996). The first detectable EBV proteins are EBNA-LP and EBNA-2 (Thompson and Kurzrock 2004, Thompson and Kurzrock 2004, Karlberg, et al. 2010).

Viral DNA polymerase accomplishes linear viral replication, which occurs during the lytic phase of the viral life cycle. There are three classes of viral lytic gene products, immediate-early (IE), early (E), and late (L). BZLF1 and BRLF1 are some of the IE products that act as transactivators of the viral lytic programme (El-Guindy, et al. 2013). Activation of lytic replication or reactivation from latency is crucial for transmission. The early products for example, BNLF2a have an inclusive range of roles, including replication, metabolism, and blockade of antigen processing, while late products tend to code for structural proteins such as the viral capsid antigens (VCA) and gene products used for immune evasion (e.g., BCRF1). The significant importance of EBV infection in B cells is that they are induced to activate their growth program and trigger differentiation into memory B cells via the germinal centre reaction. Infected memory B cells are released into the peripheral circulation (Figure 1-4). The number of infected B cells declines over time after the beginning of symptoms of primary infection

(Hadinoto, et al. 2008), but these cells are never eliminated fully (El-Guindy, et al. 2013, Zhao, et al. 2011).

1.6 EBV Latency Programmes and gene expression patterns

Latency is the state of persistent viral infection without active viral production. EBV persists mostly in the memory B-cell compartment and possibly also in epithelial cells (Thorley-Lawson, Duca and Shapiro 2008). Due to differing combinations of EBV latency gene expression in various EBV associated malignancies and EBV derived cell lines, a set of distinct EBV latency programmes have been defined (Shah and Young 2009). These have been characterised as latency Type 0, I, II and III (Table 1-1). By using different transcription programs, latent EBV genomes can multiply in dividing memory cells (type I), induce B-cell differentiation (type II), activate naïve B cells (type III), or completely restrict all gene expression in a context-specific manner (Figure 1-3) (Thorley-Lawson, Duca and Shapiro 2008).
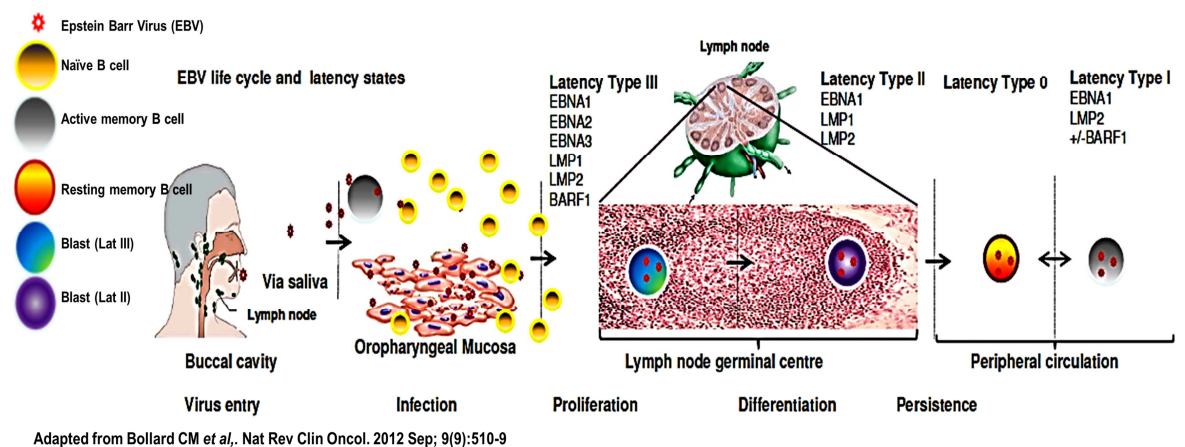
Figure 1-3: The infection life cycle and latency states of EBV

EBV infection is transmitted from host to host via saliva, and infects epithelial cells where it replicates in the oropharynx before infecting naïve B cells. Following infection of naive B cells in the lymph node, all latent genes are expressed (Latency III) and the viral latent proteins drive the B cell through subsequent proliferation and the germinal centre (GC) reaction in the absence of a cognate antigen. To establish a persistent latent infection, EBV must access the memory B cell compartment and reside within long-lived peripheral B cells where the expression of highly immunogenic viral latency antigens EBNA1, EBNA-2, and EBNA-3, are down-regulated and only few viral gene products (Latency II, Latency 0 and Latency I) as indicated in (Table 1-1) are expressed in order to escape immune detection. Terminal differentiation to plasma cells results in reactivation of the virus to the lytic cycle, expression of lytic proteins and production of infectious virus. The virus can infect B cells within the lymphoid tissue or be shed into the saliva. Adapted from (Bollard, Rooney and Heslop 2012).

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Table 1-1: EBV latency types and associated gene expression pattern.
(Bollard, Rooney and Heslop 2012, Shah and Young 2009, Young and Rickinson 2004a, Klein, Kis and Klein 2007)

Type of Latency	Viral Genes Expressed
Type 0	None Detectable
Type I	EBNA-1, EBERs, BARTs
Type II	EBNA-1, EBERs, BARTs, BARF0 LMP1,LMP2A, LMP2B
Type III	All EBV Latent Genes

After infection of naive B-cells, EBV activates a growth programme (also termed latency III) that drives B-cell proliferation, and is characterised by expression of its entire latency gene complex (Figure 1-3) which consists of eleven characterised EBV latently expressed genes, including six EBV Nuclear Antigens EBNA1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP, three integral latent membrane proteins LMP1, LMP2A and LMP2B and two small non-polyadenylated EBV encoded RNAs known as EBER1 and EBER2. There is also a further set of poorly understood transcripts known as BARTs (Murray and Young 2002) and recently discovered microRNAs (Riley, et al. 2012). Type III latency gene products, which drive B cell transformation and proliferation, are highly immunogenic. In order to escape detection by CD8+ cytotoxic T lymphocytes, the virus survives in B cells by downregulating the expression of its immunogenic proteins including the viral transcription factor EBNA-2 (Okur and Brenner 2010). Primarily B cells enter lymphoid follicles where they proliferate and express only three viral proteins (type II latency) (Thorley-Lawson, Duca and Shapiro 2008). This facilitates minor proliferation of the cells in the GC where the cell is hidden from immunosurveillance. Once leaving the GC the infected cells switch to either latency type I or type 0 to maintain infection without activating an immune response (Bollard, Rooney and Heslop 2012, Allday 2009). Terminal differentiation to plasma cells results in reactivation of the virus to the lytic cycle, expression of lytic proteins and the production of infectious virus. The virus can infect

B cells within the lymphoid tissue or be shed into the saliva (Laichalk and Thorley-Lawson 2005).
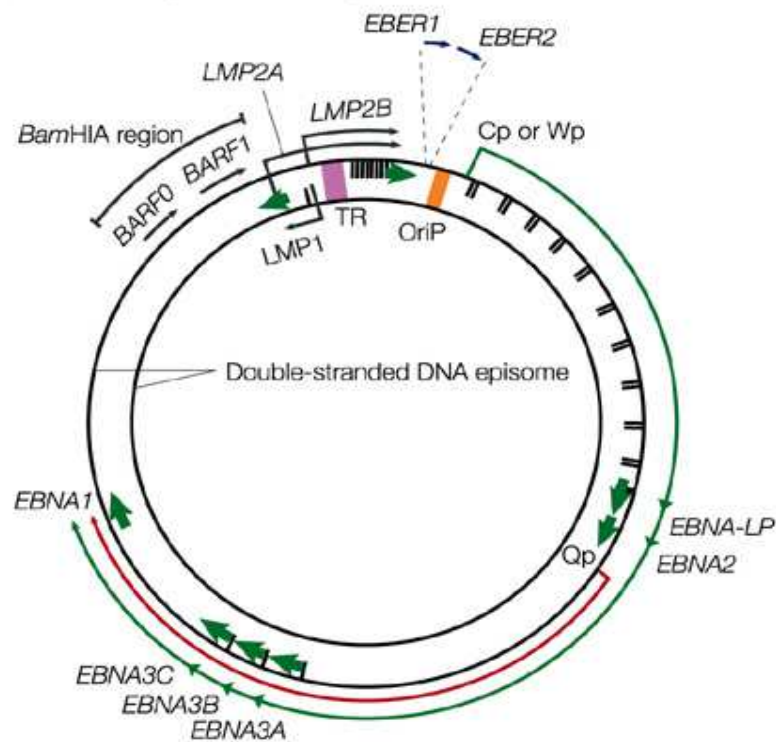
Figure 1-4: EBV Latent Genes.

Diagram of the double-stranded EBV episome showing EBV latent genes transcription. The episomal origin of replication (OriP) is shown in orange, EBNA1 binds to the OriP in a sequence specific manner to activate replication of the EBV genome. The large green arrows depict the coding regions for the six nuclear antigens (EBNA1, 2, 3A, 3B, 3C and EBNA-LP) and the three latent membrane proteins (LMP1, 2A and 2B). The highly transcribed non-coding RNAs EBER1 and EBER2 are represented at the top of the genome by the blue arrows. The BamHIA region contains the BARF0 and BARF1 transcript codes. The thin green arrow indicates the direction and transcript region utilised from the *EBNA Cp/Wp* promoter during type III latency. The EBNA latency III primary transcript is alternatively spliced to give rise to the EBV nuclear antigen-coding mRNAs. The *EBNA* Qp promoter is active during latency I and II and gives rise to *EBNA1* mRNA, as highlighted by the red arrow. (Figure adapted from (Young and Rickinson 2004a) .

1.7 The EBV-encoded nuclear antigens

1.7.1 EBNA1

EBV nuclear antigen 1 (EBNA1), a 641 amino acid protein, is the only viral protein consistently expressed in all EBV-associated malignancies and EBV-proliferating cells in healthy carriers (reviewed by (Frappier 2012)) EBNA1 is essential for lifelong viral persistence and is required for maintenance and replication of the EBV genome, through specific binding of EBNA1 to the EBV-origin of replication (oriP) (reviewed by(Young and Murray 2003a)). EBNA1 can also activate expression of critical cellular genes affecting cellular growth control and is known to possess oncogenic properties (Young and Murray 2003a, Tsimbouri, et al. 2002, Wilson and Levine 1992). This 73 kDa protein consists of a short amino-terminal region a 20-40 kDa, glycine-alanine (Gly-Ala) repetitive sequence flanked by arginine-rich sequences and a highly charged acidic carboxyl-terminal sequence (Young and Murray 2003a, Young and Rickinson 2004b). The presence of the Gly-Ala repeat domain in EBNA1 prevents processing by the proteasome, and has also been found to inhibit *EBNA1* mRNA translation. In this manner EBNA1 greatly reduces its own recognition by cytotoxic T cells, enabling infected cells to evade the immune response (Blake 2010).

1.7.2 EBNA-2

EBNA-2 is an 83 kDa transcription factor that plays a critical role in B cell immortalisation. It is a transcriptional co-activator that modulates the expression of viral and cellular genes, driving cell cycle progression from G_0 to G_1. Together with EBNA-LP, EBNA-2 is the first EBV latent gene expressed upon infection of a B cell (Reviewed in (Middeldorp, et al. 2003)). EBNA-2 can be nucleoplasm-, chromatin- and nuclear-matrix associated and localises to large nuclear granules (Petti, Sample and Kieff 1990, Hennessy and Kieff 1985). A crucial role for EBNA-2 in B cell immortalisation was discovered when a mutant strain of EBV, called P3HR1 was isolated and found to lack the ability to transform B cells in vitro. Further investigation showed that P3HR1 possessed a deletion encompassing the EBNA-2 open reading frame (ORF) and also partially that of EBNA-LP. It was soon discovered that the ability

of P3HR1 to transform B cells could be restored by the re-introduction of EBNA-2 to the genome. This implicated EBNA-2 as a vital EBV latent protein in the process of EBV-driven B cell immortalisation (Reviewed in (Thompson and Kurzrock 2004, Young, Dawson and Eliopoulos 2000)). Further studies have shown that EBNA-2 is also essential for maintaining immortalisation (Kempkes, et al. 1995a, Cancian, et al. 2011).

Structure of EBNA-2

EBNA-2A is 483 amino acids (aa) long whilst EBNA-2B is composed of 455 aa. Both contain a negatively charged highly homologous amino terminal region that facilitates homo-dimerisation (Figure 1.6). This is followed by a proline-rich domain which can contain from 10-40 successive prolines. The homology between the two types of EBNA-2 is least in the "diversity" region which then leads into the domain responsible for binding cellular CBF1/RBP-Jκ DNA-binding protein. This region is followed by an arginine-glycine rich domain that spans approximately 18 aa, and is followed by a negatively charged *trans*-activation domain that has been found to interact with a number of general transcription factors and co-activators in the cell. Finally the carboxyl terminus is responsible for nuclear localisation (Zimber-Strobl and Strobl 2001). Three regions of importance for B cell immortalisation by EBNA-2 have been identified through extensive mutational analysis: residues 94-110, encompassing the proline rich region; 280-337, containing the region required for interaction with CBF1; and 425-462, a region that harbours the acidic *trans*-activation domain(Zimber-Strobl and Strobl 2001, Ling, Rawlins and Hayward 1993, Cohen and Kieff 1991). While the transactivation domain, the nuclear localisation motif and the region which mediates promoter contact are located in the C-terminal half of EBNA-2, most of the N-terminal half of EBNA-2 does not have a critical role in transforming B cells and immortalisation, except for seven proline motif (Yalamanchili, Harada and Kieff 1996). The C-Terminal region of EBNA-2 determines the greater transforming ability of EBV type A through enhanced regulation of LMP-1 and CXCR7 expression (Cancian, et al. 2011).
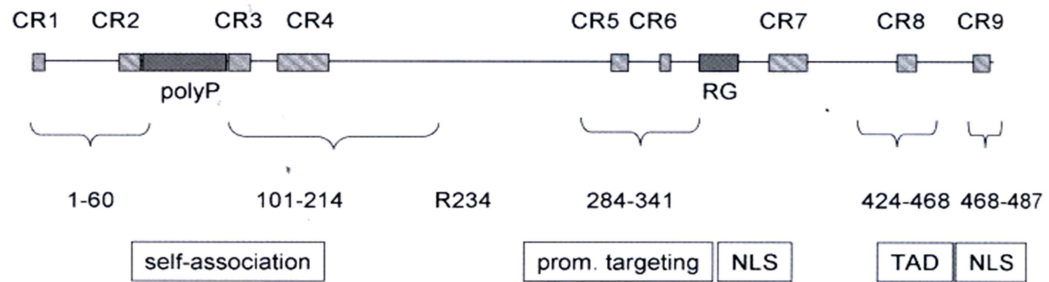
Figure 1-5: The structural domains of EBNA-2.

EBNA-2 consists of a negatively charged region at the amino-terminus, which is likely to play a role in homo-dimerisation (Dim), a polyproline region (Pro) consisting of 10-40 consecutive prolines depending on the virus strain, a diversity region with low homology between EBNA-2A and EBNA-2B, a domain responsible for interaction with RBP-Jk/CBF1 (RBP-Jκ), an arginine-glycine rich stretch (ArgGly) and a negatively charged region carboxyl terminus, which harbours a trans-activation domain (TAD), conserved regions (CR1-9), which have been defined by comparison of EBV strain type A and B, baboon and rhesus macaque lymphocryptoviruses and a nuclear localisation signal (NLS) (Figure adapted from(Zimber-Strobl and Strobl 2001)).

Table 1-2: The CR1-CR9 and their putative functions in EBNA-2 mediated transformation and immortalisation (Zetterberg,H., Rymo, L. 2005)**.**

Conserved Region	Respective Fonction
CR1	Increases the LMP1 promoter activity.
CR2- CR3	PPR is located between these two Conserved regions. The number of proline repeats differs between the two: types 1 (43 out of 45 amino acids are proline)& type 2 (14 out of 16 amino acids are proline).[25]
	Interacts with RNA helicase DP103 and SW1/SNF, chromatin-remodelling complex.
CR5-CR6	CR5, CR6, and the sequence between these, associates with RBP-JK or PU.1 in order to mediate promoter targeting.
CR4-CR5	Divergent region is located at CR4/CR5, which mediates self-association in addition to the two other regions at Amino terminal with the same function.
CR5	Interaction with SKIP.
CR6-CR7	Poly-RG motif is in between CR6 and CR7. It cooperates with CR9 at the C-terminal end of EBNA-2, for nuclear localisation signal.
CR8	This consists of an acidic activation domain, which associates with TFIIH, TAF40, TFIIB, p100, and P300, CBF, and PCAF histone acetyltransferases. Amino acids 453-466 (the core fragment) of acidic activation domain of EBNA-2 can be replaced by the herpes simplex viral protein VP16 activation domain, which indicates functional similarities.
CR9	Nuclear localisation signal and conserved Arginine-Glycine rich region.

Transcriptional regulation by EBNA-2

EBNA-2 does not directly bind DNA, rather it utilises a number of cellular transcription factors to achieve specific promoter targeting including (i) CBF1/RBP-Jκ, (ii) the ETS family protein Spi-1/Pu.1, (iii) the ATF/CRE complex, (iv) the CREB-binding protein CBP and (v) the chromatin remodelling complex SWI/SNF (Wu, Krumm and Schubach 2000, Wang, Grossman and Kieff 2000, Pegman, et al. 2006b, Ling, et al. 1994, Laux, et al. 1994, Johannsen, et al. 1995a, Henkel, et al. 1994). EBNA-2 has two principal mechanisms by which it activates EBNA-2-responsive genes: (i) it associates with its target genes to alleviate the repressive effect elicited by bound CBF1 (Figure 1-6) and followed by (ii) strong transcriptional promotion of the target gene (Figure 1-6) (Palermo, et al. 2008). EBNA-2 can also interact with members of the basal transcription machinery (BTM), including TFIIH, TFIIE, TFIIB, TAF40 and RPA70 through its acidic *trans*-activation domain (Palermo, et al. 2008, Tong, et al. 1995a, Tong, et al. 1995b, Tong, et al. 1995c). Further to these interactions, EBNA-2 has been shown to associate with the DEAD-box protein DP103 (Grundhoff, et al. 1999) which has an established role in transcriptional repression (Yan, et al. 2003). Of the BTM utilised by EBNA-2 for specific promoter regulation is the cellular DNA binding protein, *Cp* binding factor 1 (CBF1) which binds to the cognate DNA sequence 5'CGT GGG AA 3' (Nikrad, et al. 2005). CBF1 is also known as recombination signal-binding protein 1 for Jκ (RBP-Jκ). CBF1 is a ubiquitously expressed transcription factor that has been highly conserved during evolution (Zimber-Strobl, et al. 1993). CBF1 directs EBNA-2 to its target genes allowing regulation of the genes by EBNA-2. EBNA-2 drives one of its own gene promoters, the *EBNA Cp* promoter, via an upstream EBNA-2 responsive element (called the EBNA-2-responsive enhancer (E2RE) by alleviating CBF1 mediated repression (Jin and Speck 1992, Sung, et al. 1991). In the absence of EBNA-2, CBF1 acts as a co-repressor in the cell bound by a large multi-protein complex of cellular proteins which includes SMRT/NcoR, Sin3-associated protein 30 (SAP30), SIN3A, the CBF1-interacting co-repressor (CIR), histone deacetylase (HDAC) 1 and HDAC2. Another protein that can bind to this complex is Ski-interacting protein, SKIP. EBNA-2 mediates trans-activation of genes by competing with the SMRT-HDAC co-repressor complex thus relieving the target promoters of this repressor complex (Figure 1-6).

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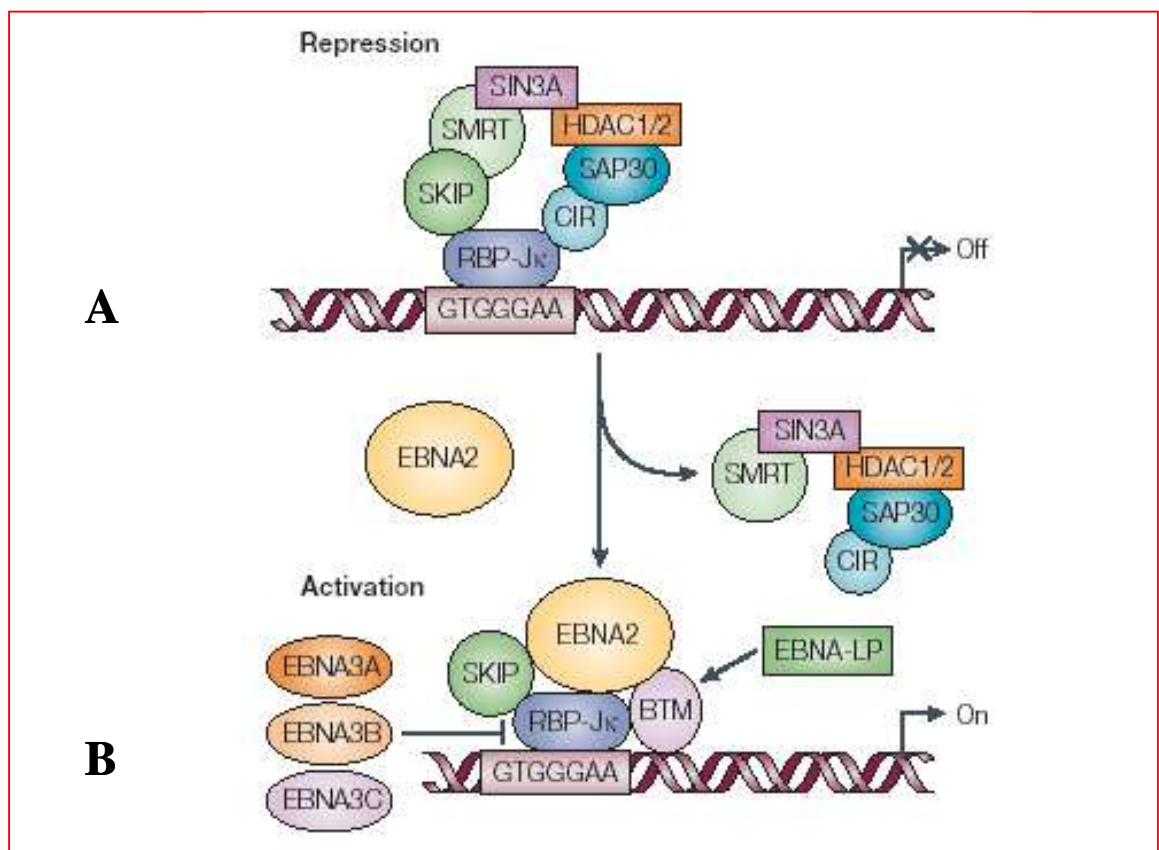
Figure 1-6: EBNA-2-mediated promoter activation (I)

(A) EBNA-2 acts as a transcriptional activator by interacting with the DNA-binding Jκ-recombination-binding protein (RBP-Jκ/CBF1) and relieving transcriptional repression that is mediated by a large cellular multi-protein complex consisting of SMRT/NcoR, SAP30, SIN3A, CIR, SKIP, HDAC1 and HDAC2. The binding of SKIP to SMRT facilitates nuclear entry of CBF1. (B) EBNA-2 abolishes CBF1-mediated repression by competing with the SMRT-HDAC co-repressor for binding to both CBF1 and SKIP. The EBNA-2 acidic domain recruits the BTM, TFIIB, TFIIH and p300 to drive transcriptional activation. (Figure adapted from (Young and Rickinson 2004a)).

Once repression is relieved, EBNA-2 brings strong transcriptional activation to the promoter (Hsieh and Hayward 1995). To activate this, EBNA-2 binds histone acetyltransferases (HATs) and SW1/SNF, the chromatin-remodelling factors to alter chromatin structure rendering the gene accessible for transcription. EBV utilises cellular RNA polymerase II (RNA pol II) for transcribing viral target genes (Figure 1-7).

EBNA-2 interacts with general transcription factors such as TFIIH and TFIIE along with the cyclin dependant kinase (CDK) 9 to initiate and maintain phosphorylation of RNA pol II. The main phosphorylation sites of the CTD are Ser-2 and Ser-5. Bark-Jones *et al,* have shown by CDK9 inhibition studies, that EBNA-2 requires CDK9 recruitment for efficient transcriptional activation (Bark-Jones, Webb and West 2006).

The CTD Ser-5 phosphorylation has also been shown to fluctuate relative to EBNA-2 expression levels, demonstrating that Ser-5 phosphorylation may be an EBNA-2-dependant process (Reviewed in (Palermo, et al. 2008)). In peptide studies CDK9 has been shown to preferentially phosphorylate Ser-5 (Ramanathan, et al. 2001), implicating a transcriptional initiation model that involves recruitment of CDK9 by EBNA-2 for the phosphorylation of Ser-5 thus activating the CTD and RNA pol II activity(Palermo, et al. 2008, Ramanathan, et al. 2001). EBNA-2 interaction with TFIIH aids phosphorylation of the CTD leading to strong sustained transcriptional up-regulation of the target gene (Palermo, et al. 2008).
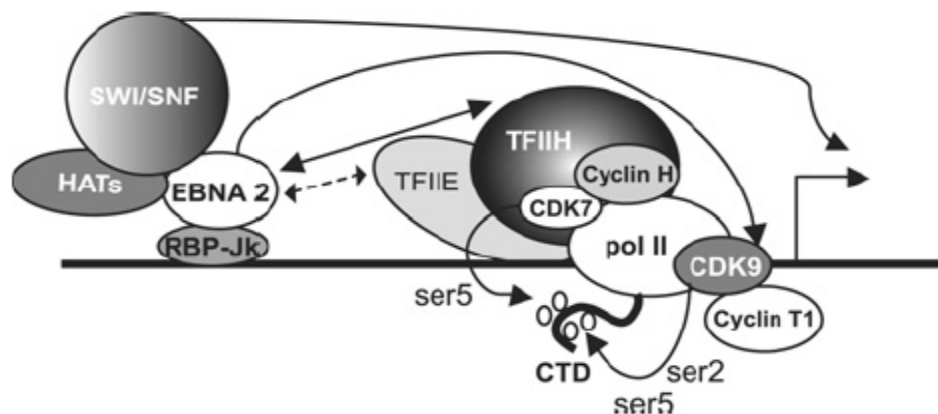
Figure 1-7: Mechanism for EBNA-2-mediated promoter activation (II).

Association with the target promoter leads to binding of EBNA-2 and the chromatin remodelers SWI/SNF along with HATs, to make the gene accessible for transcription. EBNA-2 then promotes assembly of the transcription complex by interacting with the general transcription factors TFIIH and TFIIE. Transcription initiation is activated by CDK9 stimulated phosphorylation of the Ser-5 and possibly the Ser-2 on the C-terminal domain (CTD). TFIIH also plays a role in Ser-5 phosphorylation through its bound subunit, CDK7. However, there is no evidence to suggest that EBNA-2 has a role in this process (Figure adapted from (Bark-Jones, Webb and West 2006)).

EBNA-2 functions in two ways; (i) reduce repression to allow up-regulation of its target genes, thus it binds to the repressor domain as described in (Figure 1-7) and abolishes the repression complex, (ii) EBNA-2 also brings a strong transcriptional activation domain to the promoter which plays an important role in the *trans*-activation of the target gene (Palermo, et al. 2008, Hsieh and Hayward 1995). EBNA-2 directly up-

regulates the expression of a range of viral and cellular genes, some of the most significant among these targets are those encoding the activated B cell surface marker CD23, the cellular proto-oncogene *c-myc*, the B cell differentiation marker CD21, the anti-apoptotic Bcl-2 family member *bfl-1*, the B cell specific transcription factors Runx3 and BATF, the chemokine receptor CCR7 that navigates the B cell to lymphoid organs, along with the EBV LMP1, LMP2A and LMP2B and the EBNA *Cp* promoter (Pegman, et al. 2006b, Ling, et al. 1994, Johannsen, et al. 1995a, Burgstahler, et al. 1995, Spender, et al. 2002) (Reviewed in(Kieff, E., Rickinson, A, B. 2007)). EBNA-2 has also been shown to elicit negative transcriptional regulation. Evidence from LCL studies has exposed EBNA-2 as a repressive influence on Nurr77-mediated apoptosis. This inhibition mechanism involves the interaction of the EBNA-2 transcriptional complex with Nurr77 protein, sequestering Nurr77 within the nucleus and preventing signalling to the mitochondria that would otherwise lead to cytochrome *c* release and stimulation of apoptosis (Lee, et al. 2002, Li, et al. 2000). To date EBNA-2 is known to transcriptionally repress cellular genes the immunoglobulin heavy chain locus (*IgM*) (Jochner, et al. 1996), *BCL6* and *TCL1* (Boccellato, et al. 2007). DNA microarray studies have been used to catalogue EBNA-2 cellular target genes and proteins (Maier, et al. 2006, Spender, et al. 2006, Schlee, et al. 2004c).

Latent promoter switching by EBNA-2

It is accepted that transcription through the *EBNA* W promoter (*Wp*) is an inefficient process that leads only to the expression of the promoter proximal genes, EBNA-LP and EBNA-2 (Palermo, et al. 2008, Bell, et al. 1998), (see Figure 1-4: EBV Latent Genes. for *Wp* coordinates relative to the EBNAs). The *Wp* is located in the IR1 and thus there are multiple copies of the *Wp* present on the genome. This is thought to facilitate the production of abundant EBNA-2 transcripts which are required to switch transcription from the *Wp*. Initially the *Wp* drives EBNA expression, however as EBNA-LP and EBNA-2 expression increases transcription switches predominantly to the *Cp* promoter (*Cp*) (Palermo, et al. 2008). The *Wp* itself does not appear to competently maintain transcription to allow the remaining *EBNAs* to become fully transcribed. The *Wp* however produces sufficient *EBNA-2* mRNA such that the EBNA-2 protein stimulates CDK9 dependant phosphorylation and recruits general transcription factors that activate and maintain the RNA pol II function from the *Cp* (Palermo, et al. 2008, Bark-Jones,

Webb and West 2006). The *Cp* contains an EBNA-2-responsive element (E2RE), allowing successful transcriptional initiation, promoter clearance, elongation and RNA processing of the product of interest. This yields a family of transcripts of up to 120 kb long that encodes the full range of EBNAs. Due to the inefficiency of the *Wp* activity, the switch to EBNA-2-led *Cp* transcription does not occur until approximately 48 hours after infection (Reviewed in (Palermo, et al. 2008)). EBNA-2 activation of *Cp* is required for transactivation of the remaining EBNAs (1, 3A, 3B and 3C) which are required for immortalisation.

EBNA-2 subverts the cellular Notch Signalling pathway

EBNA-2 subverts and manipulates B cell function, by usurping the mechanisms and diverse roles of Notch signalling in normal B cell physiology as well as B lymphocyte malignancy (Reviewed in (Kempkes,B., Strobl,L.J., Bornkamm,G.W., Zimber-Strobl, U. 2005, Johnson, et al. 2010)). In order to develop lymphocytes bearing functional receptors that are not harmful to the host, B cells are subjected to several checkpoints (apoptosis, proliferation or differentiation) at multiple stages of development (Cariappa, et al. 2001, Hase, et al. 2004, Rhee, et al. 2005, Mackay, et al. 2010). During B cell maturation, cell fate decisions are directed by signals derived from the microenvironment, and these interact with receptors expressed on developing B cells (Hardy and Hayakawa 2012, Wen, et al. 2005). These signals may be antigen-dependent through surface B cell receptor (BCR) on B cells (Kurosaki 2000, Kurosaki 2011) and modulated by co-stimulatory signals (Chung, et al. 2012). B cell maturation is also modulated by BCR independent signals, such as IL-4 (Sims, et al. 2005), IL-7 (Purohit, et al. 2003), IL-10 (Gary-Gouy, et al. 2002), BLyS/BAFF (Batten, et al. 2000) and Notch ligands (Cruickshank and Ulgiati 2010). Notch signalling regulates multiple aspects of lymphoid development and function and stimulates the development of marginal zone lymphocytes. It has been well documented that Notch is required for the later stages of B cell development, and Bertrand *et al*. showed Notch1 is expressed throughout human B cell development, while Notch2 is only detected in the late pre-B cell compartment (CD34$^-$CD19$^+\mu^{lo}$) (Bertrand, et al. 2000). Several viral proteins appear to utilize Notch signalling in B cells to mediate their functions, including

Kaposi's sarcoma-associated herpes virus (KSHV) and EBV (Cheng, Pekkonen and Ojala 2012).

Functional overlap of EBNA-2 and Notch

As stated previously, EBNA-2 function is elicited through the recruitment of cellular transcription factors such as CBF-1 which is a key component of the Notch signalling pathway. This CBF1-associated gene regulation usually relies upon activation of the Notch signalling cascade, however, EBNA-2 appears to act as a functional homologue of activated Notch, usurping the cellular Notch function and acting as a constitutively activated N^{IC} (Hsieh, et al. 1996, Hayward 2004). The mechanism by which EBNA-2 and N^{IC} dismantle the CBF-1 repressor complex appears to be one and the same: each protein interacts with CBF-1 and SKIP, subsequently displacing SMRT/NcoR from the complex. Both EBNA-2 and N^{IC} proceed to enlist chromatin modifying molecules, some of which overlap. EBNA-2 binds p300, PCAF and CBP (Wang, Grossman and Kieff 2000) and p100, a co-activator that stimulates TFIIE (Tong, et al. 1995b), while N^{IC} recruits GCN4, PCAF and p300. Indeed, unsurprisingly EBNA-2 and N^{IC} overlap in functional activities and target genes. Both EBNA-2 and N^{IC} can inhibit Nur77-induced apoptosis by binding to and sequestering this pro-apoptotic transcription factor (Lee, et al. 2002, Jehn, et al. 1999). Both have also been implicated in modulating the activity of a number of overlapping genes in B cells such as *Hes1*, *LMP2A (EBV)*, *BATF*, *CD21* and *IgH*. Contrastingly, Notch cannot regulate the EBNA-2 targets *LMP1*, *CD23* and *c-myc* (Reviewed in (Zimber-Strobl and Strobl 2001)). There is well documented evidence that EBNA-2 and Notch1-IC associate with different transcription factors other than CBF1, for instance PU.1 (Johannsen, et al. 1995b) and that this contributes to a different EBNA-2 versus Notch1 response. For example EBNA-LP cooperates with EBNA-2 in trans-activating the *LMP1* promoter (Nitsche, Bell and Rickinson 1997).

1.7.3 The EBNA-3 family (EBNA-3A, EBNA-3B and EBNA-3C)

The EBNA-3 family consists of 3 nuclear proteins, EBNA-3A, -3B and -3C (previously referred to as EBNA-3, EBNA-4, and EBNA-6 respectively). The nuclear proteins are encoded by alternatively spliced transcripts initiated at the *Cp* promoter (Kieff, 1996) and are composed of 944, 937 and 992 amino-acid residues, respectively (Petti *et al*, 1990). While EBNA-3A and -3C are essential for the growth transformation of lymphocytes *in vitro*, EBNA-3B is not required for B cell immortalisation under these conditions (Young and Murray 2003a). EBNA-3C can induce the up-regulation of both cellular and viral gene expression, and is also known to repress the *Cp* promoter (as reviewed in (Bark-Jones, Webb and West 2006, McClellan, et al. 2012)). EBNA-3C interacts with the retinoblastoma protein, pRb, to promote cell transformation (Skalska, et al. 2010). In addition, EBNA-3A and EBNA-3C co-operate as the main factors of drug resistance in BL cells and in the down-regulation of the pro-apoptotic Bcl-2-family member Bim (Skalska, et al. 2010). EBNA-3B has been shown to induce expression of vimentin and CD40 (Silins and Sculley 1994). All three EBNA-3 proteins can also bind CBF1, negatively regulating gene expression in the Notch pathway. This results in repression of EBNA-2 *trans*-activation, which in turn regulates the synthesis of the EBNA-3 proteins themselves (Skalska, et al. 2010). Thus the EBNA-3 proteins balance and fine-tune the action of EBNA-2 by precisely regulating CBF1 activity, thus regulating the expression of cellular and viral promoters containing CBF1 binding sites (Young and Rickinson 2004b, Skalska, et al. 2010).

1.7.4 EBNA-LP

The EBNA Leader Protein (EBNA-LP/EBNA-5) is one of the first viral proteins produced during EBV infection of B-lymphocytes (Portal, et al. 2011). EBNA-LP is encoded by the first ORF (leader sequence) of the extensively spliced primary transcript, originating from either the *Wp* or *Cp* promoter, and is composed a multi-repeat domain (W1W2) and a unique carboxyl-terminal domain (Y1Y2). *EBNA-LP* encodes a protein of variable size (20-130 kDa) depending on the number of *Bam*HI W repeats contained by a particular EBV isolate. During early infection in B cells and following expression of EBNA-LP in type I BL cell lines, EBNA-LP is distributed

throughout the nucleus, whereas in LCLs, EBNA-LP localises to promyelocytic leukemia nuclear bodies (PML NBs) (Ling, et al. 2005). Although not essential for transformation, EBNA-LP enhances the efficiency of the process (Portal, et al. 2011).

EBNA-LP is known principally as a transcriptional co-activator of EBNA-2. It has previously been shown that EBNA-LP co-operates with EBNA-2 in the up-regulation of *LMP1/LMP2* and *Cp* latency promoters in B cells and that EBNA-LP and EBNA-2 co-operative to stimulate expression of cyclin D2 in resting B cells in addition to mediating G_0 to G_1 transition during immortalisation (Peng, et al. 2005a). EBNA-LP co-activates EBNA-2 through interacting with the promyelocytic leukemia nuclear body (PML NB)-associated protein Sp100 and displacing Sp100 and heterochromatin protein 1 α (HP1α) from PML NBs (Ling *et al*, 2005). Numerous other co-factors that potentially associate with EBNA-LP have been identified including pRb, p53, hsp72/hsc73, hsp27, Hax-1, ERR1, p14ARF, DNA-Pkcs, α-tubulin, β-tubulin, prolyl-4-hydroxylase, and HA95 (Ling, et al. 2005). However, to date, no correlation has been made between the association with these factors and EBNA-LPs ability to co-operate with EBNA-2.

1.8   The EBV-encoded latent membrane proteins

1.8.1   LMP1

Latent Membrane Protein 1 (LMP1) is a major transforming protein of EBV. Several studies have shown that this protein functions as a classic oncogene and is essential for EBV-induced B-cell transformation *in vitro* (reviewed in (Young and Rickinson 2004b, Middeldorp and Pegtel 2008, Lo, et al. 2010)). LMP1 has pleiotropic effects when it is expressed in BL-derived cells, resulting in many of the phenotypic changes observed during EBV-infection, including induction of cell adhesion molecules and activation markers (Kieff, E., Rickinson, A, B. 2007), and the up-regulation of anti-apoptotic proteins Bcl-2, Mcl-1, Bfl-1, A20 and c-IAPs (D'Souza, et al. 2004, Pratt, Zhang and Sugden 2012). LMP1 also induces cellular MicroRNA miR-146a, a modulator of lymphocyte signalling pathways (Cameron, et al. 2008). This 63 kDa integral membrane phosphoprotein functions as a constitutively activated receptor (Young and Murray 2003b) and is a member of the tumour necrosis factor receptor (TNFR)

superfamily, activating several signalling pathways in a ligand-independent manner (as reviewed in (Young and Rickinson 2004b)). Functionally, LMP1 mimics the cellular growth signal that normally results from the binding of CD40 ligand (another member of the TNFR superfamily) and can partially substitute for CD40 *in vivo*, providing both growth and differentiation signals to B cells (Graham, Arcipowski and Bishop 2010). The LMP1 protein can be divided into three domains (Figure 1-8). Firstly, an amino-terminal cytoplasmic tail (amino acids 1-23), which tethers LMP1 to the plasma membrane and orientates the protein. Secondly, a *trans*-membrane region, consisting of six hydrophobic *trans*-membrane loops, which are involved in self-aggregation and oligomerisation (amino acids 24-186). Third, a long carboxyl-terminal cytoplasmic region (amino acids 187-386), which possesses most of the signalling activity of the molecule (Young and Rickinson 2004a, Young and Murray 2003a). LMP1 signals mainly from the intracellular compartments. Both oligomerisation and localisation within glycosphingolipid-rich membrane rafts are essential for the initiation of signalling which results in the activation of several signalling pathways in a ligand-independent manner that contributes to the many phenotypic consequences of LMP1 expression (Lam and Sugden 2003). At least four signalling pathways have been implicated in the function of LMP1, namely nuclear factor κB (NF-κB), c-Jun NH$_2$ terminal kinase (JNK), p38/mitogen activated protein kinase (p38/MAPK) and janus kinase/signal transducers and activators of transcription (JAK/STAT) (Shair, et al. 2007). It is the cytoplasmic C-terminus of LMP1 that is responsible for the transduction of signalling cascades that result in primary B cell transformation and phenotypic changes. Within the C-terminus of LMP1, there are at least two major activating domains (Figure 1-8), C-terminal activating region 1 (CTAR1) and CTAR2 (Young and Rickinson 2004b). CTAR1 (also known as transformation effector site 1, TES1) is located proximal to the membrane (amino acids 186-231), binds TNFR-associated factors (TRAFs), and is essential for EBV-mediated B-cell immortalisation. CTAR2/TES2, which is located near the C-terminus, supports the long-term growth of immortalised B cells and recruits the TNFR-associated death domain (TRADD) protein and receptor-interacting protein (RIP) (Eliopoulos, et al. 2003). The activities of CTAR1 and CTAR2 affect diverse signalling cascades and provide the basis for the transforming properties of LMP1. Figure 1-8 explaining the structure and function of LMP1.
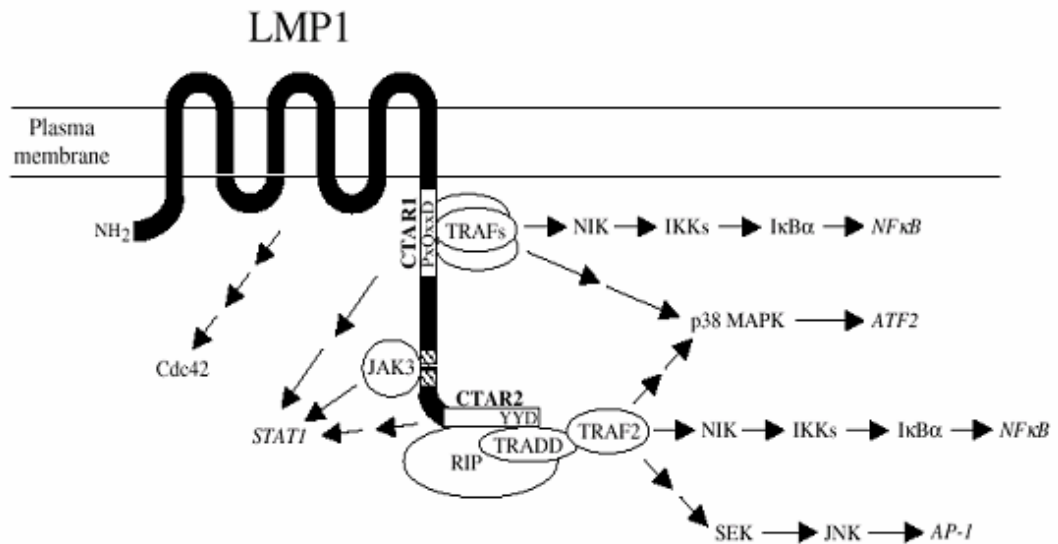
Figure 1-8: Structure and Function of LMP1.

The C-terminus of LMP1 contains at least two activating regions, referred to as C-terminus activation regions 1 and 2 (CTAR1 and CTAR2). CTAR1, which is essential for EBV-mediated B cell immortalisation, binds TRAF1, TRAF2, TRAF3 and TRAF5 and activates the NF-κB and p38 signalling pathways. CTAR2 supports the long-term growth of immortalised B cells and recruits TRADD to activated downstream signals, such as NF-κB, JNK, and p38. Both LMP1 C-terminal domains also mediate the activation of the JAK/STAT pathway, although an intermediated region has also been shown to bind JAK3 and induce STAT binding activity independently of CTAR1 and CTAR2. The *trans*-membrane domains of LMP1 are responsible for the activation of the small GTPase Cdc42 leading to cytoskeletal changes (adapted from Eliopoulos and Young, 2001).

1.8.2   LMP2A and LMP2B

Transcription of *LMP2* is regulated by two separate promoters, located 3kb apart on the viral DNA (Speck, et al. 1999, Wasil, et al. 2013)(Speck, et al. 1999, Wasil, et al. 2013). Two mRNAs that have unique 5' exons followed by eight common exons encode two distinct proteins of 2.0 and 1.7kb in length, namely LMP2A and LMP2B, respectively (Speck, et al. 1999, Wasil, et al. 2013). Thus the structures of LMP2A and LMP2B are similar; LMP2A contains an N-terminal cytoplasmic domain of 119 amino acids with eight tyrosines that are phosphorylated in LCLs, 12 *trans*-membrane domains, and a C-terminal domain of 12 amino acids. LMP2B, however, lacks the entire N-terminal cytoplasmic domain (Young and Rickinson 2004b).

The EBV LMP2A is a functional homologue of the cellular B Cell Receptor (BCR) (Schaadt, et al. 2005b), and constitutively associates with Src family kinases through its N-terminal cytoplasmic tail to activate Ras/PI3K/Akt (Moody, et al. 2005a) and mTOR (Moody, et al. 2005b) in order to signal to NF-κB (Guasparri, Bubman and Cesarman 2008). LMP2A is dispensable for LCL generation and B cell proliferation (Vrazo, et al. 2012), but seems to indorse survival through upregulation of Bcl-X$_L$ and Survivin (Portis, Dyck and Longnecker 2003). In certain cell models, LMP2A overcomes the necessity for BCR expression (Caldwell, Brown and Longnecker 2000, Caldwell, et al. 1998, Casola, et al. 2004), improves antibody production and plasma cells occurrence (Swanson-Mungerson, Bultema and Longnecker 2006), and alters tolerogenic signals prompted through the BCR on the transgenic BCR^{HEL} background (Swanson-Mungerson, et al. 2005).

LMP1 and LMP2A signalling mimics CD40 and BCR signalling, respectively, and has been suggested to modify B cell functions including the ability of latently-infected B cells to access and transit the germinal center. It has been shown recently in a transgenic mouse model that *LMP1* and LMP2A coexpression changes B cell maturation and the response to antigen. *In vitro*, *LMP1* upregulated activation markers and promoted B cell hyperproliferation, and co-expression of LMP2A restored a wild-type phenotype. It thus may be the case that LMP2A modulates *LMP1* function (Vrazo, et al. 2012). LMP2A is known to block BCR signal transduction through specific phosphotyrosine motifs in its N-terminal domain, to prohibit induction of lytic EBV infection and to promote B-cell survival. Neither LMP2A nor LMP2B are essential for EBV-induced B-cell transformation *in vitro* (Mancao and Hammerschmidt 2007, Schaadt, et al. 2005a). While most of the research to date has focussed upon LMP2A, LMP2B has been shown to colocalise with LMP2A in the membrane where the C terminus of both splice variants can interact and regulate the activity of one another (Lynch *et al*, 2002). LMP2B was shown to negatively regulate LMP2A activity by interfering with its aggregation (Rovedo and Longnecker 2007). Furthermore, LMP2B has been found to regulate susceptibility to induction of lytic EBV infection (Rechsteiner, et al. 2008).

Taken together, these data support a role for LMP2A in modifying the normal programme of B cell development to favour the maintenance of EBV latency and to prevent inappropriate activation of the EBV lytic cycle. It is also evident that LMP2B is involved in the regulation of switching from latent to lytic EBV in B cells harbouring functional EBV through impacting the activity of LMP2A (Rechsteiner *et al*, 2008).

1.9 Other EBV latent transcripts

1.9.1 EBER1 and EBER2

EBV encodes two small non-polyadenylated RNAs termed EBV-encoded RNAs 1 and 2 (EBER 1 and EBER 2), the most abundant viral transcripts in latently EBV infected cells with the exception of oral hairy leukoplakia lesions from AIDS patients and some hepatocellular carcinomas. In addition to the latent proteins, EBER1 and EBER2 are expressed in all forms of latency (Wu, et al. 2007a). EBERs 1 and 2 are uncapped, non-coding RNAs of 167 and 172 nucleotides respectively (Mahjoub, et al. 2008). The EBERs are not essential for the EBV-induced transformation of primary B lymphocytes, since recombinant EBV harbouring EBER gene deletions was able to infect but not able to transform lymphocytes (Yajima, Kanda and Takada 2005, Wu, et al. 2007b). Expression of the EBERs in BL cell lines has been found to increase tumourigenicity, promote cell survival and induce IL10 expression (Yajima, Kanda and Takada 2005, Sheikh and Qadri 2011, Iwakiri, et al. 2009) and it has been suggested that EBER-mediated inhibition of PKR (a latent, IFN-inducible Ser/Thr kinase) function might be important for viral persistence (Nanbo, et al. 2002). Furthermore, transfection of the EBER genes into the EBV-negative AKATA cell line restored the oncogenic potential that was originally present in the EBV-positive AKATA cells but was lost in the EBV-negative subclones (Nanbo, et al. 2002).

1.9.2 BARTs/ CSTs

The EBV *Bam* HI A rightward transcripts (BARTs) or complementary-strand transcripts (CSTs) are a group of abundantly expressed RNAs that are encoded by the *Bam* HI A region of the EBV genome and were originally identified in nasopharyngeal carcinoma (NPC) tumour tissues, but were subsequently found to be expressed in other EBV-associated malignancies, such as BL, HL and nasal T-cell lymphoma, as well as in the peripheral blood of healthy individuals (Reviewed in (Kieff, E., Rickinson, A, B. 2007, Young and Rickinson 2004b, Yamamoto and Iwatsuki 2012)). The protein products –if any- of these highly spliced transcripts remain to be conclusively identified. Another transcript that is generated from the *Bam* HI A region is *BARF1*, which encodes a 31 kDa protein that was originally identified as an early antigen expressed

upon induction of the EBV lytic cycle. The BARTs are often expressed in circumstances in which the EBNA-2 and EBNA-3 proteins are not synthesised, such as epithelial cell infection and in EBV-associated malignancies in immunocompetent individuals (Yamamoto and Iwatsuki 2012).

1.10 Serotypes of EBV

Limited genetic variation has been found among the EBV isolates studied to date. Indeed, there are only two designated subtypes, known as type A and type B (also called as type 1 and type 2). EBV A and B were classified originally through the analysis of two EBV isolates that presented significant differences in their EBV nuclear antigen 2 (EBNA-2)-coding regions (Chang, et al. 2009). EBV type A was characterised from an EBV genome isolated from an IM patient in North America, designated B95-8. AG987, a strain derived from an African BL case, was found to have a shorter EBNA-2 sequence and was designated EBV type B. Remarkably, the EBNA-2s of the two strains show only 55% homology at the protein level (Knipe, D.M, Howley, P.M. 2001, Lucchesi, et al. 2008b). In the cell culture-based in vitro immortalisation assay, EBV Type A has been proven to be more competent at transforming human B cells into proliferating lymphoblastoid cell lines (LCLs) than EBV type B (Rickinson, Young and Rowe 1987). The two strains show differing geographical distributions. In Africa, both strains are present at approximately the same frequency, however the type A strain predominates in Europe and the United States (Reviewed in (Lucchesi, et al. 2008b)). The two serotypes show significant variation, including the presence of three additional open reading frames (ORFs) in type B (BFRF1A, BGLF3.5 and BDLF3.5) (Lacoste, et al. 2010, Dolan, et al. 2006) and type specific differences have been demonstrated to extend to EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP and the EBERs (Rickinson, Young and Rowe 1987, Sample, et al. 1990, Arrand, Young and Tugwood 1989). However the differences in the EBNA-2 region have been proven to be the key behind the altered transforming efficiency capabilities of the two strains. In 1989, Cohen *et al* proved that the distinction between the immortalising capabilities of the two strains was EBNA-2-dependant. A recombinant type B EBV strain was developed in which the type B EBNA-2 coding region was substituted by the coding sequence for type A EBNA-2. This resulted in an increased transforming efficiency in the type B strain on a par with the type A variant (Cohen and Kieff 1991, Lucchesi, et al. 2008a).

The diminished transformation proficiency of the type B EBNA-2 compared to the type A variant, has not yet been fully elucidated. However, a number of recent findings may clarify the differing effects of the two EBNA-2s on B cell immortalisation. The EBNA-2 target LMP1 shows similar expression levels in LCLs proliferating due to either EBV type A or B. It has now been shown however, that there is a transient difference in LMP1 levels immediately following B cell infection by the two types of EBV (Lucchesi, et al. 2008b, Cohen, et al. 1989). The initial induction of LMP1 by EBNA-2 type A is a significantly earlier and more intense response than the induction initiated by EBNA-2 type B. LMP1 is a key player in B cell immortalisation, and delayed expression caused by inefficient LMP1 induction by EBV type B may be one reason why the type B variant is less proficient at B cell immortalisation than type A EBV. A small number of cellular genes were also highlighted as differentially regulated by the two types of EBV (Lucchesi, et al. 2008b).

1.11 EBV-Associated Diseases

Primary infection of EBV in early childhood results in short-term proliferation of infected B cells. The infection is usually self-limited and controlled by the strongly elevated T cell immune response. If the infection occurs in adolescence or adulthood, up to 50% T cells in the host can be specific to the virus, which may develop the clinical symptom of infectious mononucleosis (IM). EBV then persists latently in the host within long-life memory B cells (Thorley-Lawson 2001a). After primary infection, a subset of EBV positive B cells survives and emerges into the memory compartment. These cells can go for limited expansion or extrafollicular proliferation and remain in the host for a long time. The virus can be reactivated periodically and infect new hosts through virus shedding in saliva. Although the virus is normally a harmless passenger in patients without obvious immune-deficiencies, EBV-associated lymphomas are well characterised, where the host's cellular immune system fails to control EBV-induced B-cell proliferation, thus the infected B cells can transform from their latent state into malignant cells. EBV infection has been linked to lymphoproliferative disorder of B, T and NK lymphocytes such as Burkitt's lymphoma (Allday 2009), Hodgkin's lymphoma (HD) (Ambinder 2007), peripheral T cell lymphoma and nasal T/NK lymphomas that

arise in both immunosuppressed and immunocompetent hosts (Dunleavy, Roschewski and Wilson 2012).

Endemic Burkitt's lymphoma occurs frequently in young children in the equatorial regions of Africa and Papua New Guinea and has an incidence of 50-100 cases per 1,000,000 individuals (Kutok and Wang 2006). In contrast, EBV-associated sporadic lymphomas occur in children and young adults and have no specific geographic distribution, with an incidence of 2-3 cases per 1,000,000 individuals (Kutok and Wang 2006). EBV accounts for 40 - 50% of childhood non-Hodgkin's lymphomas (NHLs) and 1-2% of adult lymphomas in Western Europe and the United States. Endemic Burkitt's lymphoma is almost 100% associated with EBV, whereas, association of sporadic Burkitt's lymphoma with EBV is low (15-30% of cases) (Kutok and Wang 2006). Nasopharyngeal carcinoma (NPC) (Gullo, Low and Teoh 2008) is most common in southern China, and accounts for approximately 20% of all adult cancers. It is extremely rare in Europe and North America, with an incidence rate of less than one per 100,000 population (Shah and Young 2009, Magrath 2012). EBV has also been associated with a host of non-lymphoid malignancies including smooth muscle tumours in immunocompromised patients (Purgina, et al. 2011) and a selection of gastric carcinomas (Iizasa, et al. 2012, Paniz-Mondolfi, et al. 2013). There is evidence that EBV triggers autoimmune liver disease (Rigopoulou, et al. 2012), thyroid disease, multiple sclerosis (MS) (Tzartos, et al. 2012, Holmoy 2008b) systemic lupus erythematous (SLE), and rheumatoid arthritis (RA) (Lossius, et al. 2012). It has been observed from a diverse panel of cancers that high grade tumours are three times more likely to be EBV-positive than corresponding low grade tumours (Qin, et al. 2002, Hummel, et al. 1995)(Qin, et al. 2002). A list of EBV-associated diseases and their features are given in Table 1-3.

Table 1-3: EBV Associated Diseases

Non-Malignant EBV Associated Diseases		
Disease	Infection Site	Latency
Infectious mononucleosis (IM)	B cell	III
Chronic active EBV	B cell	III
Oral hairy leukoplakia (OHL)	Epithelium	N/A
Malignant EBV Associated Diseases		
Burkitt's Lymphoma (BL)	B cell	I
Hodgkin's Disease (HD)	B cell	II
Nasopharyngeal Carcinoma (NPC)	Epithelium	II
T/NK Lymphoma	T Cell	II
Peripheral T Cell Lymphoma	T Cell	II
Gastric Carcinoma	Epithelium	II
AIDS-Burkitt's Lymphoma	B cell	I/II
AIDS-immunoblastic or large cell lymphoma	B cell	II/III
X-linked lymphoproliferative disease	B cell	III
Post-transplant lymphoproliferative disease (PTLD)	B cell	III
AIDS-CNS lymphoma		III
Lymphatoid granulomatosis	B cell	III
Smooth muscle tumours (e.g. Leiomyosarcomas)	Mesenchymal	Various

The loss of gene promoter methylation has been suggested to play a key part in early stages of haematological malignancies including B cell malignancies (Lee, et al. 2012). DNA methylation in gene promoters has a key role for repressing gene expression, and the hypomethylation of this region is associated with gene activation (Ooi, O'Donnell and Bestor 2009, Poetsch and Plass 2011). Recent comprehensive studies indicated that the B cell transcription program is associated with gene promoter hypomethylation and overexpression of key genes in Epstein-Barr virus-associated transformation of resting B lymphocytes (RBLs) to proliferating B lymphoblastoid cells. EBV-mediated transformation and B cell activation share common pathways (Thorley-Lawson 2001a), however methylation changes associated with EBV-mediated transformation to LCLs are independent of B cell activation and hypomethylation takes place only when proliferation has started (Hernando, et al. 2013). *Hernando et al,* showed that the

maintenance of DNA methylation is less efficient in transcriptionally active regions as cells start to proliferate. B cell transcription machinery was observed to associate with the subset of genes that undergo hypomethylation and further upregulation of genes relevant in EBV-mediated transformation of B lymphocytes (Hernando, et al. 2013).

There are three main types of Burkitt's lymphoma; Endemic (mainly occurs in equatorial Africa, associates with chronic EBV and Malaria infection), Sporadic (occurring in scattered instances outside of Africa, also associates with EBV but to a lesser degree) and Immunodeficiency-associated, (usually associated with HIV infection or the use of immunosuppressive drugs, also associated with EBV infection). The B lymphocytes in BL have rearranged immunoglobulin genes and contain one of three translocations of the proto-oncogene *c-myc, c-myc/Ig* (8:14, 2:8, 8:22), and the most common translocation is from the long arm of chromosome 8 to chromosome 14, t(8:14). The complex interaction of EBV, Malaria, and HIV with B lymphocytes as cofactors in BL, enhances B cell activation and proliferation and therefore survival of B cells carrying the *c-mys/Ig* translocation (Reviews, (Thorley-Lawson, Duca and Shapiro 2008, Brady, Macarthur and Farrell 2008)).

Hodgkin lymphoma (HL) is derived from B cells and subdivided into classical HL (cHL) and nodular lymphocyte predominant HL (NLPHL) (Eberle, Mani and Jaffe 2009). Classical HL is further divided by histological measures into mixed-cellularity, nodular-sclerosis, lymphocyte-rich, and lymphocyte-depleted subtypes. These so-called classical forms of the disease are different from lymphocyte-predominant HL in NLPHL, which is not associated with EBV (Reviewed in (Banerjee 2011)). HL is distinctive amongst human B cell lymphomas because of the infrequency of the malignant cells (the Hodgkin and Reed-Sternberg (H/RS) cells) in classical HL and the lymphocyte-predominant (LP) cells in NLPHL, which usually account for 0.1% to 10% of the cells in the affected tissues (Kuppers 2009). Furthermore, H/RS cells are unique in the extent to which they have lost their B cell typical gene expression pattern, for instance when H/RS cells acquire Ig mutations during affinity maturation that results in a non-functional Ig molecule (Brauninger, et al. 2006). Deregulation of transcription factor networks plays a key role in this reprogramming process. H/RS cells show strong constitutive activity of the transcription factor NF-κB (Younes, Garg and Aggarwal 2003). Multiple mechanisms likely contribute to this deregulated activation, including signalling through particular receptors and genetic lesions. Inactivating mutations or deletion in the *TNFAIP3* tumor suppressor gene, encoding a negative regulator of NF-

κB activity, were recently identified in about 40% of patients with classical HL (Nomoto, et al. 2012). H/RS cells are latently (Type II latency) infected by EBV in about 40% of patients (Ambinder 2007), and an important role of this virus in HL pathogenesis, in particular for cases in which H/RS cells had lost the capacity to express a BCR due to destructive somatic mutation/ suggests that these B cells can be rescued by activation of this transcription factor and subsequent induction of anti-apoptotic cell genes. *Bfl-1* is an NF-κB target gene and plays a critical role in this cell context (Loughran, et al. 2011). Thus, EBV infection may provide alternative mechanisms for rescuing abnormal B cells from apoptosis and illustrates how EBV gene products can substitute for specific molecular alterations that are important for tumorigenesis.

The association between EBV infection and NPC is complex and not yet completely defined. EBV infection alone is not enough to cause NPC, since more than 95% of human population are EBV seropositive and occurrences of this cancer is rare (Shah, et al. 2000, Lin 2009). Other factors, such as a person's genes, diet, work place (exposure to formaldehyde or wood dust (Hildesheim, et al. 2001)) may contribute, along with EBV, to the development of NPC. For instance NPC is found about twice as often in males as it is in females (Xie, et al. 2013). People who live in parts of Asia, northern Africa, and the Arctic region (Devi, et al. 2004), where NPC is common, typically eat diets very high in salt-cured fish and meat. Indeed, the ratio of this cancer is falling in places where those traditional diets are now higher in fruits and vegetables (Hsu, et al. 2012b, Yu, et al. 1986).

1.12 Regulation of cell growth and survival by EBV

Suppression of the cellular apoptotic program by EBV is central to both the establishment of latent infection and the development of EBV-associated malignancies. When EBV infects it primarily encounters resting B cells in the oropharyngeal mucosal lymphoid tissue (Kieff, E., Rickinson, A, B. 2007, Sarid and Gao 2010). It drives the infected cells into cell cycle and maintains cell division, increasing the population of infected cells and allowing them to progress, via the B-cell maturation pathways, to the site of latent persistence of the virus, in resting memory B cells. The complex

mechanisms by which EBV affects cell growth and can prevent cell death are a result of a combination of EBV-induced pathways that affect both cell growth and apoptosis, and the co-operative roles of the latent genes, especially EBNA-2, EBNA-3C and LMP1 in these processes has long been established (Richards, et al. 2008).

1.12.1 EBV affects Cell Cycle Progression

Cells typically progress through the cell cycle in an orderly fashion through a growth phase (G_0/G_1), to a synthetic phase in which the DNA is copied (S), to a second growth phase (G_2), prior to cell division or mitosis (M) and back to a quiescent cell arrest phase (G_0/G_1) following cell division (Figure 1-9). Entry of a B lymphocyte into the cell cycle is a well-co-ordinated procedure. Mitogenic signals may be derived from a range of sources: from protein or polysaccharide antigens (Ags) that stimulate the B cell receptor (BCR), to pathogenic products that signal via Toll like receptors (TLRs) (Allen, Okada and Cyster 2007, Gerondakis, Grumont and Banerjee 2007, Niiro and Clark 2002). Proliferation occurs only at precise points during a B cell's development or through an immune response, when the B cells increase in reaction to Ags and form germinal centres (GCs) with T cell help (Allen, Okada and Cyster 2007). Deregulated proliferation is a trademark of autoimmunity, where autoreactive B cells react to stimulation with activation rather than anergy, and in B cell cancers, where proliferation can be detached from external controls (Cambier, et al. 2007).

