Results! Why, man, I have gotten a lot of results. I know several thousand things that won't work.
- Thomas Edison (1847-1931)

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, 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.

Signed: _____

ID No.: _____

Date: _____

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Abstract

The regulation of cellular genes by the Epstein-Barr virus.

Susan Phelan

The Epstein-Barr virus (EBV) is a common herpesvirus that establishes a lifelong silent infection in the majority of all human beings. EBV has oncogenic potential, and is linked with the genesis of some cancers including Burkitt's Lymphoma (BL), Hodgkin's lymphoma (HL), Post-Transplant Lymphoproliferative Disease (PTLD), and nasopharyngeal and gastric carcinomas. EBV infection of primary B cells induces cell activation and proliferation, an efficient process that leads to the outgrowth of permanently growing lymphoblastoid cell lines (LCLs). Central to B-cell immortalisation by EBV is the modulation of host cellular gene expression by viral gene products. The characterisation of these host-virus interactions will shed light on the mechanisms that drive cell transformation, the establishment of life-long latency and potentially the development of EBV-associated B cell malignancies. A better understanding of the EBV B cell immortalisation process may present therapeutic targets of relevance to EBV associated and non-associated B cell diseases.

Different cell signaling pathways are activated by EBV during infection of B cells, this project initially involved an exploration of the molecular basis for the up-regulation of the cellular anti-apoptotic *bfl-1* gene by the EBV products LMP1 and EBNA. LMP1 mimics the NF-κB signaling pathway, while EBNA2 is a constitutively active functional homologue of Notch. It was determined in this study that EBV can regulate *bfl-1* in either an NF-κB or Notch dependent manner. Furthermore, mutation of both the putative NF-κB and Notch binding sites in the *bfl-1* promoter is required to prevent EBV mediated regulation of *bfl-1*.

Novel host cellular genes, the expression of which is modulated by EBV, were identified in this study by infecting untouched naïve B-cells, derived

from Peripheral Blood Mononuclear Cells, with recombinant green fluorescent protein (GFP)-tagged EBV. The transcription profiles of genes associated with selected cell signaling pathways were then determined by Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) using gene panel PCR arrays, and a comparison of relative transcript levels pre- and post-infection with virus was made. The transcriptional modulation of selected novel genes was then confirmed using an established conditional LCL in which the EBV growth programme can be controlled with β-estradiol.

Several candidate genes were identified using this approach. In particular, the cellular gene *Transducin-Like Enhancer of Split 1 (TLE1)* was identified as a novel negative target of EBV. TLE1 is a nuclear co-repressor component from the cellular Notch signaling pathway. Notch signaling is a major determinant of B cell fate, and the manipulation of this pathway by EBV features prominently on the route to virus-induced cell 'immortalisation'. The role of TLE1 was investigated by establishing lymphoblastoid cell line (LCL) transfected cell pools either expressing ectopic TLE1 or in which endogenous *TLE1* was knocked down using RNAi. It was shown that the level of *TLE1* expression affected the proliferative capacity of an LCL. In addition, other genes were found to be modulated due to the presence of ectopic TLE1. This was corroborated by knockdown of *TLE1* in the same LCL. It is concluded that this host-virus interaction between EBV and *TLE1* may positively contribute to the survival of EBV-infected B-lymphocytes proliferating on the EB viral growth programme.

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Abbreviations

6-MP	6-mercaptopurine
7-AAD	7-aminoactinomycin D incorporation
β-gal	Beta galactosidase
A_{260}/ A_{280}	Absorbance at 260nm/ Absorbance at 280nm
aa	Amino acid
ABI	Applied Biosystems
ADAM	Advanced Detection and Accurate Measurement
AES	Amino Terminal enhancer of split
AHF	Allogeneic helper factors
AIDS	Acquired immune deficiency syndrome
Ala	Alanine
AML	Acute Myeloid Leukemia
AP	Alkaline phosphatase
APC	Adenomatosis polyposis coli
APS	Ammonium persulphate
Arg/Gly	Arginine–glycine
ATP	Adenosine tri-phosphate
BART	*Bam*HI A rightward transcript
BATF	B cell specific transcription factor
B-CLL	B-cell chronic lymphocytic leukaemia
B-DLCL	B-cell diffuse large cell lymphoma
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BCR	B cell receptor
BD	Becton Dickinson Biosciences
bHLH	Basic helix loop helix proteins
BL	Burkitt's lymphoma
Bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
Ca^{2+}	Calcium

CAD	Caspase activated Dnase
CAT	Chloroamphenicol acetyl transferase
CBF1	C promoter binding factor 1
CBP	CREB Binding Protein
CcN	Phosphorylation sites for CK2 and cdc2
Cdk	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase Inhibitor
cDNA	Complementary DNA
CD40L	CD40 ligand
c-FLIP	FADD-like IL-1β-converting enzyme-inhibitory protein
CGNs	Cerebellar granule neurons
CHF	Cardiovascular helix-loop-helix factor
CIDE	Cell death-inducing DFFA45-like effector
CIP	Calf intestinal phosphatase
CIR	Co-repressor of CBF-1
CLP	Common lymphoid progenitors
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus immediate-early promoter
CO_2	Carbon dioxide
Con A	Concanavalin A
Cp	C promoter
CR	Cysteine rich
CSL	CBF1/Su(H)/Lag-1/RBP-Jκ/KBF2
CSR	Class switch recombination
C_T	Threshold Cycle
CTAR	C-terminal activation region
ctBP	Binding protein to the C-terminal half of E1A
C-terminal	Carboxy terminal
CTL	Cytotoxic T leukocyte
DD	Death domain
DEAE	Diethyl aminoethyl
DEPC	