Progression through the cell cycle is regulated by many different proteins, such as tumour suppressor gene products, p53 and pRb (retinoblastoma gene product), which act within the cell cycle facilitating the repair of damaged or mutated DNA within single cells, or alternatively, induce these injured cells to undergo apoptosis (Fajas 2013, Matsumoto and Nakayama 2013). EBV exploits normal cellular pathways to regulate cell cycle progression. Indeed EBV genes can manipulate the proliferation machinery of the infected cell through the deregulation of the function of several oncogenes and tumour suppressor genes implicated in the cell cycle such as *c-myc*, *p53* and *pRb*, thus inhibiting the ability of these proteins to redirect cells with damaged or mutated DNA toward a DNA repair pathway or apoptosis. As a consequence, host cells with latent viral infection are allowed to proliferate without regulation. Although rare events, these virally-infected cells may then develop mutations induced by 'DNA-

damaging' events, including tobacco, alcohol, carcinogens, toxins, ionizing radiation, prolonged ultraviolet light/sun exposure, chemical insult, or even other viral infections which may result in the development of a malignant tumour (Flaitz and Hicks 1998).
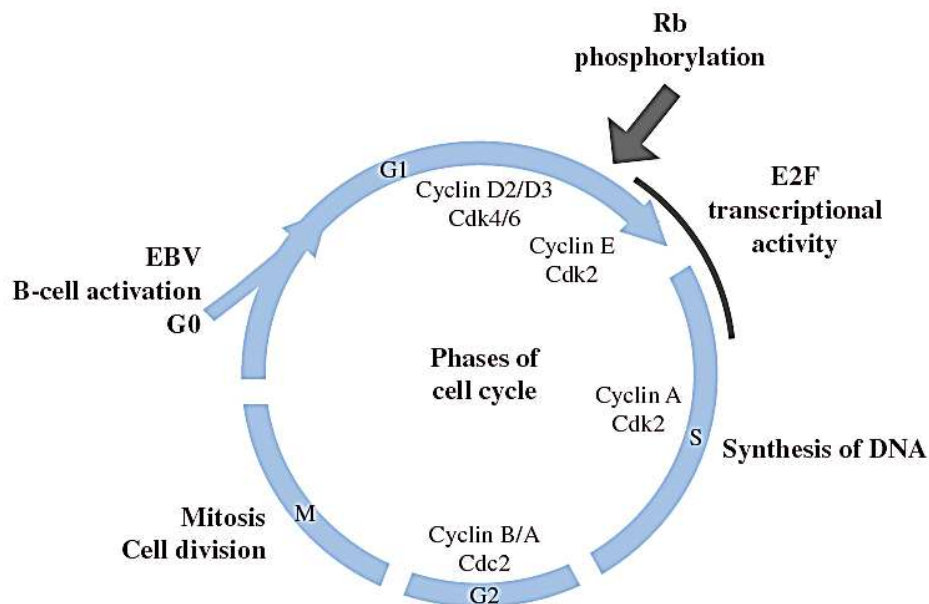
Figure 1-9: Phases of lymphocyte cell cycle

The cell cycle may be divided into four distinct phases; G1, S-phase, G2 and M-phase. The G1 phase of the cell cycle is the longest and occurs prior to DNA synthesis, which occurs during S-phase. During M-phase, cell division takes place. The progression through the cell cycle is regulated by many different proteins, some of which are illustrated. The first biochemical event observed, in early G1 phase, is the induction of the D-type cyclins and their partners, cyclin dependent kinases (cdks), cdk4 and cdk6. One of either cd4 or cd6 partners a D- type cyclin, in the absence of cyclin dependent kinase inhibitors (CDKIs), to become catalytically active. The induction of D-type cyclins is paralleled by a loss in CDKIs. The most important substrate of the cyclin D/cd4/6 complex is the family of pocket proteins, typified by pRb, the retinoblastoma susceptibility gene product. Other members of the pocket protein family include p130 and p107. These pocket proteins repress a family of transcription factors called E2F, and the E2F-pocket protein complexes also act as gene suppressors. Phosphorylation of pRb by the cyclin D/cdc4/6 complex results in the release of E2F transcription factors and transcription of E2F-responsive genes. E2F binding sites have been identified in the promoters of many genes important for cell cycle regulation and the promoters of proteins involved in DNA synthesis. In many systems, the induction of E2F transcriptional activity results in entry into the cell cycle (Adapted from Brennan, 2001).

Interactions of EBV proteins with the cell cycle

EBV facilitates the establishment of a persistent viral infection in the host by influencing pathways that control cell cycle checkpoints (G1/S, G2/M and mitotic cell cycle checkpoints) and DNA repair (Seto, et al. 2010, O'Nions and Allday 2004). Cyclin D2 is probably the first cell cycle protein to be induced following EBV infection of B cells and it is accompanied by a loss of the CDKI p27kip1(Dolcetti and Carbone 2010). Phosphorylation of the pocket protein pRb is observed approximately 6 hours later. All these events correspond to those seen during normal B cell proliferation, thus implying that EBV exploits normal cell pathways to regulate pRb phosphorylation during the cell cycle (Frost, et al. 2001a).

pRb

Retinoblastoma protein (pRb) is a central regulator of cellular proliferation, controlling entry into G_1/S in the cell cycle, mainly through its interaction with the cellular transcription factor E2F, which activates genes important in DNA synthesis (Fajas 2013). EBV has been shown to regulate pRb-modulated pathways and to drive cells through the G_1/S restriction point (Saha, et al. 2011) (Figure 1-9). Through the up-regulation of cyclins involved in the inactivation of pRb, such as cyclin D2 and cyclin A, or by directly inactivating pRb itself, EBV liberates the E2F transcription factors to drive potentially infected cells through the cell cycle. In this regard, it has been shown that EBNA-2 and EBNA-LP are sufficient to activate the expression of cyclin D2 during immortalisation of resting human B lymphocytes (Sinclair *et al*, 1994) and LMP1 is also thought to regulate cyclin D2 (Arvanitakis, Yaseen and Sharma 1995). Moreover, the *c-myc* oncogene, a direct target of EBNA-2 (Polack, et al. 1996, Schlee, et al. 2004a) has also been shown to *trans*-activate the cyclin D2 promoter (Bouchard, et al. 2001). EBV may also repress cyclin dependent kinase inhibitors in order to facilitate the inactivation of pRb. The induction of cyclin D2 is paralleled by the loss of the CDKI p27kip1 in response to EBV (Frost, et al. 2001b) and EBNA-3C has been shown to play a role in the regulation of p27kip1 (Knight and Robertson 2004). Since cyclin D2 and p27kip1 together regulate the cyclin dependent kinases, cdk4 and cdk6 activity, the link between EBNA-3C and p27kip1 suggests that EBNA-3C along with EBNA-2, EBNA-LP and LMP1 is implicated in the progression through the G_1 phase of

the cell cycle. Recent studies conducted in LCLs showed that EBNA-3C stabilizes as well as enhances the functional activity of Cyclin D1 and subsequently enhances the kinase activity of Cyclin D1/CDK6 which enables ubiquitination and degradation of pRb. Thus this complex efficiently reverses the inhibitory effect of pRb on cell growth and enables the G1/S transition in EBV transformed LCLs (Saha, et al. 2011).

Cyclin A, an activator of S phase progression, has been shown to interact with the carboxyl terminus of EBNA-3C *in vitro*. EBNA-3C has been shown to stimulate cyclin A-dependent kinase activity and rescue p27-mediated inhibition of cyclin A/Cdk2 kinase activity by decreasing the molecular association between cyclin A and p27 in cells (Knight and Robertson 2004). In addition, EBNA-3C can target the SCF^{Skp2} complex, thereby regulating the activity and stability of cyclin A/cdk2 and pRb complexes (Knight and Robertson 2004, Knight, Sharma and Robertson 2005a, Knight, Sharma and Robertson 2005b).

p53

The p53 pathway is targeted for inactivation in most human cancers either directly or indirectly, highlighting its critical function as a tumour suppressor. p53 is normally activated by cellular stress and mediates a growth-suppressive response that involves cell cycle arrest and apoptosis (Olsson, et al. 2012, Kuribayashi and El-Deiry 2008, Kuribayashi, Finnberg and El-Deiry 2008). Elevated incidences of p53 mutation have been identified in BL tumour biopsies and BL cell lines (Gaidano, et al. 1991). In addition BL cells with p53 mutations have been shown to be relatively resistant to DNA-damaging drugs such as cisplatin compared to those cell lines harboring wild-type p53 (Gaidano, et al. 1991, Simoes Magluta, et al. 2009). EBV LMP1 can protect against wild-type p53 mediated apoptosis (Fries, Miller and Raab-Traub 1996a). Moreover, LMP1 regulates p53 both at transcriptional and translational level (Li, et al. 2012). In addition, BZLF1 has been shown to interact with p53 and inhibit its *trans*-activating function in lymphoid cells (Mauser, et al. 2002) and EBNA-LP can also interact with p53 in vitro, although the functional significance of this interaction has not yet been determined (Szekely, et al. 1993). Moreover EBNA-3C has been shown to block p53-mediated apoptosis through stabilizing Gemin3, a member of DEAD RNA helicase family which exhibits diverse cellular functions including DNA transcription, recombination and repair, and RNA metabolism (Cai, et al. 2011).

c-Myc

c-Myc, a helix-loop-helix leucine zipper transcription factor, is known to be involved in numerous cellular functions such as cell cycle progression, apoptosis, cellular transformation cell proliferation, adhesion, and differentiation in a variety of different cell types. Myc promotes proliferation and growth through both the induction of genes involved in cell cycle control (including *CDK4, CDC25A, cyclin D1, D2, A*, and *E)*, and through the suppression of growth-arresting genes (such as *GADD45, p15INK4b, p21CIP1, p27KIP1, GAS1)* and induction of *(p16INK4A and p14ARF)* (Rui and Goodnow 2006, Maruo, et al. 2011). The C-terminal basic helix–loop–helix zipper domain facilitates binding to DNA sequences with the core consensus sequence 5'-CACGTG'3' known as an "E-box", and requires the association of its hetero-dimeric partner, Max (Brady, Macarthur and Farrell 2008). c-Myc also possesses an N-terminal *trans*-activation domain through which it drives the expression of a multitude of target genes (Brady, Macarthur and Farrell 2008). To date, there are approaching 1,700 genes which have been identified as Myc-responsive genes (www.myccancergene.org). However, only a minority of these have been implicated as direct target genes (Sionov 2013, Zeller, et al. 2006). c-Myc expression is required to drive cells through the G_1 into the S phase of the cell cycle (Figure 1-9). A model explaining the role of c-Myc in the apparently diverse roles of apoptosis and proliferation, predicts that c-Myc expression preferentially results in proliferation under favorable growth conditions with this activity being dependent on its apoptosis-promoting activity being blocked (Evan, et al. 1994, Jamerson, Johnson and Dickson 2000). c-Myc also regulates gene expression by upregulating microRNAs (miR-17 and miR-20a) which regulate E2F1 and cyclin D1 translation or by down regulation of a series of microRNAs (miR-30 cluster miR-15a, miR-16, tumour suppressor let-7 miRNA, miR-22, miR-23a/b, miR-26a/b, miR-29a/b/c, miR-34a, miR-146a, miR-150, and miR-195) (reviewed in (Sionov 2013)).

c-Myc over-expression has been observed in tumour cells from 91% of BL cases. Indeed, chromosomal translocations of *c-myc* into the *Ig* loci is observed in practically all BLs, and the most frequent translocation, the *c-myc/IgH* t(8; 14), accounts for approximately 80% of these. The remaining 20% of cases are split between the translocations with the *IgK* and *IgL* (t(2;8) and t(8;22) respectively). The c-*myc* gene becomes transcriptionally activated as a consequence of the chromosomal translocation and is sustained by up-stream *c-myc* regulators such as NF-κB or BCL-6 (Rui and

Goodnow, 2006). Thus c-Myc deregulation is regarded as priming normal proliferating cells for apoptosis (Sionov 2013). It is clear that c-Myc expression is deregulated in EBV-immortalised (non-tumour-derived) B cells (Cherney, Bhatia and Tosato 1994). Studies using an LCL in which the function of EBNA-2 is dependent on the presence of oestrogen have revealed that EBNA-2 is the EBV protein predominantly responsible for driving c-Myc in LCLs (Kempkes, et al. 1995b) and that *c-myc* is a direct target gene of EBNA-2 in this context (Kaiser *et al*, 1999; Moore *et al*, 2001). c-*Myc* is also induced to moderate levels by LMP1 (Schlee, et al. 2004b). In addition EBNA-3C has been shown to stabilise *c-myc* expression, and this recruitment of both c-Myc together with its cofactor Skp2 to *c-myc*-dependent promoters can enhance c-Myc-dependent transcription (Bajaj, et al. 2008).

1.13 Cytokines activated during EBV-mediated immortalisation

Following B lymphocyte activation, EBV also induces the synthesis of several cytokines including TNF, IL-1, IL-6 and IL-10. Both IL-6 and IL-10 play a significant role in the growth of EBV-transformed cells (Wroblewski, et al. 2002, Spender, et al. 2001a, Incrocci, McCormack and Swanson-Mungerson 2013b, Waldman, et al. 2008, Eliopoulos, et al. 1997). LMP1 has been implicated in the regulation of both of these cytokines via NF-κB and the p38 stress-activated protein kinase (Eliopoulos, et al. 1997, Wakisaka and Pagano 2003). Further studies indicate that IL-10 may also be regulated by the EBERs (Sheikh and Qadri 2011) and by the EBV transcription factor EB1/Zta (Mahot, et al. 2003) and LMP2A (Incrocci, McCormack and Swanson-Mungerson 2013a). The receptor molecules that IL-6 and IL-10 use to mediate their activities both activate the JAK family of tyrosine kinases, and downstream signalling cascades including STAT proteins and the PI3K pathway (Brennan 2001). Significantly, IL-10 has been shown to act as a co-factor for B cell growth (Moore, et al. 2001), and it has been demonstrated that PI3K is important for IL-10-mediated cell growth of both primary monocytes and a murine mast cell line (Antoniv and Ivashkiv 2011). Interestingly, EBV vIL-10 enhances the growth transformation of a B cell infected with EBV (Jochum, et al. 2012) and both human and EBV-encoded IL-10 can induce LMP1 in the absence of EBNA-2 (in the Daudi, P3HR1, and other BL cell lines) (Kis, et al.

2006). Furthermore, exposure of the KMH2-EBV cells to CD40-ligand and IL-4 induced LMP1 expression, in the absence of EBNA-2 and IL-10 could induce the expression of LMP1 in tonsillar B cells infected with the non-transforming, EBNA-2-deficient EBV strain P3HR1 and enhance LMP1 expression in two EBV-positive NK lymphoma lines suggesting that IL-10 might contribute to the establishment of type II EBV latency (Kis, et al. 2006).

1.14 Signalling Molecules involved in EBV-mediated immortalisation

Constitutively active signalling pathways are a common feature of many malignant cells. Thus, it is important to characterise the potential role of signalling molecules that are activated by EBV-induced cytokines and by EBV gene products themselves. The pathways activated by IL-6 and IL-10 (JAK family of tyrosine kinases, PI3K and STATs) have previously been implicated in lymphocyte growth and transformation (Rane and Reddy 2000). The STAT family of transcription factors are one substrate of the JAK family of tyrosine kinases. STATs have been shown to be active in EBV-immortalised cells (Weber-Nordt, et al. 1996) and are thought to play a role in oncogenesis (O'Shea, Holland and Staudt 2013). It has also been reported that LMP1 can activate JAK3 and STAT1 (Gires, et al. 1999) and a STAT reporter that is known to bind STAT1, STAT3 and STAT5 (Brennan 2001, O'Shea, Holland and Staudt 2013).

PI3K, a lipid kinase that is involved in lymphocyte survival and proliferation (Novak, et al. 2010, Beckwith, et al. 1996, Brennan, et al. 2002), can be activated by oncogenic Ras (Yang, et al. 2012). Reports indicate that pathways downstream of the small G protein Ras are active in EBV-immortalised cells and significantly, inhibition of PI3K using a chemical inhibitor, inhibits the proliferation of EBV-immortalised cells (Yang, et al. 2012, Castellano and Downward 2011). Interestingly, LMP2 was found to activate a PI3K-mediated pathway, Akt (Portis and Longnecker 2004). Furthermore, PI3K has also been shown to affect D-type cyclin expression in EBV-immortalised B cells (Brennan 2001), suggesting a mechanism for its role in EBV-induced lymphocyte growth.

1.15 Apoptosis

The induction of early cell death in response to viral infection would limit virus production and consequently diminish or eliminate spread of virus progeny in a population. Thus, in order to maximise their replicative capacity many viruses deregulate the normal cell cycle and secure selective advantage and biological success by developing mechanisms which either suppresses or triggers apoptosis. By preventing infected lymphocytes from undergoing apoptosis, EBV ensures survival/persistence in the host system (Spender and Inman 2011, Fu, He and Mao 2013). Apoptosis is a genetically controlled pre-programmed cellular suicide, in which individual cells are destroyed while the integrity of the surrounding tissue is preserved. While this targeted cell destruction is essential in many physiological contexts including embryogenesis, immune cell maturation and response, tissue homeostasis and in the cellular response to injury, it also functions as an emergency response to aberrant growth induced by the activation of oncogenes and viral infection and its deregulation is implicated in many pathological states(Taylor, Cullen and Martin 2008b). The process involves dramatic morphological changes including cell shrinkage, nuclear re-organisation, plasma membrane blebbing and eventual fragmentation of the cell into membrane bound apoptotic bodies (Giansanti, Torriglia and Scovassi 2011, Saraste and Pulkki 2000).

Apoptosis is orchestrated by a family of cysteine proteases known as the caspases. These molecules are activated upon receipt of divergent pro-apoptotic stimuli and play an essential role in the implementation of apoptosis coordinating the efficient dismantling and engulfment of targeted cells. Caspases are synthesised as relatively inactive precursors that require proteolytic processing in order to achieve activation (MacKenzie and Clark 2012). To date, fourteen caspases have been identified, with eleven caspases known to be expressed in humans. Depending on their involvement in the life and death of a cell, caspases are broadly divided into apoptotic or inflammatory caspases (Figure 1-10). The inflammatory caspases are involved in cytokine activation (Caspase 1, 4, 5). The apoptotic caspases are generally divided into two distinct groups; initiator caspases (Caspase 2, 8, 9, 10) at the top of the cascade, which are activated first by the action of caspase adaptor molecules, such as Fas-associated protein with death domain (FADD) or apoptotic protease-activating factor-1 (Apaf-1). These molecules instigate a caspase cascade, activating the effector caspases (Caspase 3, 6, 7) further downstream, whose function is principally to dismantle cellular structures. There are

three main apoptotic cascades activated by specific initiator caspases: the death receptor pathway, the mitochondrial pathway and the ER pathway. These are activated by caspase-8, caspase-9, and caspase-12, respectively (MacKenzie and Clark 2012, Bratton and Salvesen 2010, Gupta, Reutelingsperger and Narula 2005).
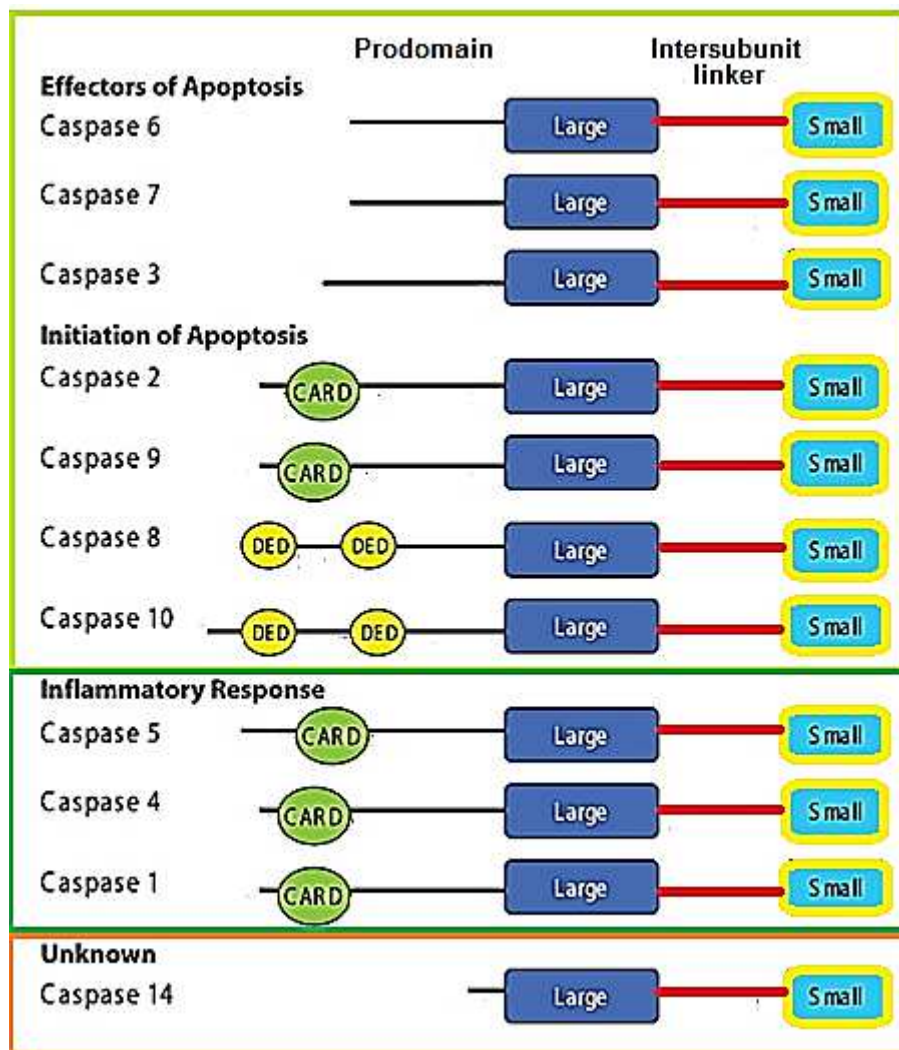
Figure 1-10: The caspase family

The major groups of caspases, apoptotic (with two main subgroups of effectors and initiators caspases) and inflammatory caspases are illustrated in figure. Caspases contain three main domains: a prodomain and large and small catalytic subunits. Activation of caspases involves the proteolytic cleavage of zymogens, the removal of the prodomain and separation of the large and small subunits. The prodomains of activator and inflammatory caspases contain protein–protein-interaction domains (such as the caspase-recruitment domain (CARD) and the death effector domain (DED)) that link them to apoptosis signalling molecules. Figure adapted from (MacKenzie and Clark 2012).

Two separate pathways of apoptotic cell death can be distinguished by the involvement of the Bcl-2 family proteins and by which caspases are required for their execution (Figure 1-11) (Giansanti, Torriglia and Scovassi 2011, Youle and Strasser 2008). The intrinsic pathway, (also called the Bcl-2-regulated or mitochondrial pathway), is activated by various developmental triggers or cytotoxic insults, such as viral infection, DNA damage and growth-factor deprivation, and is regulated by the Bcl-2 family of proteins. This pathway predominantly leads to the activation of caspase-9 but in certain cell types is known to take place in the absence of caspase-9 or Apaf-1(Youle and Strasser 2008, Hardwick and Soane 2013). The extrinsic (or death-receptor pathway) is triggered by ligation of the death receptor molecules such as Fas or tumour necrosis factor (TNF) receptor-1 (TNFR1) (members of the TNF receptor family) which harbour an intracellular death domain with the ability to recruit and activate caspase-8 through FADD at the cell surface (Youle and Strasser 2008, Schultz and Harrington 2003, Cancro 2009). This recruitment causes subsequent activation of downstream effector caspases, such as caspase-3, -6 or -7, independently of any involvement of the Bcl-2 family (described in Figure 1-11). Interestingly, it has been suggested that the two pathways may also be interconnected and that the molecules in one pathway have the ability to influence those of the other. In this regard it has been established that the extrinsic pathway can intersect the intrinsic pathway through caspase-8 cleavage-mediated activation of the pro-apoptotic BH3-only protein Bid in which the C-terminal truncated form of Bid (tBid) translocates to mitochondria from where it promotes further caspase activation through the intrinsic pathway (Caspase 9) (Tait and Green 2010).
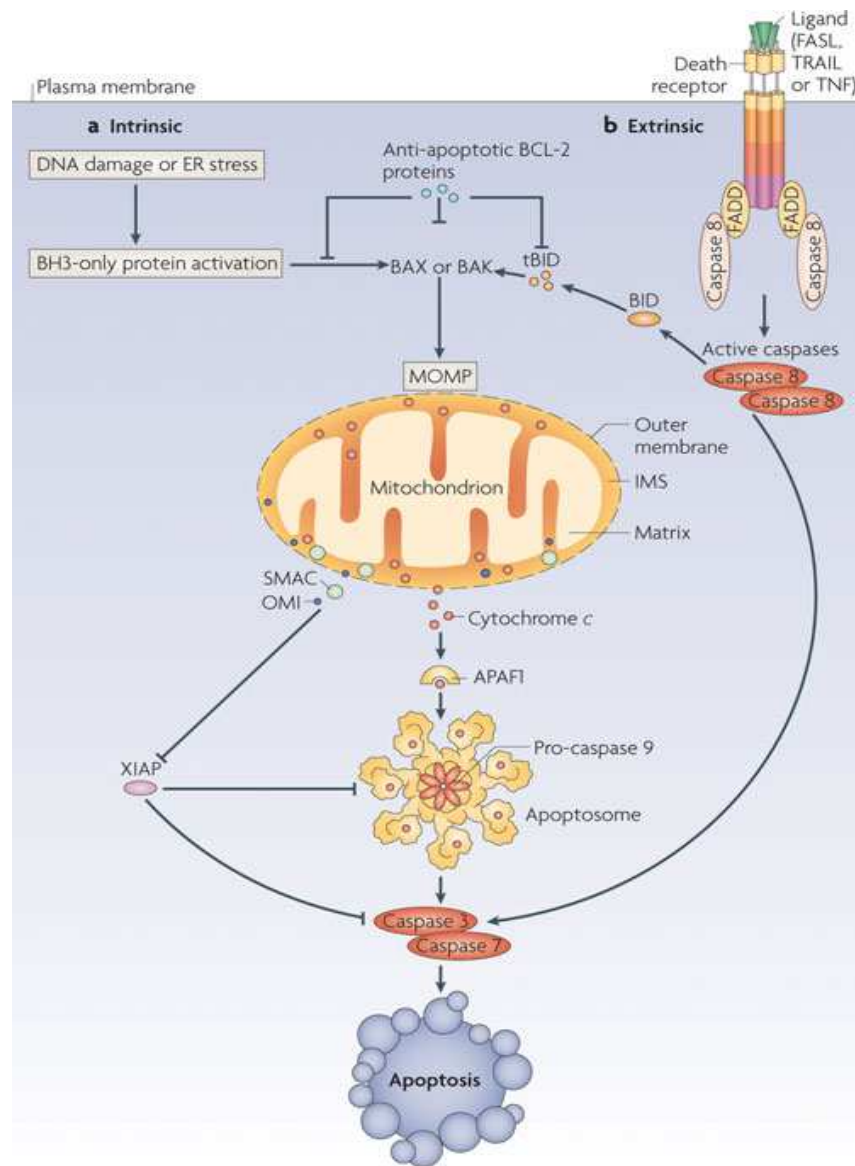
Figure 1-11: A schematic drawing depicting intrinsic and extrinsic pathways of apoptosis

Apoptosis can be triggered by cell surface receptors, (such as Fas and TNFR1) (extrinsic pathway, right), or by genotoxic agents, metabolic insults or transcriptional cues (intrinsic pathway, left). The intrinsic pathway begins with BH3-only protein induction or post-translational activation, resulting in the inactivation of some Bcl-2 family members. This relieves inhibition of Bax and Bak activation, which in turn promotes apoptosis. Some BH3-only proteins, such as Bim and Puma, may also be able to activate Bax and/or Bak (as shown by the dotted line). When activated, Bax and Bak promote cytochrome *c* release and mitochondrial fission, leading to activation of Apaf-1 into an apoptosome and activation of caspase-9. Caspases in turn cleave a series of substrates, activate DNases and orchestrate the destruction of the cell. Mitochondrial release of second mitochondrial derived activator of caspase (SMAC) and OMI neutralizes the caspase inhibitory function of X-linked inhibitor of apoptosis protein (XIAP). The extrinsic pathway can bypass the mitochondrial step and activate caspase-8 directly, ultimately leading to cell demolition. The Bcl-2 family regulates the intrinsic pathway and can modulate the extrinsic pathway when cleavage of Bid communicates between the two pathways. A detailed discussion of the Bcl-2 family can be found in Section 1.15.1 (Adopted from (Hardwick and Soane 2013)).

The extrinsic and intrinsic pathways both come together upon the execution phase, which is considered the final chapter of apoptosis, beginning with the activation of the effector caspases. Caspase-3, caspase-6, and caspase-7 function as effector caspases activating cytoplasmic endonucleases and proteases, which degrade nuclear material and cytoskeletal proteins (MacKenzie and Clark 2012, Bratton and Salvesen 2010). These effector caspases cleave various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, ultimately causing the morphological and biochemical alterations seen in apoptotic cells. Caspase-3 is thought to be the most important of the effector caspases, and is activated in response to any of the initiator caspases. Caspase-3 specifically activates the endonuclease caspase-activated DNase (CAD). In healthy proliferating cells CAD is complexed with its inhibitor, ICAD (inhibitor of caspase-activated DNase). In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Taylor, Cullen and Martin 2008a)(MacKenzie and Clark 2012, Taylor, Cullen and Martin 2008c)(MacKenzie and Clark 2012, Taylor, Cullen and Martin 2008c). CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganisation and disintegration of the cell into apoptotic bodies and facilitates disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (MacKenzie and Clark 2012, Taylor, Cullen and Martin 2008a).

Phagocytic absorption of apoptotic cells is the final phase of apoptosis. Phospholipid asymmetry and externalisation of phosphatidylserine (PS) on the surface of apoptotic cells and their fragments is a key characteristic of this stage. Although the mechanism of PS translocation to the outer leaflet of the cell during apoptosis is not well understood, research indicates that Fas, caspase-8, and caspase-3 are involved in the regulation of PS externalisation on oxidatively stressed erythrocytes (Schultz and Harrington 2003, Krysko, et al. 2008). However, it should be noted that caspase-independent PS exposure occurs during apoptosis of primary T lymphocytes. The appearance of PS on the outer leaflet of apoptotic cells then assists non-inflammatory phagocytic recognition, facilitating their early uptake and subsequent disposal (Schutters and Reutelingsperger 2010, Peng, et al. 2005b).

1.15.1 Bcl-2 Family proteins

Key players in the determination of cell survival and death are the members of the Bcl-2 family of proteins. The Bcl-2 family, both pro- and anti-apoptotic, are responsible for controlling the intrinsic pathway of apoptosis in mammals (Hardwick and Soane 2013). The founder member of this family the B-cell lymphoma-2 (*bcl-2*) gene was discovered at the t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas, where its transcription becomes excessively driven by the immuno-globulin heavy chain gene promoter and enhancer on chromosome 14 (Tsujimoto, et al. 1985, Bakhshi, et al. 1985, Arif, et al. 2009). One key early discovery that introduced a new paradigm for carcinogenesis was that over-expression of *bcl-2* does not promote cell proliferation as most previously discovered oncogenes do; rather, over-expression of *bcl-2* inhibits cell death (Vaux, Cory and Adams 1988). The Bcl-2 family of regulators function upstream of the caspase cascade and display a range of bioactivities, from inhibition to promotion of apoptosis (Hardwick and Soane 2013).

In mammals, there are at least 23 Bcl-2 family members, including Bcl-2 itself and proteins that have either three-dimensional (3D) structural similarity or a predicted secondary structure that is similar to Bcl-2 (Figure 1-12) (Young and Rickinson 2004a, Fu, He and Mao 2013, Hardwick and Soane 2013). Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 which correspond to α-helical segments (Youle and Strasser 2008, Hardwick and Soane 2013). A characteristic of the Bcl-2 family proteins is their ability to form homo- and hetero-dimers, as well as their ability to become integral membrane proteins (Youle and Strasser 2008, Hardwick and Soane 2013). The anti-apoptotic members of this family (Bcl-2, Bcl-$_{XL}$, Bcl-w, Mcl-1, A1, Boo/Diva, Bcl-B, and C. elegans CED-9) are known to contain at least three of the four Bcl-2 homology domains. The pro-apoptotic members can be further characterised into to sub-categories being either "Bax-like" in their structure (Bax, Bak, Bcl-$_{XS}$, Bok/Mtd and Bcl-$_{GL}$) i.e. containing two or three BH domains, or alternatively, the so-called "BH3-only" proteins which share homology with each other and the remainder of the Bcl-2 protein family only through the short BH3 motif (Figure 1-12) (Bad, Bik, Blk, Bid, Hrk, Bim/Bod, Bmf, Noxa, Puma and C. elegans Egl-1) (Hardwick and Soane 2013, Tait and Green 2010). Some of the Bcl-2 family members do not fit into these classical subgroups. One of them, the Bcl-G, has a BH2 and a BH3 region, and was proposed to trigger apoptosis (Guo, Godzik and Reed

2001) initially. However recently it has been shown Bcl-G possibly does not function in the canonical stress-induced apoptosis pathway, but rather has a role in protein trafficking inside the cell (Giam, et al. 2012).

Overall the structures of the BH3-only proteins seem to be unrelated and appear to lack a close evolutionary relationship to the core members of the Bcl-2 family (Aouacheria, Brunet and Gouy 2005). However, all BH3-only proteins interact with and regulate the core Bcl-2 family proteins to promote apoptosis. The BH3 domains of the Bcl-2 family members are crucial to their function indeed; just the 51 amino acids that code the domain in *bak* are enough to have a pro-apoptotic effect, while mutations of the BH3 regions in *bax, bak* and *bik* were found to render the proteins ineffective (Elangovan and Chinnadurai 1997a, Tong, et al. 2001). However recent *in vitro* studies demonstrated that mutations to Bax outside the BH3 domain interrupted interactions with prosurvival proteins and promoted apoptosis, so they concluded that Bax is directly controlled by prosurvival proteins leading to blockage of apoptosis (Czabotar, et al. 2011).
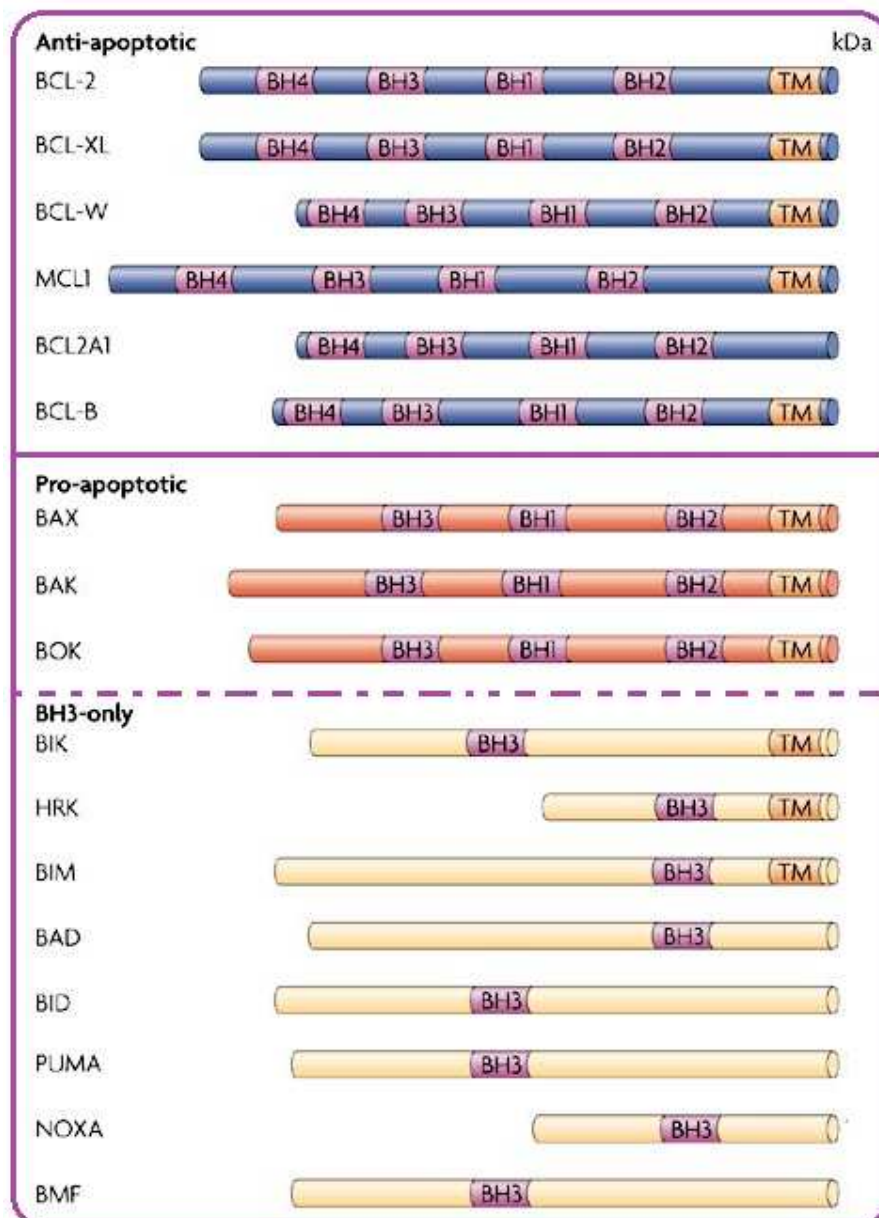
Figure 1-12: The pro-apoptotic and anti-apoptotic Bcl-2 family members.

Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 which correspond to α-helical segments. Each of the BH domains is indicated above. (Adapted from (Taylor, Cullen and Martin 2008a)).

The pro-apoptotic family members Bax and Bak are critical in the permeabilisation of the outer mitochondrial membrane (OMM) and the subsequent release of apoptogenic molecules such as cytochrome c and SMAC/DIABLO into the cytosol, which ultimately leads to caspase activation. The anti-apoptotic family members, such as Bcl-2 and Bcl-X_L, are known to inhibit the activity of Bax and Bak. A growing body of evidence indicates that BH3-only proteins de-repress Bax and Bak by directly binding and inhibiting Bcl-2 and other anti-apoptotic family members or alternatively that they may directly activate Bax and Bak (Czabotar, et al. 2011, Westphal, et al. 2011) (Figure 1-11). It has been established that both Bax and Bak promote caspase activation by their effects on mitochondria. These two pro-apoptotic proteins induce the release of proteins from the space between the inner and outer mitochondrial membranes (Westphal, et al. 2011). This process of mitochondrial outer membrane permeabilisation (MOMP) leads to the release of cytochrome c and other soluble proteins. While it is generally believed that Bax and Bak form integral membrane pores, the biochemical nature of such pores and how anti-apoptotic Bcl-2 family proteins might regulate them remains to be seen (Tait and Green 2010). During cytochrome c release into the cytosol, Bax and Bak induce mitochondria to fragment into more numerous and smaller units (Tait and Green 2010, Chipuk, Bouchier-Hayes and Green 2006, Martinou and Youle 2006).

Once the OMM has been permeabilised, soluble proteins diffuse from the intermembrane space into the cytosol, where they promote caspase activation. The best studied of these proteins is cytochrome c, which binds to Apaf-1 and leads to the assembly of the apoptosome, which can bind pro-caspase-9 or and thereby induce its activation through a conformational change in the molecule. Cytochrome c–Apaf-1-dependent activation of caspase-9 is absolutely required for neuronal and fibroblast cell-death processes (Tait and Green 2010, Chipuk, Bouchier-Hayes and Green 2006). However, lymphocytes may use alternatives to Apaf-1-, caspase-9- and cytochrome c-independent pathways, such as pro-apoptotic Bcl-2-family-member-dependent pathways for caspase activation (Hao, et al. 2005, Marsden, et al. 2002). One such pathway which is Apaf-1-independent, involves the relief of caspase inhibition by inhibitor of apoptosis proteins (IAPs), such as XIAP, which bind and neutralise certain caspases (such as caspase-9 and caspase-3). This inhibitory action of IAPs can be antagonised by the binding of SMAC/DIABLO, which is released from mitochondria after the activation of Bax and/or Bak (Youle and Strasser 2008, Fulda and Debatin 2006, Degterev and Yuan 2008).

1.15.2 BH3-only proteins

BH3-only proteins are essential for cell death initiation (Happo, Strasser and Cory 2012b). A growing body of evidence indicates that different apoptotic stimuli can activate distinct but sometimes overlapping sets of BH3-only proteins. The pro-apoptotic activity of BH3-only proteins is tightly controlled by diverse transcriptional and post-translational mechanisms to prevent inappropriate cell death (Lomonosova and Chinnadurai 2008a, Bouillet and Strasser 2002, Happo, Strasser and Cory 2012a). Puma and Noxa are transcriptionally induced in response to DNA damage by p53 for example, whereas other BH3-only proteins, such as Bid or Bad, are thought to be mainly regulated at the post-translational level (Youle and Strasser 2008, Happo, Strasser and Cory 2012a).

While the molecular basis of BH3-only protein activity is still under investigation, it is clear that BH3-only proteins have the ability to bind to a hydrophobic groove on the surface of Bcl-2-like molecules, formed by their BH1, BH2 and BH3 domains thereby antagonising their pro-survival function (Willis and Adams 2005a). This physical interaction is dependent on a functional BH3-domain within the BH3-only protein. It has been suggested that BH3 only proteins can function via at least three separate modes of action (Letai *et al*, 2002; Kuwana *et al*, 2005; Strasser, 2005) and since the signalling pathways that mediate apoptosis seem to proceed through a succession of interconnected amplification loops, it is conceivable that more than one of these processes takes place (Happo, Strasser and Cory 2012a, Strasser 2005).

In one model of BH3-only protein function, this interaction is thought to block the ability of Bcl-2-like pro-survival proteins to regulate the pro-apoptotic Bax/Bak, preventing them from undergoing oligomerisation and causing loss of mitochondrial outer membrane integrity and cell death (Happo, Strasser and Cory 2012a, Strasser 2005) (Figure 1-13 a). It has also been suggested that the BH3-only proteins and Bax/Bak-like proteins function in parallel in a mutually dependent manner (Strasser 2005, Lomonosova and Chinnadurai 2008b)(Figure 1-13 b). In an alternative model, the BH3-only proteins activate Bax/Bak directly. In support of this model tBid and Bim have this ability. Bid and Bim directly activate Bax (Du, et al. 2011), causing its insertion into the mitochondrial membrane where it becomes active. However, Bim and tBid can be bound and neutralised by Bcl-2-like proteins and the role of other BH3-only proteins in this model is to occupy Bcl-2-like proteins and stop them from sequestering

Bim/tBid. Accordingly, these BH3-only proteins (e.g., Bad and Bik) are called sensitisers (Happo, Strasser and Cory 2012b, Lomonosova and Chinnadurai 2008b) (Figure 1-13 c). It has been shown recently that in the absence of potent direct activators, like Bim and Bid, other BH3-only proteins, Bmf and Noxa, can directly activate Bax/Bak (Du, et al. 2011). Additionally a further possibility is that, to keep cells from dying, Bcl-2 (and/or its pro-survival homologues) might bind and inhibit a protein, perhaps a caspase activator (such as Apaf-1), and that apoptotic stimuli might trigger both BH3-only proteins and Bax/Bak-like proteins, which together would disrupt this binding (Happo, Strasser and Cory 2012a, Strasser 2005, Lomonosova and Chinnadurai 2008b) (as illustrated in Figure 1-16).
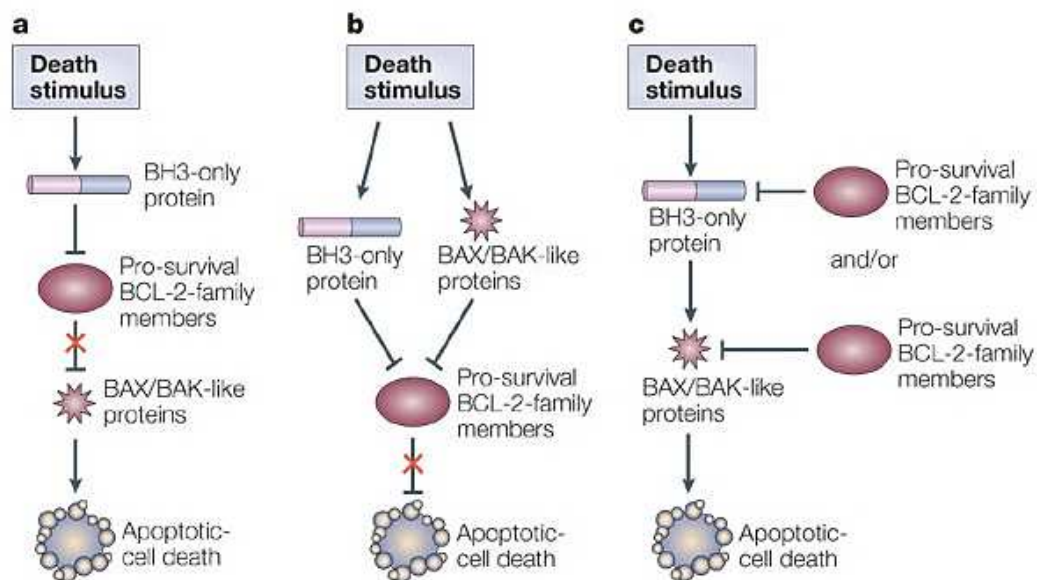
Figure 1-13: Models for interaction between BH3-only proteins, Bax/Bak-like proteins and pro-survival Bcl-2-family members in signalling in apoptosis.

Three possible models are illustrated above. (A) BH3-only proteins might inactivate Bcl-2-like pro-survival proteins, which keep cells alive by inactivating Bax/Bak-like proteins. (B) Death stimuli might simultaneously activate BH3-only proteins and Bax/Bak -like proteins, which both block the pro-survival function of Bcl-2 and its homologues. (C) BH3-only proteins might activate the pro-apoptotic activity of Bax/Bak-like proteins directly, and pro-survival Bcl-2-family members might function by binding and inactivating BH3-only proteins and/or Bax/Bak-like proteins. (Adapted from(Strasser 2005)).

BH3-only proteins have distinct (only partially overlapping) binding preferences for their pro-survival Bcl-2-like relatives (Happo, Strasser and Cory 2012a, Zhang, et al. 2011), indicating that certain BH3-only proteins can only antagonise a very distinct subset of Bcl-2-like molecules *in vivo*. For example Noxa can only block Mcl-1 and A1 while others, such as Bim and Puma, are able to bind with similar affinities to all known Bcl-2 pro-survival proteins and therefore, may have broader physiological functions than other BH3-only proteins (Happo, Strasser and Cory 2012a). Since these proteins are essential initiators of cell death pathways, understanding their regulation may provide novel therapeutic targets to prevent pathological cell death. Inhibitors of BH3-only proteins may alleviate degenerative disorders, whereas small molecule mimetics, for example ABT-737 may be used to kill cancer cells or self-antigen-specific lymphocytes in autoimmune disease (Karlberg, et al. 2010, Bodet, et al. 2011, Touzeau, et al. 2011, Reed and Pellecchia 2005).

Bik

The regulation of *bcl-2* family members by EBV is a significant research interest in the laboratory. *Bik*, in particular, was a major interest in the present study and will now therefore be discussed in detail. Bik was originally identified as a binding partner and antagonist of anti-apoptotic Bcl-2 and its adenovirus homologue, E1B 19K. Bik also displayed affinity for Bcl-X_L, Bcl-X_S and the EBV BHRF1 suggesting that Bik might be a mutual target for cellular and viral anti-apoptotic proteins (Chinnadurai, Vijayalingam and Rashmi 2008b). Bik shares only the short BH3 signature domain with other members of the Bcl-2 family and has no homology for the other Bcl-2 signature domains BH1, BH2 and BH4 (Figure 1-12).

Bik Expression

The *bik* transcript has a tissue-specific expression profile (Verma, et al. 2000)(Verma, et al. 2000) with strong expression in the kidney and pancreas in comparison to other organs (Daniel, et al. 1999). The gene is also expressed in many tumour lines (Kim, et al. 2010) and in B cells. In humans, Bik seems to play a key role in B cell homeostasis. In a gene expression profiling study of B cell germinal center reaction, *bik* transcript

was detected during naive B cell to centroblast transition and remained up-regulated in memory B cells. Increased expression of Bik was also identified in cells that had been induced to apoptosis followed by antigen receptor stimulation (by ligation of surface IgM) and TGFβ signalling induction (Verma, et al. 2000, Jiang and Clark 2001a, Spender, et al. 2009).

Bik Structure

Bik is localised on chromosome 22q13 and consists of five exons and four introns, spanning a region of 19kb. *Bik* gene encodes the 160 amino acid pro-apoptotic Bik protein that shares the distinct sequence motif with the Bcl-2 family and a characteristic C-terminal *trans*-membrane segment (Boyd, et al. 1995b, Han, Sabbatini and White 1996b) which determines its sub-cellular localisation (Elangovan and Chinnadurai 1997c) (Figure 1-14). Bik is predominantly localised to the endoplasmic reticulum (ER) and to lower extent to mitochondrial outer membrane (MOM) (Chinnadurai, Vijayalingam and Rashmi 2008b) from where it elicits its pro-apoptotic signals by regulating BAX/BAK-dependent release of Ca^{2+} from the endoplasmic reticulum stores and cooperates with other BH3-only proteins such as Noxa to cause rapid release of cytochrome *c* from mitochondria. Bik is also known to selectively inhibits BCL-X_L, BFL-1, or BCL-w through complex formation (Chen, et al. 2005), and given sufficient time, these signals lead to cell death (Zhao, et al. 2008b, Germain, et al. 2005, Chinnadurai, Vijayalingam and Rashmi 2008a, Kvansakul, et al. 2010b).

The translational start site and BH3 domain are located in exon 2 and 3, respectively (Figure 1-14). The alpha-helical BH3 region is required for both death promoting activities and dimerisation with anti-apoptotic proteins. In addition to the BH3 domain, the C-terminal domain adjacent to the TM also has been shown to be essential for optimal cell death activity. Human Bik protein has been shown to be phosphorylated on Thr(33) and Ser(35) by a casein kinase II-like kinase and mutations that prevented phosphorylation reduced the cell death activity (Verma, Zhao and Chinnadurai 2001) whereas enhanced apoptosis was the result of mutations that increased the phosphorylation (Tong, et al. 2001, Boyd, et al. 1995a, Li, et al. 2003). The C-terminal *trans*-membrane hydrophobic domain and the stop codon are contained within exon 5. The transcriptional initiation site was identified as a G residue marked as +1(Figure

1-14), located approximately 13kb from the translational start site. The gene has a long un-translated sequence in exon 1. No canonical TATA or CCAAT boxes were identified near the transcriptional initiation site (Verma, et al. 2000).
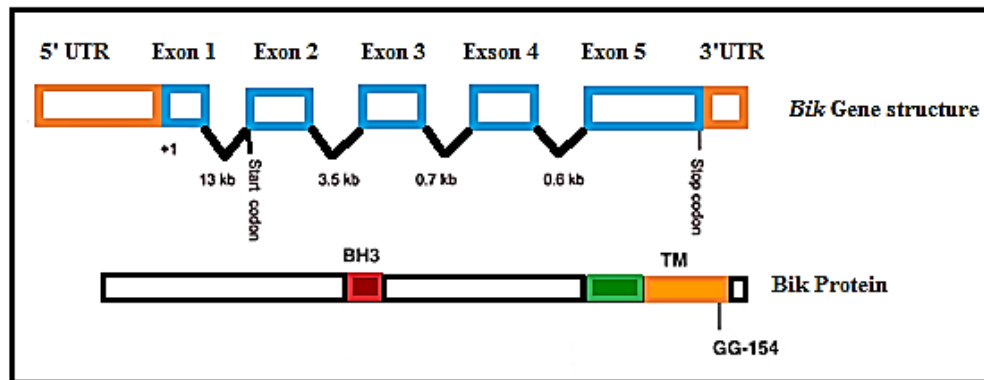
Figure 1-14: Arrangement and sizes of exons and introns in the *bik* gene (Top). Bik protein structure (Bottom). Adapted from, (Chinnadurai, Vijayalingam and Rashmi 2008b, Verma, Zhao and Chinnadurai 2001).

A role for Bik in B cell biology

Several lines of evidence suggest that the balance between pro-apoptotic Bik and the anti-apoptotic Bcl-X_L protein plays a key role in determining B cell fate and that apoptosis activation signals the up-regulation of Bik protein in order to antagonize the function of Bcl-X_L. Consistent with this hypothesis, cell surface IgM ligation (equivalent to signalling from the BCR) in human B104 B cell lymphoma cells induces Bik accumulation and increases the amount of Bik associated with Bcl-X_L ultimately leading to apoptosis through the activation of caspases (Jiang and Clark 2001a). Furthermore, the cytokine transforming growth factor-β (TGFβ)-mediated apoptosis in the Ramos B-lymphoma cell line involves the activation of caspases and is accompanied by Bik up-regulation and a simultaneous loss of Bcl-X_L expression (Spender, et al. 2009). The involvement of Bcl-X_L in regulating immature B cell apoptosis is well established (Motoyama, et al. 1995, Fang, et al. 1998) and indeed the ability of Bcl-X_L to rescue Bik induced apoptosis is also recognised (Boyd, et al. 1995c, Spender and Inman 2009a). Bik binds to and can antagonise anti-apoptotic Bcl-X_L

under physiological conditions (Jiang and Clark 2001a, Shimazu, et al. 2007). Thus it may be the relative proportion of Bcl-X$_L$ not associated with Bik that determines whether B cells survive.

Bik is also weakly down-regulated following CD27 ligation, whereas CD40 ligation (both belong to the TNF/TNFR family) resulted in a similarly weak increase in Bik levels in B cell receptor (BCR)-mediated apoptosis of Ramos cells. Thus, it is possible that CD27 signals regulate BCR-mediated apoptosis at the mitochondrial level in a manner different from CD40 signals through the alteration of the balance among Bcl-2 family members to regulate mitochondrial permeability. In addition, since CD27 and CD40 inhibit p53 activation it is conceivable that Bik might be involved in the p53-dependent apoptosis pathway in B cells (Hase, et al. 2002). The possible involvement of other pro-apoptotic Bcl-2 members such as Bax or Bak in B cell apoptosis can also not be ruled out (Chinnadurai, Vijayalingam and Rashmi 2008a). Interestingly Bik has been shown to be necessary for IFNγ-induced cell death in human airway epithelial cells (Mebratu, et al. 2008). Hence it is possible, that blocking Bik function may have a significant role in the evasion of host immune responses.

Mechanism of bik-induced apoptosis

It has been well established that Bcl-2 family proteins function at the mitochondria to prevent or promote the release of apoptogenic factors such as cytochrome *c*, AIF and SMAC/Diablo. In addition to their mitochondrial localisation, some anti-apoptotic members of the Bcl-2 family are also present at the endoplasmic reticulum (ER) and perinuclear membrane regions, suggesting a role of the Bcl-2 family proteins at sites other than mitochondria (Zhao, et al. 2007, Zong, et al. 2003). The evidence suggests that Bik may act from the ER, coupling upstream death signals to events at the surface of the mitochondria via activation of the death effectors Bax and Bak, resulting in cell death (Wei, et al. 2001, Zong, et al. 2001b).

The ER is well characterised as a calcium (Ca^{2+}) store that sequesters excess cytosolic Ca^{2+} and serves as a reservoir for Ca^{2+} signalling to maintain intracellular calcium homeostasis (Giorgi, et al. 2012). A growing body of evidence suggests that changes in intracellular Ca^{2+} homeostasis plays a significant role in the modulation of apoptosis

(Giorgi, et al. 2012, Hetz 2007). Bcl-2 family proteins regulate apoptosis by a mechanism that affects the Ca^{2+} stores within the ER, or alternatively via a mechanism that controls the apoptotic cross talk between the ER and the mitochondria. Members of the Bcl-2 family appear to differentially regulate intracellular Ca^{2+} level. Translocation of Bax, an apoptotic signalling protein, from the cytosol to the mitochondrial membrane is another step in this apoptosis signalling pathway (Reviewed in (Giorgi, et al. 2012, Hetz 2007)).

Bik is exclusively localised to the ER rather than mitochondria through its *trans*-membrane domain (Germain, Mathai and Shore 2002, Zhao, et al. 2008a). Bik can induce changes in the content of cytosolic as well as ER Ca^{2+} stores (Zhao, et al. 2008a, Mathai, Germain and Shore 2005) and this process is thought to be mediated through a Bax/Bak regulated mechanism (Giorgi, et al. 2012, Mathai, Germain and Shore 2005). The depletion of ER Ca^{2+} stores rather than the elevation of intracellular Ca^{2+} or the extra-cellular Ca^{2+} influx play an important role in Bik-induced apoptosis (Zhao, et al. 2008b). Furthermore, it has previously been demonstrated that ER Bik initiates dynamin-related protein-1 (DRP1)-regulated remodelling of mitochondrial cristae during apoptosis in a manner dependent on the transmission of Ca^{2+} to the mitochondria (Germain, et al. 2005) further supporting a role for Bik in the regulation of the ER Ca^{2+} stores.   Exactly how the over-expression of Bik affects the intracellular Ca^{2+} homeostasis remains unknown. Bik may oligomerise with the other Bcl-2 member proteins and create a non-selective or selective ion pore across the ER membrane causing leakage of Ca^{2+} from the ER Ca^{2+} stores. Alternatively, Bik protein may interact directly or indirectly with the ER Ca^{2+} pump, or other Ca^{2+} channels (Giorgi, et al. 2012, Zhao, et al. 2008a).

Upon induction of apoptosis Bax translocates from the cytosol to mitochondrial membranes where it is thought to oligomerise into an active *trans*-membrane pore (Hsu, Hsu, Wolter and Youle 1997, Desagher, et al. 1999, Wolter, et al. 1997). Bik is thought to function through the initiation of cell death signalling, whereas Bax/Bak-like proteins, function further downstream (Zong, et al. 2001a, Lindsten and Thompson 2006). Further reiterating this point is the finding that Bik induces exposure of an N-terminal Bax epitope mediating its insertion into the outer mitochondrial membrane, release of cytochrome *c*, and cell death in H1299 cells (Gillissen, et al. 2003). Since the interaction of Bik with Bax is associated with a change in conformation of Bax leading to cytochrome c release (Gillissen, et al. 2003) it would suggest that Bik functions as a

positive death effector, engaging downstream components of the cell death pathway. Furthermore, Bik can co-operate with Noxa, to release cytochrome *c* from the mitochondria and this pathway involves conformational changes in Bax leading to its activation and ultimately caspase activation (Germain, et al. 2005).

Thus it appears that Bik initiates a pathway from its location at the ER that stimulates the release of Ca^{2+} from the ER via activation of the death effectors Bax and Bak, which leads to cytochrome *c* release and signalling of apoptosis from mitochondria (Germain, et al. 2005, Germain, Mathai and Shore 2002). The collapse of membrane potential along with the release of cytochrome *c* from mitochondria is followed by the activation of caspases (Smaili, et al. 2003). Accordingly Bik, is known to elicit pro-apoptotic signals which lead to cell death by pathways which involve the activation of caspases (Zhao, et al. 2007, Germain, Mathai and Shore 2002, Mathai, Germain and Shore 2005, Gillissen, et al. 2003) which can be inhibited by the broad range caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD-fmk) (Mathai, et al. 2002, Naumann, et al. 2003, Hur, et al. 2004). In one study Bik induced the depolarisation of mitochondrial membrane potential (MMP) through a caspase-12-dependent pathway, leading to the activation of initiator caspase-9 and ultimate cell death in 293 and Hep3B cells suggesting that caspase-12 may directly or indirectly influence the intrinsic apoptosis signal transduction pathways triggered by Bik at the ER (Zhao, et al. 2007). Bik has also been shown to induce the catalytic activation of caspase-9, caspase-7 and caspase-3 processing in chemoresistant human tumour prostate and colon cell lines (Tong, et al. 2001). A model for caspase-dependent Bik-induced apoptosis can be seen in Figure 1-15.
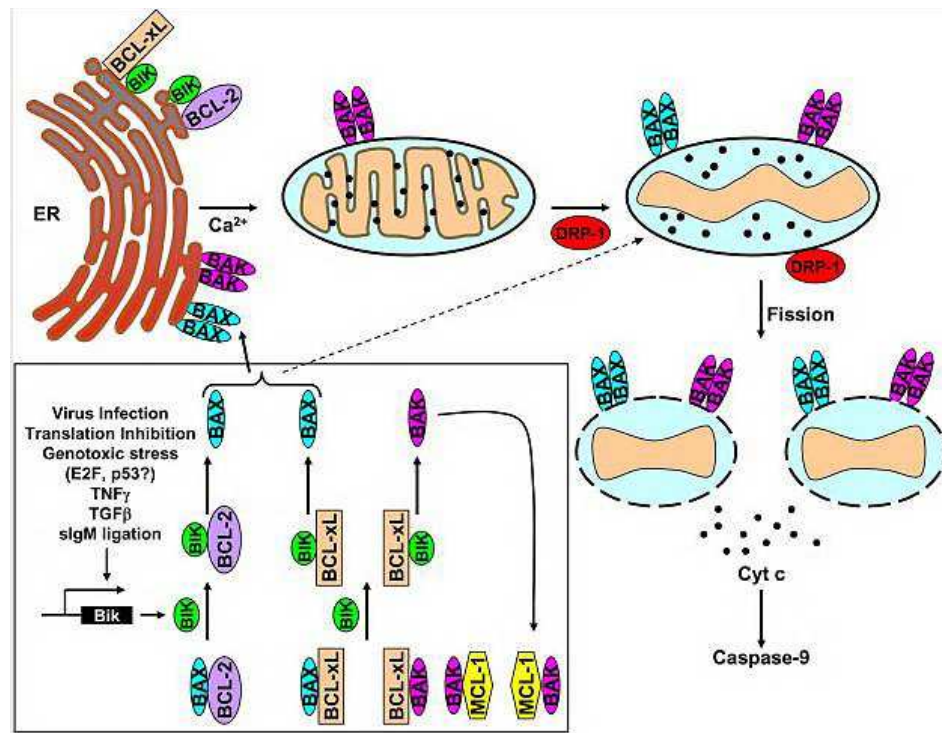
Figure 1-15: The caspase-dependent *bik*-induced apoptotic pathway.

Upon various stimuli that transcriptionally activate the expression of endogenous *bik*, Bik protein activates BAX through interaction of Bik with BCL-X_L and BCL-2, induces the depletion of ER Ca^{2+} stores, exposure of an N-terminal Bax epitope mediating its insertion into the outer mitochondrial membrane, release of cytochrome *c*, and activation of a caspase-12-dependent pathway, leading to the activation of initiator caspase-9 and ultimately cell death. Adapted from, (Chinnadurai, Vijayalingam and Rashmi 2008a).

Interestingly it has also been reported that Bik can induce cell death in a caspase-independent fashion in human melanoma cells (Oppermann, et al. 2005) and that inhibition of caspases by treatment with zVAD-fmk in the absence of Bcl-2 actually enhances Bik-induced cell death in mouse embryonic fibroblasts (MEFs) (Rashmi, et al. 2008). Interestingly a pathway in which Bik-mediated cell death induces the release of cytochrome *c* but not mitochondrial membrane potential loss and without modulating voltage-dependent anion channel activity (VDAC), (an important component of the PTP), has also been reported, suggesting that Bik may target molecules other than the PTP or act alone to induce cytochrome *c* release (Shimizu and Tsujimoto 2000). Furthermore, findings have suggested that Bik can induce cytochrome *c* release from mitochondria independently of Bax in H1299 cells. This report favoured a direct

induction of the mitochondrial apoptotic signalling cascade by *bik* and subsequent activation of caspases (Germain, Mathai and Shore 2002). It is conceivable that *bik* could be acting on separate distinct pathways, in response to different stimuli.

A mechanism of Bik-induced apoptosis regulated by Bcl-2 family members, such as Bax or Bcl-2/Bcl-X$_L$ cannot be excluded. The pro-apoptotic activity of BH3-only proteins is proposed to be governed by their propensity to interfere with at least a dual layer of protection of cells from the pro-apoptotic activity of Bak (Willis and Adams 2005b). It has previously been shown that binding of Bik inactivates Bcl-X$_L$ and releases both Bax and Bak. While the interaction between Bik and Bcl-X$_L$ seems to be stronger, Bik is also known to bind to Bcl-2 (Verma, Zhao and Chinnadurai 2001, Gillissen, et al. 2003, Elangovan and Chinnadurai 1997b), indicating that Bcl-2 is at least in part inhibited by Bik. Once the capacity of Bcl-2 to bind Bax has been surpassed, the liberated Bax protein becomes activated. Notably, the Bax inhibitors Bcl-2 and Bcl-X$_L$ are not stabilised by Bik. In contrast, Bak released from Bcl-X$_L$ upon Bik expression is still kept in check by Mcl-1 and this is enforced by the up-regulation of the Bak inhibitor Mcl-1 by Bik. Mcl-1-mediated repression of Bak thereby provides a molecular rationale for the strict Bax dependency of Bik-induced cell death. Thus it appears that Mcl-1 determines the Bax dependency of Bik-induced apoptosis (Gillissen, et al. 2007). In this instance Bik appears to act as de-repressor molecule binding to distinct anti-apoptotic proteins. The consequent de-repressed pro-apoptotic multi-domain proteins can then be counteracted by a second set of anti-apoptotic proteins. This adds a higher degree of specificity and the outcome of triggering this tightly balanced interaction network would be determined by both the specific BH3-only proteins induced by a given death stimulus and the abundance of their anti-apoptotic counterparts forming this second obstacle to apoptosis (Gillissen, et al. 2007). A model for the regulation of Bik-induced apoptosis by Bcl-2 family members can be seen in (Figure 1-16).
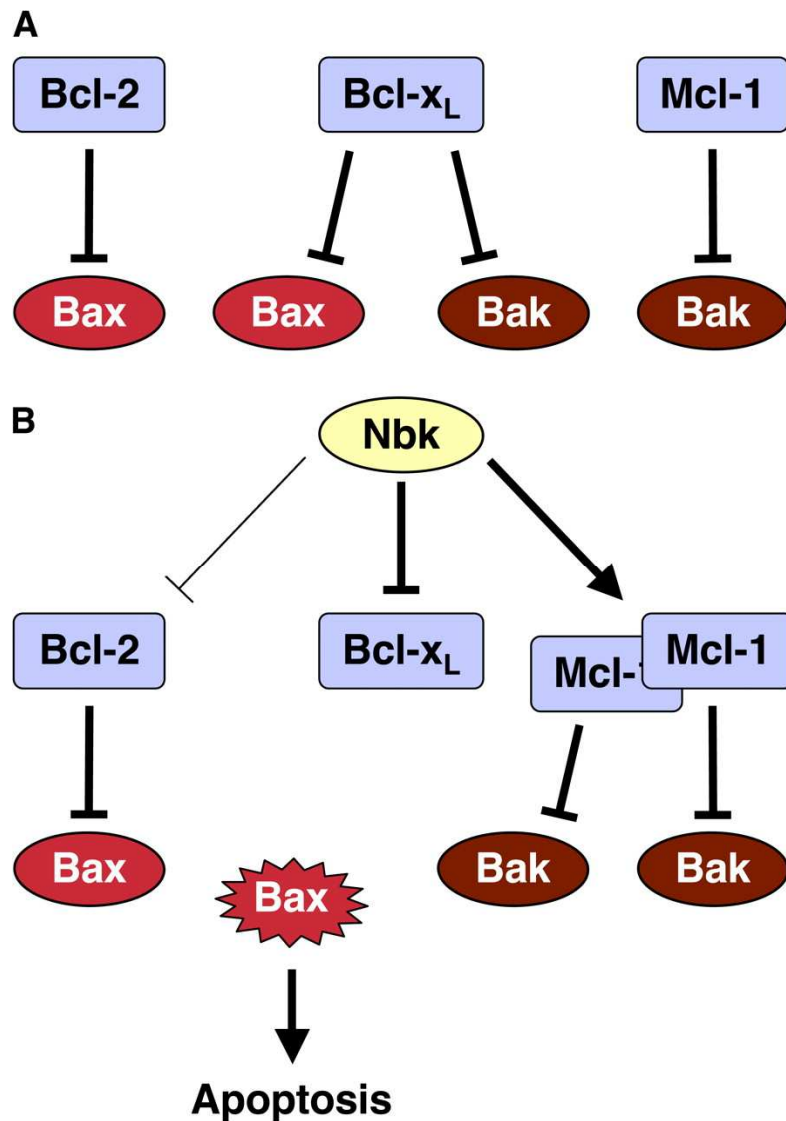
Figure 1-16: Model for the regulation of Bik -induced apoptosis by Bcl-2 family members.

(A) In healthy cells, Bax is inactivated by Bcl-2 and Bcl-$_{XL}$, whereas Bak is sequestered by Bcl-X_L and Mcl-1. (B) Bik/Nbk activates the pro-apoptotic Bax through neutralisation of the anti-apoptotic Bcl-$_{XL}$. In contrast, increased levels of the anti-apoptotic Mcl-1 keep in check Bak that was released from Bcl-X_L upon competition with Nbk. Adapted from (Gillissen *et al*, 2007).

Regulation of *Bik*

Regulation of *bik* on several different levels has been reported. Evidence of roles for transcriptional regulation, methylation, phosphorylation and protein degradation by the proteasome have all been indicated. Additionally, the specificity of the interplay between hetero-dimerising partners is also thought to be associated with *bik* control.

Indeed stringent regulation of *bik* expression at various steps is probably essential, given the fact that Bik is predicted to possess an already exposed BH3 domain and is thus considered constitutively active (McDonnell, et al. 1999). It is clear that external and internal stimuli lead to *bik* regulation at several different levels, including tissue-specific expression (Daniel, et al. 1999), transcriptional and translational control and intracellular localisation (Germain, Mathai and Shore 2002, Zhao, et al. 2008a, Mathai, et al. 2002). Evidence that *bik* is transcriptionally regulated by a number of transcription factors including p75^{NTR}, SFRP1, IgM, E4orf6, E1A, GRP78/BiP, the E2F transcription factors and TGFβ has been demonstrated (Jiang and Clark 2001a, Mathai, et al. 2002, Saltzman, et al. 1998, Tabassum, Khwaja and Djakiew 2003, Han and Amar 2004). Furthermore, apoptosis triggered by Bik can be either p53-dependent or independent depending on the induction stimulus (Mathai, et al. 2002, Han, Sabbatini and White 1996a, Bartke, et al. 2001).

The regulation of Bik function by phosphorylation has also been verified (Verma, Zhao and Chinnadurai 2001). Bik is phosphorylated on consensus CKII phosphorylation sites (Thr-33 and/or Ser-35) (Verma, Zhao and Chinnadurai 2001) an event that appears to result in a gain in function (Verma, Zhao and Chinnadurai 2001). Mutation of the phosphorylation sites, in which the threonine and serine residues were changed to alanine residues, indicated that phosphorylation of Bik is required for its apoptotic activity, without significantly affecting its ability to hetero-dimerise with Bcl-2 and Bcl-X$_L$ (Verma, Zhao and Chinnadurai 2001). The precise mechanism by which phosphorylation plays a role in the apoptotic activity of Bik is not known, although it is possible that phosphorylation of Bik may play role in interaction with other potential cellular targets or that the activity of Bik may be activated by phosphorylation in response to different apoptotic stimuli in various human tissues (Verma, Zhao and Chinnadurai 2001). A phospho-mimetic form of Bik (designated BikDD) has been shown to kill pancreatic cancer cells in an orthotopic mouse model (Xie, et al. 2007).

The methylation status of *bik* remains uncertain. While treatment with the DNA methylation inhibitor, zebularine, induced genomic de-methylation of a multiple myeloma cell line KAS-6/1, and induced the expression of Bik (Pompeia, et al. 2004), conflicting reports argue that DNMT1 mediated *bik* suppression does not involve a change in the DNA methylation state or histone modification, maintaining that DNMT1 is capable of regulating gene expression by a DNA methylation independent pathway (Milutinovic, et al. 2004). A closer look at the mechanism of action of zebularine

reveals that this drug acts by forming a complex with DNMT1 in the position normally incorporated by the base targeted for methylation (Zhou, et al. 2002). Thus, the gene targeted to disrupt methylation may also suppress Bik independently of the DNA methylation state (Pompeia, et al. 2004). Other studies have indicated a role for DNA methylation in transcriptional silencing of *bik*. Exposure to 5'-aza-2'-deoxycytidine resulted in strong up-regulation of *bik* mRNA in renal cell carcinoma (RCC) cell lines (Sturm, et al. 2006) and was shown to be induced by the histone deacetylase inhibitor, sodium butyrate in human hepatoma cells (Ogawa, et al. 2004). Increases in *bik* mRNA levels were observed in both HepG2 and Hep3B cells following treatment with 5-aza-2′-deoxycytidine or histone deacetylase inhibitor sodium butyrate given separately. Bik protein however was undetectable (Wang, et al. 1998). A combination treatment of both 5-aza-2′-deoxycytidine plus depsipeptide has shown a synergistic effect on *bik* gene induction in a variety of cancer cell lines (Dai, et al. 2006). It may therefore be the case that control of *bik* expression by epigenetic mechanisms occurs in a cell type-specific manner. The realisation that Bik may rapidly turnover during apoptosis suggests another level of control by means of Bik degradation (Marshansky, et al. 2001). It has been postulated that the accumulation of Bik protein may be a direct result of its reduced degradation in the cell (Marshansky, et al. 2001). Indeed, reports suggest that Bik is one of the mediators of proteasome inhibitor-induced apoptosis and that the proteasome inhibitors Bortezomib (Nikrad, et al. 2005) and lactacystin (Marshansky, et al. 2001) can regulate Bik expression, through stabilisation of the protein. Thus accumulation of Bik is sufficient to induce apoptosis indicating that it may be the accumulation of Bik rather than the expression per se that determines whether a cell undergoes apoptosis (Jiang and Clark 2001b).

Similar to other BH3-only proteins, Bik binds to pro-survival family members, killing cells when over-expressed (Boyd, et al. 1995a). Not unlike other pro-apoptotic members of the Bcl-2 family the BH3 domain of *bik* has proven critical for pro-apoptotic activities; dimerisation as well as for cytochrome *c* release and apoptosis (Boyd, et al. 1995a, Cosulich, et al. 1997, Hegde, et al. 1998). Since BH3-only pro-apoptotic proteins share only the BH3 domain in common (Bouillet and Strasser 2002), it has been postulated as to whether the BH3 domain is a death effector module eliciting its cell death activity through the inactivation of the anti-apoptotic proteins by hetero-dimerisation.This will result in disruption of the complex of the death suppressor with a caspase activating molecule such as Apaf-1, which in turn results in the activation of

caspases and eventually death of the cell (Boyd, et al. 1995a, Kelekar and Thompson 1998, Lutz 2000, Chittenden 2002) as represented in (Figure 1-17). Regulatory control could then be directly associated with the specificity and nature of the interplay between Bik and its hetero-dimerising partners (Han, Sabbatini and White 1996b, Elangovan and Chinnadurai 1997c). In this regard, the death promoting activity of Bik can be suppressed by co-expression of Bcl-2, Bcl-X_L and BHRF1, and E1B-19k (Elangovan and Chinnadurai 1997c, Boyd, et al. 1995a). Bik also binds with Bcl-X_S, a death-promoting protein that lacks the BH1 and BH2 domains, possibly indicative of a co-operation effect between the two proteins (Boyd, et al. 1995a). It has long been recognised that the relative levels of available Bcl-2 family dimerisation partners in the cell may shift the balance of cell fate either in favour of viability or of cell death, following exposure to an appropriate stress (Han, Sabbatini and White 1996b). The assumption that certain BH3-only ligands possess a degree of specificity for their pro-survival receptors would render apoptosis of the Bcl-2 family a sophisticated affair, with Bik functioning as a naturally dominant negative antagonist whose role in the cell is to sequester and inactivate anti-apoptotic genes.
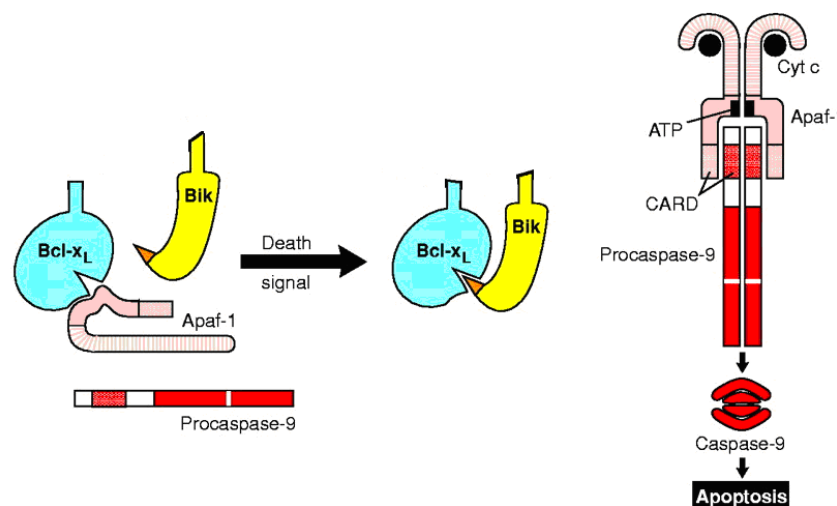
Figure 1-17: Model for Apaf-1 regulation by the Bcl-2 family.

A pro-survival member Bcl-2 family member such as Bcl-X_L may bind Apaf-1 and prevent it from activating procaspase-9 (or another initiating pro-caspase). A death signal may, for example, provoke interaction of a BH3 family member such as Bik, or perhaps a Bax family member with Bcl-X_L, preventing it from neutralising Apaf-1. In the presence of cytochrome c released from mitochondria and ATP, Apaf-1 can then binds to procaspase-9 and promote its dimerisation and activation by autocatalysis. Caspase-9 subsequently activates effector caspases. Adapted from, (Adams and Cory 1998)).

Association of Bik with anti-apoptotic proteins indeed hinders Bik function (Boyd *et al*, 1995). However, mutational analysis of the BH3 domain in *bik*, suggests that hetero-dimerisation via the BH3 domain with survival proteins alone is insufficient to explain their cell death inducing activity (Elangovan and Chinnadurai 1997c). It has also been hypothesised that sequences in the C-terminal region of Bik might also be required for the pro-apoptotic activity of Bik, in addition to the activity of the BH3 domain. The precise biochemical activity of the C-terminal region of Bik is not known but it is conceivable that the C-terminal sequences may function in concert with the BH3 domain to enforce apoptosis. Another, simpler rationalisation may be that the C-terminal region plays a role in Bik protein folding is also possible (Elangovan and Chinnadurai 1997c). Interestingly, this region is known to contain a sequence motif similar to the substrate binding motif of the caspase family, and it was postulated that this motif could modulate the substrate-caspase complexes. Indeed one study demonstrated that the *trans*-membrane domain not only decided the sub-cellular localisation of Bik, but also had an important role in Bik-induced apoptosis in Hep3B cells (Zhao, et al. 2008a).

1.16 EBV regulation of the Cellular Apoptotic Programme

A central component of the overall EBV strategy and its role in the development of related malignant disease is the ability of the viral proteins to suppress the cellular apoptotic program. In this regard, EBV has developed mechanisms that suppress the apoptotic programme of the host in order to delay cell death during the lytic stage of infection, until virion numbers have been sufficiently amplified to transmit infection to other individuals via saliva, and also to ensure persistence in the host memory B cell compartment. EBV genes have been shown to manipulate the cell survival machinery of the infected cell by directly regulating the apoptotic death machinery, specifically by inducing the expression of several anti-apoptotic proteins such as Bcl-2, Bfl-1, Mcl-1, A20, c-IAP-2 and also through the down-regulation of expression of pro-apoptotic proteins such as Bax and Bim (Laherty *et al*, 1992; Fries *et al*, 1996; Wang *et al*, 1996; (Loughran, et al. 2011, Spender and Inman 2011, Pegman, et al. 2006a, Pegman, et al. 2006a, Anderton, et al. 2008, Leao, et al. 2007).

Group I BL cell lines which display type I latency, only expressing EBNA1, EBERs and BARTs and many EBV-negative BL lines can readily be triggered into apoptosis, whereas Group III BL cell lines, which express the complete set of EBV latent proteins, are relatively resistant to a variety apoptotic triggers, including growth factor withdrawal, Ca^{2+} ionophore treatment and over-expression of the p53 tumour suppressor gene (Henderson, et al. 1991, Okan, et al. 1995). EBV-negative BL cells converted to the type III latency state by infection with the B95-8 strain of EBV also display elevated thresholds of resistance to apoptotic stimuli (Gregory, et al. 1991), thus implicating EBV latent genes in cell survival.

1.16.1 EBV up-regulates *bcl-2*

Type I BLs express little or no Bcl-2 whereas group III BL cells lines display high levels of the Bcl-2 protein (Henderson, et al. 1991, Gregory, et al. 1991). Bcl-2 is a prototype anti-apoptotic protein that interacts with the mitochondrial membrane and inhibits the action of caspases (Hardwick and Soane 2013). Transfection of individual EBV latent genes into EBV-negative BL cell lines has shown that up-regulation of Bcl-2 expression correlates with the expression of three EBV proteins; LMP1, (Henderson, et al. 1991, Rowe, et al. 1994) EBNA-2 (Finke, et al. 1992), and EBNA-3B (Silins and Sculley 1995). Significantly, over-expression of Bcl-2 in group I BL cell lines following gene transfer results in reduced apoptosis in response to a number of stimuli compared with group III cell lines and the inhibition of cell death was correlated to the amount of Bcl-2 expressed (Henderson, et al. 1991, Gregory, et al. 1991). Indeed, transfections with EBV LMP1 also resulted in enhanced survival of these cells and the ability of LMP1 to confer resistance to apoptosis to the type I BLs resulted from its ability to induce endogenous Bcl-2 expression (Henderson, et al. 1991, Gregory, et al. 1991). While LMP1 and EBNA-2 appear to up-regulate Bcl-2 expression *in vitro*, the recirculating B cells of peripheral blood which are a target of EBV already express relatively high levels of Bcl-2. EBV infection and so expression of LMP1 or EBNA-2 or the combined expression of both, does not significantly impact Bcl-2 expression. Thus it may be that the role of LMP1/EBNA-2 *in vivo* is to maintain the high constitutive level of Bcl-2 rather than to induce it (Martin, et al. 1993).

1.16.2 EBV up-regulates *A20*

A20 is an inducible zinc finger protein that confers resistance to TNFα cytoxicity (Laherty, et al. 1992) and is induced in response to a wide variety of stimuli, including cytokines such as TNF, IL-1, and CD40 as well as bacterial and viral products such as lipopolysaccharide (LPS) and human T-cell leukemia virus type I Tax in many cell types including fibroblasts, lymphocytes and endothelial cells (Beyaert, Heyninck and Van Huffel 2000). A20 is constitutively expressed in EBV-immortalised B cells and transfection experiments have demonstrated that LMP1 induces the expression of A20 in both lymphocytes and epithelial cells (Fries, Miller and Raab-Traub 1996b). Studies of the A20 promoter have shown that LMP1 transcriptionally activates the A20 gene through *cis*-acting NF-κB binding sites with a role for LMP1-inducible binding of an NF-κB-like factor to a consensus binding sites within the A20 promoter. A20 has also been identified as functioning in a negative feedback loop by inhibiting the activation of NF-κB from both CTAR1 and CTAR2 of the LMP1 protein. This effect is mediated by the binding of A20 to TRAF2 (Eliopoulos, et al. 1999).

1.16.3 EBV up-regulates *mcl-1*

Mcl-1 is a Bcl-2 family protein which can act a key molecule in apoptosis control, promoting cell survival by interfering at an early stage in a cascade of events leading to release of cytochrome c from mitochondria (Michels, Johnson and Packham 2005). Maximal Bcl-2 up-regulation by LMP1 requires 48-72 hours (Rowe, et al. 1994, Kim, Kim and Park 2012). Up-regulation of the Bcl-2 homologue Mcl-1 by LMP1 precedes the induction of Bcl-2 and is transient with levels of Mcl-1 decreasing when Bcl-2 levels begin to increase. Mcl-1 is an anti-apoptotic protein that has been shown to contribute to the prolonged existence of chronic lymphotrophic leukaemia B cells and its higher expression is correlated with resistance to chemotherapy treatment (Bolesta, et al. 2012). It is thought that Mcl-1 functions as a rapidly inducible, short-term effector of cell viability (Michels, Johnson and Packham 2005). LMP1 also prevented the decline in Mcl-1 levels in response to apoptotic stimulation triggered by elevated cyclic AMP. This effect of LMP1 was associated with a delayed cell death in the EBV-negative BL

cell line BL41. The maintenance of Mcl-1 expression by LMP1 is likely to be a crucial immediate-early response that enables cells to survive until Bcl-2 can be up-regulated (Kim, Kim and Park 2012).

1.16.4 EBV up-regulates *c-IAP2*

C-IAP2 is a multi-functional protein which regulates not only caspases and apoptosis but also controls inflammatory signalling and immunity, mitogenic kinase signalling and cell proliferation as well as cell invasion and metastasis. C-IAP2 is a member of the cellular inhibitor of apoptosis protein family (c-IAP), and was originally identified as a molecule recruited to the TNF receptor complex receptor via TRAF1 and TRAF2 (Zheng, et al. 2010). Its expression is preferentially up-regulated by TNF and other stimuli activating NF-κB including IL-1, LPS, and CD30 stimulation (Damgaard and Gyrd-Hansen 2011, Geserick, et al. 2009). Transcriptional activation by TNF or IL-1 is mediated cooperatively by two NF-κB binding sites bound by the NF-κB p50/p65 hetero-dimer. In addition, over-expression of CD40 or LMP1 resulted in the *c-IAP2* promoter-driven reporter gene activation (Hong, et al. 2000). It has been postulated that the LMP1-inducible c-IAP2 protein functions as a pro-survival factor during EBV-infection, acting independently of or in concert with TRAF1 and other LMP1-inducible anti-apoptotic proteins such as A20 and Bcl-2 (Hong, et al. 2000).

1.16.5 EBV up-regulates *bfl-1*

Studies in our laboratory have revealed the up-regulation of another anti-apoptotic gene, *bfl-1/A1* during EBV infection. The pro-survival Bcl-2-family member Bfl-1/A1 is a transcriptional target of NF-κB that is over-expressed in many human tumours and is a means by which NF-κB is known to inhibit apoptosis (D'Souza, et al. 2004, Loughran, et al. 2011). Two EBV proteins LMP1 and EBNA-2 have been shown to up-regulate expression of this cellular gene in the laboratory (Pegman, et al. 2006b, D'Souza, et al. 2004, D'Souza, et al. 2004, Loughran, et al. 2011, Pegman, et al. 2006a, D'Souza, Rowe and Walls 2000). In this regard, expression of LMP1 in EBV-negative BL cell lines

coincides with a dramatic increase in *bfl-1* mRNA (D'Souza, Rowe and Walls 2000) and *bfl-1* expression was found to protect EBV-positive cells against serum depletion-induced apoptosis. Moreover, expression of LMP1 in EBV-negative cell lines was found to *trans*-activate the *bfl-1* promoter through interactions with components of the tumour necrosis factor receptor (TNFR)/CD40 signalling pathway. This process is NF-κB dependent involving the recruitment of TNFR-associated factor 2 and is mediated to a greater extent by CTAR2 relative to the CTAR1 domain of LMP1 (D'Souza, et al. 2004). EBNA-2 also up-regulates *bfl-1* expression. EBNA-2 *trans*-activation of *bfl-1* requires CBF1 and there is an essential role for a core consensus CBF1-binding site on the *bfl-1* promoter. Interestingly *bfl-1* expression is induced and maintained at high levels by the EBV growth program in an LCL and withdrawal of either EBNA-2 or LMP1 does not lead to a reduction in *bfl-1* mRNA levels in this context. However the simultaneous loss of both EBV proteins results in a major decrease in *bfl-1* expression (Pegman, et al. 2006b, Loughran, et al. 2011).

1.16.6 EBV down-regulates *bax*.

Bax is a member of the Bcl-2 family that, together with Bak, is required for permeabilisation of the OMM (Happo, Strasser and Cory 2012a). EBV LMP1 inhibits *bax* promoter activity through activation of NF-κB signalling via CTAR-1 and CTAR-2. The *bax* promoter harbours 3 κB consensus sites (Grimm, et al. 2005) of which LMP1 is known to activate 2, namely κB2 and κB3. LMP1 induced binding of the NF-κB hetero-dimer p65/p50 to the κB2 site and of the p50/p50 homo-dimer to the κB3 site. Promoter mutation analysis revealed that the κB2 site is necessary for inhibition of *bax* promoter activity and the κB3 site for its activation. However, the activation of the *bax* promoter by LMP1 was observed only in the presence of specific inhibitors of p65/p50. In all other cases LMP1 inhibited *bax* promoter activity. Moreover, in cells with impaired Bax function or expression the anti-apoptotic activity of LMP1 is significantly reduced indicating that Bax is an important target of the anti-apoptotic activity of LMP1 (Grimm, et al. 2005).

1.16.7 EBV down-regulates *Bim*

Bim is a BH3-only protein whose over-expression in cells leads to cytochrome *c* release through the activation of Bax. All mature B cells express significant amounts of BH3-only Bim (Happo, Strasser and Cory 2012a). Infection of BL cells with EBV results in a significant down-regulation in the expression of all three isoforms of the *bim* gene (Spender and Inman 2011). This down-regulation appears not to require expression of EBNA-2 or the LMP proteins (Leao, et al. 2007) and is in fact dependent on the co-operation of EBNA-3A and EBNA-3C ((Anderton, et al. 2008). It has been reported that EBV infection may lead to post-translational modulation of Bim expression, involving the phosphorylation of BimEL by the EBV-activated kinase ERK1/2 followed by its degradation through the proteasome pathway (Fu, He and Mao 2013). However, a pathway in which EBNA-3A and EBNA-3C co-operate to regulate Bim expression at the level of transcription has also been described (Anderton, et al. 2008).

1.16.8 EBV lytic proteins encoding anti-apoptotic functions

During the lytic cycle of virus replication, repressors of apoptosis can provide a selective advantage by protecting the cell from death. EBV encodes its own gene products which can delay apoptosis in order to maximise viral production and spread of virus progeny to other individuals. These genes include BHRF1, BZLF1 and BALF1. As indicated earlier, EBV also encodes a viral homologue of IL-10 (Reviewed in (Spender and Inman 2011, Kalla and Hammerschmidt 2012, Forte and Luftig 2011).

BHRF1 promotes the survival and proliferation of lytically infected cells and the highly conserved nature of BHRF1 among different EBV isolates at both the sequence and functional level supports the proposed vital role of BHRF1 in delaying cell death, thereby maximising the production of progeny virus and facilitating the establishment of virus persistence (Kvansakul, et al. 2010a). BZLF1 has been shown to interact with p53 and inhibit its *trans*-activating function in lymphoid cells, thereby providing a mechanism for preventing p53-mediated apoptosis (Morrison, et al. 2004, Zuo, et al. 2011). The BALF1 ORF in EBV possesses significant sequence similarity to other anti-apoptotic viral Bcl-2 homologs and the cellular Bcl-2 and Bcl-$_{XL}$. A recombinant GFP-

BALF1 fusion protein co-localises with mitochondrial dyes and suppresses apoptosis but not through to promotion of cell-cycle progression, and hetero-dimerises with Bax and Bak (Bellows, et al. 2002, Hsu, et al. 2012a). While the growth stimulatory and anti-apoptotic genes induced by the initial EBV infection almost certainly prolong survival in the lytic stage of the lifecycle, it is also likely that they may augment the likelihood of the virus-infected cell progressing into the memory B-cell pool. Taken together, these interactions account, at least in part, for the ability of EBV to induce cell proliferation and survival which is essential for both EBV-induced immortalisation of B cells and establishment of life-long persistence in the memory B cell compartment.

1.17 The Germinal Centre Reaction

The Germinal Centres (GCs) are structured microenvironments of secondary lymphoid tissues [spleen, peripheral lymph nodes and mucosal-associated lymphoid tissue (MALT)] where antigen-activated B cells undergo proliferation, class switch recombination (CSR), somatic hypermutation (SHM), antigen selection, and affinity maturation (Figure 1-18) (Allen, et al. 2007). Primary B cell follicles consist of naïve B-cell regions next to areas containing T-cells, macrophages and follicular dendritic cells (FDCs). The recognition of antigen by the BCR coincide with cognate priming of T-helper cells by antigen presenting dendritic cells, which initiates the T-cell dependent differentiation of B cells. GCs come to be the main sites for generating effector plasma cells proficient in secreting high affinity immunoglobulins and for differentiation of memory B cells which facilitate a faster and effective response to antigen re-occurrence. In this instance B cells accumulate somatic hypermutations in the variable regions of their immunoglobulin genes that encode the antigen binding site. This procedure potentially increases the affinity and specificity of BCR for antigen (affinity maturation) but also imposes negative selection of autoreactive and low affinity receptors (activation induced cell death). The GC reaction is therefore a second main place of apoptotic elimination of large numbers of unwanted B cells (Spender and Inman 2011). The centroblasts differentiate further into non-proliferating centrocytes and transfer to the GC area which contains FDCs and T-cells. Centrocytes carrying the highest affinity mutated receptors outcompete others for limiting amounts of foreign antigen displayed on the FDCs and for survival signals provided by the GC T-helper cells, thus guaranteeing their continued survival and differentiation (Vinuesa, et al. 2010).

Since it's a high risk procedure for generating autoreactive or non-functional clones, B cells transferring through germinal center for development and activation are prone to programmed cell death (Kurosaki 2000, Benschop and Cambier 1999). Apoptotic pathways accountable for the removal of B cells inside the GC signal through the TGFβ receptor (Spender, et al. 2009), B cell receptors (Romero-Camarero, et al. 2013), and FAS (Reviewed in (Klein and Dalla-Favera 2008, Hao, et al. 2008)).
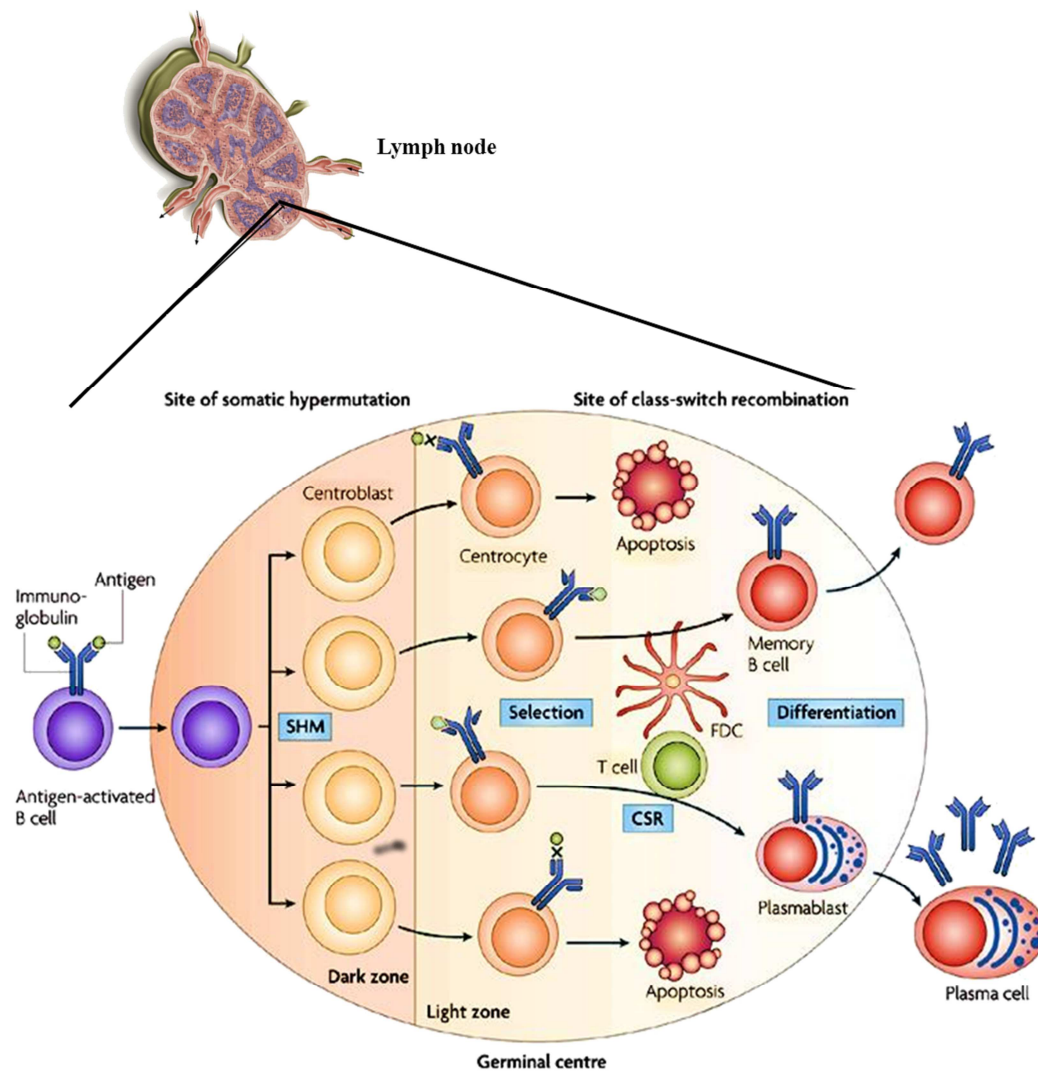
Figure 1-18: Germinal centre reaction

Antigen-activated B cells differentiate into centroblasts which then undergo clonal expansion in the dark zone. During proliferation cells go through the process of SHM. Subsequently centroblasts differentiate into centrocytes and migrate to light zone, where the modified antigen receptor, is selected for enhanced binding to the antigen by means of FDCs and T-cells. The newly generated centrocytes that produce autoreactive or non-functional antibodies undergo apoptosis and are eliminated, and the other subset of centrocytes undergo immunoglobulin CRS. Antigen selected centrocytes eventually differentiate into memory B cells or plasma cells. Adapted from, (Klein and Dalla-Favera 2008).

1.18 EBV-infected B cells and the germinal centre reaction

Central to EBV biology is the ability of the infected B cell to survive transit through a GC. Naïve B cells are the key targets of new EBV infection *in vivo*. Following primary infection of naive B cells EBV expresses its entire latency gene complex (Latency type III) thus driving expansion of the infected B cell pool. B cells enter lymphoid follicles where they proliferate and express only three viral proteins (Latency type II). EBV ensures the transit of some infected cells into the long-lived memory B cell compartment by mimicking the physiological process of antigen-driven memory-cell development during transit through a germinal centre.

EBNA-2 subverts aspects of the Notch signalling pathway thus blocking B cell differentiation and allowing cell proliferation (Lucchesi, et al. 2008a). EBNA1 binds to the latent viral DNA replication origin and maintains the viral genome in the EBV positive cells after cell division (Thorley-Lawson 2001a). LMP1 is a viral homologue of CD40 which allows activation of B cells without the need to engage with CD40L on the CD4+ T cell. LMP-1 expression mimics CD40-CD40L signal transduction and bypasses the need for CD4+ T cell help. LMP2A is a B cell receptor functional homologue. It mimics B cell receptor signalling and coupled to LMP1 signalling promotes the B cell to undergo affinity maturation, isotype switching and differentiation into memory and effector B cells (Odumade, Hogquist and Balfour 2011, Mancao and Hammerschmidt 2007).

1.19 B Cell Receptor signalling and the escape of EBV-infected B cells

BCR signalling is initiated by phosphorylation of the cytoplasmic immunoreceptor tyrosine based activation motifs (ITAMs) of CD79A/Igα and CD79B/Igβ (Figure 1-19) (Yang and Reth 2010). This phosphorylation is mainly catalysed by the Src family kinase LYN and results in the recruitment and activation of the tyrosine kinase SYK (Kurosaki 2000, Craxton, et al. 1999, Kulathu, Grothe and Reth 2009). Activation of SYK is an important event in BCR signalling causing the formation of a plasma membrane-associated signalling complex that contains multiple tyrosine kinases (including LYN, SYK and the Tec-family kinase BTK) and adaptor molecules (for instance BLNK), which mediate activation of downstream signalling pathways. Activation of phospholipase Cγ2 (PLCγ2) (Gorjestani, et al. 2011)(Gorjestani, et al. 2011) leads to the production of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Gorjestani, et al. 2011) which are required for the subsequent release of intracellular Ca^{2+} (iCa^{2+}) (Lyubchenko 2010) and activation of protein kinase C (PKC), respectively. Increased iCa^{2+} and PKC activity induce activation of (Spender and Inman 2012) mitogen-activated protein kinase (MAPK)-family kinases, including extracellular regulated kinase (ERK), c-JUN NH_2-terminal kinase (JNK), p38 MAPK, and transcription factors important for B-cell fate comprising nuclear factor of activated T cells (NFAT) and MYC (Chung, et al. 2012). Activation of inhibitor of κB kinase (IKK) leads to phosphorylation and proteasomal degradation of inhibitor of κB (IκB) and activation of the nuclear factor-κB (NF-κB) transcription factor. In contrast to p38 MAPK and JNK, ERK can also be activated independently of PLCγ2, via a RAS/ RAF1 pathway (Imamura, et al. 2009). Activation of phosphatidylinositol 3-kinase (PI3K) requires LYN-dependent phosphorylation of the cytoplasmic domain of CD19 leading to recruitment of the p85 adaptor subunit of PI3K and production of the lipid phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits a number of BCR signalling components to the plasma membrane including the serine/threonine kinase AKT where it is activated. Active AKT is important for BCR-induced survival and proliferation pathways and acts by inactivation of Bad and forkhead family transcription factors, enhanced activation of NF-κB and inhibition of GSK3, a negative regulator of Myc and D-type cyclins (Downward 2004). The activation of positive BCR signalling pathways is countered by inhibitory phosphatases including SH2 domain-containing tyrosine phosphatase-1 (SHP-1) and SH2 domain-containing phosphatidyl 5-phosphatase (SHIP)-1 and -2. These phosphatases are activated downstream of LYN

and therefore LYN plays both positive and negative roles in signal transduction via the BCR (reviewed in (Kurosaki 2000, Packham and Stevenson 2010). Figure 1-19 shows the details of these interactions.
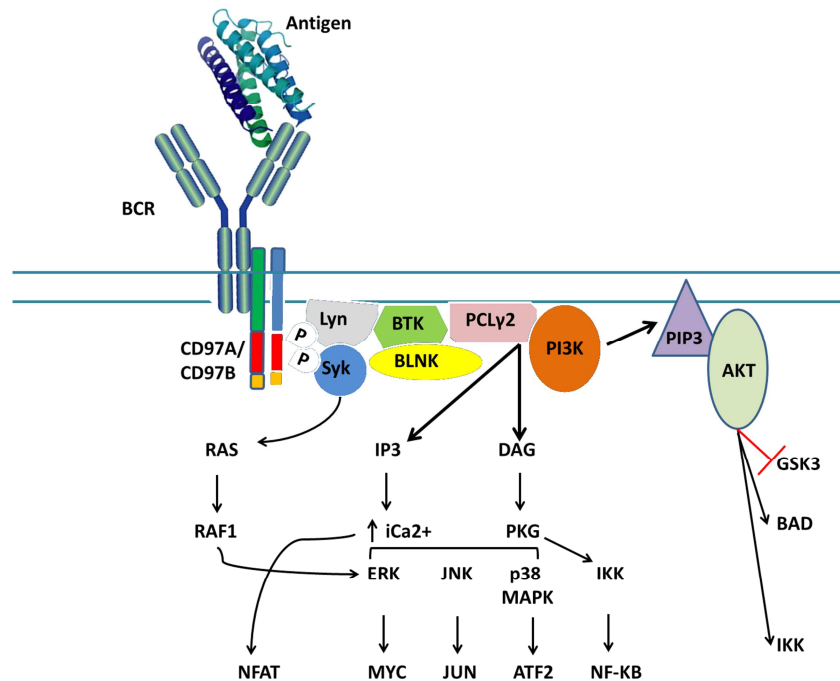
Figure 1-19: The BCR signalling pathway.

The major signalling pathways activated downstream of the BCR are shown. See text for details and abbreviations. Adapted from, (Packham and Stevenson 2010).

Studies have revealed BCR cross linking with antibodies to membrane bound IgM but not IgD on the surface of B cells results in suppression of the PI3K/pp70^{S6X} signalling pathway followed by decreased levels of cyclin D2 (Banerji, et al. 2001) and *c-myc* by NF-κB inactivation and subsequent elevation of p27^{Kip1} protein expression and its accumulation resulting in growth arrest or apoptosis (Carey and Scott 2001)(Banerji, et al. 2001). In contrast Jiang and Clark observed sIgM and sIgD ligation led to the induction of *bik* mRNA but that only sIgM ligation (and not sIgD ligation) exerted a proapoptotic effect by inducing the expression of Bik, via strong activation of PI3K (Beckwith, et al. 1996) and calcium influx resulting in apoptosis (Jiang and Clark 2001a).

Overall, EBV transformation of resting B lymphocytes to LCLs mimics antigen-induced resting B cell clonal expansion in lymph node germinal centers, where antigen binding to surface Ig (sIg) induces *c-myc*-mediated proliferation (Murn, et al. 2009) and T cell CD40 ligand (CD40L) activates B lymphocyte CD40 receptors to up-regulate NFκB, MAP kinases, and anti-apoptotic Bcl-2 family protein expression (Vrazo, et al. 2012, Elgueta, de Vries and Noelle 2010). The important point about EBV is that it encodes a homologue of the B cell receptor, LMP2A, and coupled to LMP1 signalling promotes B cells to undergo affinity maturation, isotype switching and differentiation into memory and effector B cells (Odumade, Hogquist and Balfour 2011, Mancao and Hammerschmidt 2007). LMP2A signalling does not cause B cell to grow, but delivers the tonic signal that is essential for the survival of all B cells (Thorley-Lawson 2001b).
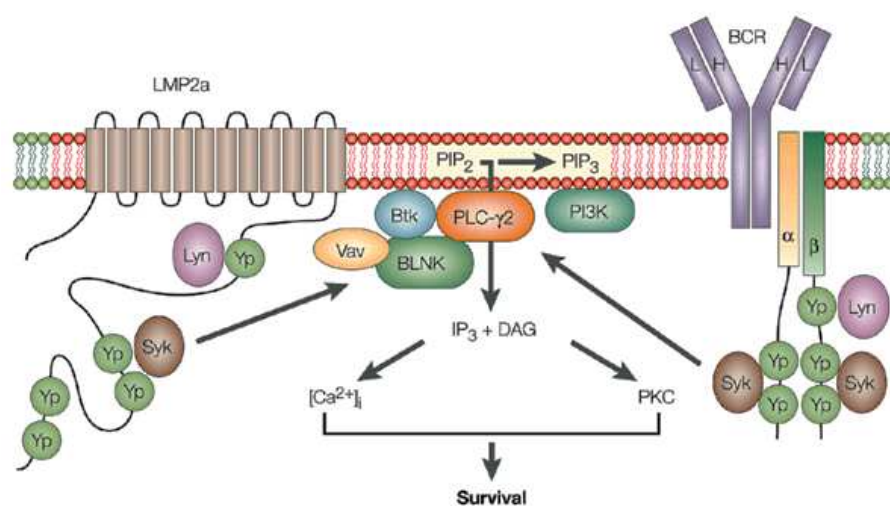
Figure 1-20: Signalling relationship between LMP2A and the BCR

LMP2A comprise of cytoplasmic amino-terminal and carboxy-terminal domains associated by 12 transmembraine sequences with no significant extracellular domain. The amino-terminal domain of LMP2A contains the same ITAMs found in the α- and β-chains of the BCR. Together LMP2A and BCR associate with LYN. Phosphrylation of tyrosine residues within the ITAM by LYN leads to recruitment of the SYK tyrosine kinase and canonical downstream BCR signalling events. Adapted from, (Thorley-Lawson 2001b).

1.20 TGFβ1 signalling in the GC

TGFβ has an apoptosis and anti-proliferative inducing capacity and this effect plays a critical role in the maintenance of B and T lymphocytes homeostasis including the fate of B cells in the GC (Schuster and Krieglstein 2002a). TGFβ, a pleiotropic cytokine, initiates a signalling cascade by binding and bringing together the membrane bound receptors, type II and type I transmembrane serine/threonine kinases (TβRI and TβRII). The complex is formed of two TβRI and TβRII. In addition endoglin and betaglican, also called accessory receptors, bind TGFβ with low affinity and present it to the TβRI and TβRII (Shi and Massague 2003). Upon TGFβ interaction with the highly active membrane bound TβRII, type I receptor (TβRI/ALk5) gets recruited and phosphorylated at its characteristic SGSGSG sequence (called the GS domain) which results in the activation of type I receptor by type II receptor. The GS domain acts as a Smad binding site and subsequently the activated form of TβRI phosphorylates and activates the receptor regulated Smads (Smad2/3) by its catalytic domain. Phosphorylated R-Smad(s) then undergo hetero-oligomerisation with common Smad (Smad4), where they translocate to and accumulate in the nucleus in conjugation with DNA binding transcriptional co-factors and transcriptional co-activators and co-repressors. This allows for positive or negative regulation of gene transcription in a cell-specific and context-dependent manner (Figure 1-21) (Shi and Massague 2003, Massague 1998).
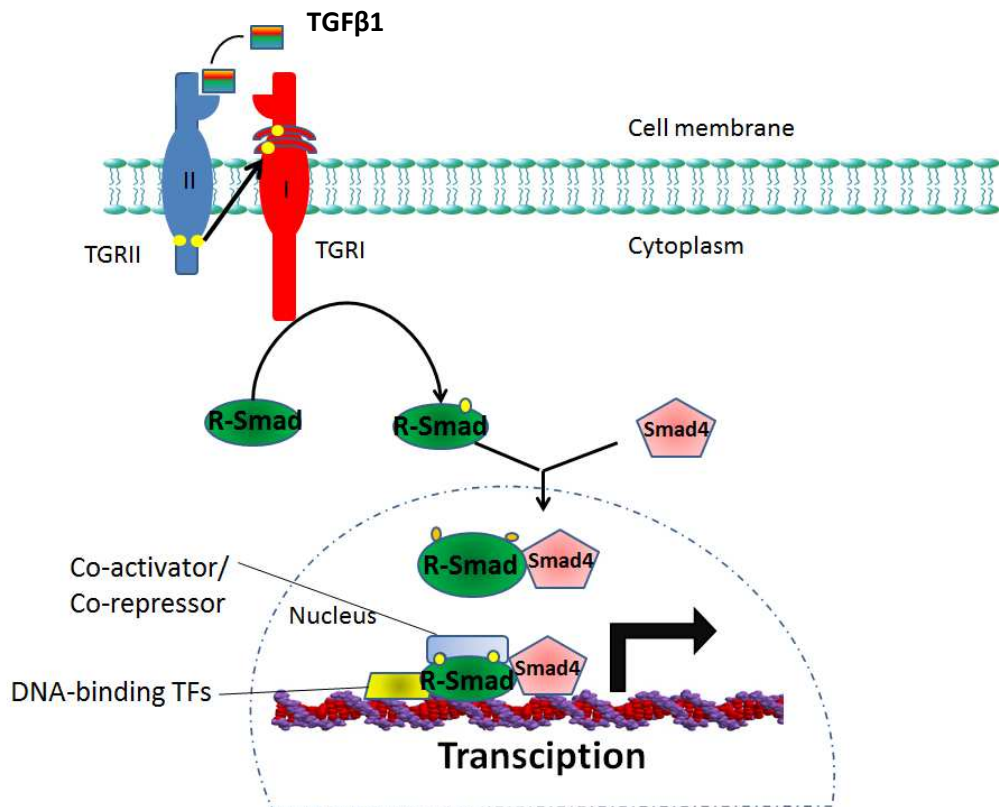
Figure 1-21: TGF-β signalling pathway

TGFβ links to the membrane-bound type-2-receptor and activates the kinase function of the type-2-receptor with subsequent stimulation of the kinase-function of the type-1-receptor. The so-called receptor-Smad-proteins (Smad 2 and Smad 3) link to the receptor complex and are phosphorylated by the type-1-receptor. The phosphorylated R-smad-proteins form a complex with the co-operative Smad 4. This complex is able to penetrate the nucleus. Here the activated R-Smads get recruited to DNA promoters with transcription factors, transcription co-activator/co-repressors and control transcription processes. Adapted from, (Ikushima and Miyazono 2011b).

Over the past 30 years, experiments designed to investigate the effect of TGFβ mediated responses in human B cell lines based on their origin, their phenotype and their EBV carrier state have suggested that the phenotypic shift of BL cells from group I toward a group III phenotype is consistent with increased resistance to the anti-proliferative and proapoptotic activities of TGFβ induced signalling (Altiok, et al. 1991). The mechanism by which EBV confers TGFβ resistance has been investigated by many groups, however it needs to be determined more clearly in a cell type and context specific way. Various mechanisms that have been suggested include; (i) loss or down regulation of the TGFβ

receptors type I/II and thus inactivation of the TGFβ signalling pathway at a very early stages, (Kumar, et al. 1991, Inman and Allday 2000, Fukuda, Kurosaki and Sairenji 2006) (ii) LMP2A inhibits TGFβ -mediated apoptosis through activation of the PI3-K/Akt pathway which results in survival signals both in B cells and epithelial cells (however this effect was not derived through alteration of expression levels of type I and type II TGFβ1 receptors) (Fukuda and Longnecker 2004), (iii) in centroblasts isolated from GCs, several of the apoptotic genes regulated by TGFβ signalling have been characterised. These include members of the Bcl-2 family acting upstream of BAX and BAK in the intrinsic apoptosis pathway. The mechanism of action of TGF-$β$ in centroblasts isolated from GCs involves the induction of an apoptotic program by transcriptional downregulation of the anti-apoptotic protein BCL-X$_L$ while also upregulating the proapoptotic BH3-only proteins PUMA and Bik. The increase in BH3-only proteins and the loss of BCL-X$_L$ expression lead to mitochondrial membrane depolarisation and intrinsic apoptosis (Spender and Inman 2011, Spender, et al. 2009).

1.21 Vitamin D

$1\alpha,25$-dihydroxy vitamin D_3 ($1\alpha,25$-$(OH)_2D_3$), the active form of vitamin D, is a key player in calcium and bone metabolism, nevertheless $1,25(OH)_2D_3$ as well has a physiological role outside its recognized role in skeletal homeostasis. Receptors for $1,25(OH)_2D_3$ are present in various immune cells, including monocytes, macrophages and dendritic cells, as well as T and B lymphocytes, thus suggesting a role for $1\alpha,25$-$(OH)_2D_3$ in both innate and adaptive immune responses (Chen,S. 2007, Pan,L. 2010). Further, immune cells express vitamin D-activating enzymes, allowing local conversion of inactive vitamin D into $1\alpha,25$-$(OH)_2D_3$ within the immune system (Morgan,J.W. 2000). It has been shown that vitamin D deficiency, especially in early life, increases the risk of autoimmune diseases later on and is associated overall with an increased risk of infections (Handel,A.E. 2010). It has long been known that multiple sclerosis (MS) is associated with an increased Epstein-Barr virus (EBV) seroprevalence and high immune reactivity to EBV and that infectious mononucleosis increases MS risk (Ascherio,A. 2007, Magliozzi,R. 2013). It has been show MS risk is associated with low vitamin D status prior to disease (Adams,J.S. 2008).

Most commonly, vitamin D_3 (cholecalciferol) synthesis occurs cutaneously following ultraviolet irradiation of skin and it can also be taken in the diet (Deeb, et al. 2005) (Figure 1.22). The *CYP27A1* gene, which encodes mitochondrial and microsomal 25-hydroxylases (25-OHase) (Haussler, et al. 1998a), hydroxylates cholecalciferol in the liver (Haussler, et al. 1998a) to 25-hydroxycholecalciferol [$25(OH)D_3$] which is subsequently 1α-hydroxylated in the kidney by mitochondrial 1α-hydroxylase (encoded by the gene *CYP27B1*), leading to the hormonally active $1\alpha,25$-$(OH)_2D_3$ (calcitriol) (Haussler, et al. 1998a). There are examples of tissue-specific regulation of vitamin D_3 synthetic enzymes in the literature. The active metabolite of vitamin D_3 (hereafter $1\alpha,25$-$(OH)_2D_3$) functions in an autocrine and paracrine manner. It has significant implications for vitamin D_3 function and signalling where it contributes to maintaining plasma calcium and phosphate homeostasis through the regulation of intestinal absorption, cell differentiation and maturation, as well as the innate immune system (Deeb, Trump and Johnson 2007a, Morris and Anderson 2010).
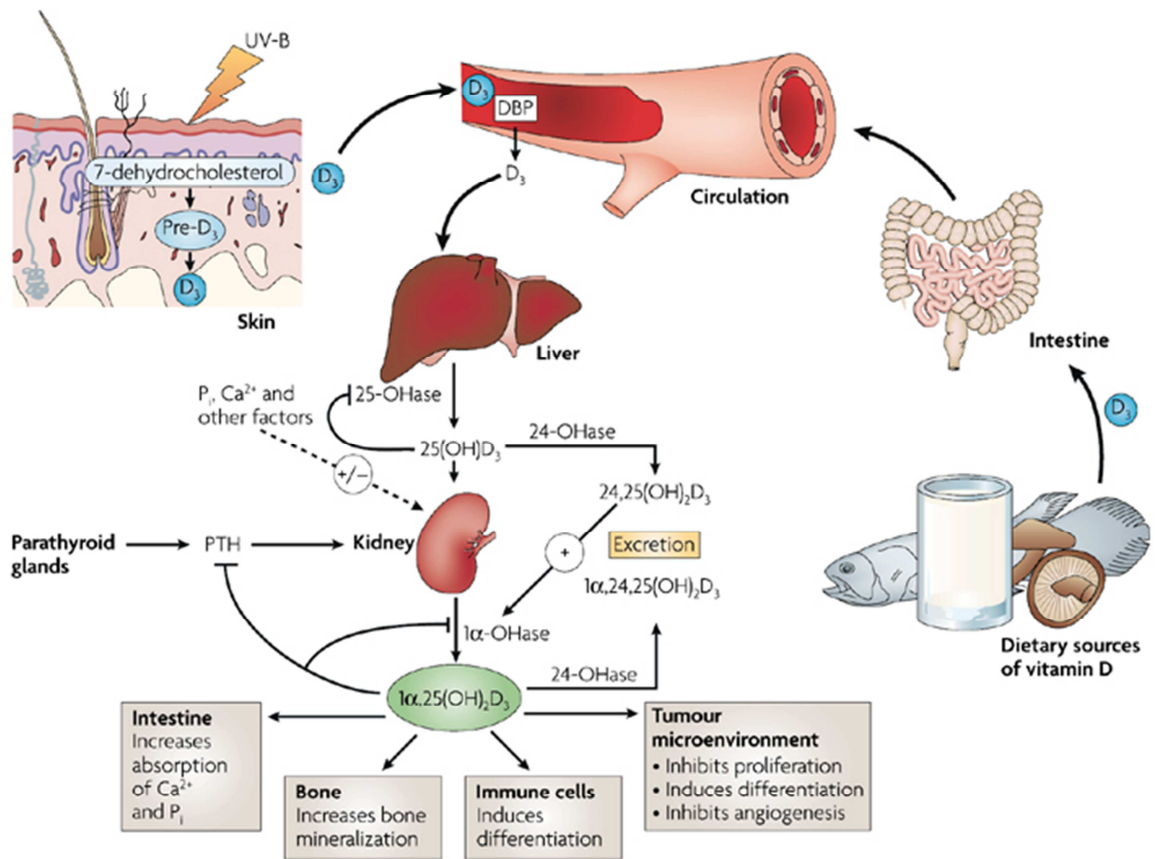
Figure 1-22: Vitamin D$_3$ metabolism

During exposure to sunlight, 7-dehydrocholesterol in the skin absorbs solar UVB radiation and is converted into cholecalciferol (vitamin D$_3$). Together with vitamin D$_3$ originating from the diet, it enters the circulation and is metabolised to 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] in the liver by vitamin D 25-hydroxylase. This is the form that circulates in the highest concentrations and reflects solar and dietary exposure. 25(OH)D$_3$ re-enters the circulation and is converted into 1α,25-(OH)$_2$D$_3$ in the kidney by 25(OH)D$_3$-1-hydroxylase, where it contributes to calcium and phosphorus homeostasis as well as modulation of the innate immune system. Adapted from (Deeb, Trump and Johnson 2007b).

Vitamin D_3 and its activated metabolites are highly lipophilic and are therefore generally found in circulation bound to vitamin D-binding protein (DBP). The main function of DBP is to transport vitamin D_3 and its metabolites. In addition, it exerts several other important physiological functions, including fatty acid transport, macrophage modulation, osteoclast activation, and chemotaxis (Gomme and Bertolini 2004, Meier, et al. 2006, Speeckaert, et al. 2006). $1\alpha,25\text{-}(OH)_2D_3$, is generally believed to enter target cells by diffusion and then form a complex with a cytoplasmic/nuclear vitamin D receptor (VDR), where it causes transcriptional activation and repression of target genes. The VDR is a member of the steroid hormone receptor superfamily and regulates gene expression in a ligand-dependent manner (Evans 1988). VDR has been found in the small intestine, colon, osteoblasts, activated T and B cells, pancreatic beta cells of the islets of Langerhans, brain, heart, skin, gonads, prostate and breast cells (Holick 2004, Morgan, et al. 2000a). $1\alpha,25\text{-}(OH)_2D_3$–VDR-dependent transcriptional activity is modulated through synergistic ligand-binding and dimerisation with retinoic X receptor (RXR). The activated $1\alpha,25\text{-}(OH)_2D_3$–VDR–RXR complex specifically binds to vitamin D response elements (VDREs) which are composed of two hexanucleotide repeats interspaced with varying numbers of nucleotides (for example, GGTCCA-NNN-GGTCCA, where N is any nucleotide; this is denoted DR3), in the promoter regions of target genes (Carlberg, et al. 1993) (Figure 1.23). The genes regulated upon binding with the VDR include those genes important for calcium metabolism such as osteocalcin, osteopontin, 25-hydroxy-vitamin D_3-24-hydroxylase enzyme (CYP24A1) and calbindin (Haussler, et al. 1998b) and also genes involved in cellular proliferation and differentiation including *c-myc* (Salehi-Tabar, et al. 2012), *c-fos, p21, p27* and *HoxA10* (Nguyen, et al. 2011).
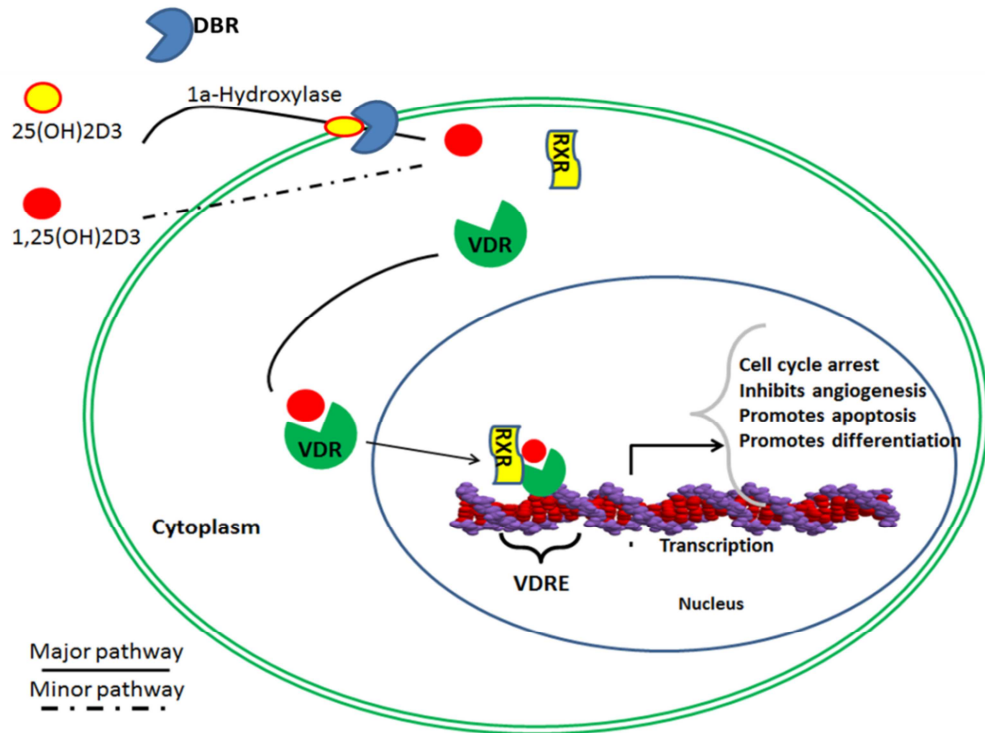
Figure 1-23: A schematic diagram of the vitaminD$_3$ signalling pathway.

25(OH)D$_3$, binds to DBP, by which it gets transported into the cell (major pathway), while 1α,25-(OH)$_2$D$_3$ is generally believed to enter target cells by diffusion (minor pathway). Binding of 1α,25-(OH)$_2$D$_3$ to VDR, results in the heterodimerisation of the VDR with the retinoid RXR. This complex then binds to the VDRE specific sequence and exerts transcriptional activation or repression of the target genes. Adapted from (Pierrot-Deseilligny and Souberbielle 2013).

1.22 Objectives of the study

The modulation of anti- and pro-apoptotic proteins by EBV plays a central role in the overall virus strategy and in the development of virus-associated malignancies. Previous work in our laboratory has demonstrated that EBV down-regulates the cellular *bik* gene in B cells proliferating due to the EBV growth programme, with a key role for EBNA-2 in this regulation. Significantly, restoration of Bik expression in LCLs leads to apoptotic cell death. The down-regulation of *bik* by EBNA-2 also highlighted the protective effect of EBNA-2 in EBV-infected cells.

Having established that the cellular *bik* gene is an important target for down-regulation by latent EBV genes in the mediation of an increased anti-apoptotic threshold of EBV-infected cells, it was the main focus of the current study to

- Establish the mechanism by which *bik* is induced and regulated in B cell lines that model the GC in response to the major eliminating signals involved in GC hemostasis.
- Define the significance of the loss of *bik* expression to the survival of EBV negative cell lines in response to induced programme cell death.
- Clarify the mechanisms by which the EBV growth programme/EBNA-2 interferes with the signalling pathways which are involved in the regulation of this proapoptotic gene.
- Investigate a role for Vitamin D$_3$ in regulating *bik* expression in B cells.

Regulation of *bik* by EBV would have implications for the biology of EBV, as EBV-mediated down-regulation of this gene may contribute to the survival of its host, since cells similar to LCLs are present in the circulation and during primary infection by the virus (D'Souza, Rowe and Walls 2000). Additionally, systematic studies into the contribution of *bik* to cell survival will provide important information about both normal B cell development and potential routes to B cell malignancy. The suppression of *bik* may contribute to the development of EBV-associated autoimmune diseases, for instance SLE and MS (Magliozzi, et al. 2013, Niller, Wolf and Minarovits 2008) and B cell malignancies, such as post-transplant lymphoproliferative disorders and BL tumours.

In summary, this thesis presents the novel finding that EBV down-regulates the cellular pro-apoptotic *bik* gene by down-regulating the transcriptional co-activator, Smad3, which also acts as the TGFβ signalling mediator. Significantly, Smad3 knockdown in EBV-negative cell lines coincided with decreased *bik* mRNA and Bik protein expression levels. The EBV latent transcription factor EBNA-2 plays a key role and this down-regulation is independent of the cellular transcription factor CBF1. Furthermore, for the first time, the *bik* gene has been identified to be the target of the $1\alpha,25\text{-}(OH)_2D_3$ intrinsic apoptotic pathway. Cholecalcitriol induced *bik* expression through a PI3K-dependent pathway. Here, it has been shown that the *bik* gene is the common target for major intrinsic apoptotic pathways in germinal centre B cell models. A broader knowledge of the role and regulation of the *bik* gene will undoubtedly enhance the understanding of EBV biology and of EBV associated diseases. Collectively, these findings suggest that modulation of *bik* expression by EBNA-2 through down-regulation of Smad3 may be a contributory factor in the development of EBV-associated B-lymphomas.

Chapter 2 :
Materials & Methods

2.1 Biological Materials

2.1.1 Cell lines

Table 2-1: Cell lines used in the study

Cell Lines	EBV Status	Cell Classification	Description
BJAB	-	Burkitt-like lymphoma cell line	BJAB is a B cell derived from a patient with EBV-negative African Burkitt's lymphoma. The cells do not contain detectable amounts of EBV DNA, nor do they express the EBV-determined nuclear antigen EBNA. However the cells have the characteristics of B-type lymphocytes and carry receptors for EBV (Menezes *et al*, 1975).
ER/EB 2-5	+	LCL	An LCL established by co-infecting B cells with a 28 kb mini-EBV plasmid that only expresses oestrogen-responsive EBNA-2, together with the EBV P3HR1 strain in which its own EBNA-2 gene is deleted (Kempkes *et al*, 1995a).
Ramos	-	Type I BL	EBV negative B Lymphocyte cell line derived from an American Burkitt's Lymphoma patient (DSMZ).

The BL-like cell line, BJAB was obtained from Professor Martin Rowe, University of Wales, Cardiff, Wales. The oestrogen-responsive cell line ER/EB2-5 was a gift from Dr. Ursula Zimber-Strobl, GSF-National Research Centre for Environment and Health, Munich, Germany. The BL cell line, Ramos (ACC 608) was bought from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH.

2.1.2 Antibodies used in the study

Table 2-2: Antibodies used in the study

Antibody	Name/Code	Description	Supplier
Mouse Anti-Bik	Mouse Anti-Human Bik Clone C33-1 (557040)	Monoclonal IgG raised against amino acids 40-114	BD Biosciences
Rabbit Anti-Smad3	Ab28379	Rabbit polyclonal IgG to Smad3 - ChIP Grade	Abcam
Mouse Anti-Smad4	Ab3219	Mouse monoclonal IgG1 [SMD46 (same as DCS-46)] to Smad4 - ChIP Grade	Abcam
Mouse Anti-β-Actin	Mouse Anti- β - Actin Clone AC-15	Monoclonal IgG raised against β-cytoplasmic actin N-terminal peptide	Sigma
Goat Anti-Mouse IgG Alkaline Phosphatase (AP) Conjugate	Anti-Mouse IgG (AP) Conjugate S3721	Affinity-purified goat anti-mouse antibody	Promega
Anti-GAPDH	(FL-335): sc-25778	Rabbit polyclonal IgG, raised against amino acids 1-335 representing full length GAPDH of human origin	Santa Cruz Biotech
Anti-Mouse-HRP	A8924	A8924 anti-mouse IgG (whole molecule) peroxidase conjugate is an affinity isolated goat polyclonal antibody and was adsorbed with rat serum proteins.	Sigma
Anti-Rabbit-HRP	A4914	Anti-Rabbit IgG (whole molecule)-Peroxidase antibody. Antibody adsorbed with human serum proteins.	Sigma

2.1.3 Bacterial strains used in the study

Table 2-3: Bacterial strains used in the study

Bacterial Strain	Genotype
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ-, Δ(*lac-pro*AB), [F´, *tra*D36, *pro*A + B + , lacl ^{q} ZΔM15]

2.1.4 Expression and Reporter Constructs

Table 2-4: Plasmids used in this study

Plasmid	Source	Description
pSG5	Stratagene	The pSG5 Vector is a eukaryotic expression vector, harbouring the SV40 promoter and the ampicillin resistance gene.
pSG5-EBNA-2	Lindsay Spender, Ludwig Institute for Cancer Research, Imperial College School of Medicine, London.	pSG5-EBNA-2 expresses the wild type EBNA-2 gene (from the EBV strain B95.8) cloned into pSG5 (Spender, et al. 2001b).
pSG5-EBNA-2 WW323SR	Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.	pSG5-EBNA-2 WW323SR is identical to pSG5-EBNA-2 except that EBNA-2 has had two tryptophan residues mutated to serine and arginine residues at positions 323 and 324 respectively. pSG5-EBNA-2 WW323SR therefore, does not bind RBP-Jκ/CBF1 (Ling *et al*, 1993a).

Table 2-5: Oligonucleotides used in this Study

Target	Primer Sequence
Bik F	5'-ggaggccctagaagaaaagactac-3'
Bik R	5'-ggaacagaggaggtaaagtgtgat-3'
Hes1 F	5'-cctcccaggatagctattg-3'
Hes1 R	5'-tttgcctgaggacttgaagc-3'
BZLF-1 F	5'-catgcagcagacattcatca-3
BZLF-1 R	5'-gacgaactgaccacaacactaga-3'

All oligonucleotides were synthesised by and obtained from MWG-Biotech, Ebersberg, Germany.

2.1.5   Bioinformatics software

A number of web-based bioinformatics tools listed in Table 2-6 were used routinely in this study.

Table 2-6: Web-based bioinformatics tools used in this study

	Source	Use
BLAST	http://www.ncbi.nlm.nih.gov/BLAST	Comparison of nucleotide or protein sequences to GenBank sequence database. Statistical significance of matches calculated.
BioSeq File Format converter	http://bioinformatics.org/JaMBW/1/2/index.html	Conversion of nucleotide sequences to other formats including FASTA and GenBank.
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php	Identification of restriction enzyme sites along a DNA sequence, and prediction of fragment sizes.
NetPrimerLaunch	http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html	Primer analysis software for prediction of primer properties including, T_m, GC content, probability of primer-dimer/hairpin formation.
TESS	http://dot.imgen.bcm.tmc.edu/	Nucleic Acid Sequence Searches, to identify putative transcription factor binding sites.
Translate tool	http://www.expasy.org/tools/dna.html	Translation of a nucleotide (DNA/RNA) sequence to a protein sequence.

2.1.6 Chemical and Molecular Biology Reagents

Table 2-7: Chemical and molecular biology reagents used in this study

Supplier	Product/Code
Abcam	ChIP kit (ab500), Mouse IgG1, Rabbit Control IgG - ChIP Grade (ab46540), Smad3 antibody-ChIP Grade (ab28379), Smad4 antibody [SMD46 (same as DCS-46)] - ChIP Grade (ab3219).
Amersham	Rainbow Molecular Weight Markers (RPN800).
Amaxa GmBH	Cell Line Nucleofector Kit T (VCA-1002), Kit V (VCA-1003).
Applied Biosystems	Bcl-2-interacting killer (apoptosis-inducing) Assay (Hs00154189_m1), Silencer® Negative Control # 1 siRNA (5 nM) (AM4611), Silencer®BIK, si1989 1 siRNA (5 nM) (4390824), Silencer®BIK, si1990 1 siRNA (5 nM) (4390824), TaqMan(R) Gene Expression Assays, Inventoried Assay ID: Hs99999905_m1 GAPDH, Custom Silencer® Select Smad3 57 and Smad3 56 (5 nM) (4390827).
ASP	Presept Disinfectant Tablets (SPR25).
BD Biosciences	Annexin V-PE kit code 559763, FACS flow (342003). Bik, Purified Mouse Anti-Human Bik, 0.5 mg/ (557040).
BDH	Bromophenol blue (44385), EDTA (280254D), Glycine (444495D), Hydrochloric acid (28507BF), Isopropanol (296946H), Methanol (29192BL), Nitrocellulose membrane (436107E), Sucrose (102745C).
Bioline	SensiMix Probe One-Step Kit 250 rxns QT725-02, SensiMix SYBR no ROX One step kit 250rxn QT235-02.
Biosciences	Bis-AcrylaGel (EC-820), HiYield Gel/PCR DNA Fragment Extraction Kit (YDF100). PBS 1X (1490-094), RPMI 1640 Glutamax (61870-010).
Cambridge Biosciences	Goat F(ab')2 anti-human IgM (2022-01).***

Eppendorf	Perfectprep Gel Cleanup (955152000).
Fermentas	GeneRuler 100bp DNA Ladder Plus (SM0323), GeneRuler 1kb DNA Ladder Plus (SM1333), PageRuler Plus Prestained Protein Ladder (SM1811), ProteoJET mammalian cell lysis reagent (K0301), ProteoJET cytoplasmic and nuclear protein extraction kit (K0311), Restriction enzymes.
Invitrogen	1 Kb DNA ladder (15615-016), 100 bp DNA ladder (15628-019), Foetal calf serum (10270-106), HEPES (15630-056), Glutamax 1640 (61870-010), RPMI 1640 (31870-025), Trypan blue (15250-061), SYBR Gold Nucleic Acid Gel Stain (S11494), SYBR Safe DNA Gel Stain (S33102).
Labkem	Nitrocellulose Membrane (436107E).
Labscan	Chloroform (A3505E).
Lennox Chemicals	IMS (B0982).
Merck	Calcium chloride (23821000), Magnesium chloride (1058321000), Potassium Hydroxide (50321000).
Msc: Medical supply Co ltd	MMLVR (M1701), Rnasin (N2111), Anti-Mouse IgG (H+L), AP Conjugate (S3721).
Mirus	Ingenio™ Electroporation Kits & Solution 0.2 cm cuvette, for 50 reactions (MIR 50115).
National diagnostics	Acrylagel (EC810), Bis-acrylagel (EC820).
Millipore	Amicon Ultrafilter (UFC8 00508), Immobilon Western Chemiluminescent HRP Substrate (WBKLSOO50), SNAP i.d. Antibody collection trays (WBAVDABTR), SNAP i.d. Double well blot holders (WBAVDBH02), Transforming Growth Factor-β1, recombinant human (GF111).*
Oxoid	Agar (L13), PBS tablets (BR14), Tryptone (L42), Yeast extract (L21).
Pierce	SuperSignal® West Pico Chemiluminescent Substrate (34080).
Promega	dNTPs (U1330), Magnesium chloride 25mM (A3511), M-MLV reverse transcriptase & RT buffer (M1701), RNasin (N2111), Wizard® PCR Preps DNA purification system (A7170), CellTiter 96® Aqueous One solution Cell Proliferation Assay (MTS)(G35882).

Roche	Leupeptin (1017128).
Qiagen	EndoFree Plasmid Max kit (12362), Qiagen® Plasmid Purification Kit (12143), Quantitect ^{TM} SYBR® Green PCR Kit (204143).
Santa Cruz Biotechnology	Anti-Rabbit IgG, HRP Conjugate (sc-2030), stored in 4°C.
Sigma-Aldrich Chemical Co.	3M sodium acetate, pH5.2 (S7899), Agarose (A5093), Albumin from bovine serum (A7906-50G), Ampicillin (A9518), Aprotinin (A4529), Ammonium Persulfate (215589), Bovine serum albumin (A9647), Copper (II) Sulphate pentahydrate (31293), DEPC (D5758), DMSO (D8779), β-oestradiol (E8875), Glucose (G7528), Glycerol (G5516), L-glutamine (G7513), Manganese Chloride (M3634), Penicillin/streptomycin (P0781), PMSF (P7626), Ponceau S (P7170), Potassium chloride (P4504), potassium phosphate monobasic (p5655) Puromycin (P8833), RNase A (R6513), RNAZap (R2020), Sodium chloride (S3014), Sodium hydroxide (S5881), Sodium phosphate (S5136), Tetracycline (T7660), TEMED (T7024), Tri Reagent (T9424), 3,3',5,5'-Tetramethylbenzidine (TMB) (T0565), Triton® X-100 (T8787), Trizma® Base (T1503), Tween 20 (P1379), Water (Molecular Grade) (W4502), Mouse Anti-β-Actin A1978, Sodium Citrate (S1804). Phenol: chloroform: isoamyl alcohol (25:24:1) (P3803), 1α,25-Dihydroxyvitamin D3 10 µG (D1530)**.
VWR International limited	Transforming Growth Factor-β1, recombinant human (514-4131)*

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* Lyophilised human recombinant transforming Growth Factor-β1 (TGFβ1) was centrifuged prior to opening and reconstituted in water at a concentration of 0.1-1.0 mg/mL, and stored at -20°C to -80°C.

** The lyophilised powder of cholecalciferol (1α,25-Dihydroxyvitamin D3 10 µg) was centrifuged prior to opening and reconstituted in 100% ethanol at a concentration of 10 µM, and stored at -80°C.

*** Anti-IgM antibody was stored at 4˚C.

2.2 DNA Manipulation*

2.2.4 DNA Storage

DNA samples were stored in Tris-EDTA (TE) buffer (pH 8.0) at 4˚C. EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimises deamidation. DNA was also stored in sterile distilled H_2O (dH_2O) at -20˚C.

2.2.2 Phenol/chloroform extraction and ethanol precipitation

The standard method to concentrate nucleic acid samples, remove proteins or change the buffers in which a sample was dissolved is a phenol/chloroform extraction followed by ethanol precipitation. DNA partitions into the aqueous phase, denatured proteins collect at the interface and lipids partition into the organic phase. Nucleic acids are routinely recovered from aqueous solutions using ethanol precipitation in the presence of sodium acetate. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution, mixed by vortexing and centrifuged for 5 min at 13,000 x g at room temperature. The upper aqueous phase was removed, avoiding any material at the interphase, and placed in a sterile microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase (to remove any residual phenol), vortexed as before, and centrifuged for 5 min at 13,000 x g at room temperature. Again the upper aqueous phase was removed to a fresh tube. One-tenth volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA to aid precipitation, mixed and followed by 2 and a half volumes of 100 % (v/v) ethanol. This mixture was vortexed and incubated at -20˚C for a minimum of 20 min. When dealing with very small quantities of DNA, samples were precipitated in ethanol at -20°C overnight. The DNA samples were then centrifuged for 20 min at 12,000 x g at 4˚C, the supernatant was removed and pellets were washed with 300 µl 70 % (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 min at 10,000 x g, the supernatant was removed and pellets were air dried for approximately 10 min. Pellets were re-suspended in an appropriate volume of sterile TE (pH 8.0) or dH_2O and were stored at 4˚C or -20˚C respectively (Chomczynski,P. 1987).

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* Preparations of all solutions used in DNA manipulation are outlined in the Appendix.

2.2.3   Restriction digestion of DNA

Restriction enzymes bind and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence, which is known as the recognition site. Restriction digestion of DNA was carried out for identification purposes, plasmid linearization or to excise fragments from plasmid DNA. Restriction digestion patterns were predicted for DNA sequences using the NEBCutter or the WebCutter 2.0 bioinformatics tool (Table 2-6). All restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). Some enzymes required the addition of Bovine Serum Albumin (BSA) to the reaction to stabilize the enzyme, (working concentration 1X). DNA digestion reactions were performed according to manufacturer's instructions (New England Biolabs) and incubated for 3 h at the optimum enzyme temperature (between 37°C and 50°C, usually 37°C).

2.2.4   Preparation of competent cells

Competence is the ability of a cell to take up extracellular DNA from its environment. *E. coli* cells are more likely to incorporate foreign DNA if the cultures are treated to make them transiently permeable to DNA. In this regard, a modified rubidium chloride (RbCl$_2$) method was employed to prepare competent cells. *E. coli* JM109 cells were streaked from a glycerol stock on to a Luria-Bertani (LB) (Appendix) agar plate and incubated at 37°C overnight. An isolated colony was then picked using a sterile inoculating loop and used to inoculate 2.5 mL of LB broth, which was incubated at 37°C in a shaking incubator at 200 rpm overnight. This culture was then used to inoculate 250 mL of sterile LB broth supplemented with 20 mM MgSO$_4$. The 250 mL culture was incubated in a 1 L flask at 37°C until the optical density (O.D.) of the culture was between 0.4 and 0.8, at 640 nm (approximately 4-5 h). The cells were then transferred to two sterile 250 mL centrifuge tubes and collected by centrifugation at 4,500 x g, 4°C for 5 min. The cells were then gently re-suspended in 0.4 of their original volume in ice-cold TFB1 (Appendix) (100 mL for 250 mL culture). Cells were kept on ice for all subsequent steps and pipettes, tubes and flasks were chilled. The re-suspended cells were then incubated on ice at 4°C for 5 min followed by centrifugation at 4,500 x g at 4°C for 5 min. Cells were then gently re-suspended in 1/25th of the

original volume of ice-cold TFB2 (Appendix) (10 mL for a 250 mL culture). These cells were then dispensed into 100 μL aliquots and incubated on ice for 1 h. Prior to storage, the aliquoted cells were snap frozen in a dry ice/isopropanol bath. *E. coli* JM109 competent cells prepared by this method and stored at -80˚C are stable for 1 year (Sambrook J, Russell DW 2001).

2.2.5   Transformation of *E. coli* with plasmid DNA

A 95 μL aliquot of competent cells was placed in a pre-chilled microfuge tube containing 5 μL DNA (~100 ng/10 μL). The contents of the tube were mixed gently by flicking and incubated on ice for 30 min, during which time an aliquot of SOC medium (Appendix) was pre-heated to 37˚C. After 30 min on ice the cells were heat-shocked in a water bath at 42˚C for 55-65 s, and were then removed immediately to ice for a further 2 min. A 900 μL aliquot of preheated SOC was added to the cells which were then incubated at 37˚C in a shaking incubator for 1 h 30 min. The cells were then collected by centrifugation for 3 min at 6,000 x g, following which 900 μL of supernatant was removed and discarded. The cells were re-suspended in the remaining supernatant and plated out, along with the controls, on LB plates containing antibiotic at the appropriate concentration, and incubated overnight at 37˚C. Plasmids carrying the antibiotic resistance gene confer this resistance to the transformed cells and thus only transformed cells will yield colonies. These colonies were subsequently used to prepare broth cultures for DNA mini-preparations (Sambrook J, Russell DW 2001).

2.2.6   Small scale preparation of plasmid DNA (Miniprep)

A single bacterial colony was used to inoculate 5 mL of LB medium containing the appropriate antibiotic and incubated with shaking (200 rpm) overnight at 37˚C. An aliquot (1.5 mL) of this culture was transferred to a sterile microfuge tube and centrifuged for 30 s at room temperature; the remainder was stored at 4˚C. The supernatant was removed from the tube using a pipette tip, leaving the cells as dry as possible. The cells were then re-suspended in 100 μL of solution I (Appendix) by vortexing. Two hundred microlitres of freshly prepared lysis solution [solution II

(Appendix)] was then added, and the tube contents were mixed by inverting the tube rapidly a number of times. Ice-cold neutralisation solution III (150 μL; Appendix) was added to stop the reaction and the tubes were vortexed gently for 10 s. The lysate was centrifuged for 5 min at 12,000 x g, and the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate. A phenol chloroform extraction and ethanol precipitation was carried out as described previously in Section 2.2.2. The pellet was air-dried, then re-suspended in 20 μL of dH$_2$O. One microlitre of DNase-free RNase A (20 μg/mL) was also added, and the mixture was vortexed, incubated at 37°C for 1 h and then stored at 4°C (Adapted from Maniatis *et al*, 1982).

2.2.7 Glycerol Stocks

Glycerol stocks enable long term storage of bacteria, since glycerol prevents water in the broth from forming ice crystals that can puncture and kill the bacterial cells. Glycerol stocks of bacterial cultures were prepared by adding 0.5 mL of a 50% (v/v) glycerol solution to 0.5 mL of the overnight bacterial culture of interest, mixing and storing at -80°C for future use.

2.2.8 Qiagen® plasmid DNA purification protocol (Midiprep)

Plasmid DNA was isolated and purified using the Qiagen® Plasmid Midi Kit. The process of plasmid isolation involves the lysis of the cells by a treatment with lysozyme followed by SDS which denatures proteins after the lysis of the membranes. The precipitation of the SDS-protein complex with high-molarity salt specifically subtracts the chromosomal DNA from the supernatant, since it remains attached to the mesosome. A glycerol stock of the bacteria of interest was streaked on an LB agar plate (containing the appropriate antibiotic) and incubated overnight at 37°C. An isolated colony from this plate was used to inoculate a 5 mL starter culture (with the appropriate antibiotic) and incubated in a shaking incubator at 225 rpm 37°C for 8 h. 100 μL of the starter culture was used to inoculate 50 mL of LB (containing the appropriate antibiotic) in a 250 mL sterile flask and incubated overnight in a shaking incubator at 37°C. It was important that the O.D. of the culture was between 1 and 1.5 at 600 nm. The bacterial

culture was transferred to a centrifuge tube and the bacterial cells were harvested by centrifugation at 6,000 x g for 15 min at 4˚C using a JA-20 rotor in a Beckman centrifuge. The supernatant was decanted and the cells were re-suspended completely in 4 mL of Buffer P1 containing RNase A (100 μg/mL). The bacterial cells were then lysed by addition of 4 mL Buffer P2 and incubation at room temperature for 5 min. Following incubation, 4 mL of pre-chilled Buffer P3 was added (to precipitate genomic DNA, protein, cell debris and SDS), mixed gently by inverting the tube 5-6 times and incubated on ice for 15 min. The mixture was then centrifuged for 1 h 30 min at 16,000 x g at 4˚C and the supernatant saved. The Qiagen-tip 100 was equilibrated by applying 4 mL of QBT buffer and allowing the column to empty by gravity. The column does not dry out at this stage, as the flow of buffer will stop when the buffer reaches the upper filter. Following equilibration, the supernatant from the previous centrifugation step was applied to the filter and allowed to flow through. The Qiagen-tip was washed with 2 x 10 mL of Buffer QC. DNA was then eluted with 5 mL of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol. The DNA/isopropanol mixture was then aliquoted and centrifuged immediately at 13,000 x g for 45 min at 4˚C and the supernatant was carefully removed using pipettes. The resulting pellet was washed with 70% (v/v) ethanol (13,000 x g for 15 min at 4˚C), allowed to air dry for 5 min and re-dissolved in 30 μL of TE or dH$_2$O. DNA was then quantified by spectrophotometric analysis, and the quality of the DNA was checked by agarose gel electrophoresis (Adapted from the manufactures protocol Qiagen® Plasmid Purification Handbook, 2005).

2.2.9   Qiagen® EndoFree plasmid purification protocol (Maxiprep)

Endotoxins are frequent contaminants in plasmid DNA prepared from bacteria. In order to improve transfection efficiency, endotoxin-free plasmid DNA was isolated and purified using the Qiagen® EndoFree Plasmid Midi Kit from Promega according to the manufacturers protocol (EndoFree® Plasmid Purification Handbook, 2005). Endotoxin-free DNA was then quantified by spectrophotometric analysis, and the quality of the DNA was checked by agarose gel electrophoresis.

2.2.10 Determination of Nucleic Acid Sample Concentration

The concentration of isolated nucleic acids can be determined spectrophotometrically. Nucleic acids absorb UV light maximally at an abosorbance of 260nm, thus optical absorbance can be used as an accurate measurement of its concentration. Pure DNA at 50 μg/mL in aqueous solution has an A_{260} of 1, while 40 μg/mL of pure RNA also has an absorbance reading of 1 at this wavelength. Absorbance is also useful as a measure of the purity of DNA. The ratio of A_{260}/A_{280} of a pure dsDNA preparation should be between 1.65 and 1.85. Higher values are often due to RNA contamination and lower values to protein and phenol contamination. Pure DNA and RNA have A_{260}/A_{280} ratios of 1.8 and 2.0 respectively. Absorbances were read on the Shimadzu UV-160A spectrophotometer using a quartz cuvette and nucleic acid concentrations were determined according to the following equations:

Concentration of DNA (μg/μL) $= \dfrac{\text{Absorbance (260 nm) x 50 x dilution factor}}{1000}$

Concentration of RNA (μg/μL) $= \dfrac{\text{Absorbance (260 nm) x 40 x dilution factor}}{1000}$

The NanoDrop® ND-1000 Spectrophotometer was also used to determine nucleic acid sample concentrations. An undiluted 1 μL sample was pipetted onto the end of a fibre optic cable (the receiving fibre). A second fibre optic cable (the source fibre) was then brought into contact with the liquid sample, causing the liquid to bridge the gap between the fibre optic ends. (The gap is controlled to both 1 mm and 0.2 mm paths by the computer). A pulsed xenon flash lamp provided the light source and a spectrometer was used to analyse the light after passing through the sample. The instrument is controlled by special software run from a PC, and the data was logged in an archive file on the PC.

2.2.11 Agarose gel electrophoresis of DNA

DNA has a negative charge in solution, and the magnitude of this charge is relative to the DNA fragment length. DNA will migrate to the positive pole in an electric field and thus DNA fragments can be separated based on size by passing through a porous gel under an electrical current. This is the basis for agarose gel electrophoresis and it is the standard method used to separate, identify, and purify DNA fragments. The appropriate quantity of agarose was added to 100 mL 1X TAE buffer based on the percentage agarose gel required. Increasing the percentage agarose (1.8-2.0%) in the gel was generally used to improve resolution of smaller DNA fragments while separation of larger DNA molecules was best achieved by using lower percentage gels (0.7-1.0%). The agarose was completely dissolved by boiling with intermittent mixing and after sufficient cooling (~60°C) the gel was cast into the Hybaid horizontal gel electrophoresis system. A comb was inserted for formation of the wells. The gel was left to polymerise before filling the chamber with 1X TAE and removing the comb. Sample buffer, containing bromophenol blue as a tracking marker (Appendix), was added to each sample before loading up to 20 μL of the sample containing the nucleic acid per well. DNA sample buffer was also added to 500 ng of a 1 Kb or 100 bp ladder, which was loaded as a size marker. The gel was run at constant voltage (5 V/cm, usually 100V), for 1 to 2 h. After completion, the gel was stained in ethidium bromide (0.5 mg/mL) for 30 min, destained in dH_2O for 15 min and viewed under UV illumination (Sambrook J, Russell DW 2001).

2.2.12 Eppendorf Perfectprep® Gel Cleanup

Extraction and purification of dsDNA fragments from TAE agarose gels was achieved using the Eppendorf Perfectprep® Gel Cleanup kit following the manufacturers' protocol (Perfectprep® Gel Cleanup Manual, 2002). The purified DNA was eluted in 30 μL of molecular biology grade water and stored at 4°C or -20°C.

2.2.13 DNA Sequencing

Sequencing of plasmid DNA was performed to ensure the nucleic acid fidelity and 'in-frame' insertion of DNA fragments following cloning. Plasmid DNA as prepared in Section 1 was quantified (Section 1) and 1-2 μg (typically 1-2 μL) was transferred to a sterile 1.5 mL microfuge tube. The DNA was lyophilised in a Savant DNA110 speed vac on the low heat setting for 15-20 min or until the solvent had evaporated. The lyophilised DNA was sent to MWG-Biotech, Ebersberg, Germany for sequencing. Sequencing forward and reverse primers were supplied by MWG-Biotech for most commercially available cloning systems. In the absence of existing sequencing primers (as in the case of the pRTS-1 vectors), a forward and/or reverse primer was designed and purchased and 100 pmol of primer was transferred to a sterile microfuge tube and sent with each sequencing reaction to MWG-Biotech. Sequencing results were obtained electronically as linear nucleotide sequences.

2.3   Cell Culture Methods

All cell culture techniques were performed in a sterile environment using a Holten laminar flow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

2.3.1   Culture of cells in suspension

All media compositions and media supplements are given in the Appendix. The cell lines BJAB, ER/EB 2-5, and Ramos were maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin. Cultures were seeded at a density of 2 x 10^5 to 5 x 10^5 cells per mL in 25 cm^2 flasks and expanded in 75cm^2 flasks. Cells were sub-cultured two or three times per week by harvesting the cells into a sterile centrifuge tube and centrifuging at 1000 x g for 5 min at room temperature. The cells were gently re-suspended in a suitable volume of fresh supplemented media and replaced into the tissue culture flask. All cell lines were incubated in a humid 5% CO_2 atmosphere at 37˚C in a Heraeus cell culture incubator.

2.3.2   Media supplements

Supplements were added to the growth media of certain cell lines either to select cells containing transfected plasmids, activate the function/or induce expression of a gene of interest or to improve cellular proliferation. The oestrogen-responsive ER/EB2-5 cell line was maintained in supplemented medium containing 1 μM oestrogen. To abolish functional EBNA-2 activity, ER/EB2-5 cells were washed 4 times in PBS, with a further 2 washes 24 h later and replaced in estrogen-free supplemented medium.

2.3.3   Cell counts

Cell counts were carried out using an improved Neubauer haemocytometer slide. Trypan blue exclusion dye was used to establish cell viability. A volume of 10 μL of trypan blue was added to 90 μL of a cell suspension and mixed. A sample of this mixture was added to the counting chamber of the haemocytometer and cells were visualised by light microscopy. Viable cells excluded the dye and remained clear while dead cells stained blue. Cell numbers were determined by multiplying the average cell count (of 3-5 individual counts) by the dilution factor (usually 1.1) and again by the volume of the haemocytometer chamber (1×10^4 cells/mL). Thus, cell counts were expressed as the number of cells per mL.

2.3.4   Cell storage and recovery

In order to prepare stocks of suspension cells for long-term storage, 1×10^7 cells in exponential phase were centrifuged, the excess media was discarded and the cells were re-suspended in 750 μL of supplemented RPMI to which 150 μL of FBS was added, and then placed on ice for 10 min. DMSO was added to a final concentration of 10% (v/v), mixed gently and transferred to a sterile cryotube. In the case of adherent cells, one confluent 75 cm^2 flask of cells was used per cell stock. Adherent cells were washed with 1X PBS, followed by trypsinisation and re-suspended in 900 μL of FBS and 100 μL of DMSO. The cells were mixed gently and added to a sterile cryotube. The cryotubes were slowly lowered into the gas phase of liquid nitrogen and then immersed in liquid nitrogen in a cryofreezer (Cooper Cryoservices). Cells were recovered from liquid nitrogen by thawing rapidly at 37˚C and transferring to a sterile centrifuge tube containing 5 mL of pre-warmed supplemented media. The cells were centrifuged at 100 x g for 5 min, then re-suspended in 5-10 mL of fresh supplemented media, transferred to a culture flask and incubated at 37˚C in 5% CO_2.

2.4   Transient transfections

Transient transfection of cells was performed by electroporation or nucleofection. In all cases, cells were seeded at a density of 2-5 x 10^5 per mL of media 24 h prior to transfection. After this time in culture, cells were counted again as it was essential for cell numbers to have almost doubled before beginning the transfection, thus ensuring that cell growth was in logarithmic phase, allowing for optimal DNA uptake during transfection. The same quantity of total DNA was used per transfection.

2.4.1   Electroporation of B lymphocytes

Transfection of Ramos and BJAB cell lines was carried out by electroporation. During the electroporation method of transfection, the application of brief high voltage electric pulses to the cells leads to the formation of nanometre-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of redistribution of membrane components that accompanies the closure of the pores. Total DNA (10-20 μg per transfection) dissolved in 30 μL TE buffer (pH 8.0) was transferred into a sterile electroporation cuvette. A total of 1 x 10^7 cells were used per transfection. Cells were centrifuged at 100 x g for 5 min and the supernatant discarded. The cells were then washed in PBS, centrifuged at 100 x g for 5 min and the supernatant again discarded. For each 1 x 10^7 cells, 220 μL of serum-free media was used for re-suspension and the cells transferred to the DNA-containing electroporation cuvettes. The cell/DNA mix was incubated at room temperature for 5 min. Each cell/DNA mix was then pulsed at 220-250 V (BJAB, 250 V and Ramos 220 V) with a capacitance of 960 μF (using a capacitance extender) and resistance set to infinity using a Biorad Gene Pulser. Immediately after electroporation the cuvettes were placed on ice until the cell/DNA mix was transferred into 5 mL supplemented media in a 6 well dish, and the contents were mixed by gently rocking back and forth. Care was taken to ensure that cells were stored no longer than 10 min after electroporation to avoid reduction in cell viability/gene transfer efficiency. Transfected cells were harvested 48 h later.

2.4.2 Nucleofection of B lymphocytes

The Nucleofector technology is based on electroporation. It consists of the Nucleofector device II (Amaxa), which delivers a cell-specific combination of electrical parameters, and Nucleofector Solutions, in which the cells are contained while the unique electrical program is executed. Optimised conditions for nucleofection of BJAB cells yielded higher percentage transfection efficiency, when compared to cells transfected using the electroporation technique and Ingenio transfection buffer. The Nucleofector solution specific to the particular cell line under investigation (Solution T/V for BJAB/IB4) was pre-warmed to room temperature and 50 mL of supplemented media was pre-warmed to 37ºC. Supplemented medium (2 mL) was aliquoted into the appropriate number of wells of a 6 well plate and pre-incubated at 37˚C/5% CO_2/humidified atmosphere. A total of ~7 μg DNA was prepared in sterile 1.5 mL microfuge tubes for each sample. For each transfection, 2.5×10^6 (IB4)/ 5×10^6 (BJAB) cells were collected by centrifugation at 90 x g for 10 min and the supernatant was discarded completely so that no residual medium remained. The cells were resuspended in room-temperature solution in question to a final concentration of 5×10^6 /100 μL (BJAB). Care was taken to ensure cells were stored in Nucleofector solution for no longer than 15 min to avoid reduction in cell viability/gene transfer efficiency. A volume of 100 μL of cell suspension was mixed with the DNA and the nucleofection sample was transferred to an Amaxa-certified cuvette, taking care to avoid bubbles. The cuvette was placed in the nucleofection chamber and pulsed on program T-016 (BJAB) or O-006 (Ramos). Immediately after this, 500 μL of pre-warmed medium was added to the cuvette. The contents were then gently transferred to the prepared 6-well plates, mixed gently and incubated at 37˚C 5% CO_2 humidified atmosphere until cells were harvested (Parametes used here are obtained from Lonza's Optimized Protocols for the Amaxa™Nucleofector™ Technology). Electroporation conditions were optimized using a protocols on the Gene Pulser MXcell electroporation system (Bio-Rad).

2.5 Flow cytometric analysis

Flow cytometry may be defined as a technology to measure properties of cells as they move, or flow, in liquid suspension. Most flow cytometers can measure two kinds of light from cells, light scatter and fluorescence. Light scatter is the interaction of light and matter. All materials, including cells, will scatter light. In the flow cytometer, light scatter detectors are located opposite the laser (relative to the cell), and to one side of the laser, in-line with the fluid-flow/laser beam intersection. The measurements made by these detectors are called forward light scatter and side light scatter, respectively. Forward light scatter provides information on the relative size of individual cells, whereas side light scatter provides information on the relative granularity of individual cells. Fluorescence is the property of a molecule to absorb light of a particular wavelength and re-emit light of a longer wavelength. The wavelength change relates to an energy loss that takes place during the process. BD FACSCalibur and BD FACSAria-II cell sorter instruments were used for these experiments and FLOWJO version 7.6.5 was used for fcs raw data analysis (According to BD Biosciences technical book).

2.5.1 Annexin V/ vital dye staining

In normal live cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, Annexin-V, is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS and can be used to identify apoptotic cells by binding to PS. Fluorescent dyes that bind to nucleotides and penetrate only damaged cellular membranes were used (vital dyes). Intercalation complexes are formed by propidium iodide or 7-amino-actinomycin (7AAD) with double-stranded DNA, which affects amplification of the fluorescence.

2.5.2   AnnexinV-PE/7AAD

The Annexin-PE Apoptosis Detection Kit (Becton Dickinson) contains recombinant Annexin-V conjugated to the fluorochrome, phycoerythrin (PE) and the vital dye 7AAD. Viable cells with intact membranes exclude 7AAD, whereas the membranes of dead and damaged cells are permeable to 7AAD. Cells that stain positive for Annexin-V-PE and negative for 7AAD are undergoing apoptosis. Cells that stain positive for both Annexin-V-PE and 7AAD are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both Annexin-V-PE and 7AAD are alive and not undergoing measurable apoptosis. For experiments performed here apoptotic cells include the sum of the cells in the upper right and lower right quadrants of flow cytometry plot of Annexin-V-PE vs 7AAD. To perform the assay, cells were harvested and washed twice in ice-cold PBS. The cells were re-suspended in 1X Annexin-binding buffer at a concentration of ~1 x 10^6 cells/mL. A volume of 5 μL of Annexin-V-PE solution and 5 μL of 7AAD solution was added to 100 μL of cell suspension. The cells were incubated at room temperature for 15 min in the dark and 400 μL of 1X Annexin-binding buffer was then added and the samples were analysed within 1 h by flow cytometry (According to the BD Biosciences Apoptosis assay kit as outlined in the product technical data sheet).

2.5.3   Cell Viability Determination using MTS Assay

An MTS assay was performed according to manufacturer's instructions (Promega). The principle behind this assay is that the MTS compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoyxphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt] is provided to cells for a fixed amount of time. The compound is reduced to formazan by live cells at a rate proportional to the number of cells present. The formazan can be detected by its absorbance at 495 nm. Changes in cell viability would be detectable by changes in absorbance detection at 495 nm.

2.6 RNA Analysis

2.6.1 RNase-free environment

Because RNA is easily degraded by ubiquitous RNases, standard procedures were employed to avoid this potential hazard (Sambrook *et al*, 1989). Prior to working with RNA, any apparatus or surfaces to be used were treated to remove RNase. The apparatus/surface was washed in detergent and rinsed well in DEPC-treated H_2O then in 100% (v/v) ethanol and finally allowed to air dry. Any apparatus to be used were also immersed in a 3% (v/v) solution of hydrogen peroxide (Sigma-Aldrich) for 15 min, and were then rinsed thoroughly in DEPC-treated upH_2O and allowed to dry. Because hands are a major source of RNase contamination, gloves were used at all times and changed frequently.

2.6.2 RNA analysis by gel electrophoresis

In order to examine RNA transcripts/ascertain the integrity of RNA, isolated samples were run on 1.5% (w/v) agarose gels. The appropriate amount of agarose was dissolved in DEPC-treated H_2O and prepared according to Section 2.2.13 The RNA samples (1 µL) were prepared for electrophoresis by adding 3 µL of RNA sample buffer (Appendix) and made up to 15 µL in DEPC-treated H_2O. The samples were heated to 65 °C for 10 min prior to loading on the gel. The gel was run in 1X TAE as described in Section 2.2.13. As ethidium bromide is included in the RNA sample buffer, the gels did not require further staining and could be visualised directly on a UV trans-illuminator.

2.6.3 RNA extraction from cultured cells

Prior to RNA isolation, the condition of cells was reviewed by phase contrast microscopy. A cell count was performed as described in Section 2.3.3.

2.6.4 RNA extraction using Tri-reagent

Cells grown in suspension were centrifuged at 100 x g for 5 min, washed once in PBS and the cells were lysed by repeat pipetting in Tri Reagent (Sigma-Aldrich). A volume of 1 mL of Tri reagent was used per 1×10^7 cultured cells. The lysate was left at room temperature for 5 min, after which time the procedure could be halted by storing samples at –80 ˚C. Phase separation was achieved by adding 200 μL of chloroform per 1 mL of lysate. The samples were covered and shaken gently but thoroughly for 15 s or until completely emulsified. Samples were incubated at room temperature for 15 min. The resulting mixture was centrifuged at 13,000 x g for 20 min at 4 ˚C. During centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase, which contained the RNA, was removed to a fresh tube and RNA was precipitated by addition of 500 μL of ice-cold isopropanol per mL of Tri reagent used initially. The samples were incubated for 10 min on ice and then centrifuged at 13,000 x g for 15 min at 4 ˚C. The resulting RNA pellet was washed using 1 mL of 75% (v/v) ethanol by inverting the tube 5 times. The pellets were then centrifuged at 13,000 x g for 5 min at 4 ˚C, and the 75% (v/v) ethanol was aspirated. Pellets were air dried and dissolved in DEPC-treated upH$_2$O. The resulting RNA preparation was heated at 60˚C and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric (Section 2.2.10) and gel electrophoretic analysis (Section 2.7.2) and the remainder of the purified RNA was stored at – 80 ˚C (Rio,D.C. 2010).

2.6.5 Total RNA isolation from cells using QIAgen RNeasy™ kit

RNA was extracted from small numbers of cultured cells ($< 5 \times 10^5$) using an RNeasy kit (QIAgen) according to the manufacturer's protocol (RNeasy Mini Handbook, 2006). An aliquot of purified RNA was removed for spectrophotometric (Section 2.2.10) and gel electrophoretic analysis (Section 2.7.2) prior to RT-qPCR.

2.6.6   Quantification of mRNA from cultured cells by RT-qPCR

The quantification of mRNA purified from cultured cells was performed in a two-step procedure. In the first step, cDNA was prepared from RNA by reverse transcription (RT) with random hexamers serving as primers. During the second step, cDNA was amplified by real time quantitative PCR (qPCR). Real-time qPCR is increasingly being adopted for RNA quantification based on its ability to detect the amount of PCR product present at every cycle (i.e. in real time), as opposed to the endpoint detection by conventional PCR methods, thus allowing the real-time progress of the reaction, especially its exponential phase, to be viewed. The real-time PCR approach is based on the detection and quantification of a fluorescent reporter, where the signal increases in direct proportion to the amount of PCR product in a reaction. TaqMan gene expression assays were used in this study for comparative gene expression analysis, normalising with *gapdh* endogenous control mRNA levels (Applied Biosystems, Table 2-9).

2.6.7   Reverse transcription

In this process mRNA was transcribed into cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. Initially, 2 μL of random hexamers was added to 2 μg RNA and the volume brought up to 10 μL with DEPC H_2O. The mixture was heated to 70˚C for 5 min, to destabilise secondary mRNA structures, and then placed on ice. Then, the RT reactants were added in the order listed in Table 2-8.

Table 2-8: MMLV RT reactants

Component	Volume
Reverse Transcriptase buffer (5X)	8 μL
dNTP mix (20 mM)	1 μL
$MgCl_2$ (25 mM)	4 μL
BSA (4 μg/μL)	1 μL
DEPC H_2O	13 μL
RNasin ribonuclease inhibitor	2 μL
M-MLV reverse transcriptase (200 U/μL)	1 μL

The reactions were placed in a Hybaid thermocycler at 37˚C for 1 h and 95 ˚C for 2 min followed by storage at 4˚C.

2.6.8 Reverse transcription quantitative PCR (RT-qPCR)

The cDNA generated by RT was quantified by real time PCR using the TaqMan gene expression assays (Applied Biosystems), which consisted of two unlabelled primers for amplifying the sequence of interest (final concentration of 900 nM each) and one dual labelled TaqMan MGB probe (6-FAM dye- and TAMRA-labelled) for detecting the sequence of interest (final concentration of 250 nM). The PCR reaction exploits the 5'-3' nuclease activity of the DNA polymerase system to cleave a TaqMan probe during PCR as illustrated in (Figure 2-1). Quantification of a cDNA target was normalised for differences across experiments/samples using an endogenous control as an active reference (*gapdh*). The PCR reaction mix was prepared for each sample (in triplicate) by addition of the reagents listed in (Table 2-9) to the individual wells of a 96-well reaction plate (Applied Biosystems) followed by the addition of 2 μL of cDNA to give a total reaction volume of 25 μL.
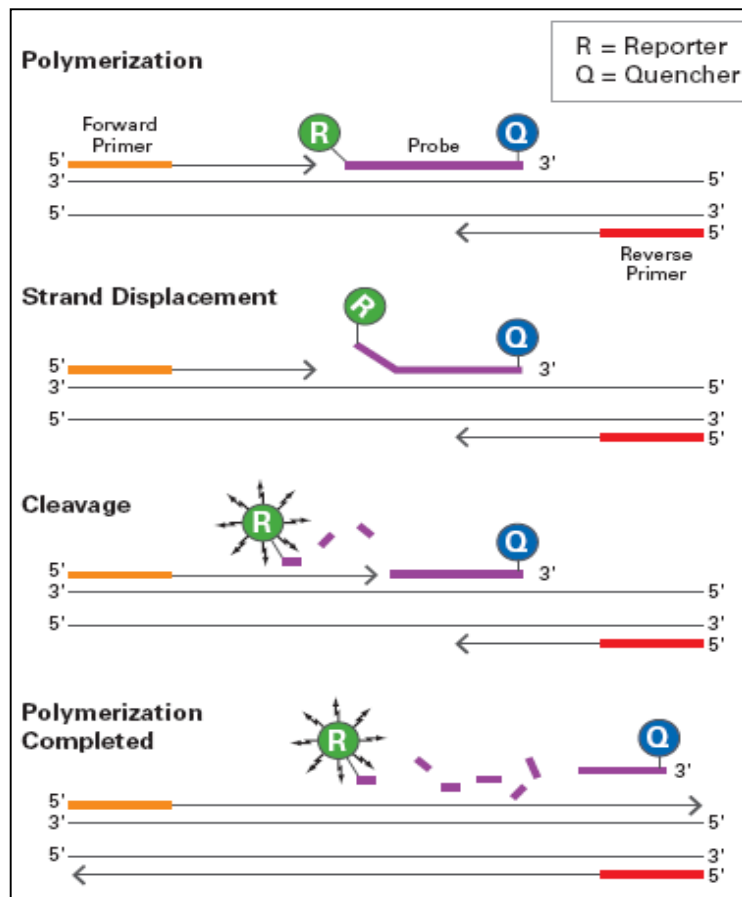
Figure 2-1: The TaqMan assay

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Lakowicz, 1983). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'–3' nucleolytic activity of the DNA polymerase system cleaves the probe between the reporter and the quencher only if the probe hybridises to the target. The probe fragments are then displaced from the target, and polymerisation of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any non-specific amplification is not detected (Adapted from Applied Biosystems White Paper on TaqMan gene expression assays http://docs.appliedbiosystems.com/pebiodocs/00106737.pdf).

Table 2-9: Real time qPCR reactants

Component	Volume/Reaction
Assay	1.25 μL
2X TaqMan PCR master mix*	12.5 μL
Nuclease Free H$_2$O	9.25 μL
Total	23 μL

The plate was covered with an optical adhesive cover and centrifuged at 2,000 x g for 2 min to eliminate air bubbles. Amplification and detection were performed with an ABI Prism 7500 sequence detection system under the following conditions: 10 min at 95 °C to activate AmpliTaq Gold DNA polymerase, and 45 cycles of 15 s at 95°C and 1 min at 60°C. During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analysing fluorescence emissions. The reporter dye (FAM) signal was measured against the internal reference dye (ROX) signal to normalise for non-PCR-related fluorescence fluctuations occurring from well to well. The threshold cycle represented the refraction cycle number at which a positive amplification reaction was measured and was set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15. The results were analysed according to the Comparative C$_T$ method ($\Delta\Delta$C$_T$) as described by (Livak and Schmittgen, 2001).

2.7 Protein Analysis

2.7.1 Preparation of cellular proteins

Proteins were isolated from both suspension and adherent cells for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by western blotting. Prior to isolation, cells were washed in PBS. Suspension cells were centrifuged at 100 x g for 5 min and the supernatant removed. Ice-cold PBS (10 mL) was added; the cells were centrifuged again at 100 x g and all of the supernatant removed. Adherent cells were washed twice with 10 mL of ice-cold PBS, and the cells were scraped into 1 mL of PBS. The crude cell suspension was centrifuged at 100 x g for 5 min. For both suspension and adherent cells, the cells were re-suspended in ice-cold suspension buffer (Appendix) using 200 μL of suspension buffer for every 1 x 10^7 cells and the cell suspension transferred to a microfuge tube. An equal volume of 2X SDS gel loading buffer (Appendix) was immediately added to the cell suspension, after which the sample became extremely viscous. The sample was then subjected to sonication for 1 min on full power (using a Vibra Cell Sonicator) to shear the DNA. The lysate was clarified by centrifugation at 500 x g for 7 min at 4°C. The supernatant (cytoplasmic protein extract) and the pellet (nuclei pellet) were aliquoted and stored at –70 ˚C until required for analysis. The ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit was also used according to the manufacturer's instructions.

2.7.2 SDS-polyacrylamide gel electrophoresis of proteins

During polyacrylamide gel electrophoresis (PAGE), proteins are driven by an applied current through a polyacrylamide gel. PAGE is carried out in the presence of the negatively charged detergent sodium dodecylsulphate (SDS), which binds to all types of protein molecules. Electrostatic repulsion between the bound SDS molecules causes the proteins to unfold into a similar rod-like shape, and since the amount of SDS bound is proportional to the molecular weight of the polypeptide and is sequence independent, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

2.7.3 Preparation of SDS-polyacrylamide gels

SDS-PAGE was performed using 10% (v/v) resolving gels and 5% (v/v) stacking polyacrylamide gels prepared as detailed in Appendix. An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water, then with dH_2O and finally wiped in one direction with tissue soaked in 100% (v/v) ethanol. The gasket was placed around the ridged plate; the plates were assembled and secured with clamps. The resolving gel was then poured to within 2 cm of the top of the larger plate and overlaid with 100% (v/v) ethanol. When set, the ethanol was removed and the stacking gel was poured. A clean comb that had been wiped in 100% (v/v) ethanol was inserted and the gel was allowed to polymerise for at least 20 min. The electrophoresis tank was filled with 1X Tris-glycine running buffer (Appendix) to a level of about 5 cm deep. After polymerisation, the gaskets and clamps were removed and the pre-poured gels were lowered into the buffer at an angle to exclude air bubbles from the gel-buffer interface. The tank was completely filled with 1X running buffer and the comb removed from the gel. Un-polymerised gel was removed by gently rinsing the wells with 1X running buffer and the wells were then straightened using a loading tip. The gel plates were fixed firmly in place with the pressure plates. The chamber formed by the inner plates (notched plate facing inwards) was filled with 1X running Buffer, the samples were loaded and the electrodes attached. The gels were electrophoresed at a constant current of 30 mA per gel until the blue dye front had reached the bottom of the gel. When complete, the plates were removed, separated and the gel was placed in transfer buffer prior to Western blotting (Section 2.8.4) (Protocol is derived from cold spring harbour protocols).

2.7.4 Western blotting

During western blotting, electrophoretically separated proteins were transferred from the polyacrylamide gel to a solid support, usually a nitrocellulose membrane, and probed with antibodies that reacted specifically with antigenic epitopes displayed by the target protein attached to the solid support. The bound antibody, unless conjugated, was detected by a secondary immunological reagent, conjugated to either the alkaline phosphatase (AP) or horseradish peroxidase (HRP) enzyme for detection.

Transfer of protein to nitrocellulose filters

Following gel electrophoresis, gels were equilibrated in transfer buffer (Appendix) for at least 15 min. Equilibration facilitated the removal of electrophoresis salts and detergents. Salts, if not removed, increase the conductivity of the transfer and the amount of heat generated during transfer. Nitrocellulose membrane was cut to the dimensions of the gel, along with 6 pieces of 3 mm filter paper that were required for the gel/membrane sandwich. Protein transfer was carried out on the Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell. A pre-soaked sheet of filter paper was placed onto the platinum anode. A pipette was rolled over the surface of the filter paper to exclude all air bubbles. This step was repeated with two more sheets of filter paper. Then, the pre-wetted blotting membrane was placed on top of the filter paper and all bubbles rolled out. The equilibrated gel was carefully placed on top of the nitrocellulose membrane, aligning the gel on the centre of the membrane. Any air bubbles were again rolled out. Another 3 sheets of pre-wetted filter were placed on top of the gel, with care taken to remove air bubbles. The cathode was placed on top of the stack and the safety cover replaced on the transfer unit. Gels were transferred for 30 min at 17 V (Protocol is derived from cold spring harbour protocols).

Staining of proteins immobilised on nitrocellulose filters

Ponceau S is a negative stain, which binds to positively charged amino acid groups of proteins. It also binds non-covalently to non-polar regions of proteins. Ponceau S staining was employed to determine whether uniform transfer of proteins to the nitrocellulose membrane had taken place. Transferred protein was detected as red bands on a white background. This staining technique is reversible to allow further immunological analysis. Following electrophoretic transfer, the nitrocellulose membrane was immersed in 20 mL Ponceau S solution (Sigma-Aldrich) and stained for 5 min with constant agitation. After proteins were visualised, the membrane was washed in several changes of dH_2O until all the stain had been washed away. The membrane was then used for immunological probing.

Immunological probing

Following Ponceau S staining, the membrane was incubated in blocking buffer (Appendix) for 3 h at room temperature followed by incubation with the appropriate primary antibody (diluted in blocking buffer, see Table 2-10) at 4 ˚C overnight.

Table 2-10: Incubation Conditions for Antibodies Used in Western Blotting

Primary Antibody	Name	Dilution In Blocking Buffer	Secondary Antibody	Dilution In Blocking Buffer
Anti-β-Actin	Anti-actin AC-15	1/10,000	AP-conjugated Anti-mouse IgG	1/20,000
Anti-Bik	Clone C33-1 (557040)	1/200	Anti-Goat-HRP	1/5000
Anti-Smad3	Ab28379	1/500	Anti-Rabbit-HRP	1/5000
Anti-EBNA-2	PE2	1/100	AP-Conjugated Anti Mouse IgG	1/5000
Anti-Smad4	Ab3219	1/500	AP-Conjugated Anti Mouse IgG	1/5000
Anti-GAPDH	(FL-335): sc-25778	1/5,000	Anti-Rabbit-HRP	1/10,000

After overnight incubation, the membrane was washed three times in TBS-T (0.1% (v/v) Tween-20 in TBS (Appendix) for 15 min. The filter was then incubated with the appropriate secondary antibody (Table 2-10) for 1 h 30 min at room temperature, followed by three 15 min washes in TBS-T. (Conjugated primary antibodies were developed following three washes in TBS-T, and needed no further incubation step). All of the above incubations were carried out with constant agitation. The membrane was then placed in a clean container and covered with 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT, Sigma) or 3,3',5,5'-tetramethylbenzidine (TMB, Sigma), which are used for the colourimetric detection of alkaline phosphatase- or hydrogen peroxidase-conjugated molecules respectively. When

incubated with alkaline phosphatase or hydrogen peroxidase, bound enzyme catalysed the production of a coloured product that was easily observable. The membrane was then rinsed in distilled water to stop the reaction and photographed.

2.8   Chromatin Immuno-precipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed by using a ChIP kit and according to the manufacturer's instructions (ab500, Abcam Inc). In brief, about 3×10^6 cells were fixed in 1% formaldehyde before lysis. Chromosomal DNA was sheared by using a sonifier (Branson 450) to an optimal DNA fragment size of 200–1000 bp. Anti-target antibody was used in the immunoprecipitation to pull down the target protein and DNA complex. Rabbit and mouse IgG were used as negative controls. After DNA purification, 0.5 µL each of input DNA, target-enriched DNA, or rabbit IgG-enriched DNA were subjected to PCR analysis. In the case of the *bik* promoter, a 420 bp fragment was amplified, PCR primers were used to investigate EBNA-2 recruitment to the BZLF1 and Hes 1 promoters. Primer sequences are listed in (Table 2-5).

2.9   Statistical analysis

Statistical analysis was performed using t test followed by paired t test two-tailed using GraphPad Prism version 5.00 for Windows, GraphPad software, La Jalla California USA, www.graphpad.com.

Chapter 3 :
EBV inhibits TGFβ1-induced apoptosis by inhibiting *bik* upregulation through the canonical Smad pathway

Transforming growth factor-β (TGFβ), a multifunctional cytokine family of growth factors, comprises more than 30 structurally related factors found in nematodes, fruit flies and humans. They include the bone morphogenetic proteins (BMPs), the closely related growth and differentiation factors (GDFs), the activins, Nodals, and transforming growth factor-beta proteins. Three almost structurally identical isoforms, TGFβ 1, 2, and 3, have been found in humans. This growth factor superfamily regulates a diverse array of cellular responses during the development and adult stages of eukaryotes (Massague 1998, Ikushima and Miyazono 2011b). The TGFβ signal transduction pathway can be modulated by many other signalling cascades and, depending on the cell type and cellular context, TGFβ can regulate and control cell growth, differentiation, embryonic development, adult stem cell differentiation, apoptosis, morphogenesis, immune regulation, wound healing and inflammation. This pathway has also been linked to cancers, fibrosis, autoimmune diseases and developmental disorders in humans (Meulmeester and Ten Dijke 2011, Inman 2011, Tian, Neil and Schiemann 2011, Chou, et al. 2010, Lasfar and Cohen-Solal 2010).

A role for TGFβ signalling in regulating the intrinsic mitochondrial apoptosis pathway during B cell development has been established. B cell subsets show significant upregulation of *bik* transcripts during differentiation as they progress through the GC reaction from naive, centroblast, centrocyte and through to memory cells (Klein, et al. 2003). In human B cells, *bik* has been identified as a direct transcriptional target of TGFβ/Smad signalling and is required for TGFβ-induced apoptosis in Burkitt's lymphoma cell lines (Spender, et al. 2009). Increases in *bik* mRNA levels have also been observed in TGFβ-treated CD77+ tonsillar B cells (centroblast), demonstrating that *bik* is also regulated by TGFβ in primary B cells. BL is an example of malignant B cells having retained the normal responses of GC B cells to external stimuli. TGFβ1 has been shown to have a growth inhibitory and pro-apoptotic effect on the EBV-negative B cell lymphoma BJAB, (Kehrl, et al. 1989) and also on Ramos, an EBV-negative BL cell line (Altiok, et al. 1991)(Spender, et al. 2009).

In the present study, experiments were therefore designed with a view to investigating a potential role and mechanism for EBV/EBNA-2 modulation of *bik* during TGFβ signalling in this cellular background. The BL-derived EBV negative cell line Ramos, which possesses a GC phenotype (group I), in addition to BJAB, an EBV negative B cell lymphoma cell line, and ER/EB2-5, a conditional LCL, were used as model systems for this purpose.

3.1 TGFβ1 induces apoptosis in a dose-dependent manner in EBV-negative B cell lines.

Ramos (Figure 3-1) and BJAB (Figure 3-2) cell lines were treated with various concentrations of TGFβ1 (Millipore, GF111). Cell survival profiles were analysed at 24 h and 48 h after this treatment by 7-AAD/Annexin-V staining and subsequent flow cytometric analysis.
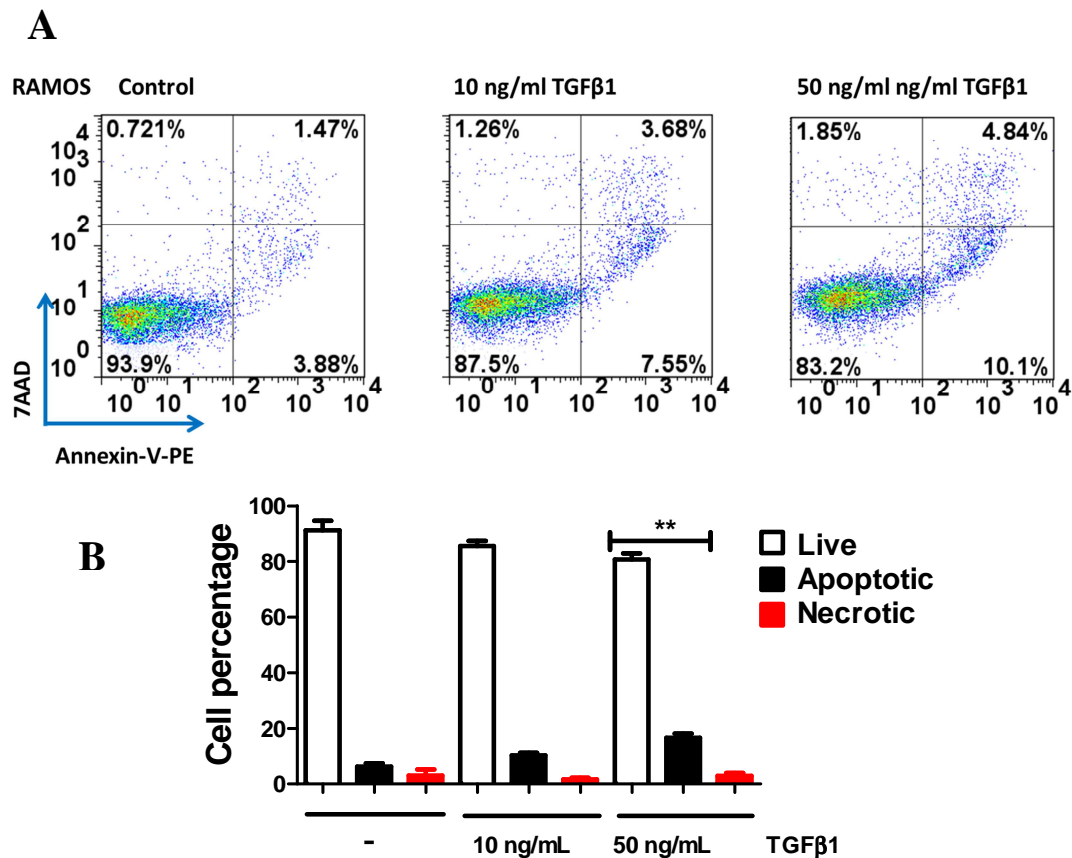
Figure 3-1: TGFβ1 induces apoptosis in Ramos cells.

(A) Representative FACS profiles of Ramos cells 24 h following treatment with various concentrations of TGFβ1, as indicated above each scatterplot. Quadrant markers were based on stained/unstained controls. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$/Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$), and the numbers shown are the percentages of cells in each quadrant. (B) Bar chart representation of the experiment shown in A (three independent experiments). Data are ±SD**P (0.001 to 0.01) vs untreated control.

A

B
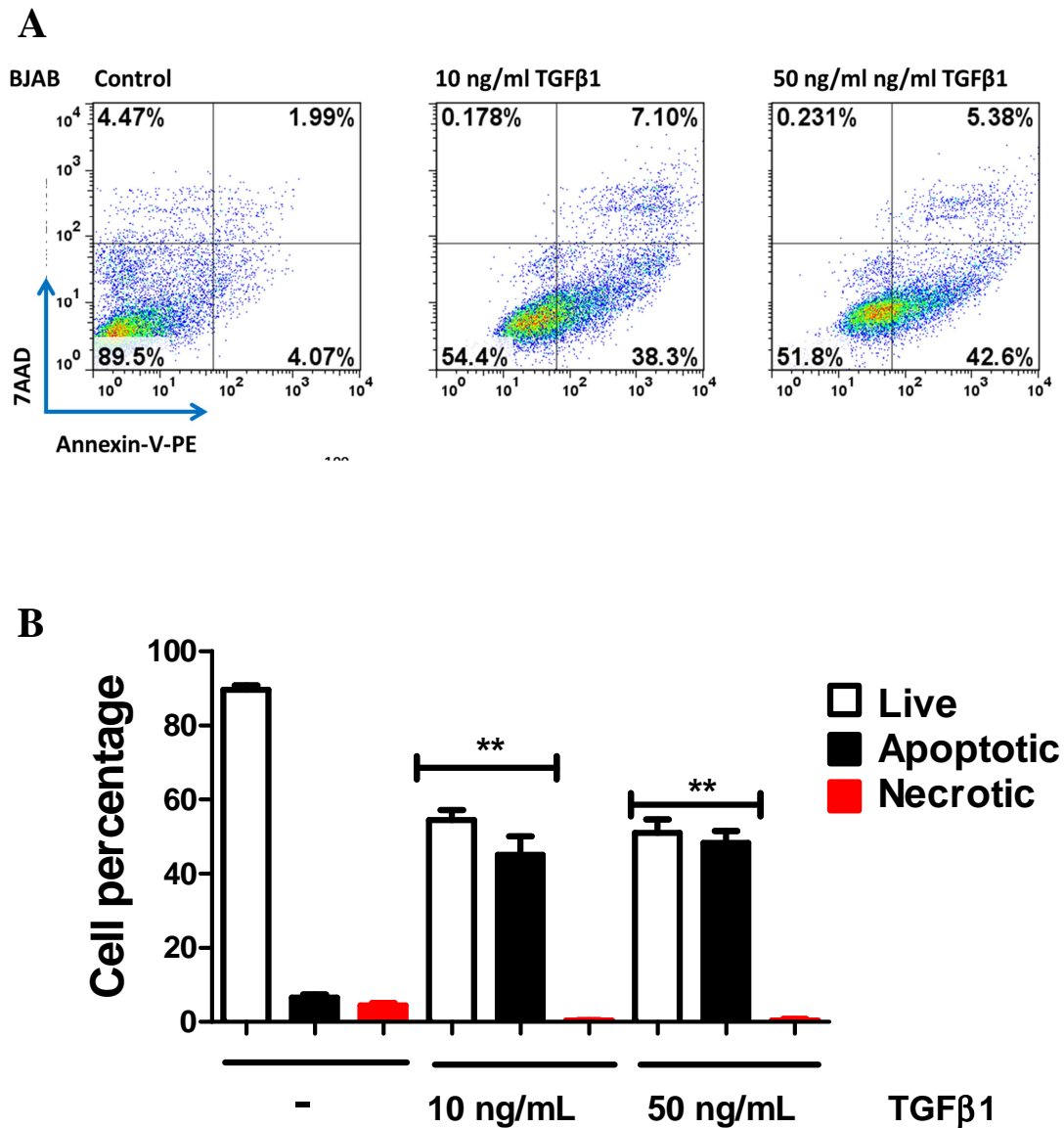
Figure 3-2: TGFβ1 induces apoptosis in BJAB cells.

BJAB cells were treated with various concentrations of TGFβ1 as indicated above each scatterplot. (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment with TGFβ1 are shown. The cells in each quadrant are as described in the legend to Figure 3-1 and the values shown represent the percentage number of cells in each quadrant. (B) Bar chart representation of the experiment shown in A (three independent experiments). Data are ±SD**P (0.001 to 0.01) vs untreated control.

TGFβ1 was observed to induce apoptosis in a dose-dependent manner. A greater proportion of BJAB cells (Figure 3-2) were seen to enter early apoptosis in contrast to Ramos (Figure 3-2) where the cells stained with Annexin-V-PE but not with the DNA vital stain, 7AAD. Programmed cell death induced by TGFβ1 ligation was a faster process in BJAB (Figure 3-2) in comparison to Ramos cells (Figure 3-1). Cell proliferation and metabolic activity was measured by cellTiter 96 Aqueous One Solution cell proliferation Assay (MTS). The overall metabolic activity was greatly decreased upon TGFβ1 treatment, by 3-fold in BJAB and to lesser extent of 1.3 fold in Ramos after 48 h (Data not shown).

3.2 TGFβ1 mediates apoptosis by upregulting *bik* mRNA and protein

In order to investigate if TGFβ1 exerts its apoptotic effect by regulation of *bik*, Ramos and BJAB cells were treated with 25 ng/mL or 100 ng/mL TGFβ1 (Millipore, GF111) for 24 h. Total RNA and protein were harvested and relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h after treatment. *Bik* mRNA levels were assayed in triplicate and normalised to *gapdh* mRNA levels. Relative quantification levels were calculated relative to the control untreated samples. Bik protein expression was analysed by Western blot and Bik protein relative density values were obtained using ImageJ.
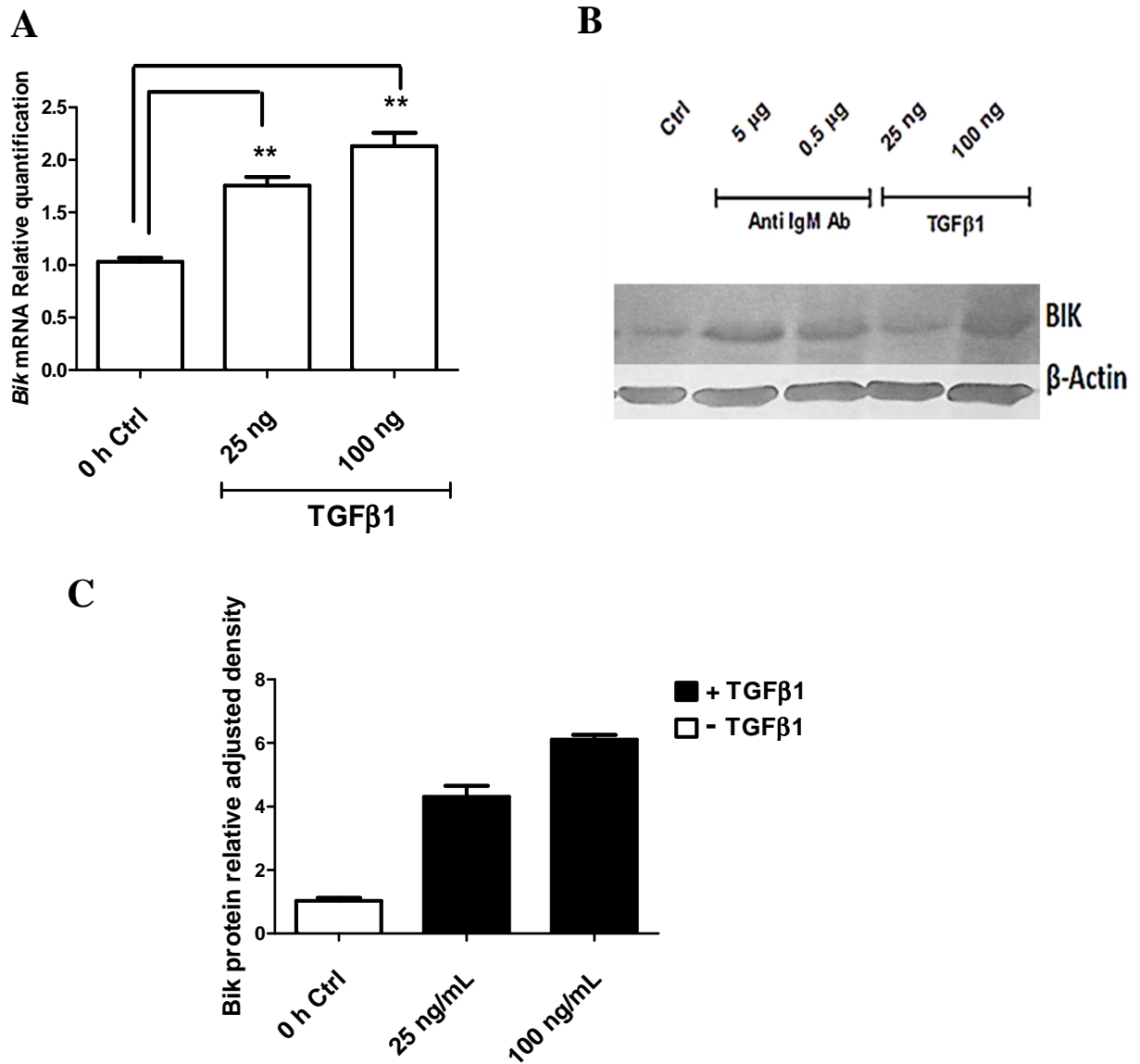
Figure 3-3: *Bik* mRNA and protein expression levels rise in response to TGFβ1 treatment in Ramos.

Ramos cells were treated with 25 ng/mL or 100 ng/mL TGFβ1. (A) Relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h after treatment. *Bik* mRNA levels were assayed in triplicate by RT-qPCR and normalised to *gapdh* mRNA levels. Relative quantification levels were calculated relative to the control. Data are ±SD **P (0.001 to 0.01) of three independent experiment. (B) Representative Western blot showing Bik protein. *Trans*-activation of *bik* in response to BCR ligation (anti-IgM) is also shown (see section B above). (C) Bik protein relative density values (from (B)) were obtained using ImageJ and were normalized to untreated control. (ref: Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.)

A

B
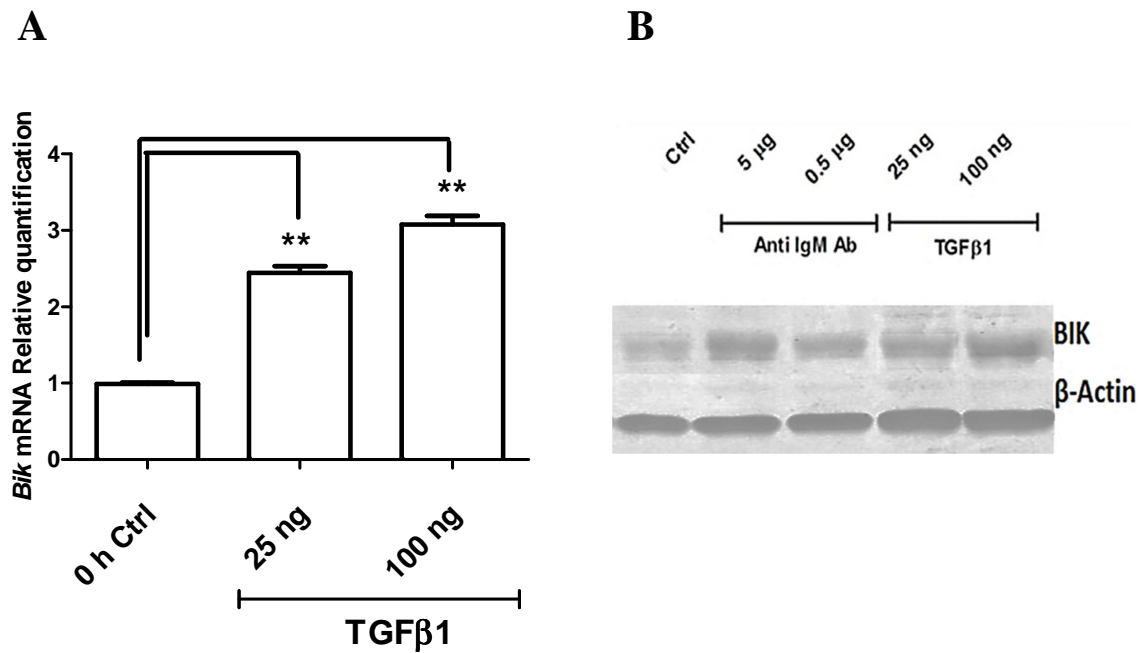
Figure 3-4: *Bik* mRNA and protein expression levels rise in response to TGFβ1 treatment in BJAB.

BJAB cells were treated with 25 ng/mL or 100 ng/mL TGFβ1 (A) Relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h following treatment with TGFβ1 as described in the legend to (Figure 3-3). Data are ±SD **P* (0.001 to 0.01) of three independent experiment. (B) Representative Western blot showing Bik protein expression at the same time point. *Trans*-activation of *bik* in response to BCR ligation (anti-IgM) is also shown (see section B above).

Bik mRNA levels increased in both Ramos (Figure 3-3 A) and to a greater extent in BJAB (Figure 3-4 A) cells following treatment with TGFβ1, in a dose-dependent manner. Bik protein levels also increased in both cell lines (Figure 3-3 B and C; Figure 3-4 B and C). However, this effect was more profound in BJAB. Bik is a phosphoprotein and it is phosphorylated on consensus Casein Kinase II (CKII) phosphorylation sites (Thr-33 and/or Ser-35). Phosphorylation is required for efficient Bik-mediated apoptosis (Verma, Zhao and Chinnadurai 2001). Bik appears as a double band, 27 kDa/26k Da in size, in some cell lines and a single band in others. Western blots using the Ramos cell line showed a single band for Bik, whereas BJAB demonstrated a double band. When the latter was stimulated with TGFβ1 ligand, it was the higher molecular weight band that was affected.

3.3 Transient *bik* down-regulation inhibits apoptosis induced by TGFβ1

In order to investigate if *bik* is essential for TGFβ1-mediated apoptosis, the *bik* gene was transiently knocked down in Ramos (Figure 3-5) and BJAB(Figure 3-6) cells using siRNA specific to *bik* (siRNA1980 and siRNA1990). In these transfection experiments, both anti-*bik* siRNAs were able to inhibit *bik* expression as reflected in decreased *bik* mRNA and protein levels, whereas the control siRNA did not. Twenty four hour after transfection, siRNA-transfected cells were then left untreated or treated with 10 ng of TGFβ1 for 24 h. The cell survival profile was determined after 24 h by 7AAD/Annexin-V-PE staining and subsequent flow cytometric analysis. As can be seen (Figure 3-7 and Figure 3-8) TGFβ1 was again seen to induce apoptosis in both cell lines. This effect was more pronounced in BJAB, which had 36.9% ±SD 4.530 apoptotic cells when compared to Ramos (26% ±SD 7.365 apoptotic). In transfected cells a significant level of apoptosis was observed before treatment, (which can be attributed to the harsh transfection conditions). Enforced downregulation of *bik* resulted in an increase in the percentage of viable cells in both cell lines (approximately 10%) before TGF β1 treatment. Transient *bik* knockdown was seen to protect cells from apoptosis mediated by TGFβ1 in Ramos and BJAB cells. This is in contrast to the control siRNA transfection where TGFβ1 induced apoptosis in Ramos (from 50% ±SD 3.717 before to 82% ±SD 4.028 after) (Figure 3-7), and BJAB (from 42% ±SD 2.101 to 72% ±SD 4.319 after) (Figure 3-8). Total Bik protein level was also monitored 24 h after TGFβ1 treatment in both cell lines after *bik* transient knockdown. Transient *bik* knockdown was seen to inhibite the TGFβ1-mediated Bik induced upregulation opposed to cells transfected with control siRNA (Figure 3-11).

A
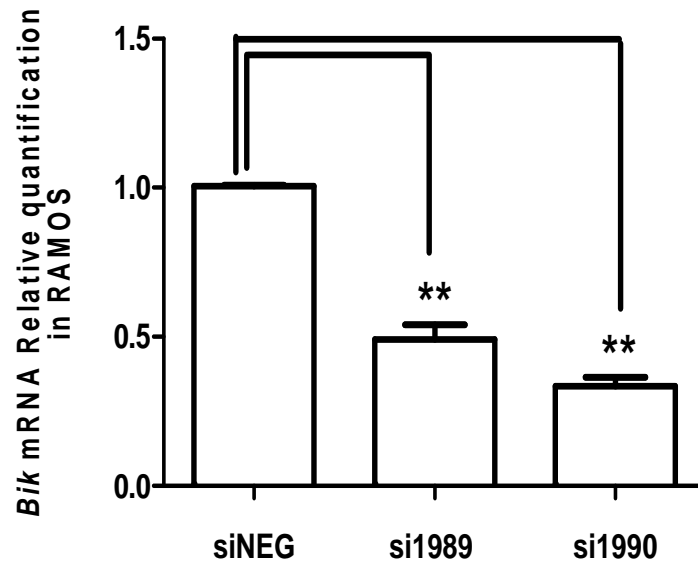
B
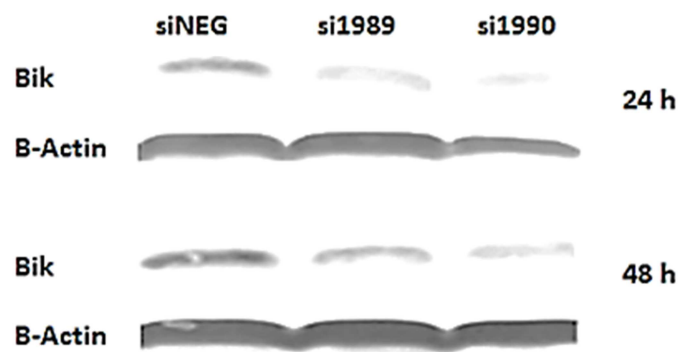
Figure 3-5: Transient *bik* knockdown in the Ramos cell line.

Ramos cells were transfected with two different anti-*bik* siRNAs (si1989, si1990), or negative control siRNA (siNeg). (A) The level of *bik* mRNA shown for 24 h post transfection was quantitated by RT-qPCR as before. Relative quantification levels were calculated relative to the siNeg control. Data are ±SD **P* (0.001 to 0.01) of three independent experiment. (B) Representative Western blot showing Bik protein expression from extracts prepared at 24 h (upper panel) and 48 h (lower panel) following transfection of Ramos cells.
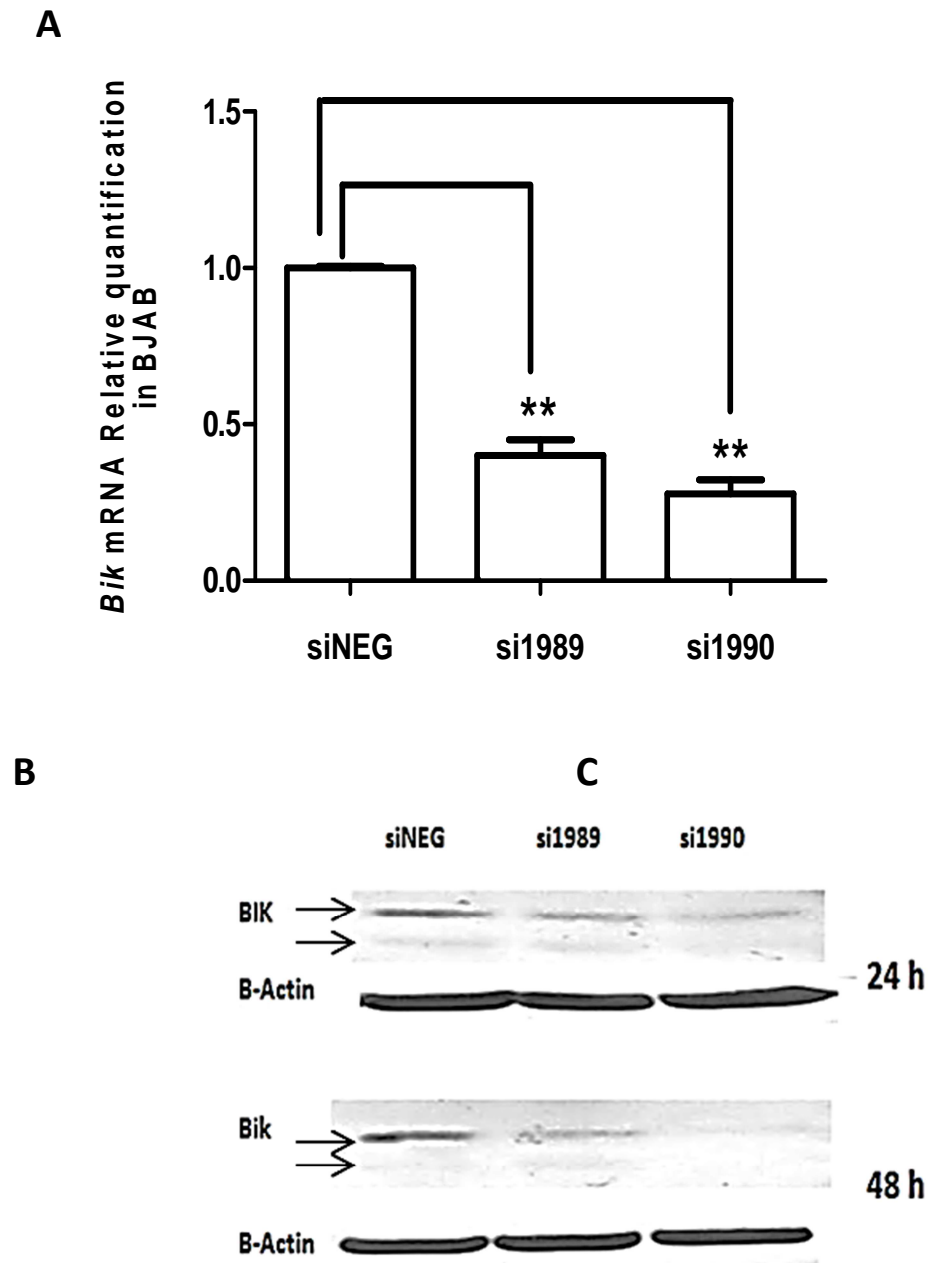
Figure 3-6: Transient *bik* knockdown in the BJAB cell line.

BJAB cells were transfected with two different anti-*bik* siRNAs (si1989, si1990*),* or negative control siRNA (siNeg). (A) The level of *bik* mRNA shown for 24 h post transfection was quantitated by RT-qPCR as before. Relative quantification levels were calculated relative to the siNeg control. Data are ±SD **P* (0.001 to 0.01) of three independent experiment. (B) Representative Western blot showing Bik protein expression from extracts prepared at 24 h (upper panel) and 48 h (lower panel) following transfection of BJAB cells.

A
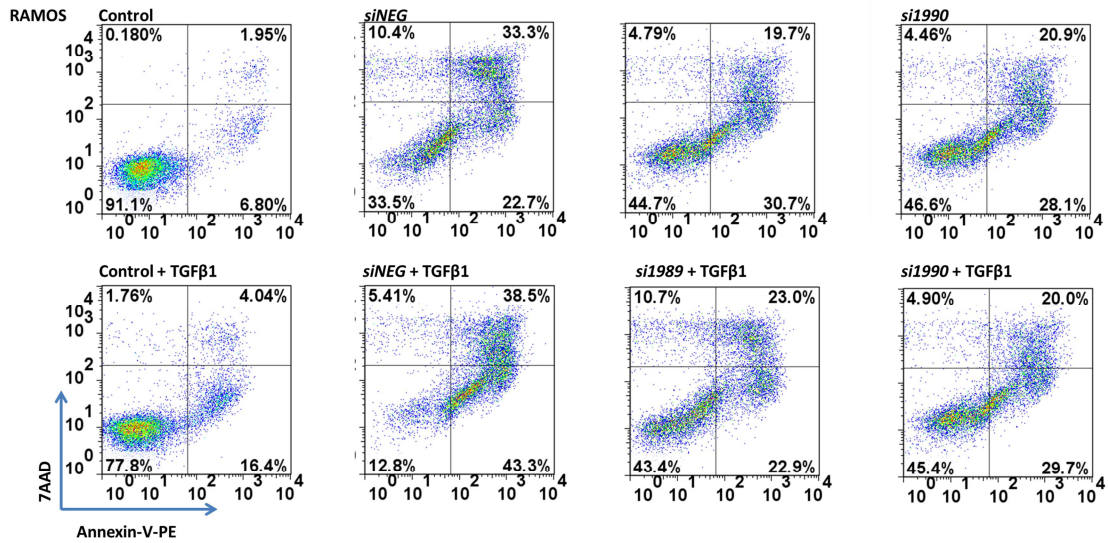
B
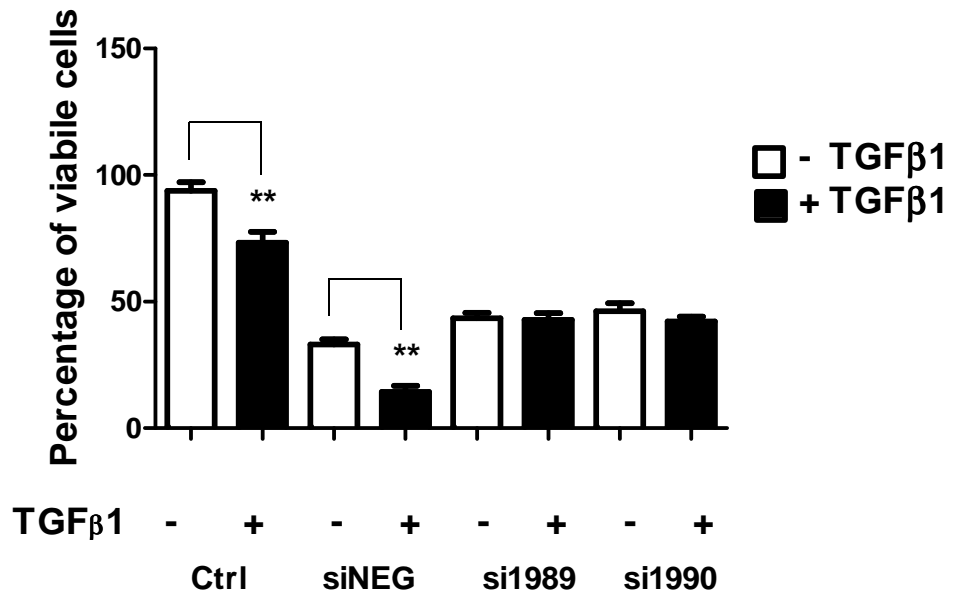
Figure 3-7: *Bik* knockdown in Ramos leads to a reduction in the loss of cell viability due to TGFβ1 treatment.

Ramos cells were transfected with each of two different *bik* siRNAs (si1989, si1990) or negative control siRNA (siNeg). (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment with TGFβ1. The cells in each quadrant are as described in the legend to Figure 3-1 and the values shown represent the percentage number of cells in each quadrant. (B) Bar chart representation of the percentage of viable cells in experiment shown in A (three independent experiments). Data are ±SD **P (0.001 to 0.01).
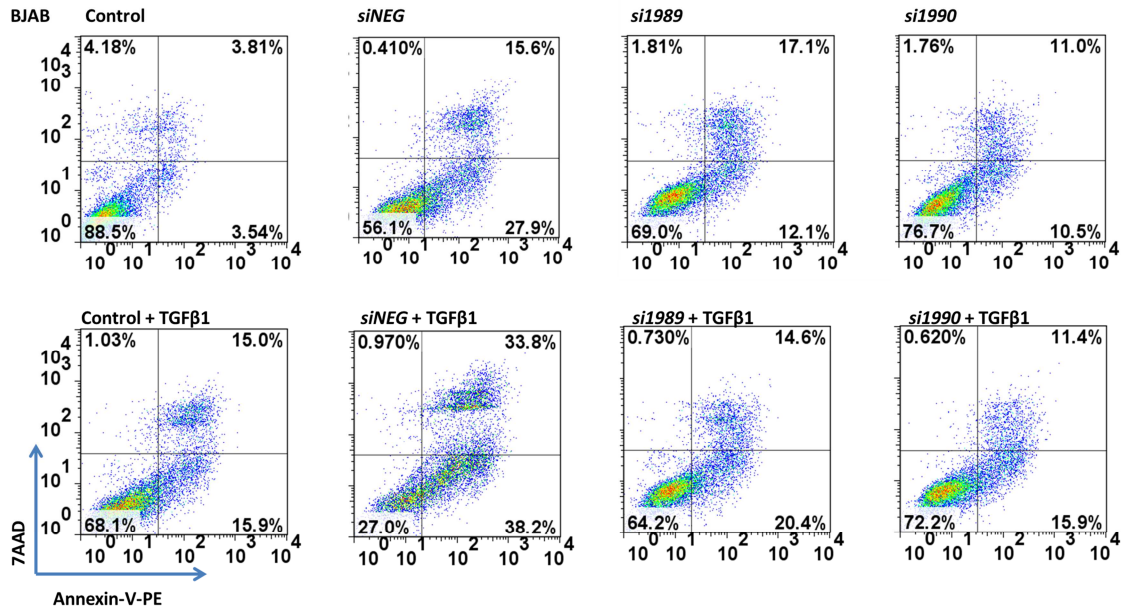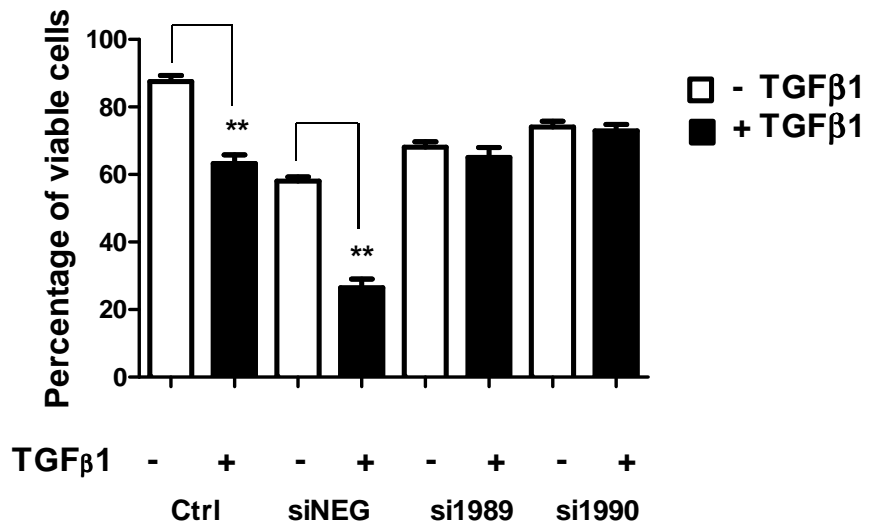
Figure 3-8: *Bik* **knockdown in BJAB leads to a reduction in the loss of cell viability due to TGFβ1 treatment.**

BJAB cells were transfected with each of two different *bik* siRNAs (si1989, si1990) or negative control siRNA (siNeg). (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment with TGFβ1. The cells in each quadrant are as described in the legend to Figure 3-1 and the values shown represent the percentage number of cells in each quadrant. (B) Bar chart representation of the percentage of viable cells in experiment shown in A (three independent experiments). Data are ±SD **P (0.001 to 0.01).

Cell proliferation was also monitored 24 h after TGFβ1 treatment in both cell lines after *bik* transient knockdown using the cellTiter 96 Aqueous One Solution cell proliferation assay (MTS). It can be seen that *bik* knockdown led to an overall increase in cell metabolic activity in untreated transfected cells in both cell lines and a partial reversal of the growth inhibitory effect of TGFβ1 when compared to cells transfected with siNeg in the case of Ramos cells (Figure 3-9). *Bik* down-regulation due to specific siRNA treatment significantly restored the cell metabolic rate that was lost due to TGFβ1 in BJAB (Figure 3-10). The proliferative effect of *bik* knockdown was more evident in BJAB than Ramos, where cells transfected with anti-*bik* siRNA and treated with TGFβ1 are seen to have an increase in cell proliferative activity in comparison to the untreated samples (Figure 3-10).
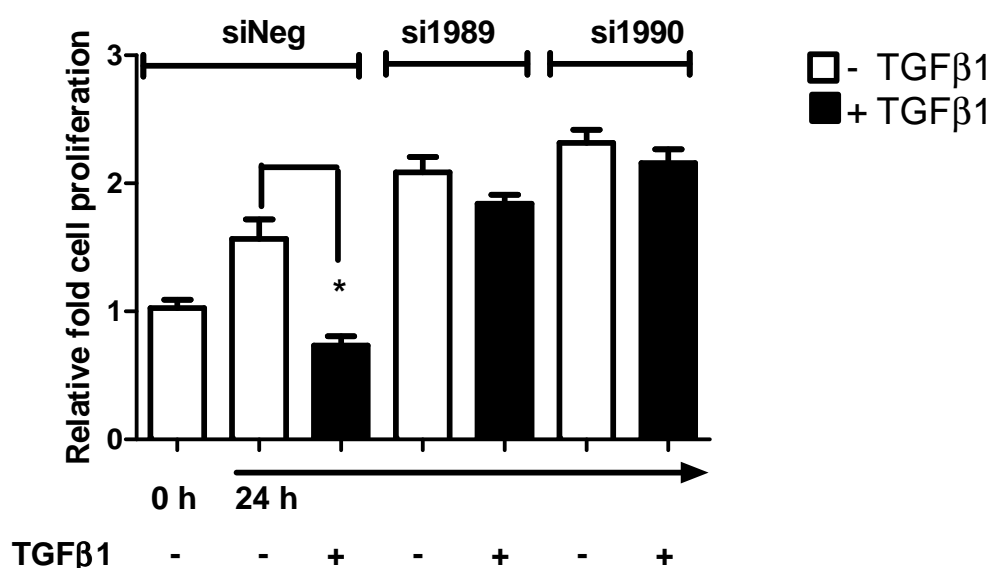
Figure 3-9: *Bik* knockdown leads to a decrease in the loss of metabolic activity seen upon treatment of Ramos with TGFβ1.

Proliferation of RAMOS cells following transfection with *bik* siRNAs (si1989 & si1990) and scrambled siRNA (siNeg) and in response to treatment with 10 ng of TGFβ1 was monitored at the time points indicated using the cellTiter 96 Aqueous One Solution cell proliferation assay (MTS). The proliferation of transfected untreated cells (white bars) and treated with TGFβ1 (black bars) was measured relative to the 0 h untreated sample. Data are ±SD *$P \leq 0.05$ of three independent experiment.
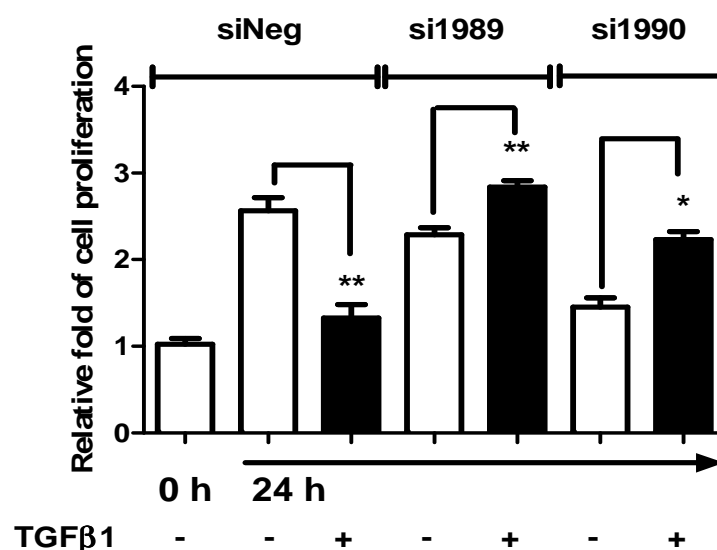
Figure 3-10: *Bik* **knockdown leads to a decrease in the loss of metabolic activity seen upon treatment of BJAB with TGFβ1.**

The proliferation of BJAB cells that were transfected with *bik* siRNAs (si1989 & si1990), and scrambled siRNA in response to 10 ng/mL of TGFβ1 was measured at the indicated time point using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS). The proliferation of transfected cells untreated (white bars) and treated with TGFβ1 (black bars) was measured relative to 0 h untreated sample. Data are ±SD *P ≤ 0.05 and **P (0.001 to 0.01) of the independent experiment.
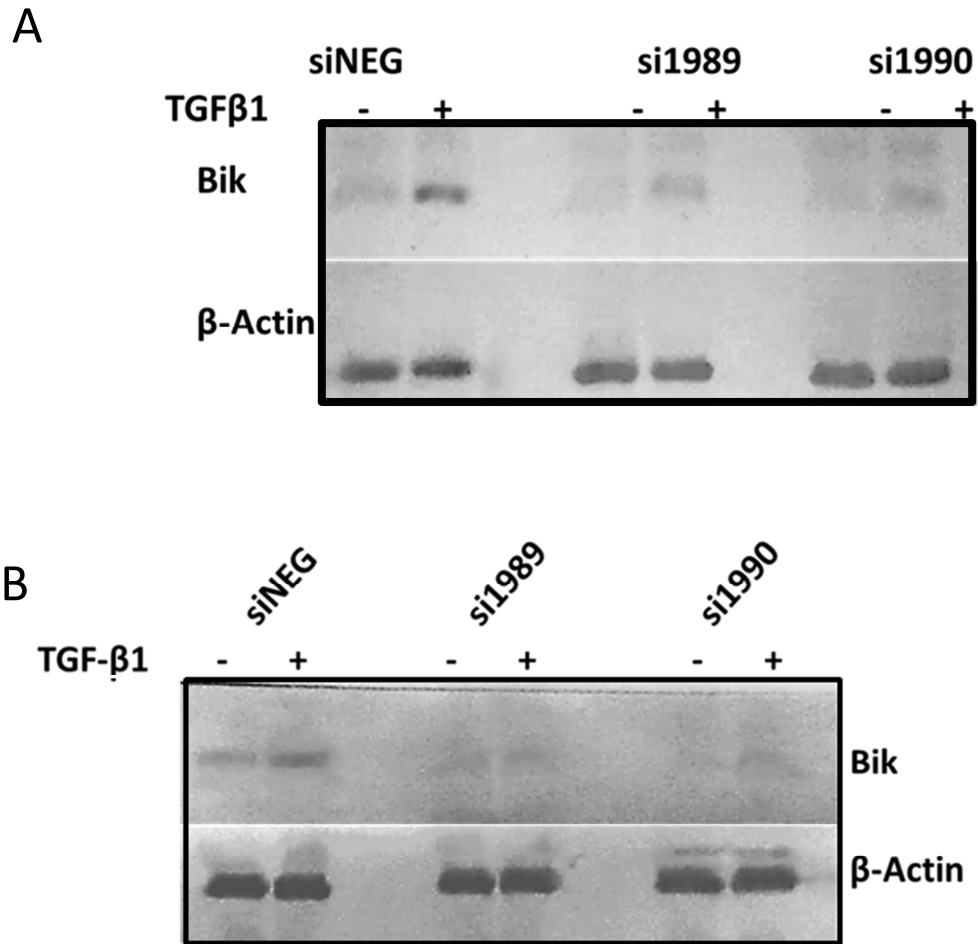
Figure 3-11: *Bik* **knockdown inhibited the TGFβ1-mediated Bik protein induction.**

Cells were transfected with each of two different *bik* siRNAs (si1989, si1990) or negative control siRNA (siNeg). Twenty four hour after transfection cells were treated with 10 ng/mL of TGFβ1. Representative Western blot showing Bik protein expression from extracts prepared at 24 h following treatment of (A) BJAB and (B) Ramos cells with TGFβ1.

3.4 Ectopic EBNA-2 inhibits TGFβ1-mediated apoptosis in Ramos cells.

It has been shown recently that EBNA-2 down-regulates the expression of *bik* (D. Walls, unpublished results). Therefore, experiments were set up in order to investigate if EBNA-2 expression might inhibit the observed pro-apoptotic effects of TGFβ1. RAMOS cells were transfected with 1µg each of plasmids expressing either EBNA-2 or the non-CBF1-binding EBNA-2 mutant, WW323SR. Forty-eight hours after transfection, cells were treated with 10 ng TGFβ1. The cell survival profile was then determined 24 h after treatment using 7AAD/Annexin-V-PE staining and subsequent flow cytometric analysis by FACS Calibur.

It can be seen that ectopic EBNA-2 or its non-CBF1 binding mutant, EBNA-2 WW323SR in Ramos cells partially rescued these cells from apoptosis induced as a result of harsh conditions of transfection (Figure 3-12). Treatment of the control transfected cells with TGFβ1 efficiently led to apoptosis and significantly decreased the percentage of viable cells (from 30% ±SD 1.656 before to 10% ±SD 2.198 after). However, ectopic EBNA-2 or its non-CBF1 binding mutant, EBNA-2 WW323SR, decreased the effect of TGFβ1-mediated apoptosis (Figure 3-12).
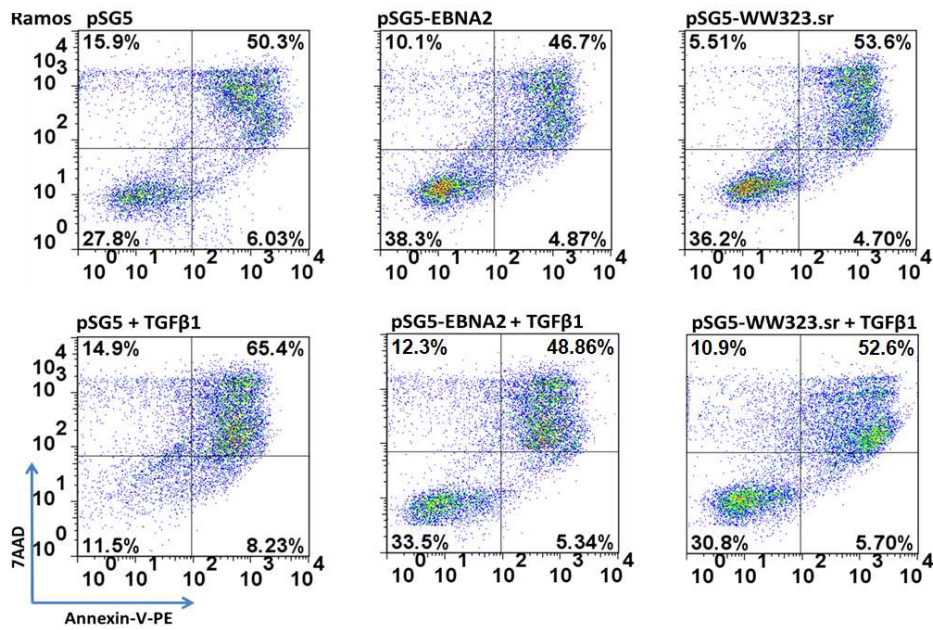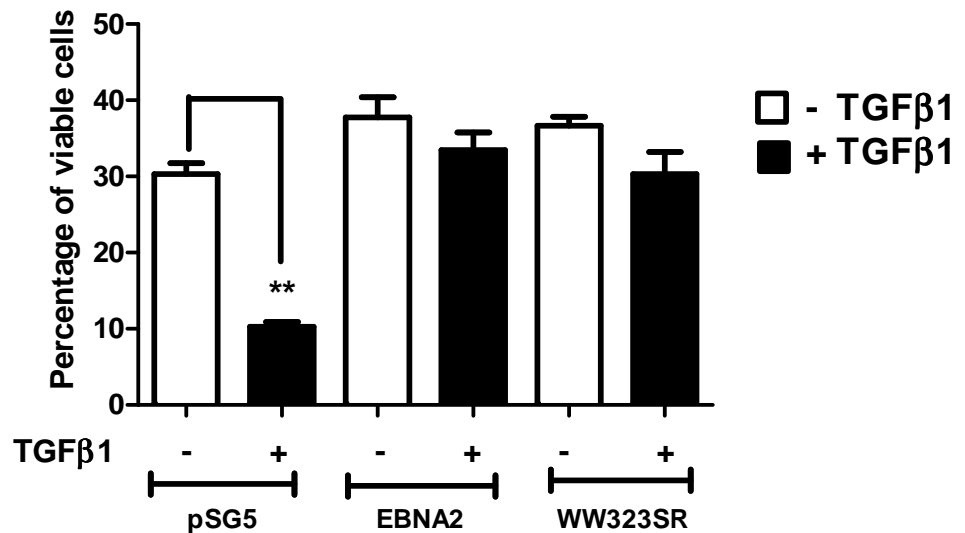
Figure 3-12: EBNA-2 rescues cells from apoptosis induced by TGFβ1 in Ramos cells.

RAMOS cells were transfected with 1µg of each plasmid as indicated underneath the graph. Forty-eight hours after transfection, cells were treated with 10 ng TFGβ1. (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment with TGFβ1. The cells in each quadrant are as described in the legend to Figure 3-1 and the values shown represent the percentage number of cells in each quadrant. (B) Bar chart representation of the relative percentage of viable cells in the experiment shown in A (average of three independent experiments). Data are ±SD **P (0.001 to 0.01).

3.5 TGFβ1 mediates apoptosis through the canonical Smad pathway

It was observed here that TGFβ1 was able to induce the expression of *bik* mRNA and protein in Ramos (Figure 3-3) and BJAB (Figure 3-4), which coincided with apoptosis and an overall decrease in metabolic activity in the cell population.

It has been shown elsewhere that TGFβ1-mediated signalling leads to the recruitment of the Smad protein complex, Smad2/3/4, to the *bik* promoter, coinciding with activation of that promoter and an increase in the level (Figure 1.12). In order to investigate the Smad3 protein level, its location in the cell and the associated relative *bik* expression in response to TGFβ1 mediated signalling, Ramos cells were treated with or without 10 ng of TGFβ1 for a duration of 24 h. RT-qPCR showed elevated *bik* mRNA in response to TGFβ1 (Figure 3-13 A). Over 24 h, the *bik* mRNA basal level first decreased between 0 and 4 h and began increasing to its basal level at the 24 h time point in the absence of TGFβ1, however the transcriptional level of *bik* mRNA was higher at both time points in the presence of TGFβ1 (Figure 3-13 A).

As described before, TGFβ1 binding to its receptors results in activation of receptor-regulated R-Smads (Smad2/3) by phosphorylation of the XserX motif in their C-terminus, leading to their translocation from the cytoplasm to the nucleus. Hence, subsequent translocation of Smad3 from the cytoplasm to the nucleus is indicative of Smad3 activation, reflecting TGFβ1 signalling.

In order to confirm activation of the TGFβ1 signalling pathway, Smad3 protein expression levels in nuclear and cytoplasmic fractions were evaluated by Western blot before and after treatment with TGFβ1. The level of Smad3 protein was detected using anti-Smad3 and was normalised to the levels of Gapdh protein. It can be seen that from the 0 to 24 h time points, the level of Smad3 protein starts to decrease in the cytoplasmic fraction and accumulates in the nuclear fraction in the presence of TGFβ1. This event was most clearly seen at the 4 hour time point, when comparing the two cytoplasmic fractions in the presence and absence of TGFβ1 (Figure 3-13 B). It can be concluded from the observations made, that Smad3 translocated from the cytoplasm to the nucleus in response to TGFβ1 signalling.

A
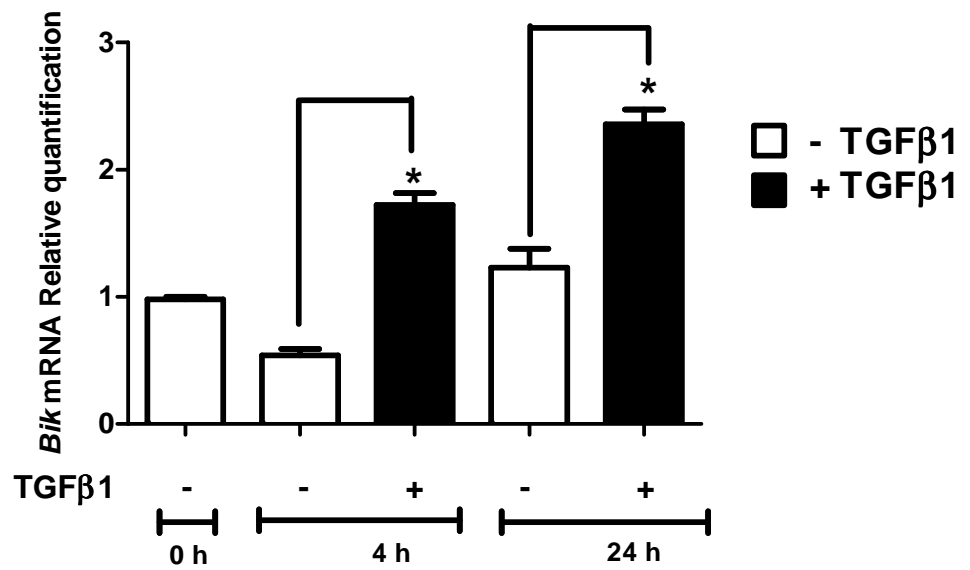
B
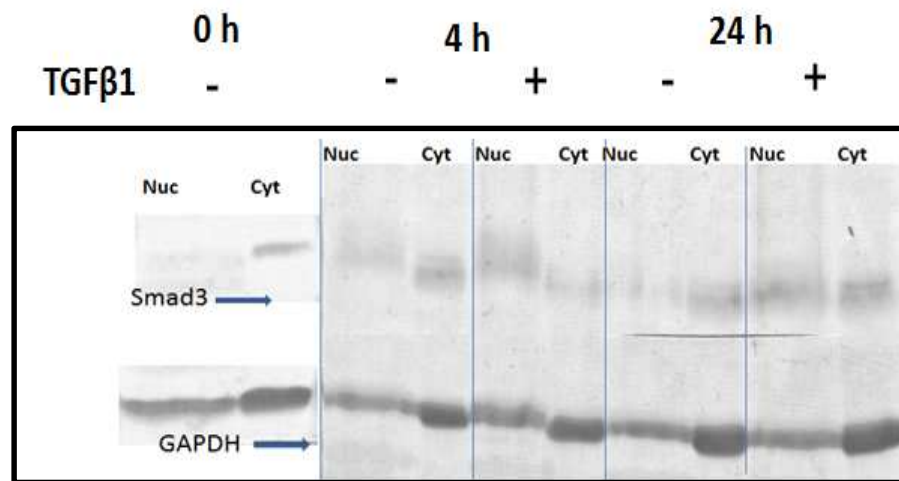
Figure 3-13: TGFβ1 induces *bik* and the translocation of Smad3 from the cytoplasm to the nucleus in Ramos cells.

Ramos cells were treated without or with 10 ng of TGFβ1 for different lengths of time as indicated. (A) RT-qPCR shows elevated *bik* mRNA levels at 0 h, 4 h and 24 h in the presence of TGFβ1 as indicated. (B) Western blot of Smad3. Protein extracts of nuclear and cytoplasmic fractions were prepared and the level of Smad3 protein was detected using anti-Smad3 (ab28379). Data are ±SD *$P \leq 0.05$.

The extent of recruitment of Smad3 and Smad4 to the *bik* promoter in the presence and absence of TGFβ1 was next investigated. Binding of Smad3 and Smad4 to the *bik* promoter was determined by chromatin immunoprecipitation (ChIP) assay. Ramos and BJAB cells were treated with 10 ng TGFβ1 for 4 h. Cells were then treated with 1% formaldehyde in order to cross-link endogenous nuclear proteins and DNA. Samples of sonicated chromatin were then individually immunoprecipitated with anti-Smad3 (ab28379), anti-Smad4 (ab3219), no antibody (beads only) and isotype control IgG respectively. DNA isolated from immunoprecipitated material was then amplified by Q-PCR using primers that target a 420-bp sequence from the human *bik* promoter (Spender, et al. 2009) (Figure 3-14 C). These experiments showed that the level of Smad3 bound to the *bik* promoter increased ten-fold in Ramos cells following treatment with TGFβ1 (Figure 3-14 A). This effect was also observed in BJAB cells but the magnitude of the increase was just two-fold (Figure 3-14 B).
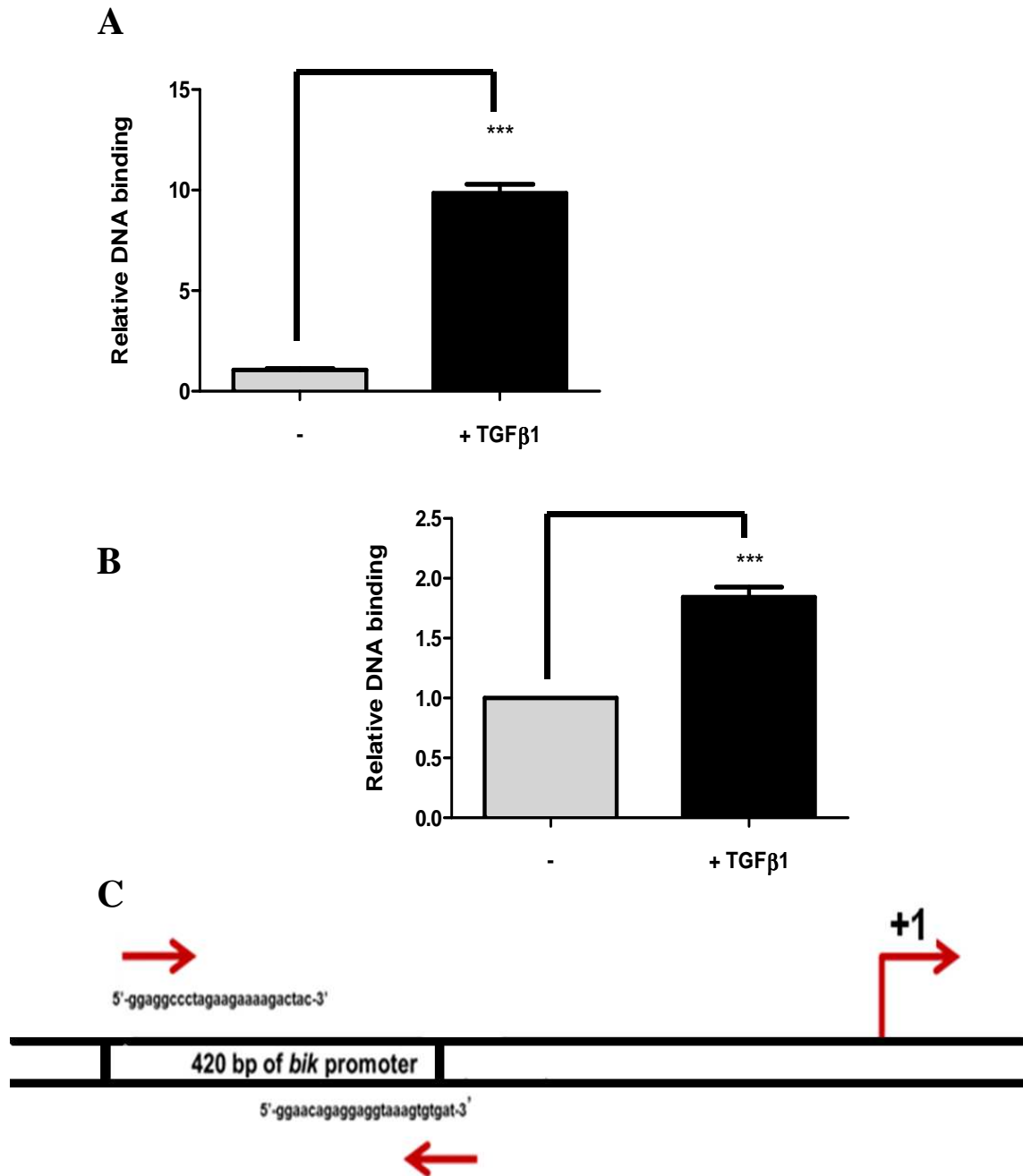
Figure 3-14: ChIP assay for Smad3 showing increased binding of Smad3 to the *bik* promoter in BJAB and Ramos cells following treatment with TGFβ1.

Recruitment of Smad3 to the *bik* promoter was determined by chromatin immunoprecipitation (ChIP) analysis. (A) Ramos and (B) BJAB cells were treated without (grey bar) or with 10 ng/mL TGFβ1 (black bar) for 4 h. Cells were then treated with 1% formaldehyde to cross-link endogenous proteins and DNA. Samples of sonicated chromatin were immunoprecipitated with anti-Smad3 (ab28379, no antibody (beads only) and isotype control IgG respectively. DNA isolated from immunoprecipitated material was amplified by Q-PCR with primers to amplify the 420 bp sequence of human *bik* promoter. (C) Smad binding region previously identified within the human *bik* promoter (Spender, et al. 2009). Straight arrows indicate position of PCR primers used in ChIP assays shown in A and B; the bent arrow shows the location of the *bik* transcription start site. Data are ±SD ***$P < 0.001$.

In order to investigate any potential interplay between EBNA-2 and TGFβ1-mediated Smad recruitment to the *bik* promoter, the EBV-negative BL cell line Ramos was used. Ramos cells were transfected with plasmids expressing either EBNA-2 or the corresponding empty vector, pSG5. Forty eight hours after transfection, 10 ng TGFβ1 was added for a duration of 4 h and then cells were harvested and treated with 1% formaldehyde. Samples of sonicated chromatin were separately incubated with anti-Smad3, anti-Smad4 and anti-EBNA-2 antibodies. DNA isolated from immunoprecipitated material was amplified by Q-PCR with primers to amplify a 420-bp sequence upstream of human *bik* promoter that has previously been shown to interact with Smads [(Spender, et al. 2009) Figure 3.14C].

The following observations were made regarding Ramos cells expressing EBNA-2, when compared to their counterparts' transfected with empty vector: (i) the level of Smad3 bound to the *bik* promoter decreased ten-fold in the presence of EBNA-2. (ii) EBNA-2 was detectable by ChIP on the *bik* promoter in cells expressing EBNA-2 (Figure 3-15).
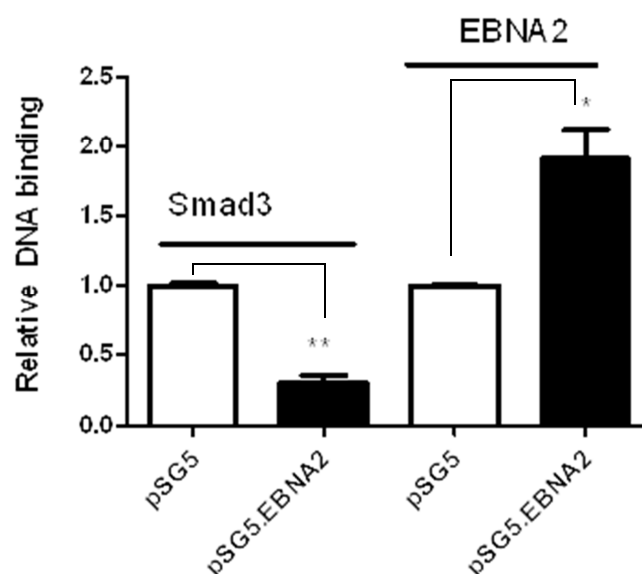
Figure 3-15: EBNA-2 expression leads to a reduction in the level of Smad3 bound to the *bik* promoter in Ramos cells

Ramos cells were transfected with pSG5 (white bars) or pSG5EBNA-2 (black bars). Forty eight hours after transfection, 10 ng/mL TGFβ1 was added for duration of 4 h. Cells were then harvested and ChIP was performed as described before. DNA isolated from immunoprecipitated material with anti-Smad3 and anti-EBNA-2 was amplified by Q-PCR. Data are ±SD *$P \leq 0.05$, **P (0.001 to 0.01).

Ramos and BJAB cells were then transfected with 1μg pSG5 or plasmids expressing either EBNA-2 or its non-CBF1-binding mutant, WW323SR. Forty-eight hours after transfection, cells were treated with 10 ng TGFβ1 for 4 h. Cells were harvested and treated with 1% formaldehyde to cross-link endogenous proteins and DNA. Samples of sonicated chromatin were then individually incubated with anti-Smad3 and anti-Smad4 or isotype control antibodies. DNA isolated from immunoprecipitated material was amplified by Q-PCR as described before. Total RNA was also extracted and RT-qPCR was used to detect the *bik* mRNA level in Ramos (Figure 3-16) and BJAB (Figure 3-17).

These transfections showed that ectopic expression of EBNA-2 or its non-CBF1 binding mutant, WW323SR, resulted in down-regulation of *bik* mRNA. Treatment with TGFβ1 led to up-regulation of *bik* mRNA in Ramos (by 7-fold Figure 3-16 C) and BJAB cells (by 9-fold Figure 3-17 C) transfected with the empty vector, pSG5. Whereas in both cell lines expressing EBNA-2 or its mutant, WW323SR, TGFβ1 treatment was seen to have no significant effect on *bik* mRNA expression level (Figure 3-16 C and Figure 3-17 C).

TGFβ1 treatment of control (data not shown) and transfected cells with pSG5 led to significant increases in binding of Smad3 to the *bik* promoter in Ramos (approximately 2.6-fold Figure 3-16 A) and in BJAB (by 4.8-fold Figure 3-17 A) and Smad4 in Ramos (by 1.8-fold Figure 3-16 B) and in BJAB (by 3.1-fold Figure 3-17 B) to the *bik* promoter where the same samples showed *bik* mRNA up-regulation in response to this treatment (Figure 3-16 C and Figure 3-17 C). It could also be seen that *bik* down-regulation due to ectopic EBNA-2 or its non-CBF1 binding mutant coincided with less Smad3 and Smad4 binding to the *bik* promoter in the presence of TGFβ1 (Figure 3-16 A and B) (Figure 3-17 A and B).
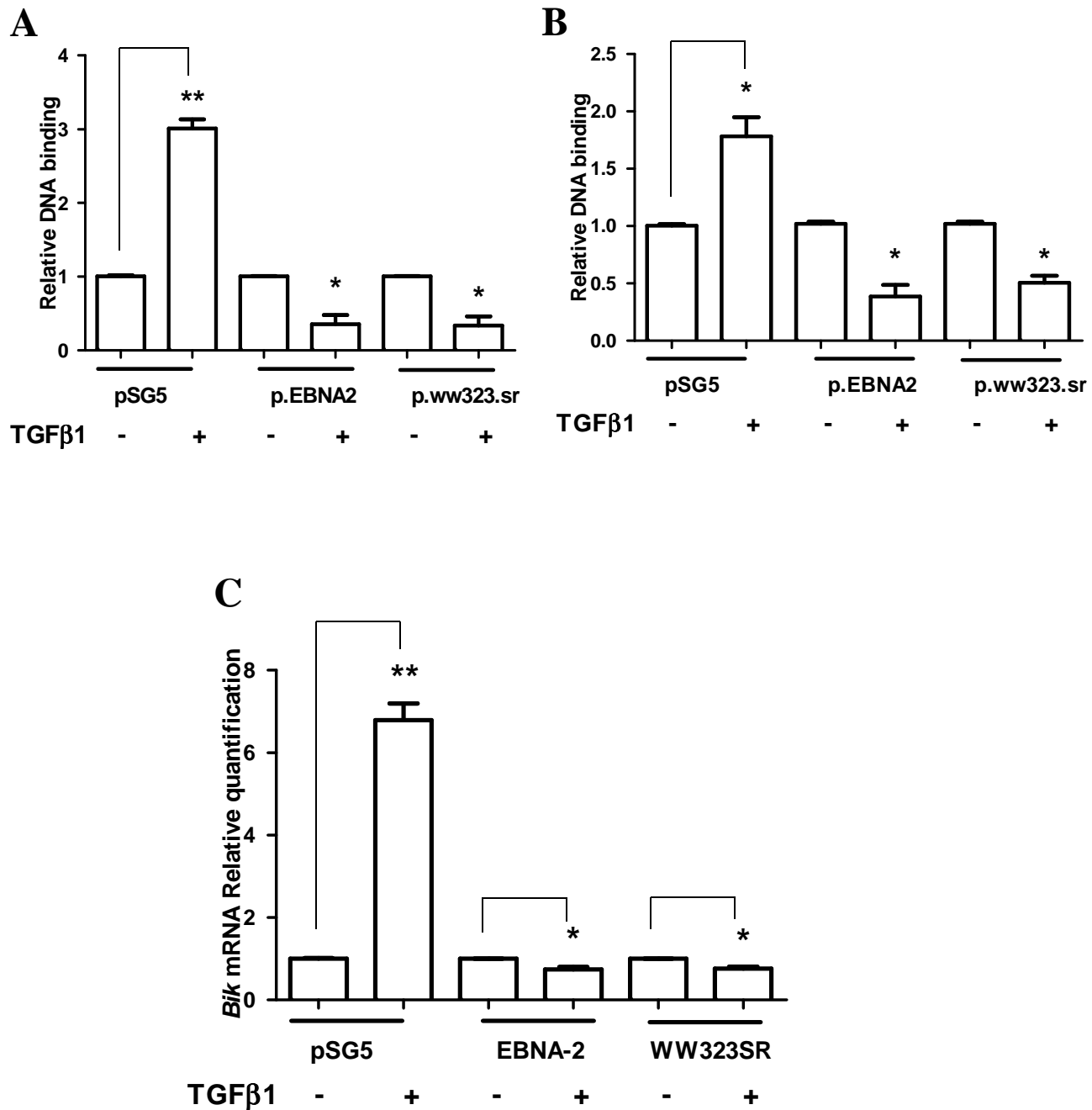
Figure 3-16: EBNA-2 abrogates the TGFβ1-mediated recruitment of Smad3/4 to the *bik* promoter and subsequent *bik* trans-activation in Ramos cell.

Ramos cells were transfected with pSG5 or EBNA-2 or WW323SR. Forty-eight hours after transfection, cells were treated with and without 10 ng/mL TGFβ1 for a duration of 4 h. Cells were then harvested and immunoprecipitated with (A) anti-Smad3 and (B) anti Smad4. DNA isolated from immunoprecipitated material was amplified by Q-PCR as before with primers to target the 420-bp sequence upstream of the human *bik* promoter transcription start site. (C) Represents relative RT-qPCR analysis for *bik* expression in the presence or absence of TGFβ1 in transfected cells. Data are ±SD *$P \leq$ 0.05, **P (0.001 to 0.01).
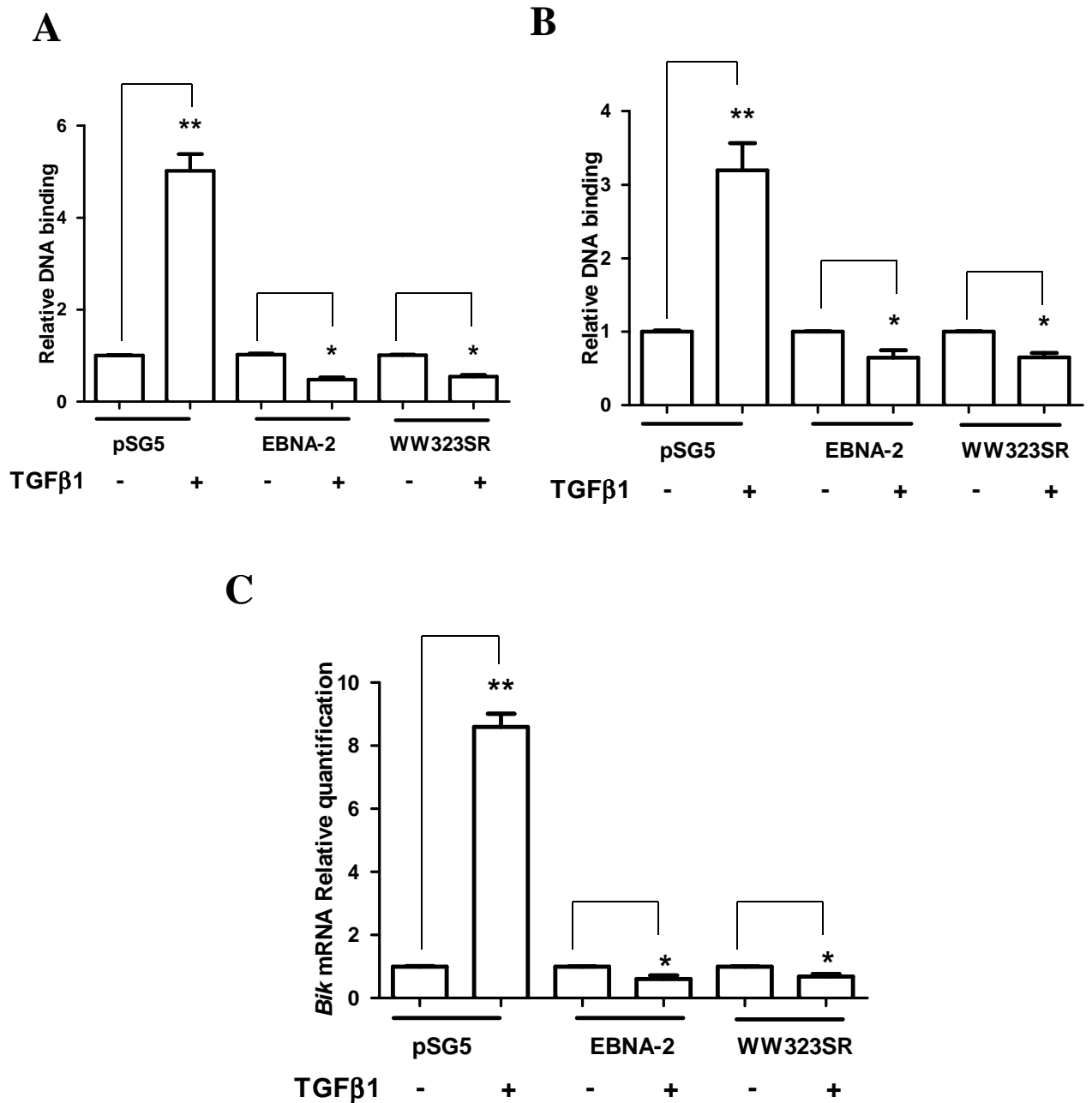
Figure 3-17: EBNA-2 abrogates the TGFβ1-mediated recruitment of Smad3/4 to the *bik* promoter and subsequent *bik* transactivation in BJAB cell.

BJAB cells were transfected with pSG5 or EBNA-2 or WW323SR. Forty-eight hours after transfection, cells were treated with (+/black) and without (-/white) 10 ng/mL TGFβ1 for a duration of 4 h. Cells were then harvested and immunoprecipitated with (A) anti-Smad3 and (B) anti Smad4. DNA isolated from immunoprecipitated material was amplified by Q-PCR as before with primers to target the 420-bp sequence upstream of the human *bik* promoter transcription start site. (C) Represents relative RT-qPCR analysis for *bik* expression in the presence or absence of TGFβ1 in transfected cells. Data are ±SD *$P \leq 0.05$, **P (0.001 to 0.01).

Total protein was harvested from the same experiment shown in (Figure 3-18) and analyzed for Smad3 expression by Western blot. The Smad3 protein level was significantly lower in BJAB cells expressing either ectopic EBNA-2 or its non-CBF1 binding mutant, WW323SR (Figure 3-18 A and B). These results indicated a role for EBNA-2 in inhibiting TGFβ1-mediated effects by Smad3 down-regulation and that the EBNA-2-CBF1 interaction was not essential for this effect.
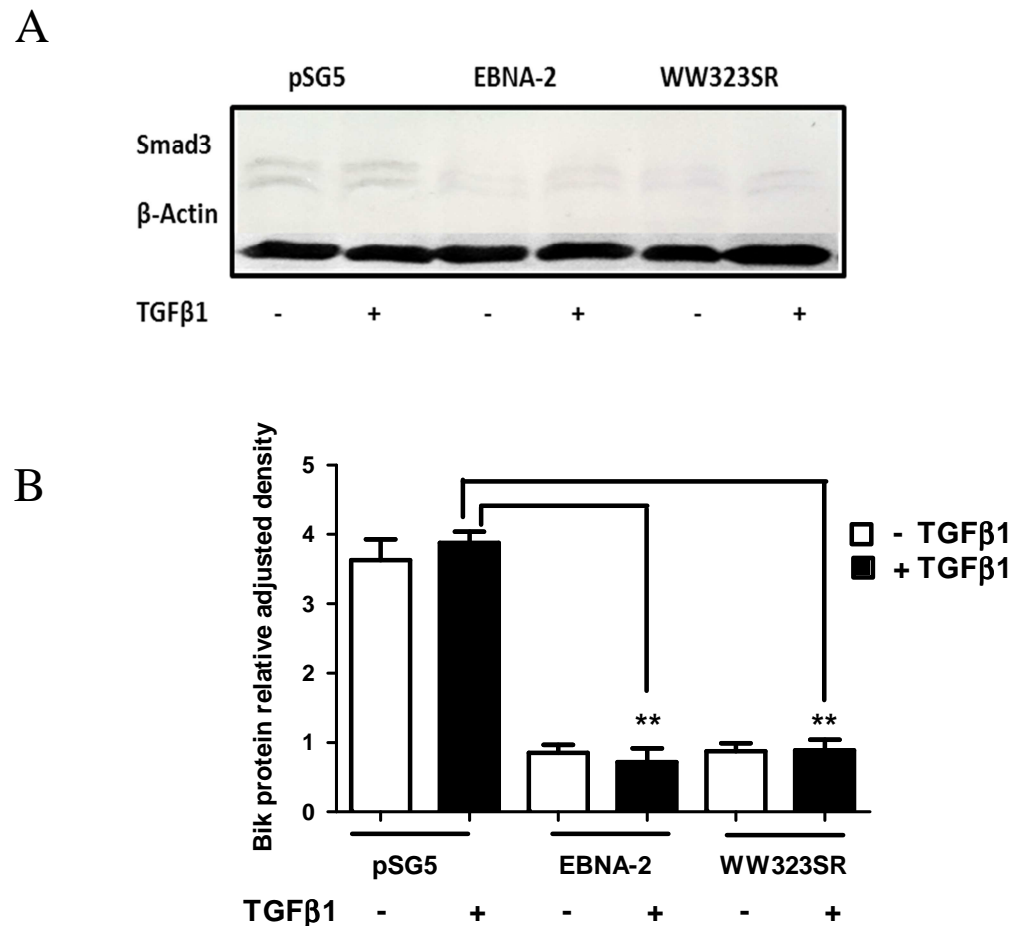
Figure 3-18: Inhibition of TGFβ1-mediated Smad3 up-regulation by EBNA-2 and WW323SR in BJAB.

BJAB cells were transfected with pSG5, EBNA-2 or WW323SR. Forty-eight hours after transfection, cells were treated with 10 ng TGFβ1 for 4 h. (A) Cells were then harvested and whole cell lysate was prepared and subjected to Western blot analysis determining the Smad3 and β-actin protein. (B) Bik protein relative density values shown were obtained from ImageJ and were normalised to untreated cells transfected with pSG5.

Next, siRNA technology was used to directly down-regulate endogenous *Smad3* so as to evaluate its contribution to the transcriptional regulation of *bik*. In this regard, Ramos and BJAB cells were treated with two different anti-*Smad3* siRNAs (si56 and si57) or negative control siRNA (siNeg). Twenty-four hours after transfection cells were treated with 10 ng/mL TGFβ for a further 4 h. Transfected cells stimulated with TGFβ1 were then harvested and analysed for Smad3 and Bik protein and *bik* mRNA levels.

It can be seen that both anti-*Smad3* siRNAs significantly inhibited Smad3 expression, whereas the control siRNA (siNeg) did not (Figure 3-19 A and B upper panel). *Smad3* knockdown also coincided with a moderate decrease in the level of Bik protein in response to TGFβ1 (Figure 3-19 A and B middle panel). Treatment of transfected cells with anti-*Smad3* siRNA reversed the TGFβ1 stimulatory effect on *bik* in comparison to cells transfected with control siRNA (siNeg) (Figure 3-19 C and D). These observations imply a direct regulatory role for Smad3 in regulating *bik* expression.
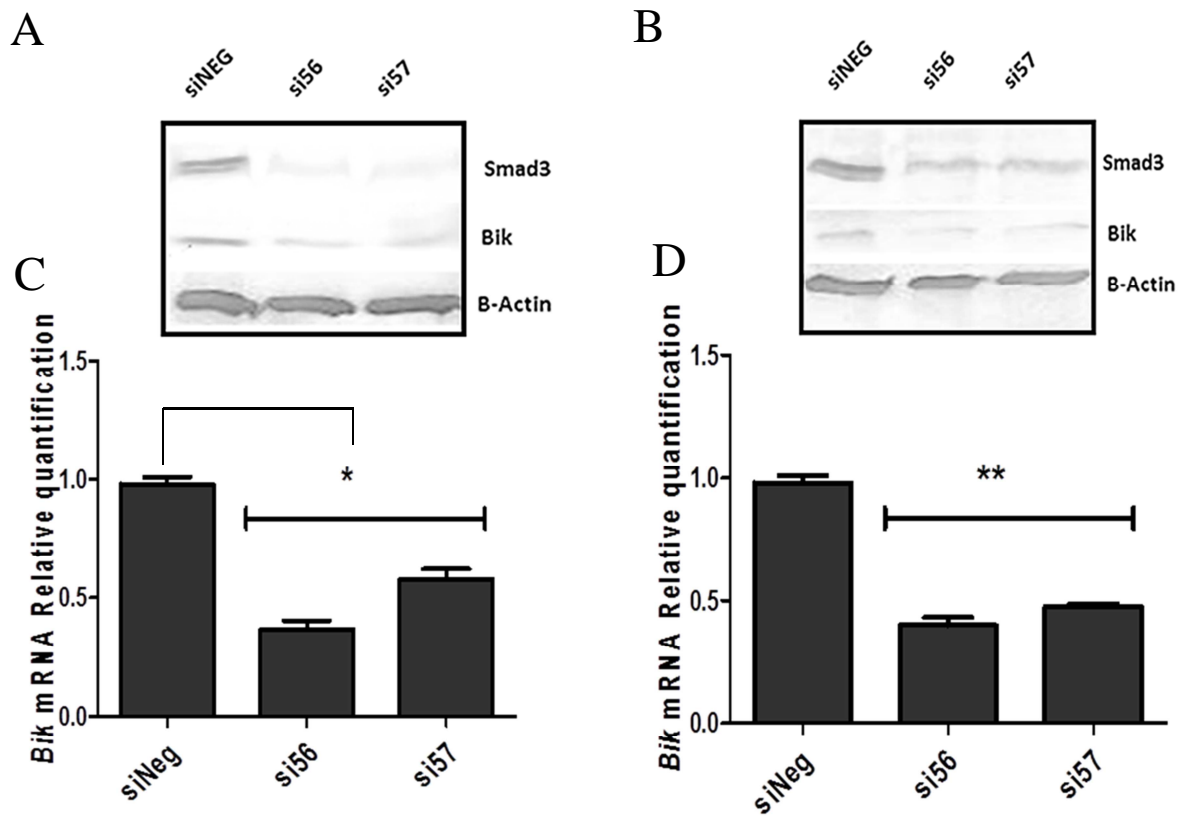
Figure 3-19: Knockdown of *Smad3* results in *bik* down-regulation.

BJAB and Ramos cells were transfected with two different anti-*Smad3* siRNAs (si56 and si57) or negative control siRNA (siNeg). Twenty-four hours after transfection, cells were treated with 10 ng/mL TGFβ1 for an additional 4 h. Western blots showing Smad3 (upper panel), Bik (middle panel) and β-actin (lower panel) in BJAB (A) and Ramos (B). Relative *bik* mRNA level were determined by RT-qPCR using RNA prepared 24 h after treatment of transfected BJAB (C) and Ramos cells (B). Fold differences were calculated relative to *bik* level in the siNeg transfected cell pool (assigned value of 1). Data are ±SD *$P \leq 0.05$, **P (0.001 to 0.01).

3.6 The EBV growth programme diminishes TGFβ1-mediated recruitment of Smad3/4 to the *bik* promoter

3.6.1 Bik is down-regulated by EBNA-2 in ER/EB 2-5 cells.

ER/EB2-5 is a conditional LCL in which the function of EBNA-2 can be controlled by the presence or absence of oestrogen (Kempkes, et al. 1995b). This was achieved by fusing the N-terminus of EBNA-2 to the hormone-binding domain of the oestrogen receptor. Essentially, this ER/EBNA-2 fusion gene was then used to complement the EBNA-2 deletion in the EBV P3HR1 viral genome (Kempkes, et al. 1995b). The resulting 'conditional' LCL, designated ER/EB2-5, generated after immortalising resting B cells with this recombinant EBV, therefore requires the presence of oestrogen for EBNA-2 function and hence cell proliferation. Addition of oestrogen results in EBV-infected B cells with a phenotype similar to those found in a typical LCL. Cells growing in the absence of oestrogen cease to proliferate due to the expression of only non-functional ER/EBNA-2 and the shut-off of the EBV growth programme (see Figure 3-20).
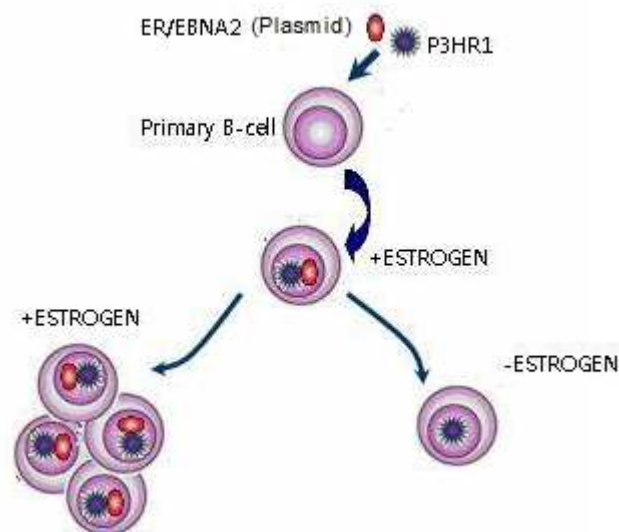
Figure 3-20: Schematic diagram of ER/EB2-5.

ER/EB2-5 is an LCL that contains a mini-EBV plasmid expressing a chimeric EBNA-2 oestrogen receptor fusion protein plus the P3HR1 viral genome, thus complementing the latter's EBNA-2 deletion. EBNA-2 expression and proliferation are dependent on the presence of oestrogen. Adapted from, (Kohlhof, et al. 2009).

In order to address a role for EBNA-2/Smads in *bik* regulation in this context, as opposed to BL-derived cell lines, ER/EB2-5 cells were cultured in the presence and absence of oestrogen. In these cells, EBNA-2 function, and therefore EB virus-driven cell proliferation, is dependent on oestrogen. Cells were harvested at 0, 24, 48, 72 and 96 h and Western blot analysis (Figure 3.21 A) confirmed that ER/EB2-5 cells grown in the absence of oestrogen, only expressed non-functional ER/EBNA-2, while cells grown in the presence of oestrogen expressed increased ER/EBNA-2 with an apparent higher molecular weight of 120 kDa, confirming the activation of ER/EBNA-2 (due to phosphorylation) and the ensuing transcriptional activation of its own gene. Changes in *bik* mRNA levels due to EBNA-2 expression over time in ER/EB2-5 are shown in Figure 3.21 B. *Bik* mRNA levels were seen to increase dramatically over time (44-fold at 48 h) in ER/EB2-5 cells upon inactivation of EBNA-2. The amount of *bik* mRNA was also seen to greatly increase by 72 h (80-fold when compared to time 0 h). A reduction in *bik* levels (approximately 12.5-fold) was observed 48 h following the addition of oestrogen to the culture medium (Figure 3.21 B). A further decrease was seen at 72 h (0.7-fold), and levels of *bik* mRNA remained stably-repressed from this point on, similar to that observed at 0 h in cycling cells. It has been shown elsewhere that TGFβ1 is able to reactivate the viral lytic cycle (Iempridee, et al. 2011). Thus, in order to rule out the effect of EBV lytic gene expression on the Smad canonical signalling pathway, ER/EB2-5 cells were left untreated with TGFβ1 in this experiment. ER/EB2-5 cells grown in the absence or presence of oestrogen for periods of 48 and 72 h were harvested, fixed and ChIP was performed for Smad3 and Smad4 as described before. DNA isolated from immunoprecipitated material was detected by Q-PCR as described before. It can be seen that expression of activated ER/EBNA-2 abrogated the observed TGFβ1-induced increase in Smad3 (Figure 3.21 C) and Smad4 (Figure 3.21 D). This effect was best seen at the 48 h time point.
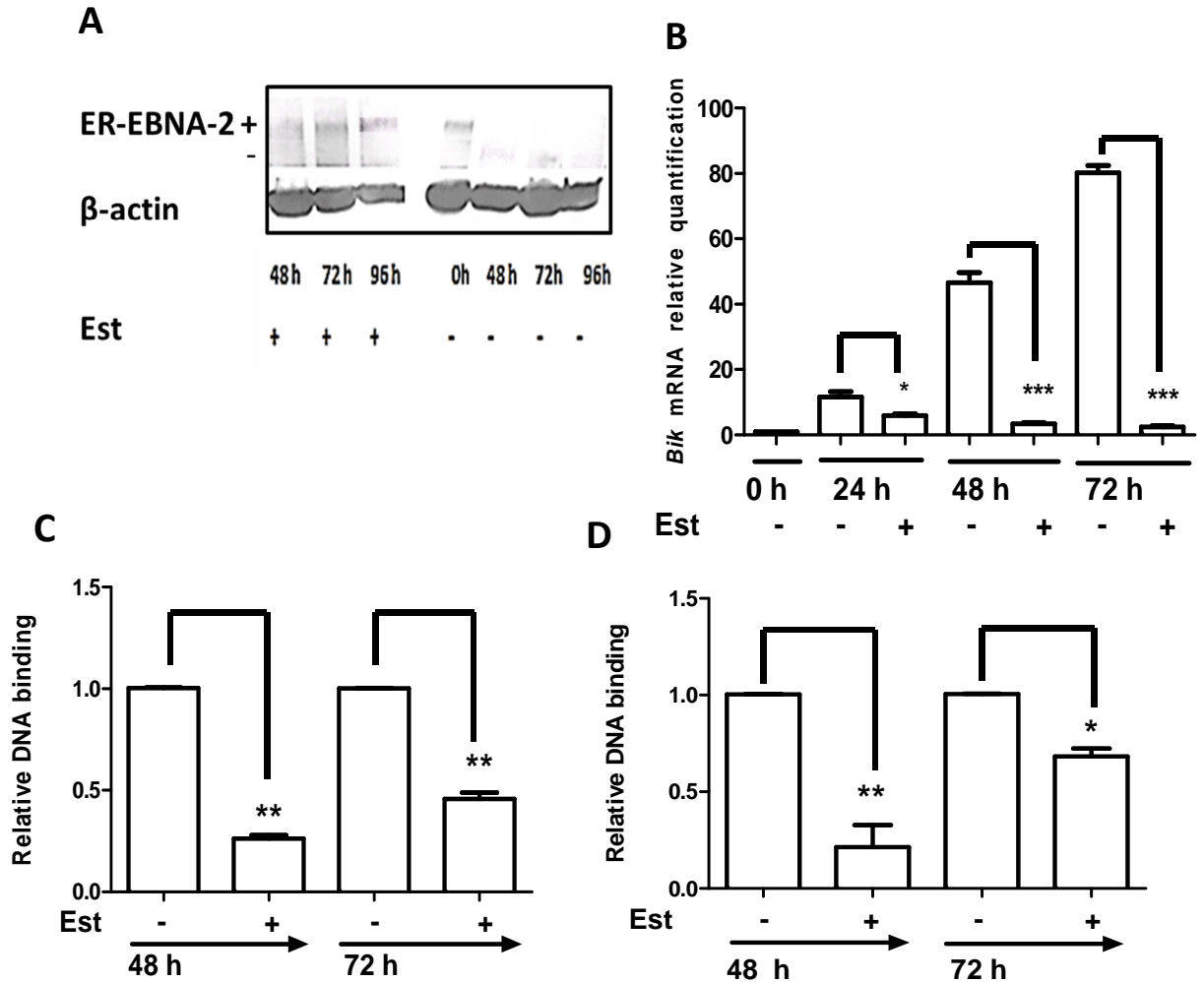
Figure 3-21: Repression of *bik* and decreased Smad3/4 recruitment to the *bik* promoter in response to the activation of ER/EBNA-2 in the conditional LCL ER/EB2-5.

ER/EB2.5 cells were cultured in the presence (active/cycling) and absence (EBV growth programme is repressed) of oestrogen. Cells were harvested at indicated time points from both activated and repressed phenotype. (A) Western blots indicating the expression of ER/EBNA-2 and β-actin proteins. (B) RT-qPCR analysis showing relative *bik* mRNA levels. Cells were harvested at 48 h and 72 h time points and ChIP was performed as described before. Chromatin was immunoprecipitated with (C) anti-Smad3 antibody and (D) anti-Smad4 antibody. DNA isolated from immunoprecipitated material was amplified by Q-PCR as described before. Data are ±SD *$P \leq 0.05$, **P (0.001 to 0.01), ***$P < 0.001$.

To investigate if EBNA-2 might be interacting with the *bik* promoter in the absence of extraneous TGFβ1, ER/EB2-5 cells were cultured in the presence and absence of oestrogen for a period of 48 h, chromatin was then harvested and ChIP was performed as described before. It can be seen that the level of Smad3 binding to the *bik* promoter decreased by 0.6-fold in the presence of oestrogen (EBV growth programme-dependent cell proliferation) and that EBNA-2 interaction with the *bik* promoter was increased by 1.3-fold (Figure 3-22).
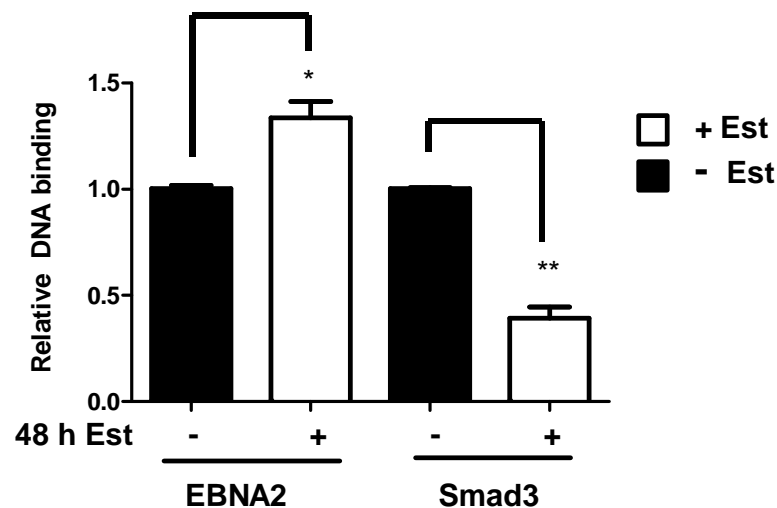
Figure 3-22: Less Smad3 is bound to the *bik* promoter during the EBV growth programme in ER/EB2-5 cells.

ER/EB2-5 cells, cycling in the presence and absence of oestrogen for 48 h, were harvested and ChIP was performed as described before with anti-Smad3 and anti-EBNA-2 antibodies. DNA isolated from immunoprecipitated material was amplified by Q-PCR as before. Data are ±SD *$P \leq 0.05$, **P (0.001 to 0.01).

In order to investigate a potential role for the EBV growth programme in regulating Smad3 levels, protein lysates were prepared from suppressed/cycling ER/EB2-5 cells at 48 h, 72 h, and 96 h and in the absence of extraneous TGFβ1. It was clear, both from whole cell extracts and nuclear fractions, that Smad3 protein levels had decreased overall in response to the activation of the EBV growth programme (Figure 3-23).
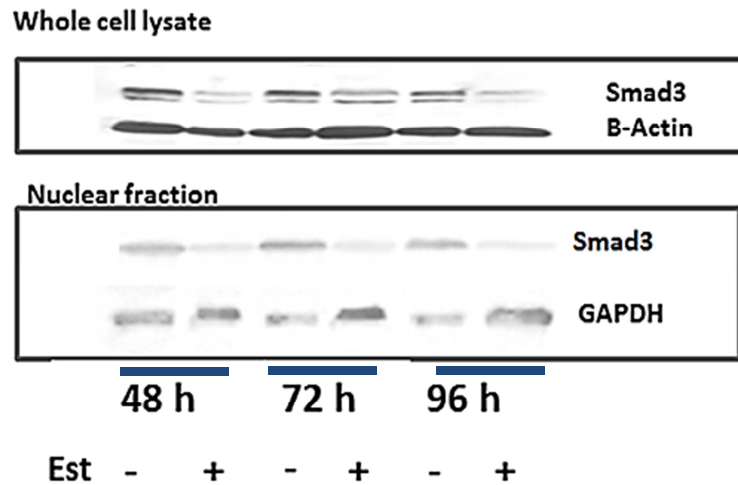
Figure 3-23: Modulation of Smad3 protein expression by the EBV growth programme in the conditional LCL ER/EB2-5.

Protein extracts were prepared from ER/EB2-5 cells cultivated in the presence (activation of EBV growth programme) and absence (suppression of EBV growth programme) of oestrogen at the time points indicated. The figure shows Western blots of Smad3 and GAPDH and β-actin proteins using whole cell lysate (top panel) and nuclear fractions (bottom panel).

Summary

Transforming growth factor beta, a member of the TGFβ superfamily of ligands, has highly pleiotropic properties which are determined by the dose and duration of signal in addition to the type and state of the target cell.

Here, evidence is presented to show that TGFβ1-mediated cell death coincided with the up-regulation of the pro-apoptotic BH3-only Bik in both Ramos and BJAB cell lines and that transient *bik* knockdown reversed the apoptotic effects mediated through TGFβ1 signalling. Furthermore, transient *bik* knockdown was seen to reverse the reduction in cell metabolic activity that was mediated by TGFβ1. TGFβ1-induced cell death is dependent on canonical Smad proteins in these cell lines, by prompting the translocation of Smad3 from the cytoplasm to the nucleus (indicative of Smad-dependent TGFβ signalling). ChIP assays showed that in the presence of TGFβ1, Smad3 and Smad4 were recruited to the *bik* promoter and subsequent RT-qPCR analysis confirmed that this coincided with an increase in *bik* mRNA levels. As recent findings in our laboratory indicated an association between EBNA-2 and *bik* down-regulation, experiments were designed to investigate a possible role for Smad3 in this process. Expression of ectopic EBNA-2 and its non-CBF1 binding mutant in Ramos cells had a pro-survival effect when apoptosis was induced through TGFβ1 signalling. Furthermore, subsequent ChIP analysis showed that less Smad3 and Smad4 was bound to the *bik* promoter in both cell lines in the presence of TGFβ1, and that this effect was not dependent on the ability of EBNA-2 to interact with CBF1. In BJAB, ectopic expression of EBNA-2 or its non-CBF1 binding mutant led to a decrease in the level of total Smad3. The level of Smad3 protein did not increase in response to TGFβ1 stimulation, as opposed to the case when BJAB cells were transfected with control plasmid (pSG5).

The conditional LCL ER/EB2-5 was used to investigate the EBV/*bik* interaction in a setting in which the EBV growth programme could be switched on and off. ER/EB2-5 cells proliferating due to EBV showed a significant decrease in *bik* mRNA when compared to cells where the EBV growth programme was suppressed. *Bik* down-regulation in ER/EB2-5 cells proliferating in the presence of oestrogen coincided with decreased levels of Smad3 and Smad4 bound to the *bik* promoter when compared to ER/EB2-5 cells, where the EBV growth programme was suppressed. In parallel to these observations, the total level of Smad3 protein was seen to be decreased in ER/EB2-5

cells proliferating due to the EBV growth programme. These results were obtained in the absence of extraneous TGFβ1, as addition of this hormone has been shown to activate the EBV lytic cycle (Iempridee, et al. 2011, di Renzo, et al. 1994). It was also illustrated here that in the presence of TGFβ1 there was an increase in EBNA-2, along with a decrease in Smad3, recruited to the *bik* promoter in Ramos cells expressing ectopic EBNA-2. Likewise, in ER/EB2-5 cells proliferating due to the EBV growth programme, the level of EBNA-2 recruitment to the *bik* promoter increased when compared to ER/EB2-5 cells where the EBV growth programme was suppressed.
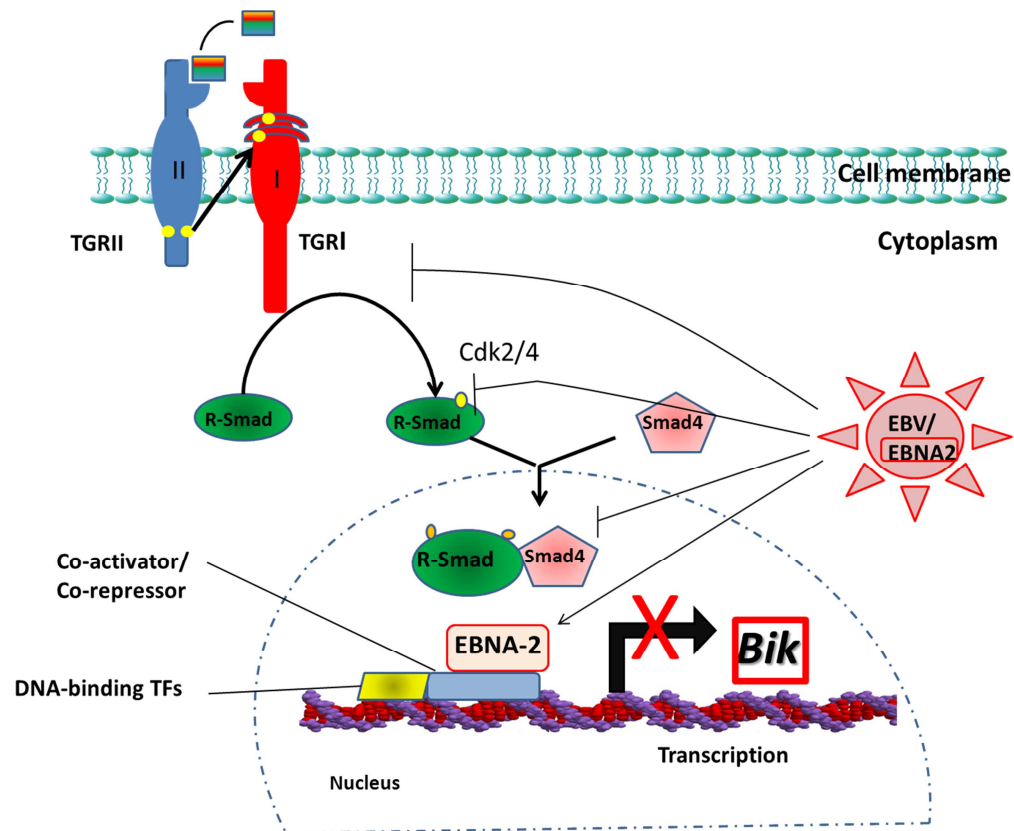
Figure 3-24: A model for EBV inhibition of TGFβ1-mediated cell death.

TGFβ triggers a signalling cascade through interaction with two transmembrane serine/threonine kinase receptors, TβR1 and TβR2. The main intracellular mediators of these receptors are a family of proteins known as Smads. Here it has been shown that in the presence of EBV growth programme or ectopic EBNA-2, the basal level of receptor regulated Smad (Smad3) protein is decreased (possible mechanisms have been indicated in figure; see Chapter 6) resulting in decreased recruitment of Smad3 to the *bik* promoter.

In summary, the mechanism of action of TGFβ1 in Ramos and BJAB was seen to partly involve the induction of an apoptotic program via transcriptional up-regulation of the pro-apoptotic protein Bik, in a manner dependent on the Smad canonical pathway. The increase in the level of this BH3-only protein contributed to intrinsic apoptosis. TGFβ1-mediated apoptotic effects were diminished in cells transiently expressing EBNA-2 as a likely consequence of Smad3 protein down-regulation and a reduction in the level of Smads bound to the *bik* promoter. Furthermore, evidence was generated to suggest that EBNA-2 may be interacting with the *bik* promoter. Moreover, it was seen that the EBV growth programme repressed *bik* through down-regulation of the basal level of Smad3. This coincided with a decreased level of *bik* promoter-bound Smad3 and an increased level of EBNA-2 recruited to the *bik* promoter (Figure 3-24).

Chapter 4 :
The EBV Nuclear Antigen-2 rescues Ramos cells from BCR-induced apoptosis

Germinal centres are highly structured microenvironments essential for the clonal expansion of antigen-specific germinal centre B lymphocytes, followed by somatic hyper mutation (SHM) and isotype switching. Since it's a high risk procedure for generating autoreactive or non-functional clones, B cells transferring through germinal centres for development and activation are prone to programmed cell death initiated from the B cell receptor (BCR), FAS and TGFβ receptor signalling pathways, unless they receive sufficient pro-survival signals, e.g. from CD40 ligand (Kurosaki 2000, Benschop and Cambier 1999). Studies have revealed that BCR cross-linking with antibodies to membrane-bound IgM but not IgD, on the surface of B cells results in suppression of the PI3K/pp70^{S6X} signalling pathway (Banerji, et al. 2001). In contrast, (Jiang and Clark 2001b) have reported that both sIgM and sIgD ligation lead to the induction of *bik* mRNA, but only the former was shown to exert a pro-apoptotic effect, with an increase in Bik protein levels and strong activation of PI3K (Beckwith, et al. 1996) and calcium influx (Jiang and Clark 2001a). Bik plays a decisive role in programmed cell death induced by toxins, cytokines (TGFβ, IFN-γ) (Spender, et al. 2009), BCR cross-linking (Jiang and Clark 2001a) and viral pathogenesis (Boyd, et al. 1995a)(Chinnadurai, Vijayalingam and Rashmi 2008a). Significant up-regulation of *bik* transcripts has been reported in germinal centre B cells during differentiation from naive (IgD+, CD27–) to centroblast transition and its level remained elevated in memory B cells, hence it is likely to be an important factor for maintenance of B cell homeostasis (Klein, et al. 2003).

Previously, it has been shown that EBV-infected cells survive apoptotic signals through the interaction of the early lytic protein, BHRF1 (Landais, et al. 2005) with Bik protein (Boyd, et al. 1995a). EBV transformation of resting B lymphocytes to LCLs mimics antigen-induced resting B cell clonal expansion in lymph node germinal centres, where antigen binding to surface Ig (sIg) induces *myc*-mediated proliferation (Murn, et al. 2009) and T cell CD40 ligand activates B lymphocyte CD40 receptors to up-regulate NF-κB, MAP kinases, and anti-apoptotic Bcl-2 family protein expression (Elgueta, de Vries and Noelle 2010). Initiation of apoptotic signalling pathways via BCR modulates the expression of Bcl-2 family members to regulate the intrinsic mitochondrial apoptosis pathway, which can be opposed by appropriate survival signals. Controlling expression of the Bcl-2 family of proteins is therefore critical in regulating the GC reaction.

BCR ligation in the absence of co-stimulatory ligand-receptor interactions is important for the elimination of autoreactive B cells. BCR engagement with anti-IgM antibody is a model for antigen-induced clonal deletion, with apoptosis induced as a consequence of surface IgM (sIgM) cross-linking. In human B cells, *bik* is a downstream target of BCR signalling (Jiang and Clark 2001a, Craxton, et al. 1999, Craxton, et al. 2000). In B104 lymphoma cells, which possess the phenotype of mature B cells, sIgM engagement was shown to correlate strongly with sustained *bik* induction and apoptosis (Jiang and Clark 2001a). Therefore, experiments were set out to investigate whether *bik* repression could confer a survival advantage on BL-derived cells following BCR engagement. The BL cell line Ramos and the B cell lymphoma-derived BJAB are examples of malignant cells that have retained the normal response of GC B cells to BCR stimulation (Young and Staudt 2013).

4.1 BCR cross-linking in EBV-negative B cell lines leads to the induction of apoptosis

BJAB and Ramos cells were seeded at 5 x 10^5 cell/mL 24 h prior to treatment with three different concentrations of anti-IgM antibody. Twenty-four hours after antibody treatment, apoptosis was measured as described previously (in chapter 3). Apoptosis was seen to have been induced in both cell lines in a dose-dependent manner (Figure 4-1 and Figure 4-2). It was observed that Ramos cells (Figure 4-1) were more sensitive to apoptosis induced by anti-IgM ligation than BJAB cells (Figure 4-2), since 1 µg/mL anti-IgM antibody in Ramos resulted in 55% ±SD 3.593 of cells undergoing apoptosis whereas in BJAB this number increased to 14% ±SD 2.600. This is not due to lack of or low expression of sIgM as several groups have shown that BJAB expresses sIgM (Singer and Williamson 1980, Guerreiro-Cacais, Levitskaya and Levitsky 2010).
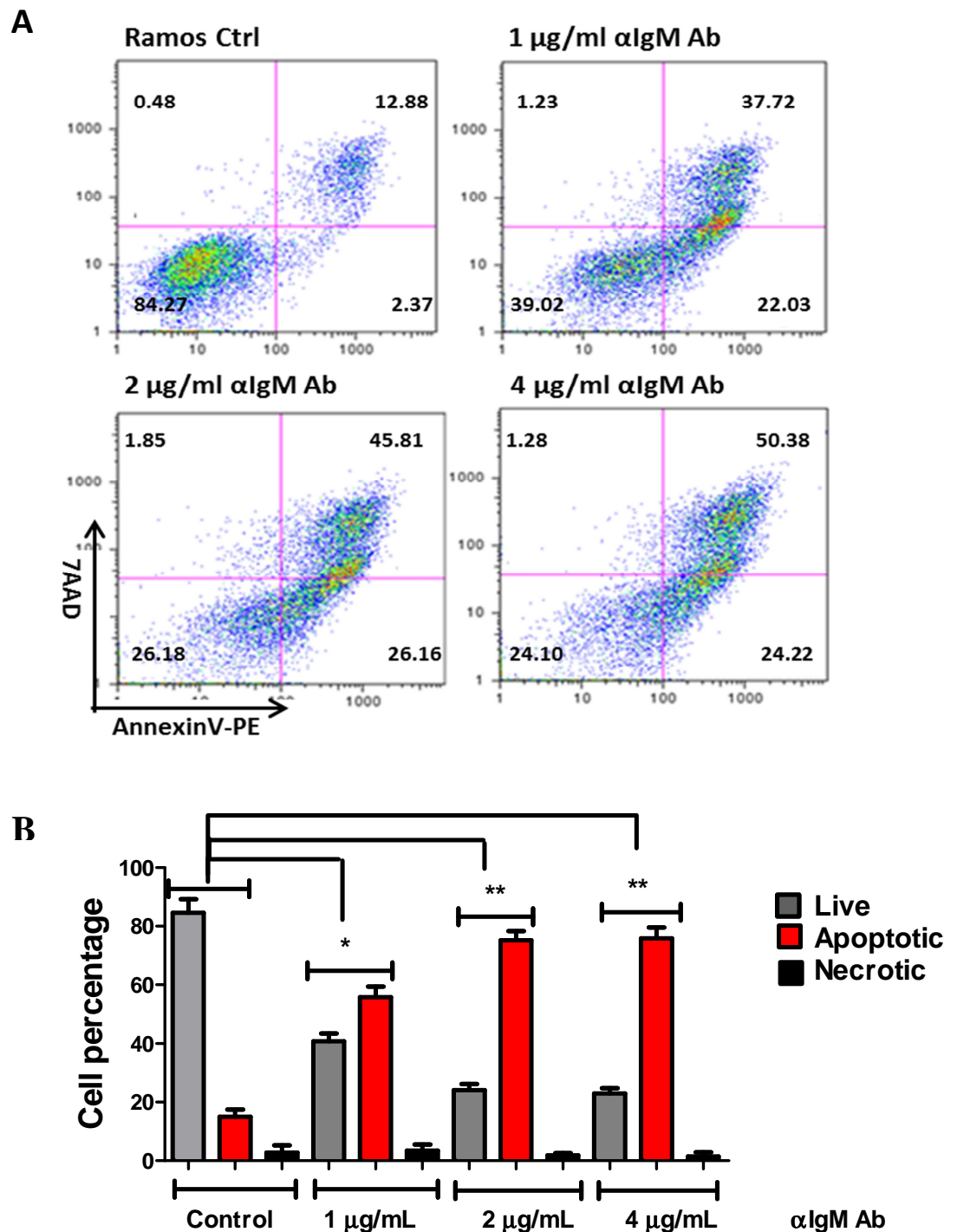
Figure 4-1: BCR cross linking in Ramos cells leads to apoptosis.

Ramos cells were treated with various concentrations of anti-IgM antibody, as indicated above each scatterplot, for a duration of 24 h. Cell survival profiles were analysed using 7AAD/Annexin-V staining and subsequent flow cytometric analysis. (A) Quadrant markers were based on stained/unstained controls. Cells in the lower left quadrant are viable (7AAD$^-$/Annexin-V$^-$), cells in the lower right quadrant are early apoptotic (7AAD$^-$/Annexin-V$^+$), cells in the upper right quadrant are late apoptotic (7AAD$^+$/Annexin-V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7AAD$^+$/Annexin-V$^-$). (B) Values presented as a bar chart reflect the percentage of cells in each quadrant for triplicate experiments. Data are Mean ±SD *$P \leq 0.05$ and **P (0.001 to 0.01).
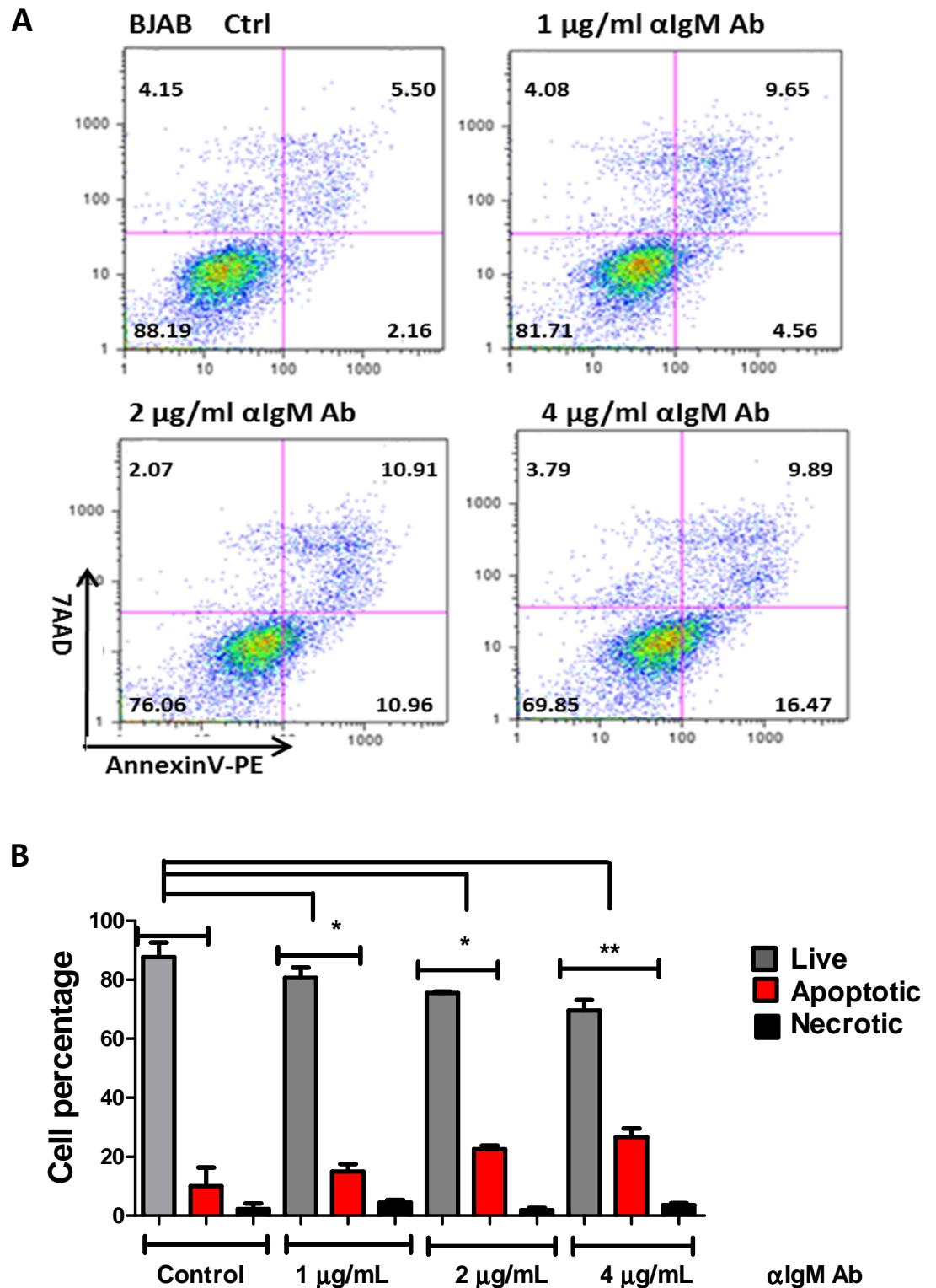
Figure 4-2: Apoptosis was induced by BCR cross-linking in BJAB cells.

BJAB cells were treated with 1, 2, and 4 µg/mL of anti-IgM antibody for 24 h. (A) Cell survival profile was analysed using 7AAD/Annexin-V staining and subsequent flow cytometric analysis as described in (Figure 4-1) legend. (B) Values presented as a bar chart reflect the percentage of cells in each quadrant for triplicate experiments. Data are Mean ±SD *$P \leq 0.05$ and **P (0.001 to 0.01) of three independent experiment.

4.2 BCR ligation leads to *bik* induction

In order to investigate if BCR cross-linking exerts its apoptotic effect by regulation of *bik*, Ramos and BJAB cells were treated with 0.5 µg/mL or 5 µg/mL anti-IgM antibody for a duration of 24 h. Total RNA and protein were harvested and relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h after treatment. *Bik* mRNA levels were assayed in triplicate and normalised to *gapdh* mRNA levels. Bik protein expression was analysed by Western blot.

Bik mRNA levels were seen to increase in both Ramos (Figure 4-3) and to a lesser extent in BJAB cells (Figure 4-4) following treatment with anti-IgM antibody, and in a dose-dependent manner. Bik protein levels increased also in both cell lines (Figure 4-3 B and Figure 4-4 C; B and C), with a more pronounced effect in Ramos. Bik is a phosphoprotein and it is phosphorylated on consensus *Casein Kinase II* (CKII) phosphorylation sites (Thr-33 and/or Ser-35). Phosphorylation is required for efficient Bik-mediated apoptosis (Verma, Zhao and Chinnadurai 2001). Bik appears as a 27 kDa/26 kDa double band in some cell lines and a single band in others. Western blot analysis of the Ramos cell line showed a single band for Bik protein, whereas BJAB demonstrated a double band. When Ramos was stimulated with the TGFβ1 ligand, it was the higher molecular weight band that was seen to be affected. These observations are consistent with what has already been reported in the literature (Jiang and Clark 2001a, Spender and Inman 2009a).

A

B
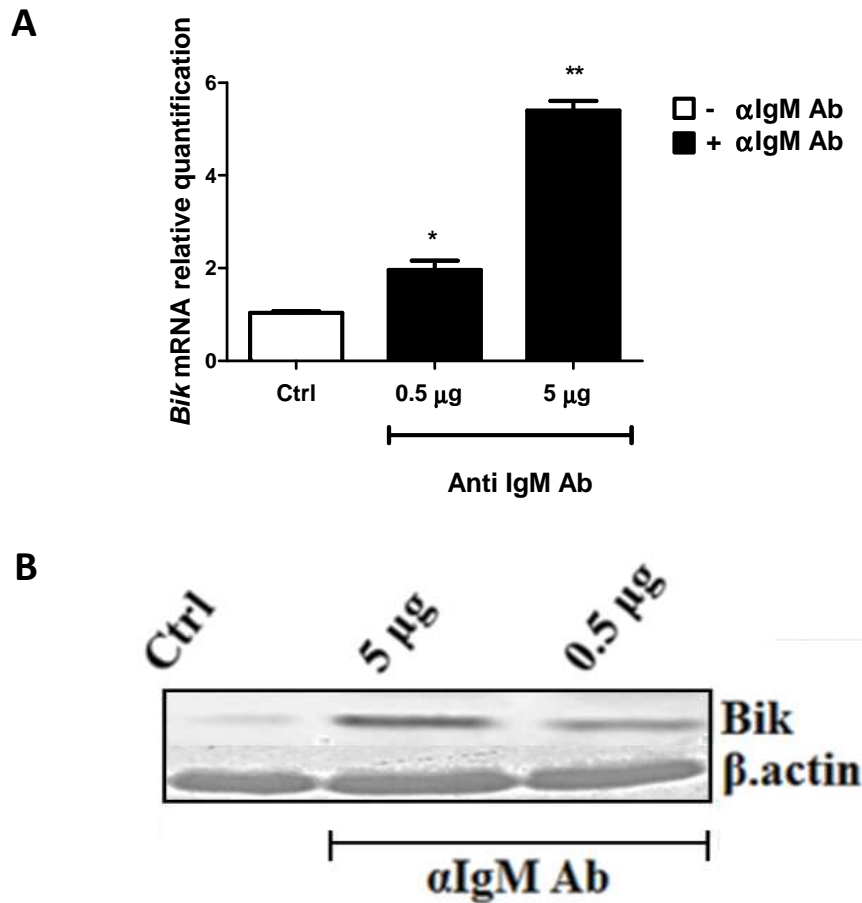
Figure 4-3: BCR ligation with anti-IgM antibody leads to *bik trans*-activation in Ramos.

Ramos cells were treated with 0.5 µg/mL and 5 µg/mL anti-IgM antibody. (A) Relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h after treatment. *Bik* levels were assayed in triplicate and normalised using *gapdh*. Relative quantification levels were calculated relative to the control. Data are Mean ±SD *$P \leq$ 0.05 and **P (0.001 to 0.01) vs untreated control. (B) Western blot showing Bik protein expression. (C) Bik protein relative density values shown were obtained from ImageJ and were normalised to untreated control.
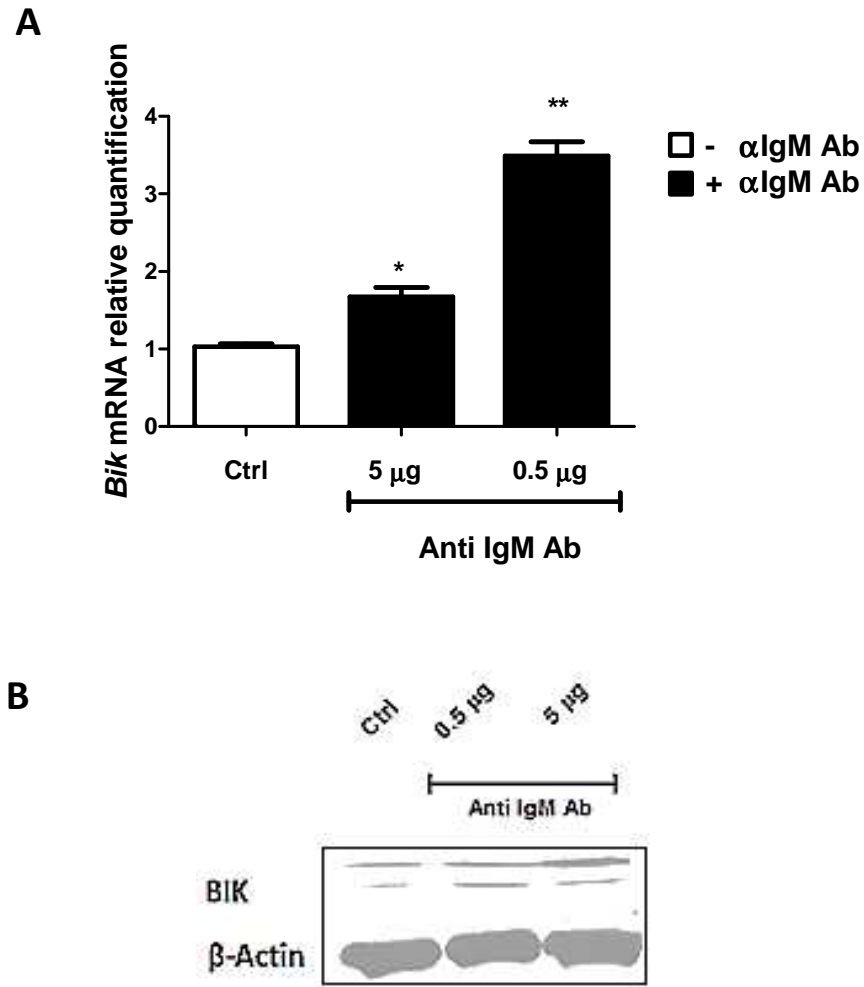
Figure 4-4: BCR ligation with anti-IgM antibody leads to *bik trans*-activation in BJAB

BJAB cells were treated with 0.5 µg/mL and 5 µg/mL Anti-IgM antibody. (A) Relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h after treatment as described in the legend of (Figure 4.3). Data are Mean ±SD *$P \leq 0.05$ and **P (0.001 to 0.01) vs untreated control. (B) Western blot showing Bik and β-actin protein expression.

4.3 Transient *bik* knockdown does not significantly inhibit anti-IgM-induced apoptosis in EBV negative cell lines

In order to investigate to what extent *bik* is involved in BCR-mediated cell death, *bik* was transiently knocked down in Ramos (Figure 4-5) and BJAB (Figure 4-6) cell lines using anti-*bik* siRNAs (si1989 and si1990). Protein extracts at 24 h and 48 h post transfection were prepared from both cell lines and were analysed by Western blot. RNA extracts were prepared at 24 h post transfection and the level of *bik* mRNA expression was analysed by RT-qPCR as described before. In transfection experiments with Ramos and BJAB, both anti-*bik* siRNAs were shown to inhibit *bik* expression, as reflected in decreased *bik* mRNA and protein levels. In contrast, the control siRNA siNeg did not affect Bik expression (Shown in chapter 3, Figure 3.5 and Figure 3.6). Cells that were transfected with siRNAs were then left untreated or treated with 0.5 µg/mL of anti-IgM antibody. The cell survival profile was determined 24 h following treatment by 7AAD/Annexin-V-PE staining and subsequent flow cytometric analysis. It can be seen that treatment with αIgM led to the induction of apoptosis in both cell lines, with a more pronounced effect seen in Ramos cells (Figure 4-5 and Figure 4-6). In the transfected cell population, significant apoptosis was observed before treatment with/without anti-IgM antibody (Figure 4-5 and Figure 4-6), and transient *bik* down-regulation appeared to rescue cells from transfection-induced apoptosis by approximately 20% (Figure 4-5 and Figure 4-6). Transient *bik* knockdown was seen to decrease the overall apoptotic level but it did not have a pronounced effect in significantly inhibiting apoptosis mediated by BCR cross-linking in both cell lines (Figure 4-5 and Figure 4-6). It has previously been shown in purified immature and mature B cells from wild type, $bim^{-/-}$, and *vav–bcl*-2 transgenic mice that BCR cross-linking mediates apoptosis through induction of both Bim (Enders, et al. 2003b) and Bik (Jiang and Clark 2001b) pro-apoptotic proteins. Thus, while it appears that *bik* knockdown had a pro-survival effect, the induction of Bim might be masking the actual effect.

A
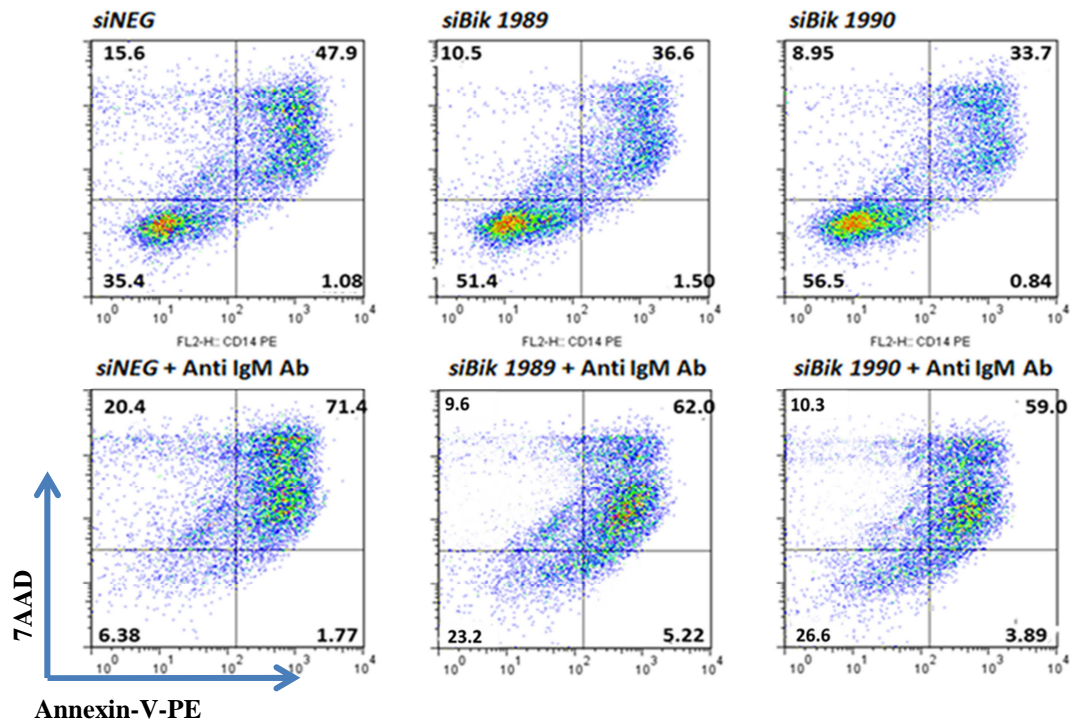
B
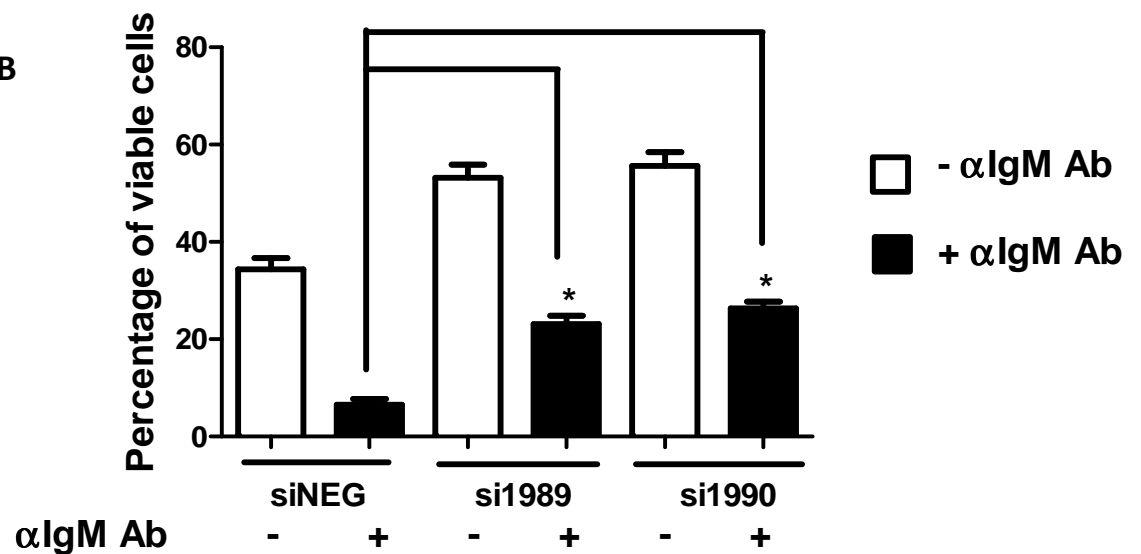
Figure 4-5: Transient *bik* knockdown does not significantly inhibit anti-IgM-induced apoptosis in Ramos.

Ramos cells were transfected with each of two different *bik* siRNAs (si1989, si1990) or negative control siRNA (siNeg). Twenty-four hours following transfection, cells were treated with 0.5 μg/mL of anti-IgM antibody for duration of 24 h. (A) Cell survival profile was analysed using 7AAD/Annexin-V staining and subsequent flow cytometric analysis as described in (Figure 4-1) legend. (B) Values presented as a bar chart reflect the percentage of viable cells for triplicate experiments. Data are Mean ±SD *$P \leq 0.05$.

Figure 4-6: Transient *bik* knockdown does not significantly inhibit anti-IgM-induced apoptosis in Ramos.

BJAB cells were transfected with each of two different *bik* siRNAs (si1989, si1990) or negative control siRNA (siNeg). Twenty-four hours following transfection, cells were treated with 0.5 μg/mL of anti-IgM antibody for duration of 24 h. (A) Cell survival profiles were analysed using 7AAD/Annexin-V staining and subsequent flow cytometric analysis as described in (Figure 4-1) legend. (B) Values presented as a bar chart reflect the percentage of viable cells for triplicate experiments. Data are Mean ±SD *$P \leq 0.05$ and**P (0.001 to 0.01).

4.4 Ectopic EBNA-2 inhibits BCR-induced apoptosis in Ramos cells

As previously described, recent findings in our laboratory indicated that Bik repression appears to be a feature of LCLs and BL cells expressing all the viral latency-associated proteins. These results suggested that *bik* is a negative transcriptional target of the EBV latent protein, EBNA-2 (Chapter 1). In order to investigate to what extent EBNA-2 expression in EBV negative cells can protect these cells from apoptosis induced by BCR cross-linking, a co-transfection strategy involving pMaxGFP with one of pSG5-EBNA-2, pSG5-EBNA-2 WW323SR or the corresponding control vector pSG5, was therefore adopted in order to realise this goal. Forty-eight hours after transfection, cells were treated with 0.5 µg/mL anti-IgM antibody for a duration of 24 h and apoptosis was measured in the population of cells expressing GFP by using Annexin-V-PE/7AAD staining. It can be seen that expression of EBNA-2 rescued Ramos cells from antigen-receptor-mediated apoptosis with 42% ±SD 5.033 of cells being viable and that the EBNA2-CBF1 interaction was not essential for this effect with 44% ±SD 5.000 viable cells, compared to Ramos cells transfected with control vector with decreased viability (17% ±SD 5.508) (Figure 4-7). The large proportion of necrotic cells present is due to harsh transfection conditions. It has been shown previously in our laboratory that EBNA-2 negatively regulates the expression of *bik*. In addition, EBNA-2 *trans*-activates the expression of the pro-survival Bcl-2 family members, *bcl-2* and *bfl-1* (Pegman, et al. 2006a). Also, EBNA2 has been shown to bind Nur77 and protect against Nur77-induced cell death (Lee, et al. 2002).
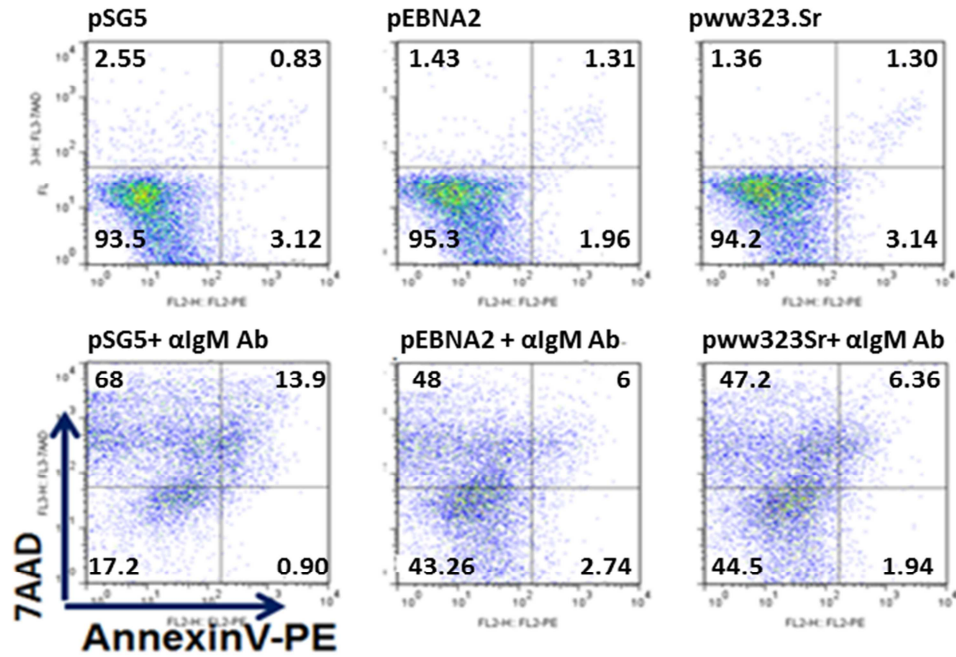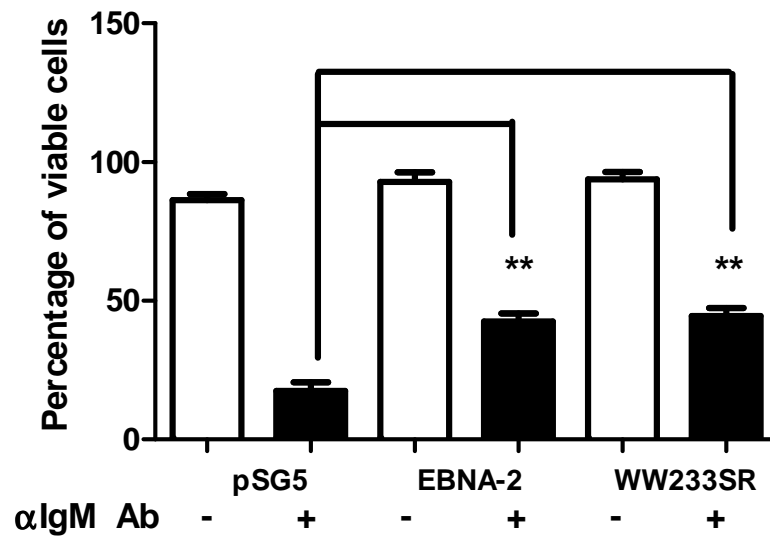
Figure 4-7: EBNA-2 inhibits apoptosis induced by BCR cross-linking in Ramos

Ramos cells were co-transfected with 0.5 µg pMaxGFP and 0.5 µg of each plasmid as indicated underneath the graph. Forty-eight hours after transfection, cells were treated with 0.5 µg/mL anti-IgM antibody. (A) Represents the cell viability/apoptosis FACS profiles 24 h after treatment with anti-IgM antibody. The cells in each quadrant are as described in the legend to Figure 4-1 (B) Bar chart representation of the percentage of viable cells in experiment shown in A (average of three independent experiments). Data are ±SD **P (0.001 to 0.01).

A similar experiment was set up in order to investigate if ectopic EBNA-2 reduces the rate or delays the onset of apoptosis induced by BCR cross-linking in BJAB (not shown). BJAB cells were co-transfected with pMaxGFP and one of pSG5-EBNA-2, pSG5-EBNA-2 WW323SR or the corresponding control vector pSG5. Poor transfection efficiency (less than 10%) and a high rate of apoptosis due to transfection was observed in all cases. Several factors were changed in order to improve the transfection efficiency and cell viability (data not shown). Three transfection reagents were used (TransIT-LT1, SuperFect, TransIT-2020) instead of the Ingenio Electroporation Kit, which was used with either the Nucleofector (Amaxa) or Electroporator (Bio-Rad) instruments. In addition, total DNA concentrations (0.5 µg, 1 µg, and 2 µg), GFP expression plasmids (pEGFP-N1 and pMaxGFP) and the presence of antibiotics in culture medium (penicillin/streptomycin –P/S) were also investigated in order to optimize the transfection efficiency and increase the cell viability. In general, transfection efficiency increased moderately (from 10% up to 12%) when using pEGFP-N1 and cell viability increased significantly when electrical current and P/S were not used. Transfection efficiencies were higher when BJAB cells were electroporated or nucleofected compared to when these cells were transfected with TransIT-LT1, SuperFect, and TransIT-2020 reagents. However, cell viability was significantly lower. BJAB cells were efficiently tranfectable with siRNA and cell viability was not seen to be as low as when they were transfected with plasmids.

4.5 The involvement of PI3K-dependent pathway in BCR-mediated *bik* mRNA expression, and apoptosis.

The activity of phosphatidylinositol-3 kinase (PI3K) is essential for normal BCR-mediated responses, developmental progression in B lymphopoiesis and for the survival of mature B cells (Xu, et al. 2012, Vigorito, et al. 2004). Experiments carried out elsewhere showed that the expression of Bik protein is significantly increased after sIgM ligation in B104 B lymphoma cells, and furthermore that Bik protein expression is PI3K-dependent, since treatment with a PI3K inhibitor, wortmannin (Wm) blocked both sIgM-mediated Bik protein production and apoptosis (Jiang and Clark 2001a). In contrast, other studies have shown that inhibition of PI3K accelerates apoptosis in BL cell lines including Ramos (Curnock and Knox 1998, Spender and Inman 2012a).

In order to investigate if the PI3K pathway plays a role in the regulation of *bik* following BCR stimulation of GC-derived cells (as opposed to B104 cells, which are derived from a mature B cell), the intrinsic apoptotic pathway was again induced in Ramos and BJAB cells by treating the two cell lines with anti-IgM antibody. The PI3K pan-inhibitor, wortmannin (10 nM), was added 30 min prior to BCR cross-linking with 0.5 μg/mL anti-IgM antibody and a*poptosis* was then assessed as before using Annexin-V-PE*/*7-AAD double *staining*.
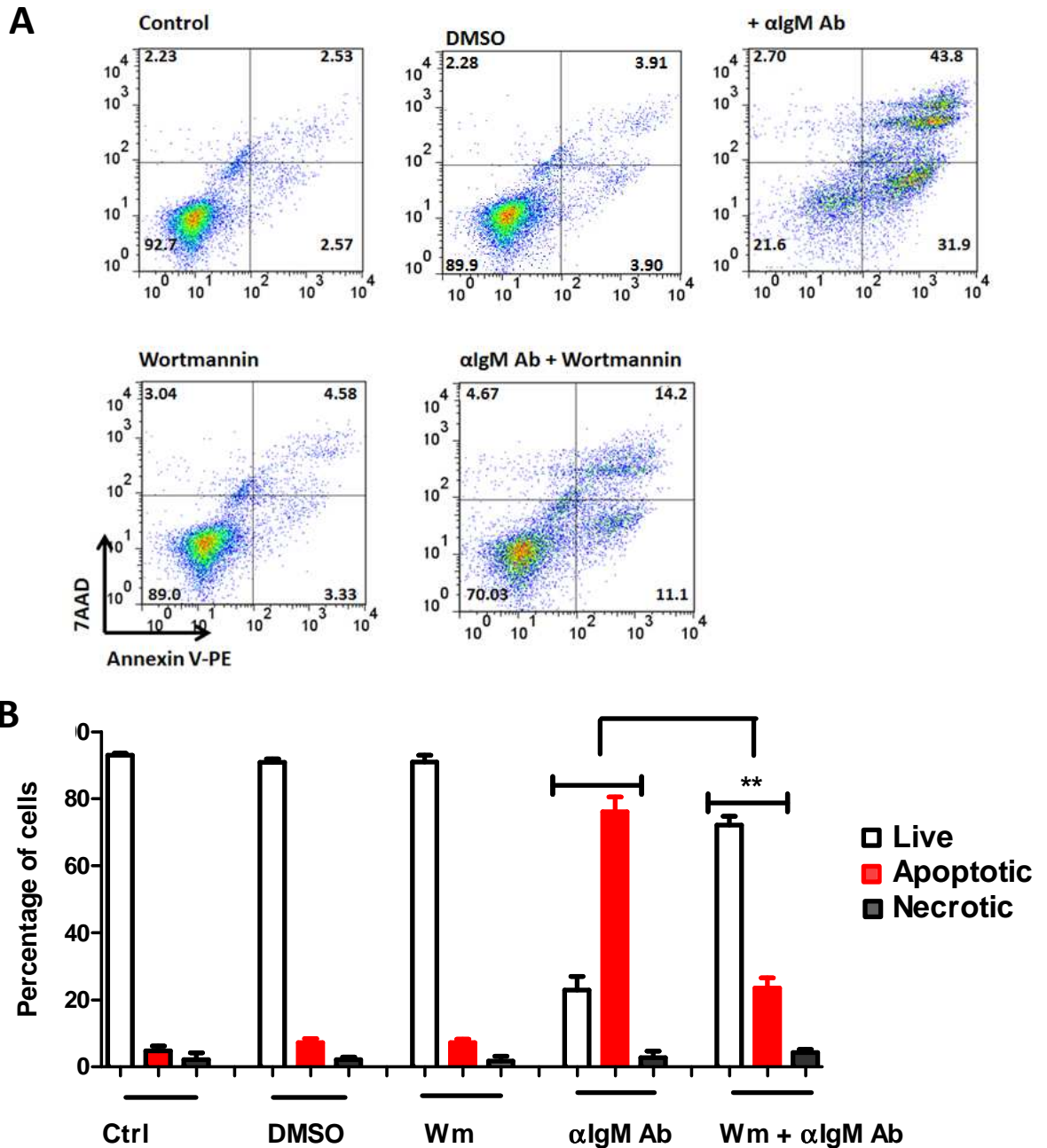
Figure 4-8: The effect of PI3K inhibition on BCR cross-linking mediated apoptosis in Ramos cells.

Ramos cells were treated with wortmannin 30 min before BCR stimulation with anti-IgM antibody. Apoptosis was assessed using 7AAD/Annexin-V double staining by flow cytometry. (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment are shown. The cells in each quadrant are as described in the legend to Figure 4-1 and the values shown represent the percentage of cells in each quadrant. (B) Bar chart represents the percentage of the cells in experiment shown in A (average of three independent experiments). Data are ±SD **P (0.001 to 0.01).

Treatment of Ramos cells with wortmannin or vehicle did not have any obvious effect on cell viability (Figure 4-8), whereas apoptosis was increased when BJAB cells were treated with wortmannin (25% ±SD 2.000) when compared to untreated cells (9.9% ±SD 1.504) (Figure 4-9). It can be seen that BCR cross-linking with anti-IgM antibody for 24 h led to 75% ±SD 5.000 of Ramos cells undergoing apoptosis (Figure 4-8).In comparison, BCR cross-linking mediated a lower level of apoptosis in BJAB cells (23% ±SD 3.512) (Figure 4-9). Treatment of Ramos cells with wortmannin inhibited the apoptotic effect induced by BCR cross-linking with anti-IgM antibody and resulted in 23% ±SD 3.060 of cells being apoptotic (Figure 4.8). In contrast, in BJAB cells, wortmannin did not have any significant impact on delaying or decreasing apoptosis induced by BCR cross-linking (Figure 4-9).
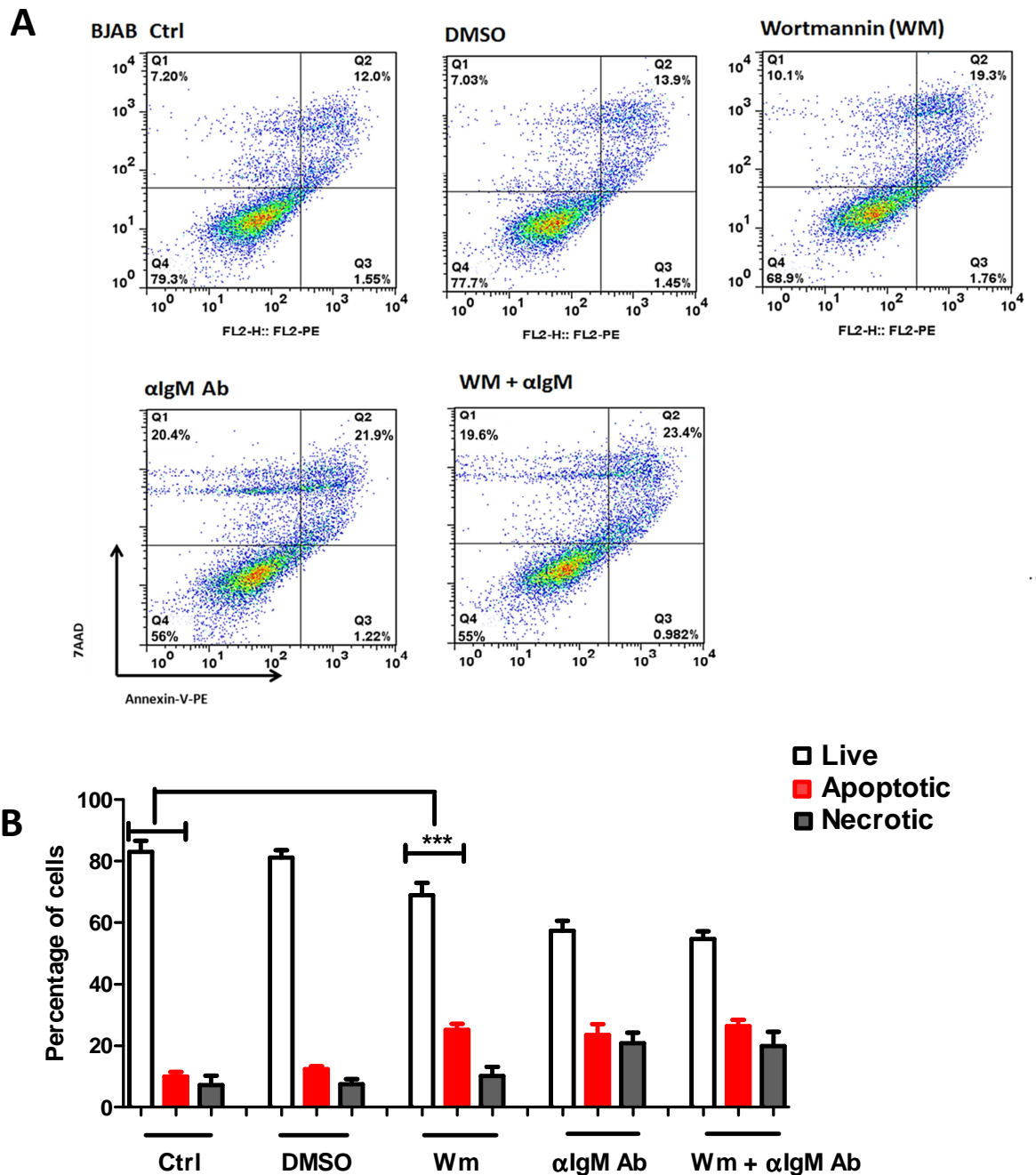
Figure 4-9: The effect of PI3K inhibition on BCR cross-linking mediated apoptosis in BJAB cells.

BJAB cells were treated with anti-IgM antibody. Wortmannin was added 30 min before anti-IgM antibody stimulation. Apoptosis was assessed using 7AAD/Annexin-V-PE double staining by flow cytometry. (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment are shown. The cells in each quadrant are as described in the legend to (Figure 4-1) and the values shown represent the percentage of cells in each quadrant. (B) Bar chart representation of the cells in experiment shown in A (average of three independent experiments). Data are ±SD ***$P < 0.001$.

In order to investigate a role for the PI3K signalling pathway in the *trans*-activation of *bik* upon BCR cross-linking, total RNA from the same experiment was also extracted and analysed for *bik* expression by RT-qPCR. As can be seen, *bik* mRNA was increased by approximately 6.3-fold in Ramos (Figure 4-10 A) and by 3.1-fold in BJAB (Figure 4-10 B) in the presence of 0.5 µg/mL anti-IgM antibody. However, when PI3K was inhibited by wortmannin, prior to BCR stimulation, *bik* mRNA expression was decreased by 3.1-fold in Ramos (Figure 4-10 A) and by 1.5-fold in BJAB cells (Figure 4-10 B).
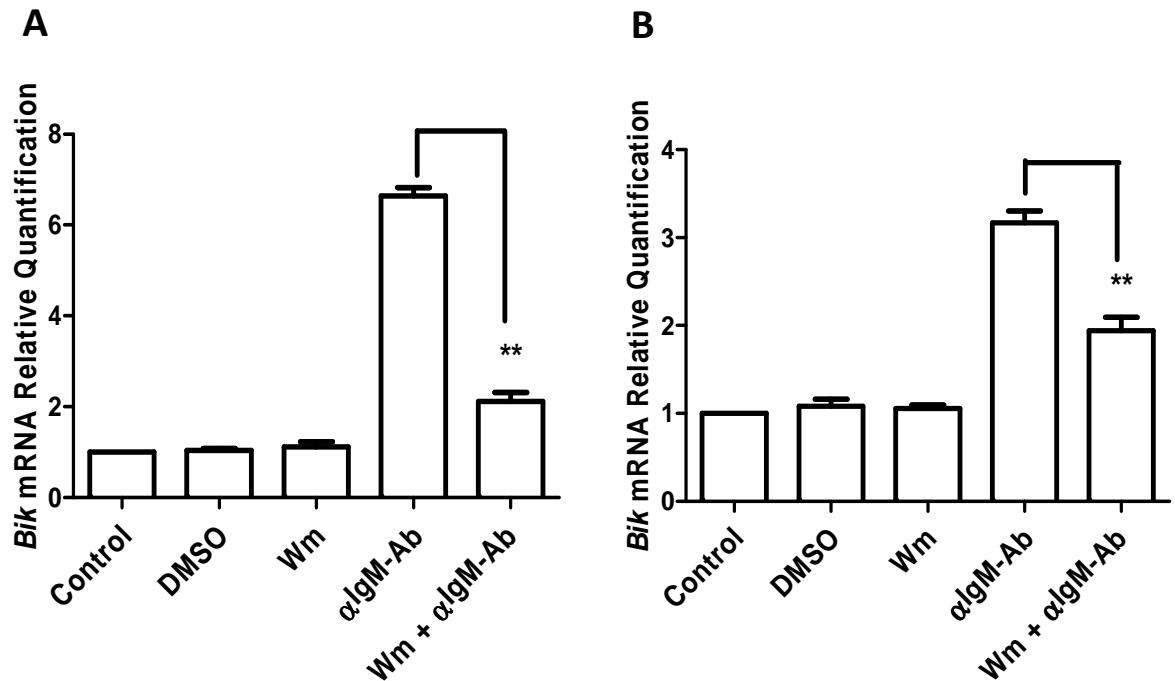
Figure 4-10: BCR cross-linking requires PI3K signalling pathway for *bik* mRNA *trans*-activation.

Apoptosis was induced in (A) Ramos and (B) BJAB cells by treating these cells with anti-IgM antibody. The PI3K pan-inhibitor, wortmannin, was added 30 min before anti-IgM antibody stimulation. Relative *bik* mRNA levels were detected by RT-qPCR using RNA prepared 24 h after treatment. *Bik* mRNA levels were assayed in triplicate and normalised to *gapdh* mRNA levels. Relative quantification levels were calculated relative to the control assigned a value of 1. Data are Mean ±SD **P (0.001 to 0.01).

Overall, BCR cross-linking led to the significant induction of apoptosis which coincided with *bik trans*-activation in Ramos cells. An intact PI3K signalling pathway seemed to be required for the BCR cross-linking mediated effect in this cell line, as inhibition of PI3K by wortmannin significantly decreased the apoptotic effect mediated by BCR cross-linking and decreased expression of *bik*. However, BCR cross-linking in BJAB cells induced less apoptosis in contrast to Ramos cells which also coincided with *bik trans*-activation. Although PI3K inhibition also led to *bik* mRNA down-regulation (to a lower extent than that observed in Ramos cells), PI3K pathway inhibition was seen to have no significant effect on preventing or enhancing apoptosis due to BCR cross-linking.

Summary

Here, Ramos and BJAB were used to study the molecular events associated with GC B cell receptor-ligation-induced apoptosis. Similarly to TGFβ1, signalling via the BCR induced apoptosis more profoundly in Ramos when compared to BJAB, which coincided with an increase in Bik protein and mRNA levels in a dose-dependent manner. Transient *bik* knockdown was not sufficient to inhibit the apoptosis mediated through BCR cross-liking in both EBV-negative cell lines. Moreover, ectopic EBNA-2 or its non-CBF1 binding mutant, EBNA-2 WW323SR, rescued Ramos cells from the apoptotic effects mediated through BCR cross-linking (Figure 4-11). Here, it has been observed that inhibition of the PI3K pathway by wortmannin led to a decreased expression level of *bik* mRNA and the viability of Ramos cells stimulated with anti-IgM antibody was augmented. However, while PI3K inhibition in BJAB cells was seen to coincide with decreased *bik* mRNA level, no significant effect was seen in reversing the apoptotic effects induced upon BCR stimulation.
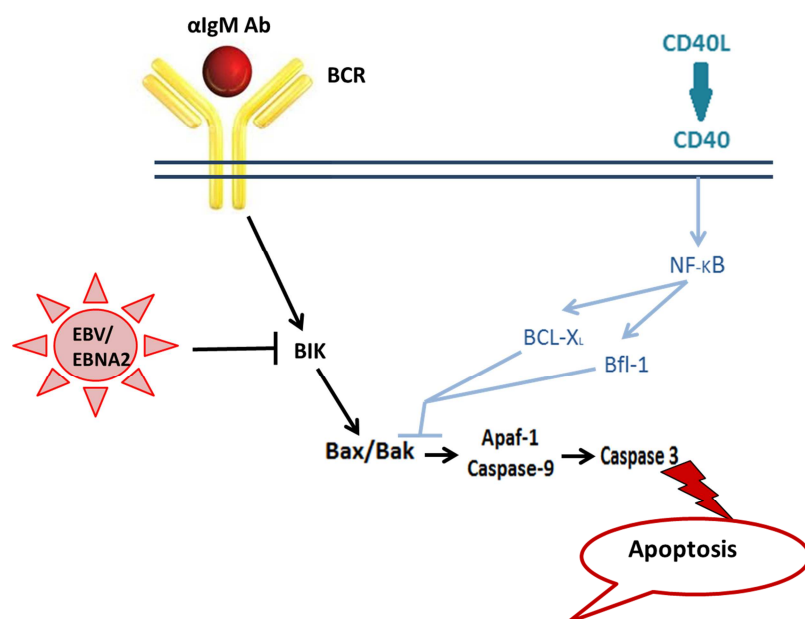
Figure 4-11: A model for inhibition of BCR-induced apoptosis by EBV.

BCR engagement in the absence of survival signals (CD40 engagement) can promote apoptotic signal transduction pathways such as activation of effector caspases and expression of pro-apoptotic genes (*bik* and *bim).* Here, it has been shown that EBNA-2 negatively modulates *bik trans*-activation following BCR cross-linking and significantly inhibiting apoptosis.

Chapter 5 :
A co-operative effect of 1α,25-(OH)$_2$D$_3$ on BCR- and TGFβ1-mediated apoptosis

A role for $1\alpha,25\text{-(OH)}_2D_3$ in B cell homeostasis

Vitamin D_3 has received increased attention recently for its pleiotropic actions on many chronic diseases, including cancer (Holick 2004, Fleet, et al. 2012), cardiovascular disease, autoimmune disease, diabetes (Adams and Hewison 2008), and neurologic disease (Garcion, et al. 2002, Holick 2007). It has been estimated that vitamin D_3 regulates the expression of over 900 genes (for review see (Wang, et al. 2005)). Investigations on B cell responses have found that $1\alpha,25\text{-(OH)}_2D_3$ inhibited the on-going proliferation of activated B cells and induced apoptosis without having any effect on initial cell division. The generation of plasma cells and post-switch memory B cells was significantly inhibited by $1\alpha,25\text{-(OH)}_2D_3$, although the up-regulation of genetic programs involved in B cell differentiation was only modestly affected (Chen, et al. 2007). B cells express mRNAs for proteins involved in vitamin D_3 activity, including CYP27B1, CYP24A1, and the VDR, each of which has been seen to be regulated by $1,25\text{(OH)}_2D_3$. Importantly, $1\alpha,25\text{-(OH)}_2D_3$ has been shown to up-regulate p27, but not p18 and p21, both of which may be important in regulating the proliferation of activated B cells and their subsequent differentiation. These findings indicate that $1\alpha,25\text{-(OH)}_2D_3$ may play an important role in the maintenance of B cell homeostasis and that the correction of vitamin D_3 deficiency may be useful in the treatment of B cell-mediated autoimmune disorders (Chen, et al. 2007). B cell reactivity with $1\alpha,25\text{-(OH)}_2D_3$ is a characteristic universally shared by naive, germinal centre and memory B cell subpopulations, and cellular activation rather than differentiation, is more defining of their biological reactivity to $1\alpha,25\text{-(OH)}_2D_3$ (Morgan, et al. 2000b). The apoptotic effects of $1\alpha,25\text{-(OH)}_2D_3$ are cell-specific and context-dependent and are mediated by modulating different signalling pathways, cell cycle regulators and Bcl-2 protein family members (Fleet, et al. 2012). Vitamin D_3 has been shown to induce apoptosis in prostate cancer cells by the intrinsic pathway and by down-regulating Bcl-2, Bcl-xL but not Bax and Bad (Guzey, Kitada and Reed 2002). It has been shown that $1\alpha,25\text{-(OH)}_2D_3$ has a direct effect on the B cell response. It inhibits the on-going proliferation of activated B cells and induces their apoptosis, and also the generation of class switched memory B cells, plasma cell differentiation, and Ig production (Chen, et al. 2007).

Here, experiments were designed to address (i) if $1\alpha,25\text{-}(OH)_2D_3$ is able to induce apoptosis in Ramos and BJAB -B cell lines that are used as GC B cell models (ii) whether the occurrence of such apoptosis coincides with upregulation of *bik,*, and (iii) the possible co-operation of $1\alpha,25\text{-}(OH)_2D_3$ with TGFβ1 and BCR signalling pathways in regulating apoptosis and *bik*.

5.1 Apoptotic effect of $1\alpha,25\text{-}(OH)_2D_3$

In order to investigate a potential apoptotic effect of $1\alpha,25\text{-}(OH)_2D_3$, Ramos cells were treated with 10 nM and 50 nM concentrations of $1\alpha,25\text{-}(OH)$ for 24 h and 48 h. Apoptosis was then measured by Annexin-V-PE/7AAD staining, followed by flow cytometry. It was observed that apoptosis was induced in a dose-dependent manner. This effect was best seen at the 48 h time point where both 10 nM and 50 nM had similar effects, with an approximately 3-fold increase in the apoptotic cell population when cells where treated with 50 nM $1\alpha,25\text{-}(OH)_2D_3$ compared to control (Figure 5-1). The effect of FCS on the apoptotic activity of $1\alpha,25\text{-}(OH)_2D_3$ was then examined. The induction of apoptosis was maximal when cells were cultured with media supplemented with 5% FCS (data not shown).

A

Ramos

B
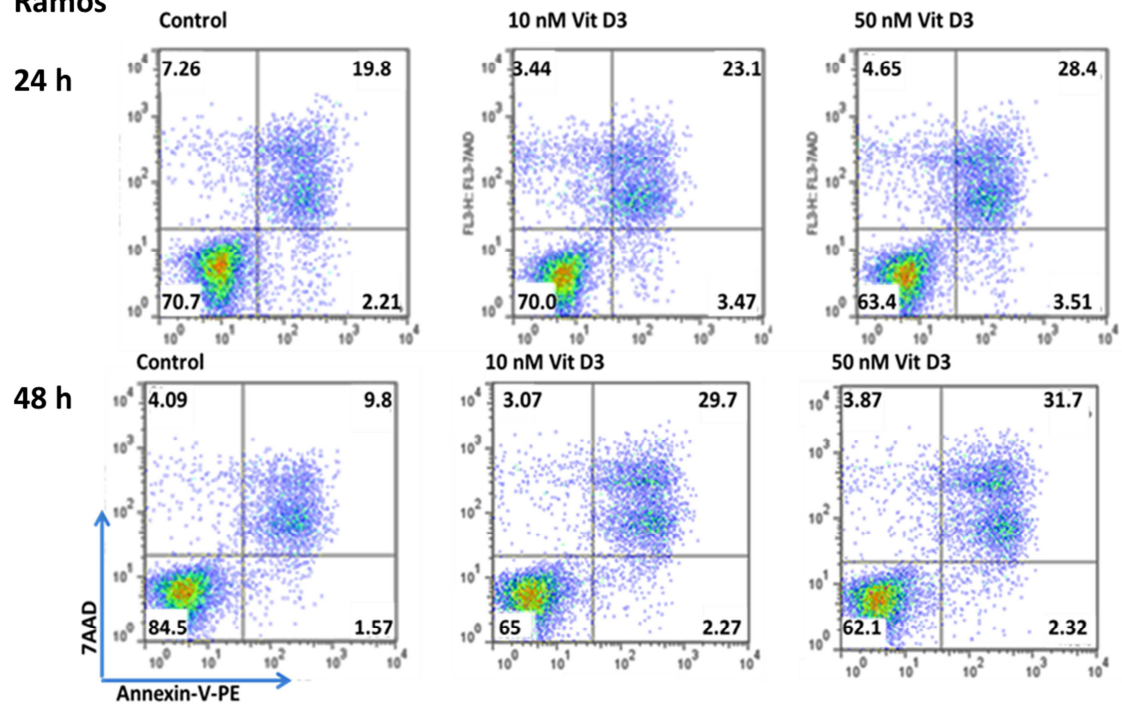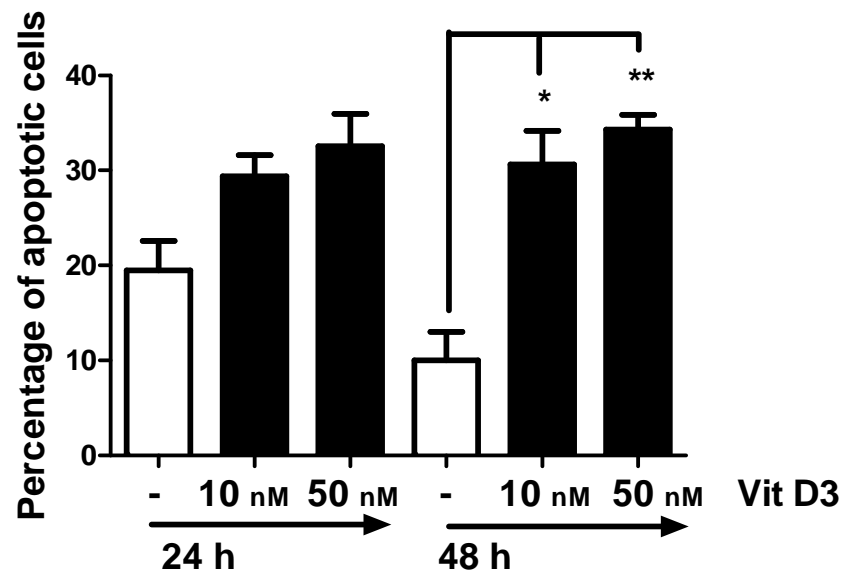
Figure 5-1: 1α,25-(OH)₂D₃ induces apoptosis in Ramos cells.

Ramos cells were treated with 10 nM and 50 nM of 1α,25-$(OH)_2D_3$ for 24 and 48 h. (A) Representative cell viability/apoptosis FACS profiles at 24 h and 48 h following treatment. The cells in each quadrant are as described in the legend to (Figure 4.1) and the values shown represent the percentage number of cells in each quadrant. (B) Values presented as a bar chart reflect the percentage of cells that were undergoing apoptosis (three independent experiments). Data are ±SD *$P \leq 0.05$ and **P (0.001 to 0.01).

5.2 $1\alpha,25$-$(OH)_2D_3$ -mediated apoptosis coincides with *bik trans-activation*

Ramos cells were then treated with 0.1 nM, 1 nM, 10 nM, and 100 nM of $1\alpha,25$-$(OH)_2D_3$ for 24 and 48 h. Total RNA and protein were harvested at the indicated time points for RT-qPCR and Western blot analysis (Figure 5-2). It can be seen that the *bik* mRNA expression level significantly increased in response to $1\alpha,25$-$(OH)_2D_3$ in a dose-dependent manner relative to untreated control. A 4-fold increase in the level of *bik* mRNA can be seen 24 h after treatment with 10 nM of the $1\alpha,25$-$(OH)_2D_3$ (Figure 5-2 A). In addition, Western blot analysis showed a 6-fold increase in Bik protein expression at the 24 h time point in response to 10 nM of $1\alpha,25$-$(OH)_2D_3$ (Figure 5-2 B).

A

B
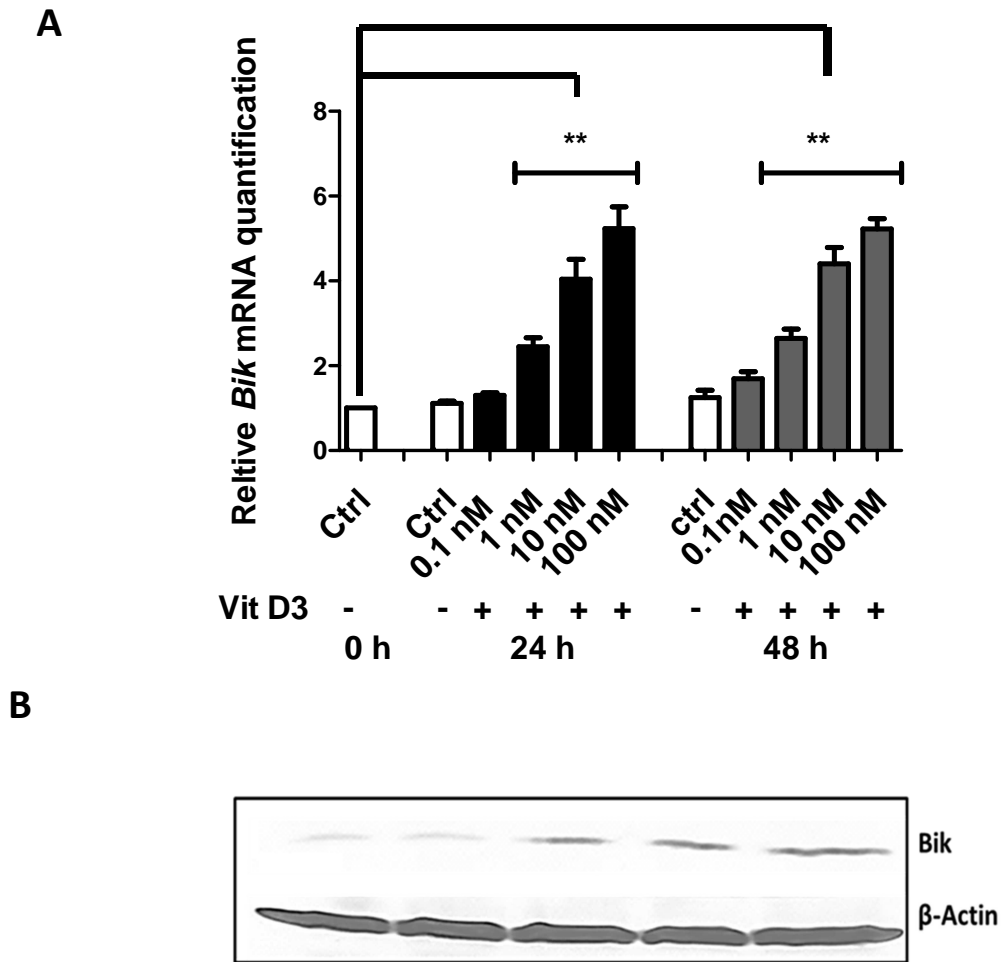
Figure 5-2: *Bik* **mRNA and protein levels increase in response to 1α,25-(OH)$_2$D$_3$ treatment (Ramos cells).**

(A) Relative *bik* mRNA levels were detected by RT-qPCR, as described in the legend to Figure 4.3, using RNA prepared at 0 h, 24 h and 48 h following treatment with three different concentrations of 1α,25-(OH)$_2$D$_3$ as indicated below each bar chart (average of three independent experiments) Data are ±SD**P* (0.001 to 0.01). (B) Western blot showing Bik protein expression for the 24 h time point in response to 0.1 nM, 1 nM, and 10 nM of 1α,25-(OH)$_2$D$_3$.

5.3 1α,25-(OH)$_2$D$_3$ and TGFβ1 independently and co-operatively induce apoptosis in Ramos and BJAB cells

A number of different experiments have demonstrated cross-talk between the TGFβ1 and vitamin D$_3$ signalling pathways (Adams and Hewison 2008, Subramaniam, et al. 2001). The activities of nuclear hormone receptors are mediated, at least in part, through the Smad proteins. Smad3 for example, a downstream component of the TGFβ signalling pathway, interacts with and acts as a co-activator of VDR (Subramaniam, et al. 2001). 1α,25-(OH)$_2$D$_3$-induced phosphorylation of Smad2 and Smad3 proteins is an indirect effect of 1α,25-(OH)$_2$D$_3$ and is dependent on TGFβ (Daniel, et al. 2007). Smad3 binds the VDR-RXR heterodimer in a ligand-dependent manner, in which an intact Smad MH1 domain is required, in contrast to many other Smad interactions, which are mediated by the MH2 domain (Leong, et al. 2001). This interaction is strongly inhibited by the inhibitory Smad, Smad7 but not by Smad6 (Yanagi, et al. 1999).

In order to address a possible co-operation between the 1α,25-(OH)$_2$D$_3$ and TGFβ signalling pathways in the GC cell context, Ramos and BJAB were treated with either 10 nM 1α,25-(OH)$_2$D$_3$ or 10 ng/mL TGFβ1 alone, or co-stimulated with both together for 24 and 48 h. Each treatment was again observed to induce apoptosis and combined treatments resulted in higher rates of apoptosis when compared to the relevant individual treatments (Figure 5-3 and Figure 5-5). The rate of apoptosis was increased by 5.5-fold after 48 h in Ramos cells (Figure 5-3 A and B) and by 7-fold in BJAB cells (Figure 5-5 A and B) co-stimulated with 10 ng/mL TGFβ1 and 10 nM 1α,25-(OH)$_2$D$_3$, when compared to untreated control at the same time point. Total RNA and protein were extracted 24 h and 48 h after treatment. *Bik* mRNA levels were seen to increase in cells treated with either TGFβ1 or 1α,25-(OH)$_2$D$_3$. Apoptosis was increased 4-fold in Ramos cells (Figure 5-4 A) and by more than 2.7-fold in BJAB cells (Figure 5-6 A) when cells were co-stimulated with TGFβ1 and vitamin D$_3$ together. The level of Bik protein was also seen to rise in response to either 1α,25-(OH)$_2$D$_3$ or TGFβ1 treatment alone and Bik expression was increased when cells were co-stimulated with both of these stimuli (Figure 5-4 B and Figure 5-6 B).
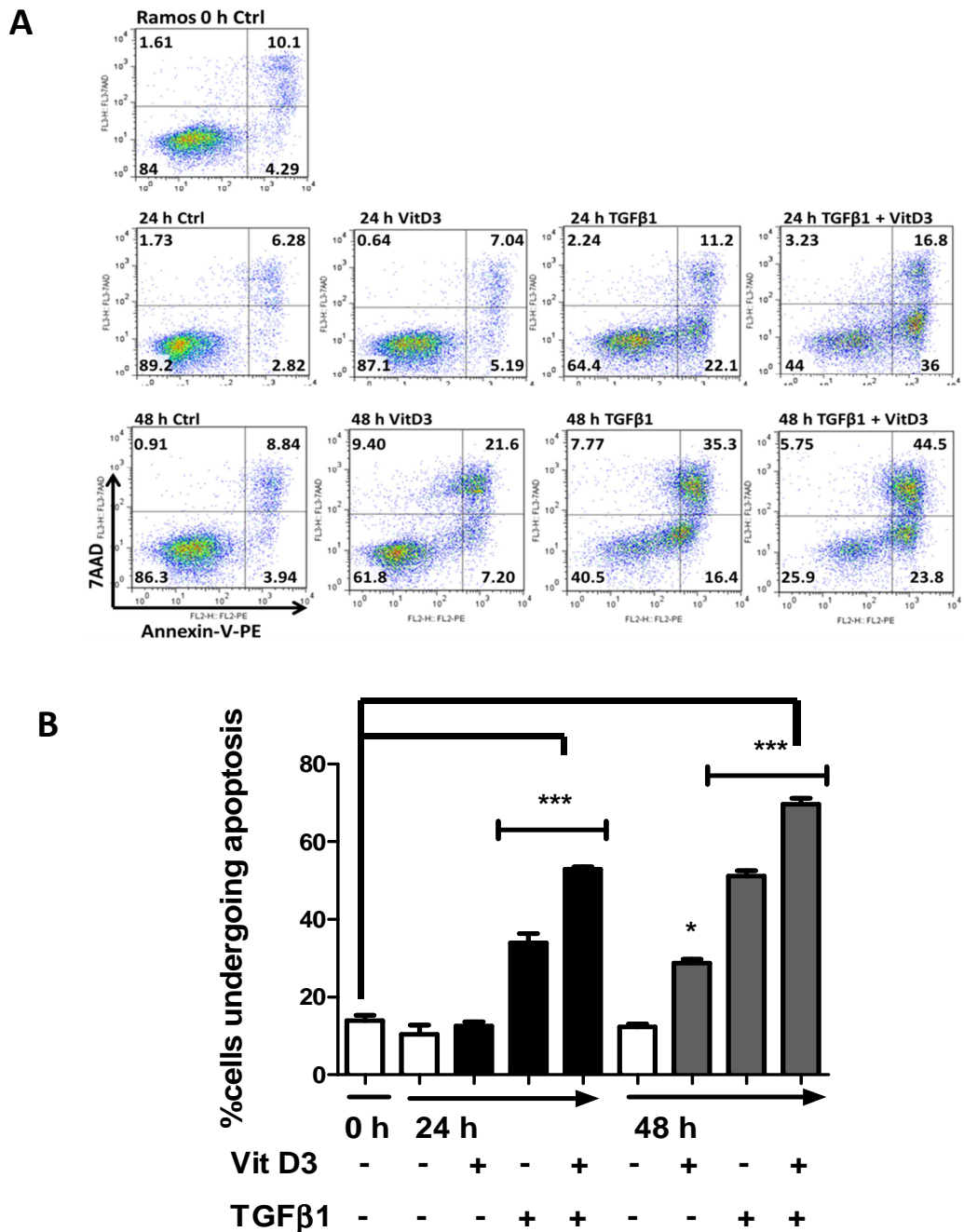
Figure 5-3: 1α,25-(OH)₂D₃ and TGFβ1 independently and co-operatively induce apoptosis in Ramos cells.

(A) Representative FACS profiles of Ramos cells 24 h and 48 h following treatment with stimuli as indicated above each scatterplot. The cells in each quadrant are as described in the legend to Figure 4.1 and the values shown represent percentage number of cells in each quadrant. (B) Values presented as a bar chart reflect the percentage of cells undergoing apoptosis (three independent experiments). Data are ±SD *$P \leq 0.05$ and ***$P < 0.001$.
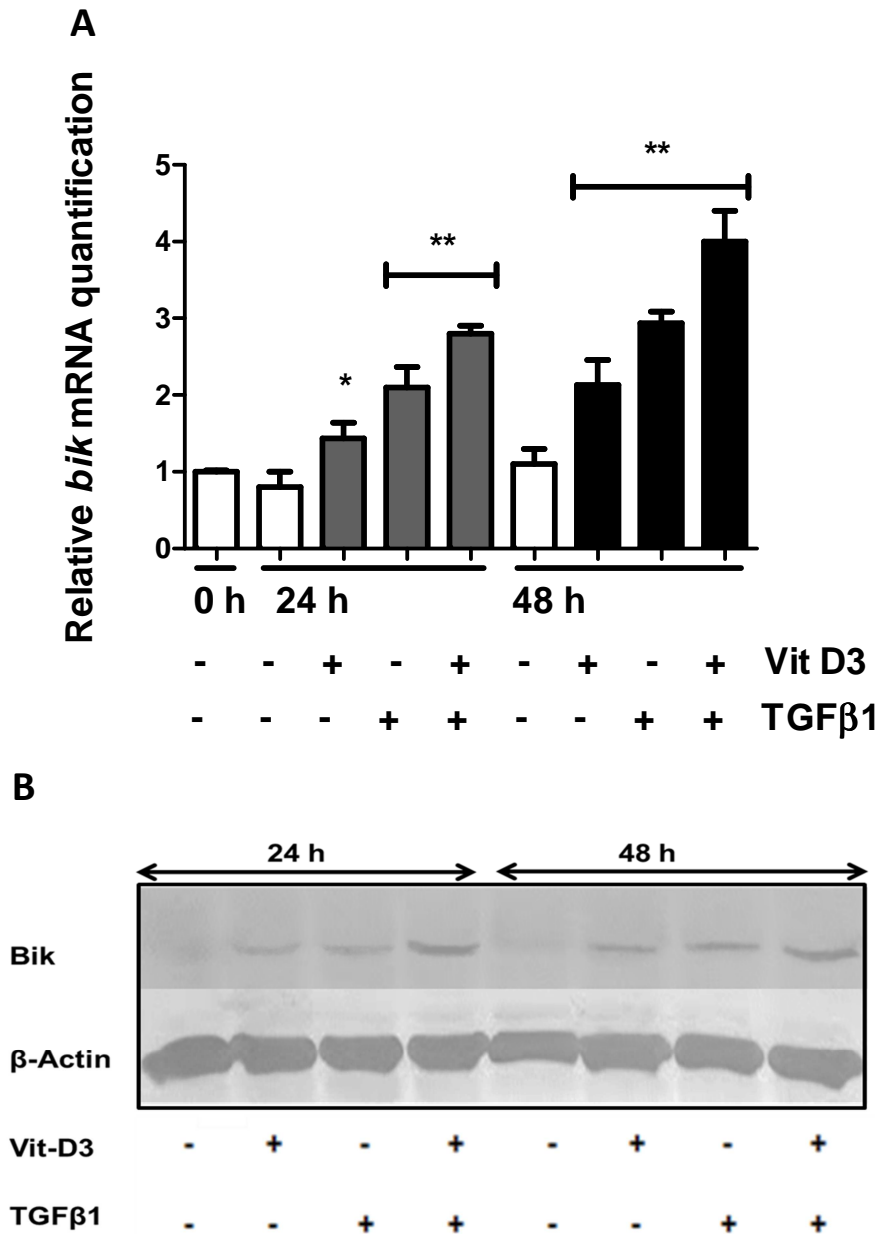
Figure 5-4: 1α,25-(OH)₂D₃ and TGFβ1 independently and co-operatively *trans*-activate *bik* in Ramos cells.

Ramos cells were treated with 10 nM 1α,25-(OH)$_2$D$_3$ and/or 10 ng/mL TGFβ1 for 24 and 48 h. (A) Relative *bik* mRNA levels were detected by RT-qPCR, as described in the legend to Figure 4.3, using RNA prepared at indicated time points following treatment with different stimuli as indicated below each bar chart. (B) Represents Western blot showing Bik and β-actin protein expression for 24 h and 48 h time points. Data are ±SD *$P \leq 0.05$ and **P (0.001 to 0.01).
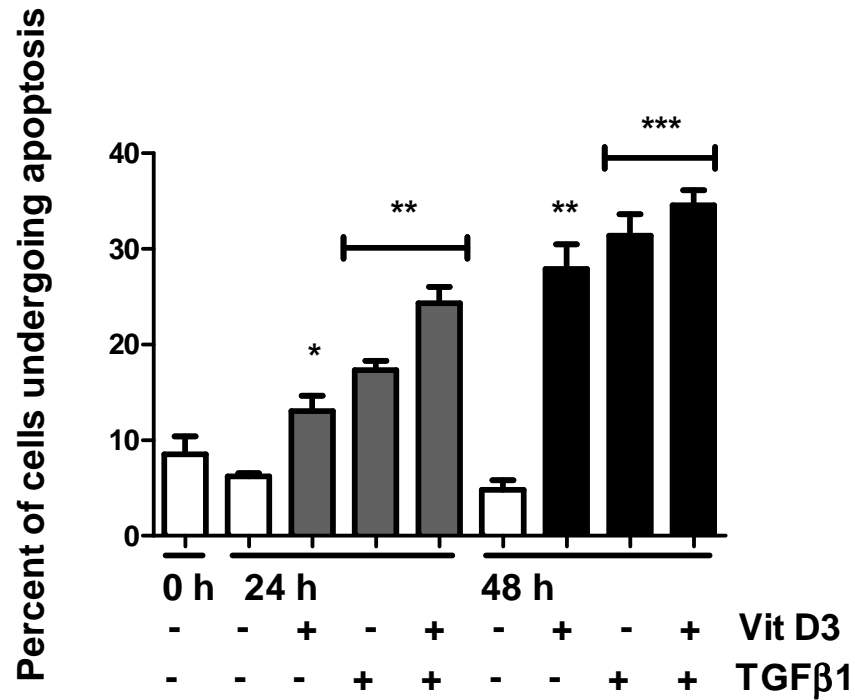
Figure 5-5: 1α,25-(OH)₂D₃ and TGFβ1 independently and co-operatively induce apoptosis in BJAB cells.

BJAB cells were treated with 10 nM 1α,25-(OH)$_2$D$_3$ and/or 10 ng/mL TGFβ1 for 24 and 48 h. Cell viability/apoptosis was measured 24 h after treatment by FACS as described in the legend to Figure 4.1 and values presented as a bar chart reflect the percentage of cells undergoing apoptosis for 24 h and 48 h after treatment. Data are ±SD *$P \leq 0.05$, **P (0.001 to 0.01), and ***$P < 0.001$.
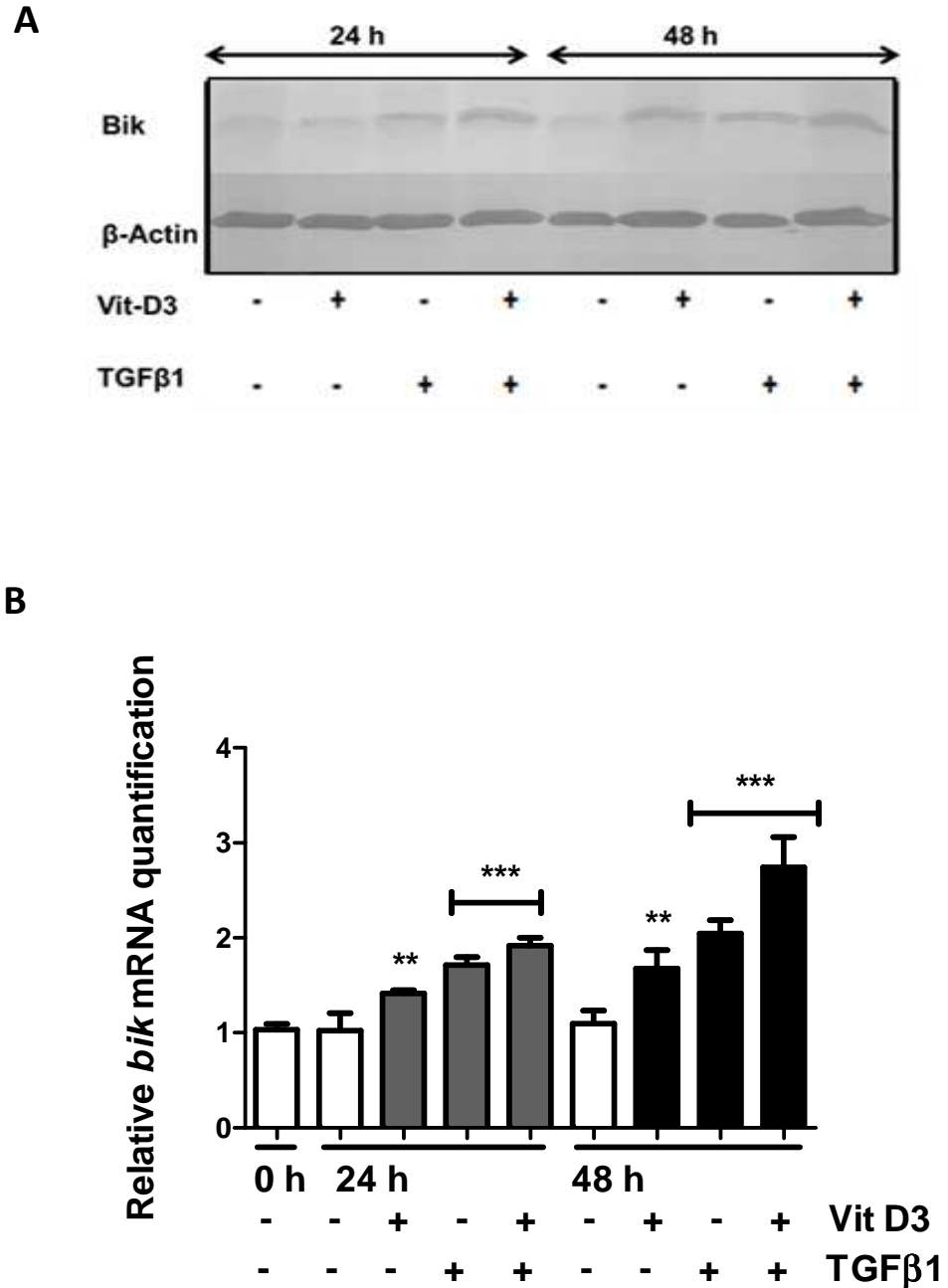
Figure 5-6: TGFβ1 and 1α,25-(OH)$_2$D$_3$ independently and co-operatively *trans*-activate *bik* in BJAB cells.

BJAB cells were treated with 10 nM 1α,25-(OH)$_2$D$_3$ and/or 10 ng/mL TGFβ1 for 24 h and 48 h. (A) Relative *bik* mRNA levels were detected by RT-qPCR, as described in the legend to Figure 4.3, using RNA prepared at the indicated time points following treatment with different stimuli as indicated below each bar chart. (B) Represents Western blot showing Bik and β-actin protein expression for 24 h and 48 h time points. Data are ±SD **P (0.001 to 0.01) and ***P < 0.001.

5.4 Co-operation between the BCR, 1α, 25-(OH)$_2$D$_3$ and TGFβ1 signalling pathways on induction of apoptosis in Ramos and BJAB cells

In order to investigate if 1α,25-(OH)$_2$D$_3$ co-operates with BCR and TGFβ1 signalling pathways in inducing apoptosis in a GC cell context, Ramos and BJAB cell lines were treated with 1α,25-(OH)$_2$D$_3$, TGFβ1 and anti-IgM antibody separately or in combination (as indicated in Figure 5-7 and Figure 5-8) for a period of 48 h. Cell survival profiles were then determined as before by 7-AAD/Annexin-V staining and flow cytometric analysis. These three stimuli were again seen to induce apoptosis in Ramos and BJAB when used individually (Figure 5-7 and Figure 5-8). Among the three stimuli, in order, anti-IgM antibody had the strongest and TGFβ1 and 1α,25-(OH)$_2$D$_3$ were observed to have the weakest effect in induction of apoptosis in Ramos cells (70% ±SD 1.854, 44% ±SD 2.147 and 30% ±SD 2.108 apoptotic respectively, see Figure 5-7). Co-stimulation of Ramos cells with TGFβ1 and anti-IgM antibody led to a significant induction of apoptosis (94% ±SD 3.858), more than was the case when each stimulus was used individually (Figure 5-7). Combined treatment of Ramos cells with 1α,25-(OH)$_2$D$_3$ and anti-IgM antibody led to 84% ±SD 3.494 apoptosis (Figure 5-7). Similarly, co-stimulation with TGFβ1 and 1α,25-(OH)$_2$D$_3$ resulted in 52% ±SD 2.152 of cells being apoptotic (Figure 5-7). Overall therefore, it can be seen that 1α, 25-(OH)$_2$D$_3$ co-operated with anti-IgM antibody and to lesser extent with TGFβ1 to increase the level of induced apoptosis in Ramos cells (Figure 5-7). On the other hand, it was observed that, at the various effector concentrations used, TGFβ1 had the strongest effect in induction of apoptosis in BJAB cells (44% ±SD 1.955 apoptotic), followed by anti-IgM antibody (35% ±SD 1.206 apoptotic) and 1α, 25-(OH)$_2$D$_3$ (31% ±SD 1.952 apoptotic) (Figure 5-8). Co-stimulation of BJAB cells with 1α,25-(OH)$_2$D$_3$ and TGFβ1 led to 50% ±SD 2.082 of cells undergoing apoptosis, more than was the case when each stimulus was used individually. Similarly, combined treatment of BJAB cells with TGFβ1 and anti-IgM antibody further increased the percentage of apoptotic cells to 57% ±SD 2.608 when compared to the individual treatment with TGFβ1 and anti-IgM antibody (Figure 5-8). Combined treatment of BJAB cells with 1α, 25-(OH)$_2$D$_3$ and anti-IgM antibody was also seen to have a co-operative effect as 46% ± SD 1.311 of cells were apoptotic after this co-stimulation.
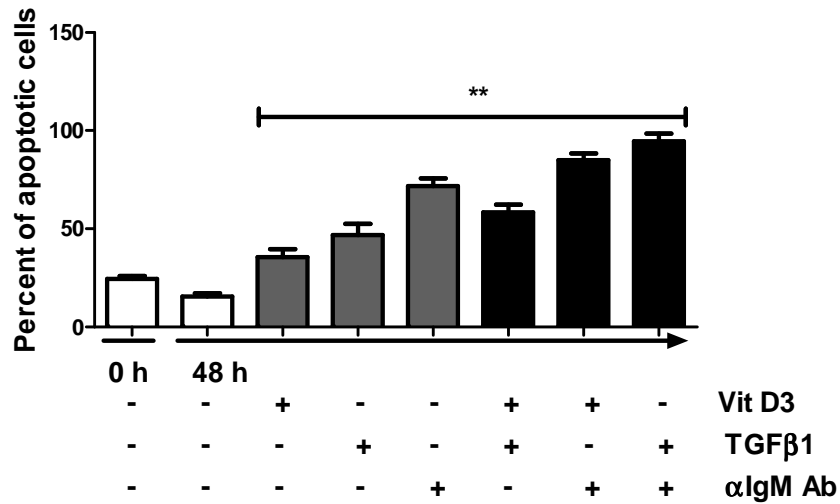
Figure 5-7: Co-operative effects of TGFβ1, 1α,25-(OH)₂D₃ and anti-IgM antibody on the induction of apoptosis in Ramos.

Ramos cells were treated with various stimuli alone or together as indicated below each bar chart, for a duration of 48 h. (A) Representative cell viability/apoptosis FACS profiles 24 h and 48 h after treatment. The cells in each quadrant are as described in the legend to Figure 4.1 and the values shown represent percentage number of cells in each quadrant. (B) Values presented as a bar chart reflect the percentage of cells undergoing apoptosis (average of three independent experiments). Data are ±SD **P (0.001 to 0.01) (See Table 5-1: Statistical significance).

Figure 5-8: Co-operative effects of TGFβ1, 1α,25-(OH)₂D₃ and anti-IgM antibody on the induction of apoptosis in BJAB.

BJAB cells were treated with various stimuli alone or together, as indicated below each bar chart, for a duration of 48 h. (A) Representative cell viability/apoptosis FACS profiles 24 h and 48 h after treatment. The cells in each quadrant are as described in the legend to Figure 4.1 and the values shown represent percentage number of cells in each quadrant. (B) Values presented as a bar chart reflect the percentage of cells undergoing apoptosis (average of three independent experiments). Data are ±SD **P (0.001 to 0.01) (See Table 5-1: Statistical significance).

Table 5-1: Statistical significance of apoptosis induced by various stimuli in Ramos and BJAB.

Statistical significance of apoptosis induced by various stimuli.			(Ramos)	(BJAB)
0 h vs 48 h control			-	-
48 h Ctrl	vs	every each treatment	**	**
Vit D$_3$	vs	Vit D$_3$ + TGFβ1	**	**
Vit D$_3$	vs	Vit D$_3$ + αIgM Ab	**	**
TGFβ1	vs	Vit D$_3$ + TGFβ1	**	**
TGFβ1	vs	αIgM Ab + TGFβ1	**	**
αIgM Ab	vs	αIgM Ab + TGFβ1	**	**
αIgM Ab	vs	αIgM Ab + Vit D$_3$	**	**

Total RNA from this experiment was prepared 48 h after treatment and *bik* mRNA levels were determined by RT-qPCR. It could be seen that the degree of apoptosis correlated with the extent of *bik* mRNA induction. It was observed that *bik* mRNA levels increased in response to anti-IgM antibody, TGFβ1 and 1α,25-(OH)$_2$D$_3$ treatments alone or in combination, as indicated in Figure 5-9. As was observed previously (Figure 5-6), treatment of cells with 1α,25-(OH)$_2$D$_3$, TGFβ1, and anti-IgM antibody coincided with *bik trans*-activation. 1α,25-(OH)$_2$D$_3$ induced *bik* to a greater extent in Ramos (2-fold) than in BJAB (1.3-fold) (Figure 5-9 A and B respectively). 1α,25-(OH)$_2$D$_3$ also co-operated with TGFβ1 in inducing *bik* to a similar extent in both Ramos and BJAB (3.4-fold and 3.6-fold respectively). When 1α.25-(OH)$_2$D$_3$ was used together with anti-IgM antibody, the level of *bik* mRNA increased 5.5-fold in Ramos compared to a 4-fold increase in BJAB (Figure 5-9 A and B).

Overall, the co-operative effect of 1α,25-(OH)$_2$D$_3$ with TGFβ1 and anti-IgM antibody treatments in induction of apoptosis was more pronounced in Ramos compared to BJAB cells. Apoptosis was seen to coincide with *bik trans*-activation in response to individual or combined treatments in both cell lines.
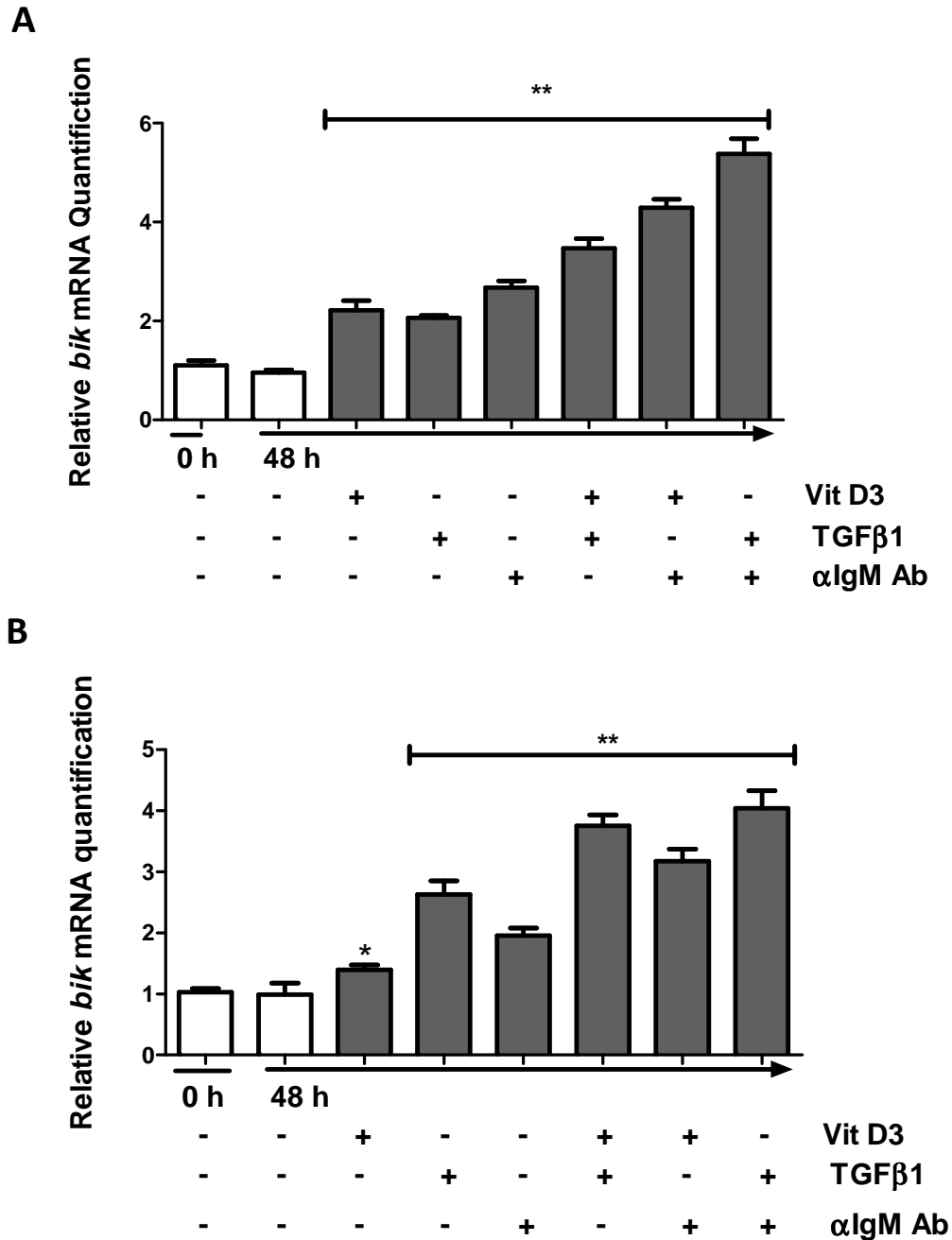
Figure 5-9: Co-operative effects of TGFβ1, 1,25-(OH)$_2$D$_3$ and anti-IgM antibody on *bik trans*-activation in Ramos and BJAB cells.

(A) Ramos and (B) BJAB cells were treated with various stimuli alone or together, as indicated below each bar chart, for a duration of 48 h. Relative *bik* mRNA levels were detected by RT-qPCR, as described in the legend to Figure 4.3 using RNA prepared at indicated time points following treatment with different stimuli as specified above. Data are ±SD *P < 0.05 and **P (0.001 to 0.01) (See Table 5-2: Statistical significance of *bik trans-activation* by various stimuli in Ramos and BJAB..

Table 5-2: Statistical significance of *bik trans*-activation by various stimuli in Ramos and BJAB.

Significance of *bik trans*-activation by various stimuli.	(Ramos)	(BJAB)
0 h vs 48 h control	-	-
48 h Ctrl vs every each treatment Except 48 h Ctrl vs Vit D$_3$	** **	** *
Vit D$_3$ vs Vit D$_3$ + TGFβ1	**	**
Vit D$_3$ vs Vit D$_3$ + αIgM Ab	**	**
TGFβ1 vs Vit D$_3$ + TGFβ1	**	**
TGFβ1 vs αIgM Ab + TGFβ1	**	**
αIgM Ab vs αIgM Ab + TGFβ1	**	**
αIgM Ab vs αIgM Ab + Vit D$_3$	**	**

5.5 A role for PI3K signalling in BCR-, TGFβ1-, and 1α,25-(OH)$_2$D$_3$-mediated apoptosis

PI3K has diverse roles in the adaptive and innate immune system. Vitamin D$_3$ has been shown to be involved in activation of the PI3K/Akt signalling pathway (Deeb, Trump and Johnson 2007b, Hughes, et al. 2008). Also, the PI3K pathway is required for sustained Bik protein production upon BCR stimulation (Jiang and Clark 2001b). TGFβ1 has also been shown to activate PI3K in a non-Smad-dependent pathway (Zhang 2009).

Here, experiments were designed to investigate a role for PI3K in the intrinsic apoptotic pathways mediated by BCR cross-linking, TGFβ1, and 1α,25-(OH)$_2$D$_3$. Cells were treated with 10 nM wortmannin (Wm) 30 min prior to individual treatments for 48 h with TGFβ1, 1α,25-(OH)$_2$D$_3$, and anti-IgM antibody or dual sets of combined treatments, as indicated below each bar chart (Figure 5-10-Figure 5-13). It was observed that PI3K inhibition in Ramos cells significantly decreased BCR cross-linking and vitamin D$_3$ mediated apoptosis to 17% ±SD 2.541 and 8%±SD 1.002 respectively, while TGFβ- mediated apoptosis was moderately increased to 26% ±SD 1.311 (Figure 5-10 B).

Co-stimulation with anti-IgM antibody/1α,25-(OH)$_2$D$_3$ led to 77% ±SD 1.692 apoptosis compared to the control. Interestingly, this effect was reversed after inhibition of PI3K signalling, and led to a substantial decrease in apoptosis (34% ±SD 2.380) (Figure 5-11 B). Combined treatment of Ramos cells with anti-IgM antibody/TGFβ1 after PI3K inhibition led to a decrease in the overall level of apoptosis (from before 80% ±SD 2.409 down to 65% ±SD 2.476 after PI3K inhibition) (see Figure 5-11 B for interpretation here). Combined stimulation with 1α,25-(OH)$_2$D$_3$/TGFβ1 resulted in a moderate increase in the percentage of apoptotic cells (37% ±SD 2.062) and treatment of cells with wortmannin was seen to have no significant effect in altering the level of apoptosis (32% ±SD 2.585) (Figure 5-11 B).
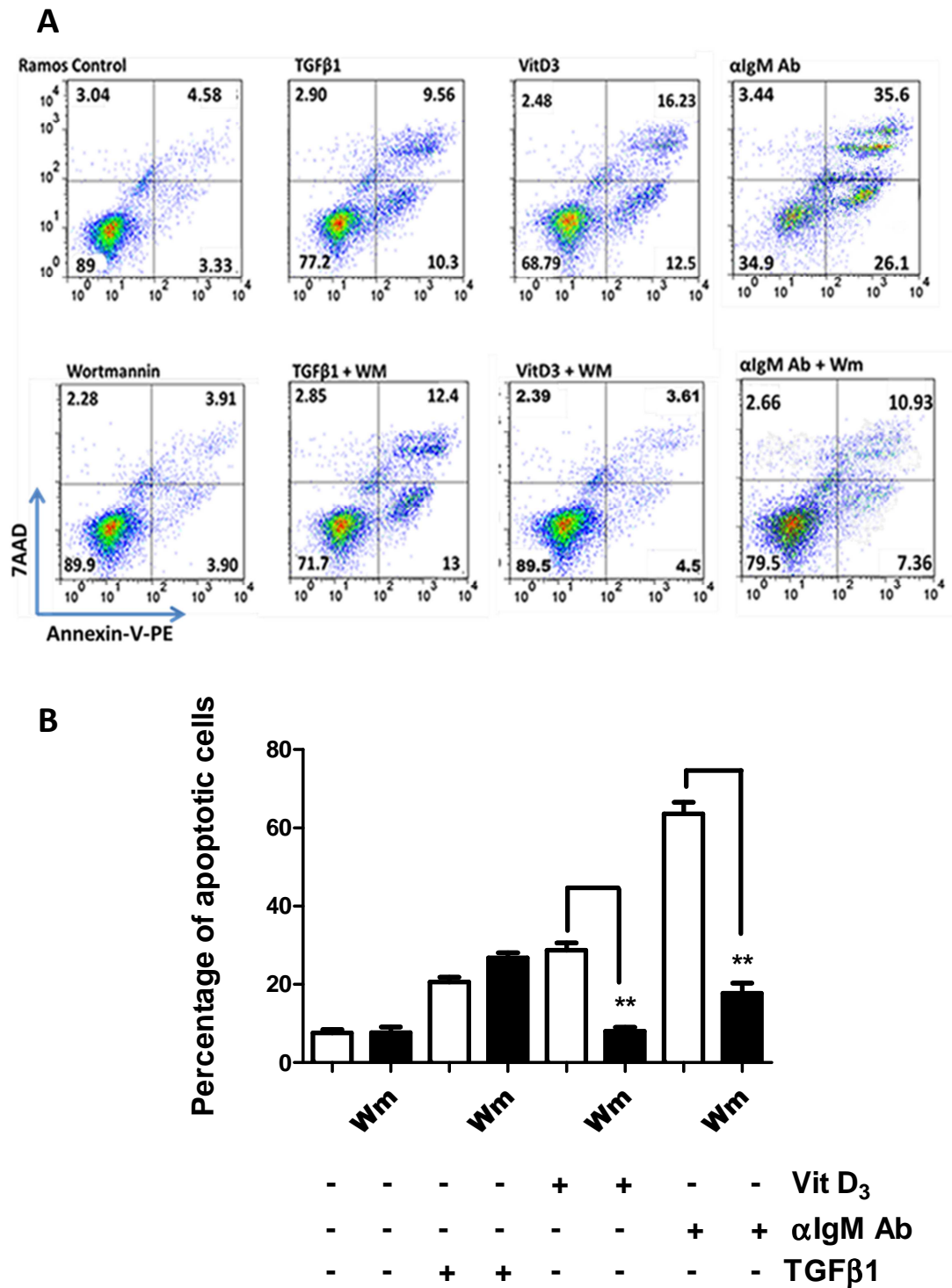
Figure 5-10: The effect of wortmannin on BCR cross-linking, TGFβ1 and 1α,25-(OH)2D3-mediated apoptosis in Ramos cells.

Ramos cells were treated with (black bar) or without (white bar), wortmannin 30 min before apoptosis was initiated with the specified stimuli as indicated above each scatter plot. (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment with TGFβ1. The cells in each quadrant are as described in the legend to Figure 3-1 and the values shown represent the percentage number of cells in each quadrant. (B) Bar chart representation of the percentage of apoptotic cells in the cell viability/apoptosis FACS assay 24 h after treatment (average of three independent experiments). Data are ±SD **P (0.001 to 0.01).
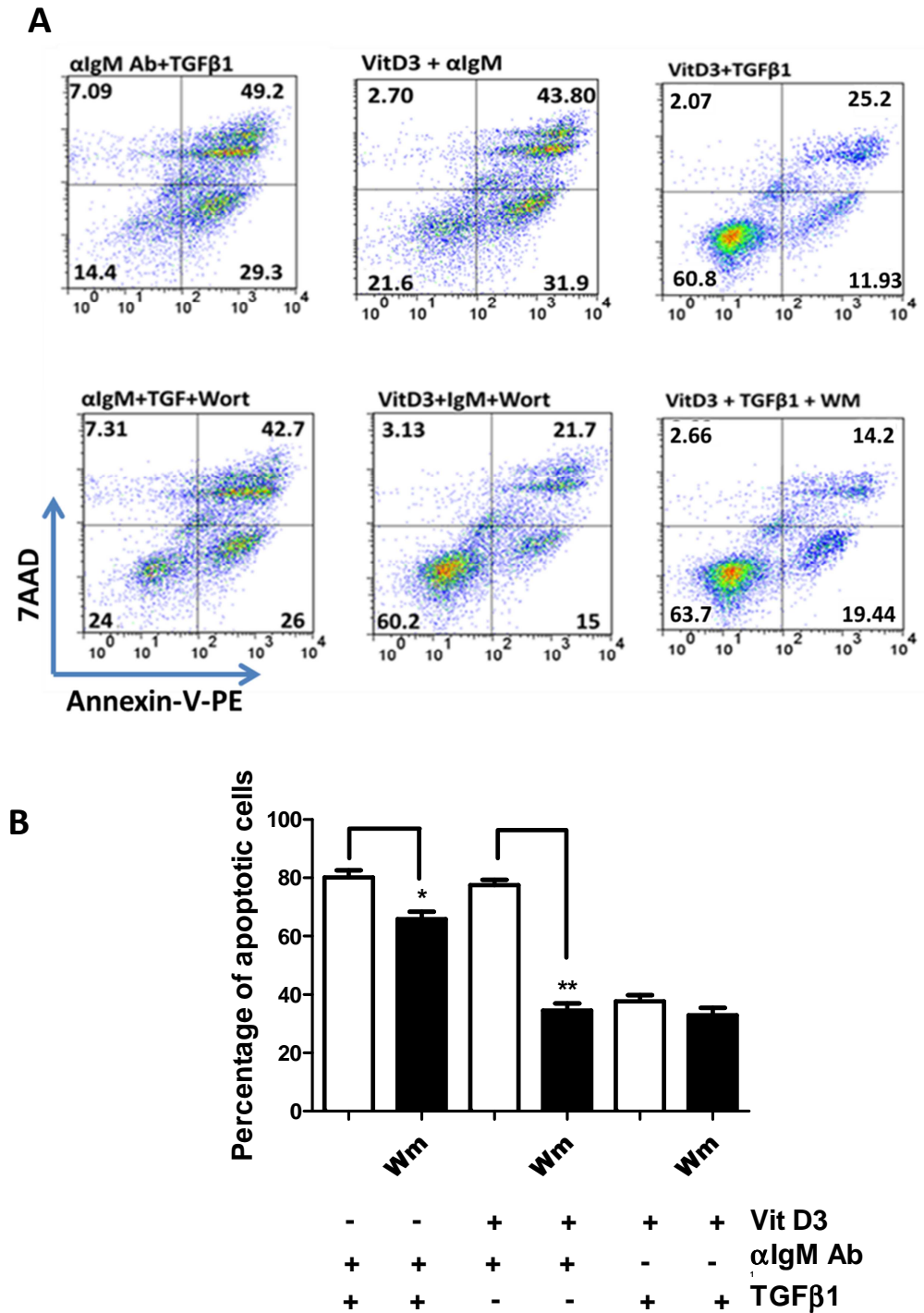
Figure 5-11: The effect of wortmannin on BCR cross-linking, TGFβ1 and 1α,25-(OH)2D3 co-operative-mediated apoptosis in Ramos cells.

Ramos cells were treated with (black bars) or without (white bars), wortmannin 30 min before, apoptosis was initiated with the specified stimuli as indicated above each scatter plot. (A) Representative cell viability/apoptosis FACS profiles 24 h after treatment with TGFβ1. The cells in each quadrant are as described in the legend to Figure 3-1 and the values shown represent the percentage number of cells in each quadrant. (B) Bar chart representation of the percentage of apoptotic cells in the cell viability/apoptosis FACS

assay 24 h after treatment (average of three independent experiments). Data are ±SD *P ≤ 0.05 and **P (0.001 to 0.01).

In comparison to the results seen for the Ramos cells (Figure 5-10 and Figure 5-11), inhibition of the PI3K pathway in BJAB cells (Figure 5-12) resulted in overall increased apoptosis (from 24% ±SD 1.607 before to 42% ±SD 2.082 after). A significant increase in apoptosis was observed when these cells were stimulated with TGFβ1 after inhibition of PI3K (76% ±SD 2.000) (Figure 5-12). There were no detectable changes in the level of apoptosis due to PI3K inhibition when cells were treated with anti-IgM antibody or 1α,25-(OH)$_2$D$_3$ (Figure 5-12). When BJAB cells were co-stimulated with anti-IgM antibody/TGFβ1 after PI3K inhibition, a significant increase in the relative rate of apoptosis was seen when compared to untreated cells (from 64% ±SD 2.589 before to 85% ±SD 3.686 after). Wortmannin had a similar effect on BJAB cells when they were treated with TGFβ1/vitamin D$_3$ (from 68% ±SD 3.092 before to 85% ±SD 1.504 after). Co-stimulation with anti-IgM antibody and 1α,25-(OH)$_2$D$_3$ however, resulted in no significant changes after wortmannin treatment (Figure 5-13).

It has previously been shown elsewhere, that Akt is constitutively phosphorylated in BJAB cells, and that the PI3K specific inhibitor LY294002 mediated Akt de-phosphorylation and subsequently the cell growth inhibition (Mori and Sairenji 2006). This therefore might explain why BJAB cells are responding differently to PI3K inhibition when compared to the Ramos cell line.
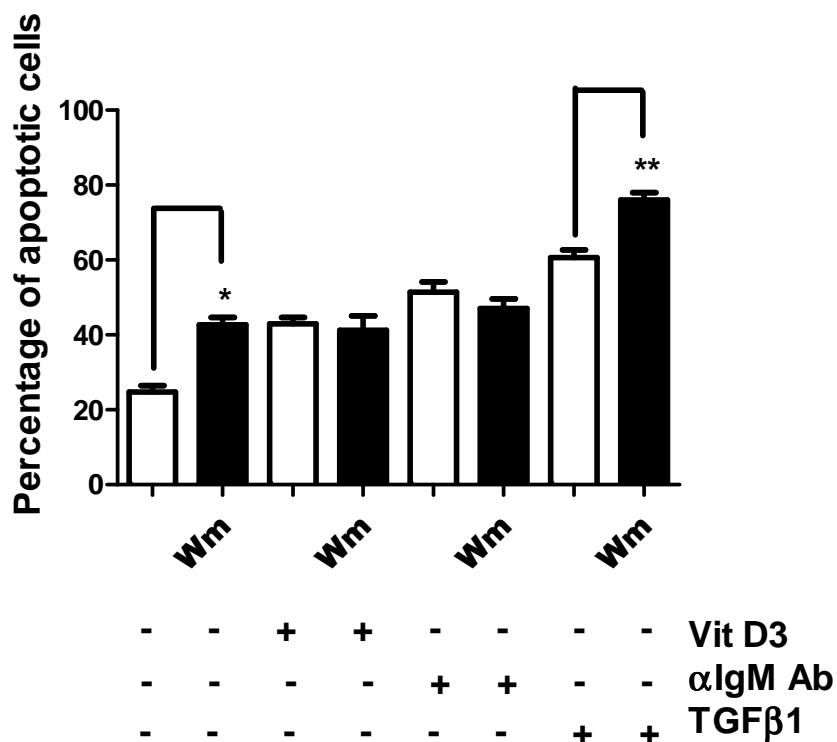
Figure 5-12: The effect of wortmannin on the BCR cross-linking, TGFβ1 and 1α,25-(OH)2D3 mediated apoptosis in BJAB cells.

BJAB cells were treated with (black bar) or without (white bars) wortmannin, 30 min before apoptosis was initiated with the specified stimuli as indicated above. Bar chart representation of the percentage of apoptotic cells in the cell viability/apoptosis FACS assay 24 h after treatment (average of three independent experiments). Data are ±SD *P ≤ 0.05 and **P (0.001 to 0.01).
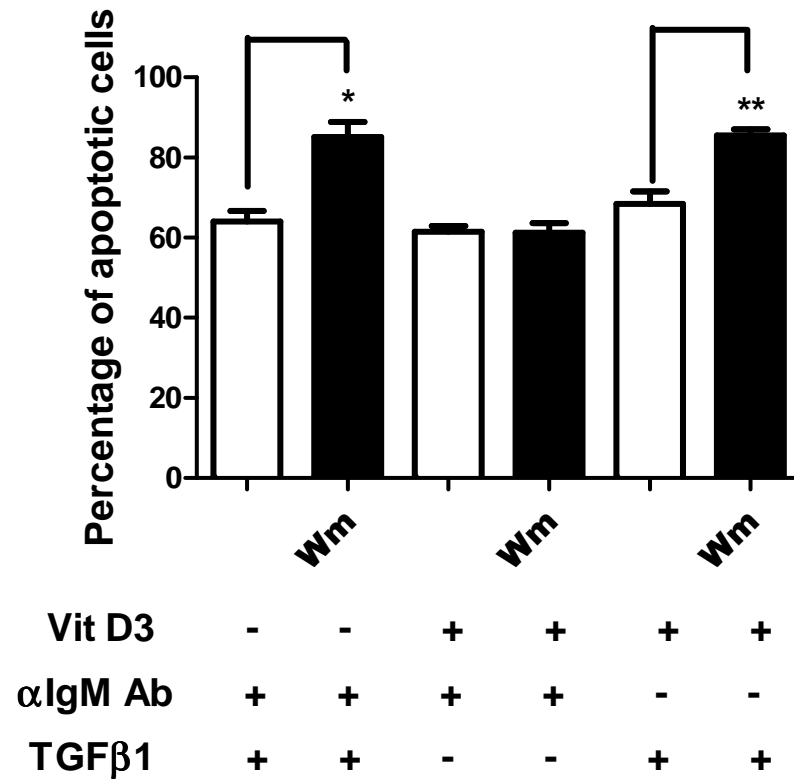
Figure 5-13: The effect of wortmannin on BCR cross-linking, TGFβ1 and 1α,25-(OH)2D3 mediated apoptosis in BJAB cells.

BJAB cells were treated with (black bar) or without (white bars) wortmannin, 30 min before apoptosis was initiated with the specified stimuli as indicated above. Bar chart representation of the percentage of apoptotic cells in the cell viability/apoptosis FACS assay 24 h after treatment (average of three independent experiments). Data are ±SD **P (0.001 to 0.01).

Summary

In conclusion, it has been shown here that the stimulation of the EBV-negative cell lines Ramos (BL-derived), and BJAB (B cell lymphoma-derived) with $1\alpha,25$-$(OH)_2D_3$ led to apoptosis. It was also shown that $1\alpha,25$-$(OH)_2D_3$-mediated apoptosis coincided with *bik* mRNA and Bik protein up-regulation in these cell lines. $1\alpha,25$-$(OH)_2D_3$ was seen to co-operate with anti-IgM antibody and TGFβ1 to further increase apoptosis in Ramos and to lesser extent in BJAB. Augmentation of apoptosis in GC model cell lines through combined treatment with $1\alpha,25$-$(OH)_2D_3$/TGFβ1 was seen to coincide with *bik trans*-activation.
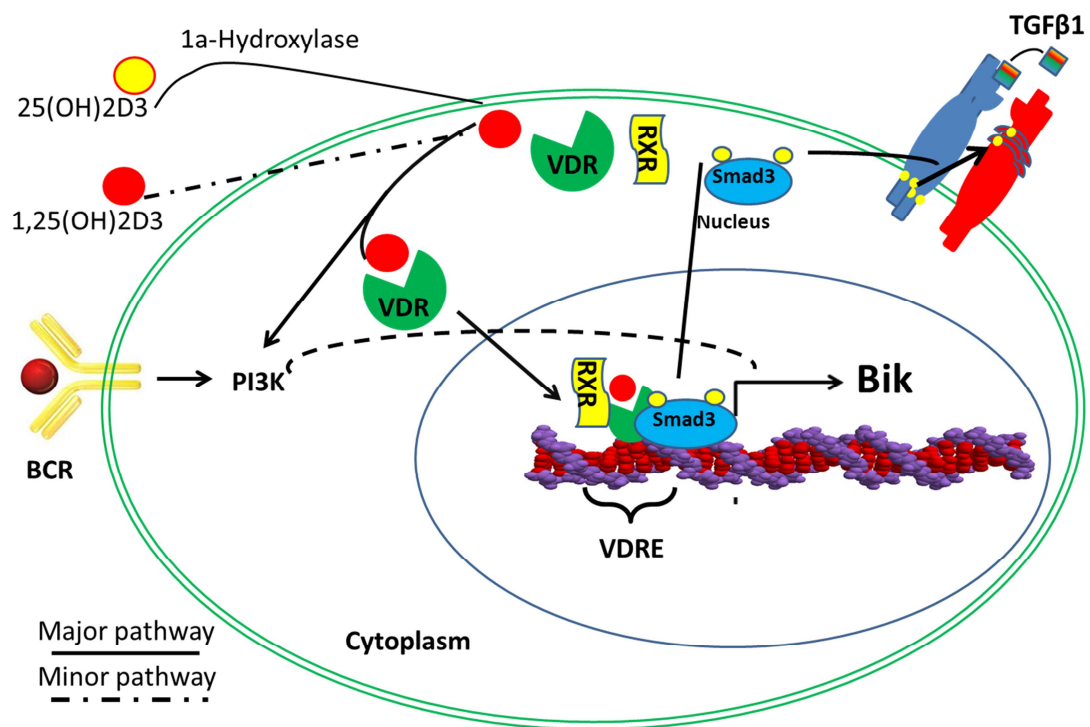
Figure 5-14: A model for possible cross-talk between $1\alpha,25$-$(OH)_2D_3$, TGFβ1, and BCR signalling pathways resulting in *bik trans*-activation and its inhibition bye EBV. See text for discussion of the interactions involved.

Wortmannin treatment alone was seen to decrease the viability of BJAB cells, but no effect was seen on Ramos cells. On the other hand, no significant changes in the level of apoptosis were seen in BJAB cells treated with an anti-IgM antibody and $1\alpha,25$-$(OH)_2D_3$ individually or together after inhibition of PI3K pathway. However inhibition of the PI3K pathway was seen to increase the apoptotic effects mediated by TGFβ1 in these cells. When BJAB cells were co-treated treated with TGFβ1/$1\alpha,25$-$(OH)_2D_3$, apoptosis was increased compared to the counterpart experiment in which PI3K pathway was not inhibited. It was observed that pre-treatment with wortmannin led to a significant decrease in the proportion of Ramos cells undergoing apoptosis, when triggered to do so with anti-IgM antibody and $1\alpha,25$-$(OH)_2D_3$, individually or in combination. Whereas inhibition of the PI3K pathway and subsequent treatment with TGFβ1 was seen to increase the rate of apoptosis in Ramos cells. The rate of apoptosis induced by combined treatment with $1\alpha,25$-$(OH)_2D_{3/}$TGFβ1 after the PI3K pathway was inhibited, showed no detectable change in Ramos cells.

Overall $1\alpha,25$-$(OH)_2D_3$ might selectively sensitise Ramos and BJAB cells to the major GC intrinsic apoptosis pathways emanating from TGFβ1 and BCR cross-linking, in part through *trans*-activating *bik*. The PI3K signalling pathway plays an important role in the $1\alpha,25$-$(OH)_2D_3$ and anti-IgM antibody with regard to the induction of apoptosis and possibly *bik* regulation in these cells (Figure 5-14).

Chapter 6 :

General discussion

Many viruses exploit and manipulate the differentiation state of infected cells, promote or block cell cycling and make use of a variety of mechanisms to evade innate cellular anti-viral responses and encourage cell survival (reviewed in (Hayward 2004)). Since apoptosis is regulated by functional pro-apoptotic and anti-apoptotic proteins, it is not surprising that EBV controls the cellular response to apoptosis by regulating both pro- and anti-apoptotic genes. Following infection of naïve B cells, EBV induces the naïve B cell activation and proliferation to become proliferating lymphoblasts which express the EBV growth programme (latency III) orchestrated by the viral transcription factor EBNA-2 (Sinclair, et al. 1994b). EBNA-2 subverts aspects of the Notch signalling pathway, thus blocking B cell differentiation, and allowing cell proliferation (Lucchesi, et al. 2008a). EBNA-2 induction of LMP-1, which mimics constitutively active CD40 signal (Uchida, et al. 1999) and LMP-2A, a second mimic of functional BCR, (Thorley-Lawson 2001a) drive the infected cell to proliferate and survive the germinal centre reaction (Babcock, Hochberg and Thorley-Lawson 2000, Wahl, et al. 2013). EBV residing within the memory and the long-lived peripheral B cell compartment down-regulate the expression of highly immunogenic viral latency antigens, and only few viral gene products (Latency II, Latency 0 and Latency I) are expressed (reviewed in Chapter 1). EBNA1 binds to the latent viral DNA replication origin and maintains the viral genome in the EBV positive cells after cell division (Thorley-Lawson 2001a). In order to survive apoptotic pathways responsible for eliminating B cells within GCs (which signal through the TGFβ receptor and BCRs), EBV employs substantial control over the expression or function of the Bcl-2 family members (reviewed in Chapter 1 and (Spender and Inman 2011)).

EBNA-2 acts as a pleiotropic modulator of viral and cellular genes that are involved in growth control, and plays a decisive role in the induction and maintenance of proliferation of the infected cell. EBNA-2, stimulates B cell survival and growth proliferation in the setting of EBV infection (reviewed in (Hayward 2004)). This viral transcription factor does not bind DNA directly but it is recruited to its site of action through complex and cell-context dependent interactions with cellular proteins including CBF1 (also known as RBP-Jk, a molecular component of the cellular Notch signalling pathway) and others (reviewed in (Zimber-Strobl and Strobl 2001, Hayward 2004)). EBNA-2 also regulates the expression of genes encoding cell surface and soluble proteins that modulate the cell's interaction with the environment, such as the receptors CD21, CD23, and CCR7 and the cytokines TNF-α, LT-α and IL-18 (Burgstahler, et al. 1995, Spender, et al. 2001b, Calender, et al. 1990, Pages, et al.

2005), in addition to cellular genes that are implicated in intrinsic growth and survival regulation, like *cyclin D2*, *c-myc, bfl-1* and *c-fgr)* (Kaiser, et al. 1999, Sinclair, et al. 1994a, Knutson 1990). Furthermore, in a study which Sindbis virus infection was used to induce Nur77 synthesis and apoptosis, it was demonstrated that EBNA-2 could bind to Nur77 and protect against Nur77-induced cell death (Lee, et al. 2002).

In humans, Bik seems to play a key role in B cell homeostasis. It has been shown that B cell subsets undergo significant upregulation of *bik* during differentiation as they progress through the GC reaction from naïve, centroblast, centrocyte and through to memory cells. It has been reported that the EBV Bcl-2 homolog, BHRF1 blocked apoptosis induced by Bik (Boyd, et al. 1995a). However it was subsequently shown that the inhibitory effect of BHRF1 was not due to direct interaction with Bik but rather due to the inactivation of Bak which, unlike Bax, is required for Bik-mediated apoptosis (Shimazu, et al. 2007). Interestingly, Bik also mediates host cell suicide in response to protein synthesis shutoff (Shimazu, et al. 2007), a process commonly observed following viral infection, and induced by the EBV early lytic gene BGLF5 (Rowe, et al. 2007). Bik has also been seen to be required for IFN-γ-induced cell death in human airway epithelial cells (Mebratu, et al. 2008).

In our laboratory, preliminary RNA multi-riboprobe nuclease assays had indicated a possible correlation between the EBV growth programme and the down-regulation of the pro-apoptotic *bik* gene in BL-derived cell lines and LCLs. Subsequently, it was also shown in our laboratory that EBV down-regulates *bik*. Significantly, restoration of Bik expression in the LCL IB4 induced a pathway upstream of the caspases, stimulating the induction of apoptosis that is dependent on the functional BH3 domain of Bik. In line with literature and previously in our laboratory, PBMC-derived untouched naïve B cells were infected with EBV, and any further decrease in the already low level of *bik* mRNA that was present prior to infection was not observed (D Walls; personal communication). It is possible therefore, that blocking Bik upregulation may have an important role in evasion of host immune responses. These results demonstrated that *bik* is transcriptionally down-regulated by EBNA-2 in EBV-negative cell lines and LCLs and that neither c-Myc nor the ability of EBNA2 to interact with CBF1 are required for this effect (D Walls; personal communication). This finding was the first indication of the transcriptional down-regulation of a pro-apoptotic gene by EBV. To date EBNA-2 is known to transcriptionally repress the activity of two other cellular genes, the immunoglobulin heavy chain locus (IgM) and *c*-myc (Maier, et al. 2006, Lucchesi, et al.

2008a). EBNA-2 has previously been implicated in the modulation of the anti-apoptotic *bcl-2* (Finke, et al. 1992). Significantly the overexpression of Bcl-2 in group I BL cell lines resulted in diminished apoptosis in response to a number of stimuli, and cell death inhibition was directly correlated to the amount of Bcl-2 expressed (Henderson, et al. 1991, Milner, Johnson and Gregory 1992). It has also been shown in the laboratory that EBNA-2 up-regulates the anti-apoptotic *bfl-1*. EBNA-2 *trans*-activation of *bfl-1* requires CBF1, and *bfl-1* expression is induced and maintained at high levels by the EBV growth programme in an LCL (Pegman, et al. 2006a).

Here, the mechanism and potential role of the *EBV*-bik interaction was investigated in a context that is relevant to EBV namely following the triggering of intrinsic apoptotic pathways by TGFβ1, BCR, and 1α,25-(OH)$_2$D$_3$ in a GC B cell setting. EBV negative cell lines which are used as GC B cell models were used for this purpose along with a conditional LCL, ER/EB2-5, in which the EBV growth programme, driven by EBNA2, could be switched on and off.

6.1 EBV inhibits TGFβ1-induced apoptosis by inhibiting *bik* upregulation through the canonical Smad pathway

TGFβ signalling mediates a wide range of biological activities in development and disease. The TGFβ1 ligand signals through heterodimeric type I and type II receptors that are members of the serine/threonine kinase family (Juarez and Guise 2011). Here it was shown that stimulation of the EBV-negative B cell lines, BJAB and Ramos with TGFβ1 led to induction of apoptosis. This effect was more evident in BJAB in comparison to the Ramos cells. The induction of apoptosis by TGFβ receptor ligation coincided with increased expression of *bik* in both cell lines. Transient *bik* knockdown by RNAi in both cell lines led to decreased apoptosis as induced by TGFβ1. Ramos cells transiently expressing EBNA-2 or its non-CBF1 binding mutant, EBNA-2 WW323SR, were also observed to have decreased apoptosis upon TGFβ1 receptor ligation compared to cells transfected with control vector.

It was also confirmed here that treatment of Ramos cells with TGFβ1 induced the translocation of Smad3 from the cytoplasm to the nucleus, as determined by Western blot. Elevated Smad3 levels were also seen to coincide with increased levels of *bik* mRNA in Ramos and BJAB. On the other hand, Smad3 knockdown resulted in a decreased level of *bik* mRNA and protein. ChIP analysis indicated that upon TGFβ1 ligation, the level of Smad3 and Smad4 recruitment to the *bik* promoter increased, which coincided with elevated *bik* mRNA levels in Ramos and BJAB cell lines. Conversely TGFβ1 treated Ramos and BJAB cells transiently expressing EBNA-2, or its non CBF-1 binding mutant, EBNA-2 WW323SR, led to a decreased level of Smad3 and Smad4 and also coincided with increased degree of EBNA-2 recruitment to the *bik* promoter. This observation was correlated with a low level of *bik* mRNA expression as determined by RT-qPCR. Similarly, in the conditional LCL ER/EB2-5 cells proliferating on EBV growth programme, the level of Smad3 bound to the *bik* promoter was decreased and EBNA-2 was seen to be recruited to the *bik* promoter. This also coincided with a later observation that the total level of Smad3 protein was decreased in ER/EB2-5 cells proliferating due to EBV growth programme. Moreover, it was also seen that Smad3 levels were decreased in EBV negative BJAB cells expressing ectopic EBNA-2 or its mutant, WW323SR. It has been cited that TGFβ1 can induce apoptosis and growth arrest depending on the cell type and the context (Ikushima and Miyazono 2011a, Schuster and Krieglstein 2002b). Several mechanisms have been suggested

through which EBV infected cells may confer resistance to TGFβ-mediated apoptosis or growth inhibition (Spender and Inman 2011, Horndasch, et al. 2002).

It has been observed that the loss, or down-regulation, of the TGFβ receptors type I/II results in inactivation of the TGFβ signalling pathway (Kumar, et al. 1991, Inman and Allday 2000, Fukuda, Kurosaki and Sairenji 2006). However, receptor down-regulation does not always account for the resistance of cells to TGFβ mediated effects, as was seen in LCLs treated with TGFβ (Horndasch, et al. 2002). In that study, the complete TGFβ signalling cascade was intact and a loss of signalling was not required for antagonizing the TGFβ-mediated effects (Horndasch, et al. 2002). In addition to this down-regulation of type I/II receptor expression (Inman and Allday 2000, Fukuda, Kurosaki and Sairenji 2006) which is associated with insensitivity to TGFβ, there are also further checks and controls related to TGFβ gene expression which act to abolish its function. Furthermore, it has also been observed in B cells and epithelial cells, that LMP2A mediated activation of the PI3K/Akt pathway confers resistance to TGFβ-mediated effects, but not the down-regulation of these receptors (Fukuda and Longnecker 2004).

TGFβ exerts its anti-proliferative effects through the targeting of regulatory proteins in the cell cycle machinery, for example the dephosphorylation of pRb (the tumour suppressor retinoblastoma protein). However, this effect was abolished in the presence of EBV or the LMP1 (Arvanitakis, Yaseen and Sharma 1995, O'Nions and Allday 2003). It was observed that LMP1 was responsible for the induced expression of cyclin D2, which is not expressed in normal B cells. It has been suggested that constitutive induction of the cell cycle regulators by LMP1 can lead to pRb hyper-phosphorylation and uncontrolled cell proliferation (Arvanitakis, Yaseen and Sharma 1995, O'Nions and Allday 2003). Among the latent genes, EBNA-2 as well as LMP1 was shown to induce the expression of cyclin D2 (Spender, et al. 2001, Sinclair, et al. 1994).

LMP1 is known to be universally expressed in NPC cells. In these EBV-infected epithelial cells LMP1 is involved in blocking TGFβ-mediated effects. LMP1 has been shown to prevent TGFβ-induced cell cycle arrest by suppressing the TGFβ-induced expression of the transcription factor ATF3. Deprivation of ATF3 induction enables TGFβ-mediated expression of Id1, which would otherwise be inhibited by ATF3 (Kang, Chen and Massague 2003). The presence of Id1 then inhibits TGFβ-induced cytostasis (Lo, et al. 2010). Induction of Id1 is possibly dependent on cell type. It has been

reported that TGFβ represses Id1 expression in epithelial cells (Kang, Chen and Massague 2003). Also, it has been shown that TGFβ induces the expression of Id1 in both the Ramos and BL-41 cell lines (Bakkebo, et al. 2010). However, ATF3 does not appear to be a TGFβ target in B cells, even when B cells are not infected with EBV. Also, while Id1 protein is up-regulated by TGFβ in B cells, growth arrest can proceed uninterrupted in the presence of Id1 (Spender and Inman 2009b). In the case of the EBV-infected gastric epithelial cell line, GT38, it has been shown that the TGFβ1/MAPK/p21 pathway is required for TGFβ1-mediated cytostasis, and that constitutive MAPK phosphorylation induced by LMP1 conferred resistance to TGFβ mediated growth arrest in this cell line (Fukuda, et al. 2002).

A better understanding of the detailed molecular mechanisms by which Notch and TGFβ signalling interact may shed a light on the EBNA-2 interaction with the TGFβ signalling cascade. Phosphorylated Smad proteins bind to p300 in TGFβ mediated signalling (Nishihara, et al. 1998, Pouponnot, Jayaraman). It has been proposed that p300 forms the bridge linking the Smad complex to the transcriptional apparatus of the cell. Both Notch (Masuda, et al. 2005) and LMP1 (Mori, et al. 2003) have been shown to abrogate TGFβ-mediated growth inhibitory effects, by sequestering p300 from receptor activated Smads. In addition, the three viral transcription factors vIRF, (Kaposi sarcoma herpes virus), E1A (adenovirus), and EBNA-2 (EBV) are known to *trans*-activate *myc* through a cellular cofactor at the PRF element (plasmacytoma repressor factor) which is located on the *myc* promoter. While all three have the ability to activate the *myc* promoter, each has a unique set of co-adaptor profiles, which can result in activation or repression of the target gene. It has been observed that CBP, but not the 300/CBP-associated factor (P/CAF) and p300 promote vIRF *trans*-activation, whereas p300 but not P/CAF and CBP suppress EBNA-2 *trans*-activation. In the case of E1A, P/CAF promotes *trans*-activation while p300 and CBP have a suppressive effect (Jayachandra, et al. 1999). Smad3 phosphorylation at Thr8, Thr179, and Ser213 via cdk2/4 in the nucleus has been reported to inactivate Smad3 (Matsuura, et al. 2004). It has been shown elsewhere that cdk2 and cdk4 are active in the presence of EBNA-2 (Kempkes, et al. 1995a) and hence, it may be the case that the effect of EBNA-2 on Smad3 might be mediated by these kinases. It has previously been shown in a BL cell line (CA46) that TGFβ exerts G_1 cell cycle arrest by transcriptional repression of E2F-1, where *c-myc* is not repressed, (due to chromosomal translocation) and that CDKIs are not induced. This is a good example of TGFβ cell-type specific activity (Spender and

Inman 2009b). Interestingly, EBNA-2 has been shown to induce E2F upregulation (Helin, et al. 1992) (Kempkes, et al. 1995a).

TGFβ signalling is one of the key signalling pathways required for elimination of unwanted B cell in the GC. In order to establish a lifelong persistent infection, EBV infected cells may confer resistance to TGFβ-mediated apoptosis or growth inhibition in GCs through several mechanisms (as described above) (Spender and Inman 2011, Horndasch, et al. 2002). EBV exerts significant control over the expression of the *bcl-2* family. *bik* is a direct target of TGFβ1 signalling in B cells (Spender and Inman 2011, Spender, et al. 2009).. The results presented here show for the first time that EBNA-2 inhibits *bik* by effecting a decrease in the level of Smad3 protein, and consequently the level of Smad3 interacting with the *bik* promoter, and that this transcriptional co-activator from the TGFβ signalling pathway is key to maintaining *bik* expression in the cell lines examined.

6.2 EBNA-2 decreases the BCR-induced apoptosis

BCR aggregation due to antigen binding promptly activates the Src family kinases (reviewed in (Romero-Camarero, et al. 2013, Craxton, et al. 1999)). Signals originating from this complex activate multiple cascades that lead to changes in cell metabolism, gene expression and cytoskeletal organisation. The complex BCR signalling pathway leads to diverse effects, including cell survival, apoptosis, proliferation, and also differentiation into antibody-producing cells or memory B cells. The consequence of the response is determined and modulated by the maturation state of the cell, the nature of the antigen, the degree and duration of BCR occupancy, and also signals from other receptors and transmembrane proteins such as CD45, CD19, CD22, PIR-B, FcγRIIB1 (CD32), CD40, the IL-21 receptor, and BAFF-R (Kurosaki 2011, Chung, et al. 2012). Overall, the EBV transformation of resting B lymphocytes to LCLs mimics antigen-induced resting B cell clonal expansion in lymph node germinal centres, where antigen binding to surface Ig (sIg) induces *myc*-mediated proliferation (Murn, et al. 2009) and T cell CD40 ligand activates B lymphocyte CD40 receptors to up-regulate NF-κB, MAP kinases, and anti-apoptotic Bcl-2 family protein expression (Elgueta, de Vries and Noelle 2010). It is noteworthy that EBNA-2 is the mediator responsible for downregulating sIgM as was observed in a study conducted using LCLs and BL cell lines conditionally expressing EBNA-2 (Jochner, et al. 1996b). It has been shown by Jiang,A. *et al,* that membrane bound IgM and IgD ligation in B104 cells (a mature B cell phenotype Chen,W. 1999) led to the induction of *bik* mRNA, but that only sIgM ligation resulted in apoptosis due to the expression of *bik*, via strong activation of PI3K (Beckwith, et al. 1996) and calcium influx (Jiang and Clark 2001a).

Here it has been shown that BCR stimulation by an anti-IgM antibody led to a significant increase in apoptosis in Ramos cells and to lesser extent in the BJAB cell line. It is important to note here that BJAB is an EBV-negative B lymphoma cell line with a non-translocated *c-myc* (Marchini, Longnecker and Kieff 1992)*,* while the BL-derived Ramos cell line carries the translocated *c-myc* gene. Subsequent Western blot and RT-qPCR analysis revealed elevated levels of Bik protein and mRNA in both cell lines when treated with various concentrations of anti-IgM antibody. It has been shown elsewhere that anti-IgM antibody treatment of BL Ramos and B cell lymphoma BJAB cells modulates the expression and protein stability of *c-myc,* promoting cells to undergo apoptosis (Kaptein, et al. 1996, Gururajan, et al. 2005), whereas *c-myc-*

deficient B cells are resistant to apoptosis by BCR triggering (Murn, et al. 2009). It was observed in the present study that apoptosis was induced due to harsh transfection conditions and that transient *bik* knock-down led to an overall increase in the cell survival rate in both Ramos and BJAB after transfection. Although transient *bik* knockdown was seen to decrease the overall level of apoptotsis it did not however have a pronounced effect on the rate of apoptosis induced by BCR cross-linking in both cell lines. It has previously been shown that BCR cross-linking mediates apoptosis through induction of both Bim and Bik (Jiang and Clark 2001b, Enders, et al. 2003a) and so the expression of Bim may be the reason for the retention of sensitivity to apoptosis under the consitions used. It was also demonstrated here that ectopic EBNA-2 and its non-CBF1 binding mutant, EBNA-2 WW323SR, could rescue Ramos cells from antigen-receptor-mediated apoptosis when compared to cells transfected with control vector. These observations indicated that EBNA-2 mediated effects on *bik* in this context occurred independently of its ability to interact with the cellular transcription factor CBF1.

In Ramos cells in which the PI3K pathway was inhibited by wortmannin, apoptosis was significantly decreased when the BCR was cross-linked with anti-IgM antibody. Whereas in BJAB cells wortmannin, did not have any obvious effect on apoptosis induced by BCR cross-linking. However, BJAB cells treated with wortmannin did exhibit a decrease in cell viability when compared to the control cells in which the PI3K pathway was not inhibited. *Bik* mRNA increased in both Ramos and BJAB cells following treatment with anti-IgM antibody and PI3K inhibition with wortmannin reversed this effect in Ramos. Although PI3K inhibition also coincided with decreased *bik* mRNA levels in BJAB cells in response to anti-IgM antibody, the extent of *bik* down-regulation was less significant to what that observed in Ramos cells.

PI3K confers a survival signal to cells, allowing them to survive several apoptotic stimuli (Kalimuthu and Se-Kwon 2013). It has been shown that the inositol phosphatases and tensin homologue (PTEN) negatively regulate PI3K-mediated survival and proliferation of hematopoietic cells by inducing the G_1 cell cycle arrest and decreasing the level of cyclin D2 (Huang, et al. 2007) and cyclin D3 (Zhu, et al. 2001). PTEN is a tumor suppressor gene that is mutated in human cancers. Deletion of PTEN has been shown to result in B cells that exhibit enhanced survival and express more MCL1 and less Bim. These cells also expressed low amounts of $p27^{kip1}$ and high amounts of cyclin D3 and therefore appeared to undergo proliferative expansion

(Miletic, et al. 2010). Many tumour cells are seen to express elevated levels of PI3K products following deletion of the phosphatase PTEN, activation of Ras or expression of autocrine growth factors. Accordingly, these cells are reasonably resistant to apoptosis. The principal mediator in this pathway is Akt, a PI3K activated protein kinase (Niiro, et al. 2012). Akt has been shown to have direct effects on the apoptosis machinery, for instance affecting the pro-apoptotic Bcl-2 related protein, BAD (Datta, et al. 1997). It has also been seen to affect the transcriptional response to apoptotic stimuli, for instance by regulating Forkhead transcription factors, and by influencing the activity of the p53 family (Downward 2004). In addition, novel connections between the metabolic effects of Akt and its control on cell survival have also been made, as LY294002 (a PI3K specific inhibitor) down-regulated Akt phosphorylation and cell proliferation in a dose-dependent manner (Downward 2004). The survival kinase PKB/Akt was highly activated in PTEN-deficient splenic B cells (Suzuki, et al. 2003). It has been shown that BJAB has no detectable PTEN protein (Pauls, et al. 2012) and that it has elevated PIP_3 and PIP_2 (Marshall, et al. 2002), with constitutive phosphorylation of AKT, whereas Ramos cells have been shown to express a very low level of PTEN protein but no detectable pAKT (Mori and Sairenji 2006, Cheung, et al. 2007).

In summary it was observed that apoptosis was induced in Ramos and BJAB upon BCR cross-linking. Apoptosis in these cells coincided with upregulation of *bik*. BCR-mediated *bik* upregulation was likely to be dependent on an intact PI3K pathway in Ramos and to lesser extent in BJAB as was in evidence following use of the PI3K pan inhibitor, wortmannin. The use of a more specific PI3K inhibitor would clarify this issue. The B cell response following BCR cross-linking is dependent on the differentiation stage of the B cells. As it has been shown, cross-linking of BCR induces proliferation of resting tonsillar follicular mantle (FM) B lymphocytes but prompts programmed cell death in the Ramos cell line (Padmore, Radda and Knox 1996). Moreover, the balance between the pro- and anti-apoptotic Bcl-2 family members directs cellular fate.

6.3 A co-operative effect of 1α,25-(OH)$_2$D$_3$ on BCR- and TGFβ1-mediated apoptosis

EBV infection is usually kept under tight control by EBV-specific immune responses, especially by cytotoxic CD8^{+} T cells which remove proliferating and lytically-infected B cells (Hislop, et al. 2007). Impaired CD8^{+} T cell control of EBV infection leads to accumulation of EBV-infected autoreactive B cells in the target organs where they generate autoantibodies and provide co-stimulatory survival signals to autoreactive T cells which would otherwise be eliminated in the target organ by apoptosis (Pender 2012). It has been shown that the level of vitamin D modulates the immune response to EBV (Holmoy 2008a). Although there are no reports regarding the direct effect of vitamin D on CD8^{+} T cells, they are known to express the highest level of VDR compared to other immune cells (Smolders, et al. 2008).

B cell reactivity with 1α,25-(OH)$_2$D$_3$ is a characteristic universally shared by naïve, germinal centre and memory B cell subpopulations, and cellular activation, rather than differentiation, is more defining for their biological reactivity to 1α,25-(OH)$_2$D$_3$ (Morgan, et al. 2000b). The effects of 1,25-(OH)$_2$D$_3$ on B cell responses includes; (i) the inhibition of the on-going proliferation of activated B cells and subsequent apoptosis, (ii) the inhibition of the generation of plasma cells and post-switch memory B cells, and (iii) up-regulated expression of the p27, but not of p18 and p21, that possibly will be important in regulating the proliferation of activated B cells and their following differentiation(Chen, et al. 2007). Vitamin D receptor decreases NF-κB activation by interfering with nuclear transcription factor NF-κB (p65 and p105). Therefore, the VDR prevents co-stimulatory signal transduction in naïve B cells, namely by decreasing CD40 signalling (Geldmeyer-Hilt, et al. 2011). Vitamin D plays a part in regulating autoantibody production. It has been shown that treatment of EBV-infected cells with 1,25-(OH)$_2$D$_3$, the immunoregulatory hormone, inhibited the production of IgM and IgG (Provvedini, et al. 1986). Also a direct inhibitory effect of 1α,25-(OH)$_2$D$_3$ on IgE production by human B cells has been reported (Heine, et al. 2002). Recently it has been shown that 1,25-(OH)$_2$D$_3$ induced a reduction of memory B cells and anti-DNA antibodies in systemic lupus erythematosus patients (Terrier, et al. 2012). In a study conducted by Yenamandra,S.P. *et al*, the expression profile of more than forty nuclear receptors were compared in the naïve and EBV-transformed B lymphocytes, freshly infected B cells and long-term cultured LCLs (Yenamandra, et al.

2009). *Nr2F2* and *RARA* were among the genes that were the most up-regulated and ERB, NUR77, PPARG, RXR-B and VDR were the most down-regulated in LCLs (Yenamandra, et al. 2009, Yenamandra, Klein and Kashuba 2009). It has been shown recently that EBNA-3 blocks the activation of VDR-dependent genes and protects LCLs against vitamin-D3-induced growth arrest and/or apoptosis by binding to VDR (Yenamandra, et al. 2010). These findings are evidence that $1,25\text{-}(OH)_2D_3$ may play an central role in the maintenance of B cell homeostasis and that an improvement of vitamin D insufficiency may be useful in the treatment of B cell-mediated diseases.

Here it has been shown that apoptosis was induced in Ramos and BJAB cells by $1\alpha,25\text{-}(OH)_2D_3$. It was observed for the first time that, $1\alpha,25\text{-}(OH)_2D_3$ mediated apoptosis coincided with up-regulation of *bik* in both cell lines. Higher levels of apoptosis were induced in Ramos cells through BCR cross-linking and to lesser extend with $1\alpha,25\text{-}(OH)_2D_3$ or TGFβ1. On the other hand, in BJAB cells TGFβ1 was observed to have the greater impact on the induction of apoptosis in comparison to anti-IgM antibody and $1\alpha,25\text{-}(OH)_2D_3$ treatment. Co-treatment of Ramos cells with $1\alpha,25\text{-}(OH)_2D_3$/anti-IgM antibody was seen to have a strong co-operative effect regarding the induction of apoptosis compared to when these cells were co-treated with $1\alpha,25\text{-}(OH)_2D_3$/TGFβ1. Similarly apoptosis was induced strongly in Ramos cells when co-treated with anti-IgM antibody/TGFβ1. In comparison when BJAB cells were co-treated with either sets of $1\alpha,25\text{-}(OH)_2D_3$/anti-IgM, $1\alpha,25\text{-}(OH)_2D_3$/TGFβ1, or anti-IgM antibody/TGFβ1 overall apoptosis was increased when compared to BJAB cells that were treated individually with each effector.

The importance of the PI3K pathway in BCR-, TGFβ- and vitamin D_3- mediated effects and its possible involvement in the cross-talk between the intrinsic signalling pathways was then investigated by using wortmannin. PI3K inhibition alone led to no significant changes in the viability of Ramos cells. However, the apoptotic effects of anti-IgM antibody and $1\alpha,25\text{-}(OH)_2D_3$ were significantly decreased, whereas the TGFβ1 apoptotic effect was enhanced when Ramos cells were treated with wortmannin. It was observed that when anti-IgM was used in combination with $1\alpha,25\text{-}(OH)_2D_3$ apoptosis was increased in Ramos cells to a level greater than that achieved with either effector alone Wortmannin was seen to decreased this overall increase of apoptosis which was achieved due to co-treatment of Ramos cells with anti-IgM antibody/$1\alpha,25\text{-}(OH)_2D_3$. A similar pattern, but of a lesser magnitude was observed when Ramos cells were co-stimulated with $1\alpha,25\text{-}(OH)_2D_3$/TGFβ1 or anti-IgM antibody/TGFβ1. Thus the PI3K pathway, possibly plays a role in BCR- and $1\alpha,25\text{-}(OH)_2D_3$-mediated apoptosis and

might mediating cross-talk between $1\alpha,25$-$(OH)_2D_3$ and BCR-signalling. Moreover an intact PI3K pathway appears to be required for *bik trans*-activation in Ramos cells in response to $1\alpha,25$-$(OH)_2D_3$ and BCR cross-linking.
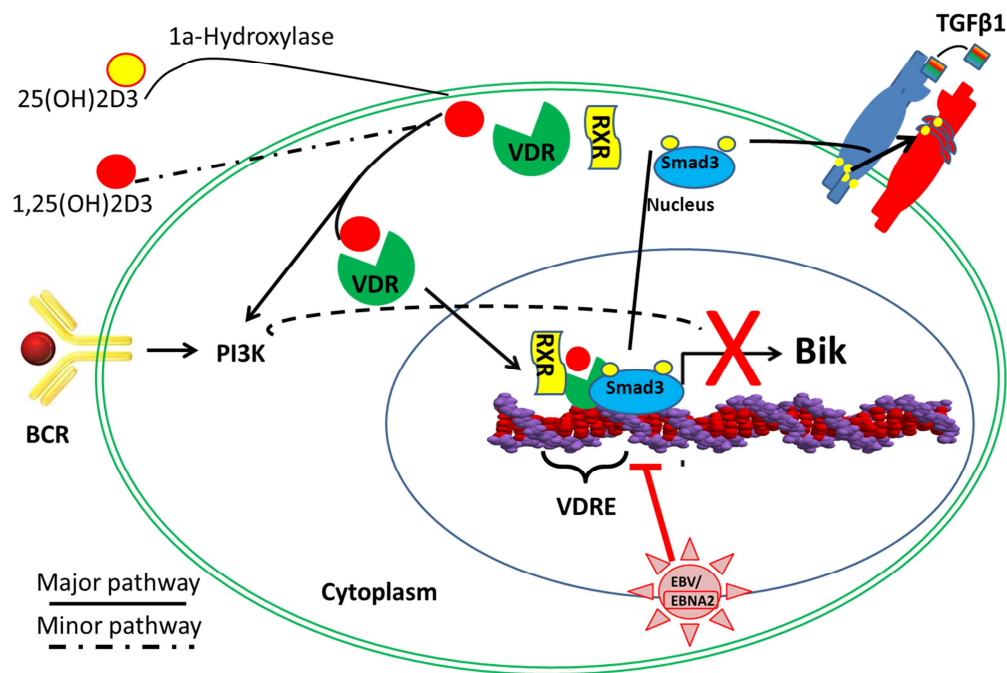
Figure 6-1: A model for possible cross-talk between $1\alpha,25$-$(OH)_2D_3$, TGFβ1, and BCR signalling pathways resulting in *bik trans*-activation and its inhibition bye EBV.

In BJAB cells treatment with wortmannin led to an overall decrease in cell viability. Unlike in Ramos cells, this drug did not display any pro-survival effect when BJAB cells were treated anti-IgM antibody or $1\alpha,25$-$(OH)_2D_3$. It was observed that TGFβ1-mediated apoptosis was enhanced when BJAB cells were treated with wortmannin. Moreover wortmannin enhanced apoptosis in BJAB cells co-stimulated with TGFβ1/$1\alpha,25$-$(OH)_2D_3$ when compared to co-stimulated cells in the absence of wortmannin. In contrast in BJAB cells, inhibition of PI3K had no significant effect on the extent of apoptosis when cells were co-stimulated with anti-IgM antibody/$1\alpha,25$-$(OH)_2D_3$. Although $1\alpha,25$-$(OH)_2D_3$ was able to induce apoptosis in Ramos and BJAB cells, $1\alpha,25$-$(OH)_2D_3$ might be using mediators known to be important in other intrinsic apoptosis-associated pathways, for example Smad3 (TGFβ) and PI3K (BCR). A number

of different experiments have demonstrated the relationship between the transforming TGFβ and vitamin D_3 signalling pathway (Adams and Hewison 2008, Subramaniam, et al. 2001). Smad3, a downstream component of the TGFβ signalling pathway, interacts with VDR as a co-activator of VDR (Subramaniam, et al. 2001). It has been shown that the 1α,25-$(OH)_2D_3$-mediated phosphorylation of Smad2 and Smad3 is an indirect effect of 1α,25-$(OH)_2D_3$ and is dependent on the presence of the TGFβ (Daniel, et al. 2007). Smad3 has been shown to bind to the VDR-RXR heterodimer in a ligand-dependent manner (Leong, et al. 2001). Vitamin D_3 has been shown to be involved in the activation of the PI3K/Akt signalling pathway (Deeb, Trump and Johnson 2007a, Hughes, et al. 2008) and PI3K pathway has been shown to be required for sustained Bik protein production upon BCR stimulation (Jiang and Clark 2001a). Additionally TGFβ1 has also been shown to activate PI3K in a non-Smad-dependent pathway (Zhang 2009). The accumulating evidence provided above indicates an interconnected network of apoptotic signals potentially emanating from TGFβ1, BCR and 1α,25-$(OH)_2D_3$, and that the interactions implied above may be important to achieve the optimal selection by apoptotic elimination of unwanted germinal centre B cells and maintaining homeostasis. It remains possible that the EBV growth programme inhibits the existing cross-talk by modulating the key mediators (Smad3 or PI3K) of these major apoptotic signalling pathways and increasing the apoptotic threshold (Figure 1-6). These molecular and cellular pathways should not be considered in isolation. They should be viewed as an array of interconnecting signals, all contributing to the final outcome, thereby allowing fine control of the cell fate and homeostasis.

Conclusions

EBV infection impacts on cell death and survival pathways in GC B cells. Interconnected signalling pathways, regulate the apoptosis of GC B cells. Establishment of a persistent latent EBV infection demands that the infected host B cells transit through the GC where networks of pro-apoptotic signalling molecules implement a harsh selection system over the differentiating B cells. Few B cells survive this course to differentiate fully. To guarantee the EBV-infected host cell survival, EBV has its own array of pro-survival mechanisms which can potentially dominate external stimuli promoting cell death such as, TGFβ, BCR cross-linking and apoptosis-induced activation by $1\alpha,25\text{-}(OH)_2D_3$ which are mediators of intrinsic apoptotic pathways.

One mechanism by which EBV promotes tumourigenesis is by altering the balance between pro- and anti-apoptotic proteins thereby leading to enhanced cell survival. In this context, previous studies have demonstrated that the viral latent genes EBNA3A, EBNA3C and LMP1 can regulate expression of members of the cellular Bcl2 family. This work identifies an additional pathway used by EBV to promote cell survival, namely repression of the cellular pro-apoptotic gene *bik* by EBNA2. The current work describes a novel mechanism by which EBV promotes cell survival, and which extends our understanding of how EBV modulates the expression of Bcl2 family proteins. In this context, Bik is particularly important given its potential role in B cell survival during B cell differentiation within germinal centres. *Bik* has also been used as a therapeutic molecule in gene therapy based approaches to treat difficult cancers (Chinnadurai,G. 2008). These results may have important implications for the pathogenesis of EBV associated disease such as IM and PTLD and the *bik/*EBV interaction could be a novel target in antiviral strategies.

In summary, this thesis presents the novel finding that EBV down-regulates the cellular pro-apoptotic *bik* gene by down-regulating the transcriptional co-activator, Smad3, which is also a TGFβ signalling mediator. Significantly, Smad3 knockdown in EBV negative cell lines coincided with decreased level of Bik. A lower basal Smad3 expression level was observed in the conditional LCL ER/EB2-5, and also EBV negative Ramos and BJAB cells expressing ectopic EBNA-2. The EBV latent transcription factor EBNA-2 plays a key role in *bik* modulation, independently of its ability to interact with the cellular transcription factor CBF1. Furthermore for the first time *bik tran*-activation has been observed to coincide with apoptosis induced by the

$1\alpha,25\text{-}(OH)_2D_3$. The PI3K was observed to potentially have a role in *trans*-activating *bik* in both Ramos and BJAB cells upon $1\alpha,25\text{-}(OH)_2D_3$ and BCR cross-linking induced apoptosis. Here it has been shown that the *bik* gene is a common target for the major intrinsic apoptotic signalling pathways in the GC reaction and that EBV negatively regulates its expression. As a consequence, EBV-infected naïve B cells undergoing clonal expression may be more resistant to such key apoptotic triggers and evade physiological elimination in order to transit through the GC reaction. Collectively, these findings suggest that *bik* modulation by EBV through down-regulation of Smad3 may be an important EBV-host cell interaction during EBV infection and a contributory factor in the development of EBV-associated B lymphomas.

Chapter 7 :
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Appendix

Solutions for DNA Manipulation

Storage of DNA

0.5 M EDTA (pH 8.0)

186.1 g   EDTA

800 mL dH_2O

The pH was adjusted to 8.0 by addition of NaOH pellets and the volume adjusted to 1 L with dH_2O. The solution was sterilised by autoclaving and stored at room temperature.

TE buffer (pH 8.0)

10 mM   Tris-Cl (pH 8.0)

1 mM   EDTA (pH 8.0)

Solution for Bacterial Growth Media

Ampicillin stock solution

A stock solution of ampicillin was made up to a concentration of 100 mg/mL in dH_2O.

The stock solution was filter sterilised and stored at -20˚C.

LB broth

5 g   Tryptone

2.5 g   Yeast extract

5 g   NaCl

The volume was adjusted to 500 mL, followed by autoclaving for 20 min at 15 lb/sq. and storage at 4˚C.

LB broth with ampicllin

Ampicillin was added to autoclaved LB broth to a final concentration of 100 μg/mL and stored at 4˚C.

LB agar

5 g	Tryptone
2.5 g	Yeast extract
5 g	NaCl
7.5 g	Agar

The volume was adjusted to 500 mL with dH_2O, followed by autoclaving and agar plates were stored at 4˚C.

LB agar with antibiotics

Antibiotics were added to autoclaved LB broth to a final concentration of 100 μg/mL (Ampicillin), after cooling the LB agar to ~50˚C. Plates were stored at 4˚C.

SOB medium

10 g	Tryptone
2.5 g	Yeast extract
0.025 g	NaCl
5 mL	KCl (250 mM)

The pH was adjusted to 7.0 with 5 M NaOH.

The volume was adjusted to 500 mL with dH_2O and the medium autoclaved.

2.5 mL of 2 M $MgCl_2$ was added after cooling the broth to 5˚C and the medium was stored at 4˚C.

SOC medium

98 mL   SOB medium

2 mL   1 M glucose (filter sterilised)

Stored at 4˚C.

Solutions for Preparation of Competent Cells

1 M MgSO$_4$

24.65 g	MgSO4.7H20
100 mL	dH20

Sterilised by filtering and stored at room temperature.

TFB1

30 mM	potassium acetate
10 mM	CaCl$_2$
50 mM	MnCl$_2$
100 mM	RbCl
15%	glycerol

The pH was adjusted to 5.8 with 1 M acetic acid, and the solution filter sterilised and stored at room temperature.

TFB2

100 mM	MOPS (pH 6.5)
75 mM	CaCl$_2$
10 mM	RbCl
15%	Glycerol

The pH was adjusted to 6.5 with 1 M KOH, and the solution filter sterilised and stored at room temperature.

e

Solutions for DNA preparations

Buffer P1 (Re-suspension buffer)

50 mM   Tris-Cl (pH 8.0)

10 mM   EDTA (pH8.0)

100 μg/mL   RNase A

Stored at 4°C following addition of RNase A.

DNase-free RNase

RNase A (1 mg/mL) in upH$_2$O.

Heated to 100°C for 30 min.

Cooled slowly and stored at -20°C.

Buffer P2 (Lysis buffer)

200 mM   NaOH

1% (w/v)   SDS

Stored at room temperature.

Buffer P3 (Neutralisation buffer) (3 M potassium acetate)

29.6 g   potassium acetate

50 mL   dH$_2$O

11.5 mL   glacial acetic acid

Adjust volume to 100 mL with dH$_2$O.

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Stored at room temperature.

f

Buffer QBT (Equilibration buffer)

750 mM   NaCl

50 mM   MOPS (pH 7.0)

15% (v/v)   Isopropanol

0.15% (v/v)   Triton-X 100

Stored at room temperature.

Buffer QC (Wash buffer)

1 M   NaCl

50 mM   MOPS (pH 7.0)

15% (v/v)   Isopropanol

Stored at room temperature.

Buffer QF (Elution buffer)

1.25 M   NaCl

50 mM   Tris-Cl (pH 8.5)

15%   Isopropanol

Stored at room temperature.

50 % (v/v) Glycerol

25 mL   glycerol

25 mL dH_2O

The solution was autoclaved and stored at room temperature.

Solutions for Agarose Gel Electrophoresis

50X TAE (Tris-acetate/EDTA electrophoresis buffer)

242 g	Tris base
57.1 mL	Glacial acetic acid
100 mL	0.5 M EDTA (pH 8.0)

The volume was adjusted to 1 L with dH_2O and the buffer was stored at room temperature.

1X TAE (Working solution)

20 mL	50X TAE
980 mL	dH_2O

Stored at room temperature.

Agarose gel loading dye

40% (w/v)	Sucrose
0.25% (w/v)	Bromophenol blue

Stored at room temperature.

Solutions for Cell Culture

Media and Supplements

Supplemented RPMI 1640 (500 mL)

440 mL	RPMI 1640
50 mL	Foetal bovine serum (decomplemented at 50˚C for 30 min)
5 mL	200 mM L-glutamine
5 mL	penicillin/streptomycin (10 mg/mL)

Estrogen (β-Estradiol)

A 20 mM solution was prepared in 100% ethanol and stored at -20˚C.

Phosphate buffered saline (PBS)

5 tablets were dissolved in 500 mL dH_2O to give a 1X working concentration of PBS, and the solution was sterilised by autoclaving.

Solutions for RNA Analysis

DEPC-treated H$_2$O

1 mL   DEPC

1000 mL   dH$_2$O

The mixture was left in a fume cupboard overnight, followed by autoclaving.

RNA loading buffer

50%   Glycerol

1 mM   EDTA (pH 8.0)

0.25%   Bromophenol blue

0.25%   Xylene cyanol FF

1 μg/μL   Ethidium bromide

Solutions for Protein Analysis

Solutions for Protein Isolation

Suspension buffer

0.1 M	NaCl
0.01 M	Tris-Cl (pH 7.6)
0.001 M	EDTA (pH 8.0)
1 µg/mL	leupeptin
1 µg/mL	Aprotinin
100 µg/mL	PMSF

Stored at 4˚C

Leupeptin

A stock solution of leupeptin was made to a concentration 2 mg/mL in dH_2O and stored at –20˚C.

Aprotinin

0.1 M stock solution of aprotinin was made up in dH_2O and stored at -20˚C.

PMSF

A stock solution of PMSF was made up in isopropanol and stored at -20˚C in the dark.

2X SDS loading buffer

100 mM	Tris-Cl (pH 7.6)
4% (w/v)	SDS
20% (w/v)	Glycerol
10% (v/v)	2-mercaptoethanol
0.2%	Bromophenol blue

Stored at room temperature.

Solutions for SDS-PAGE/ Western Blotting

Table A: 10% (v/v) resolving gels and 5 % (v/v) stacking polyacrlyamide gels

Component	10 % Resolving Gel (mL)	5 % Stacking Gel (mL)
Acrylagel	3.33	0.42
Bis-Acrylagel	1.35	0.168
1.5 M Tris (pH 8.8)	2.5	0
1 M Tris (pH 6.8)	0	0.312
dH$_2$O	2.61	1.5475
10% (v/v) SDS	0.10	0.025
10% (v/v) APS	0.10	0.025
TEMED	0.01	0.0025
Total	**10 mL**	**2.5 mL**

5X Tris-glycine running buffer

15.1 g	Tris base
95.4 g	glycine (pH 8.3)
50 mL	10 % (w/v) SDS

Made up to 1L with dH_2O and stored at room temperature.

1X Tris-glycine running buffer

200 mL	5X Tris-glycine running buffer
800 mL	dH_2O

Transfer buffer

750 mL	dH_2O
2.9 g	Glycine
5.8 g	Tris base
3.7 mL	10 % (w/v) SDS
200 mL	Methanol

Adjusted volume to 1 L with dH_2O and stored at 4˚C.

1X Tris buffered saline (TBS)

6.1 g	Tris base
8.8 g	NaCl
800 mL	dH_2O

The pH was adjusted to 7.5 with HCl and the volume adjusted to 1 L. Stored at room temperature.

m

TBS-T

1 L	1X TBS
1 mL	Tween 20

Stored at room temperature.

Blocking Buffer

5 g	Non-fat dry milk powder
100 mL	TBS-T

Stored at 4 ˚C.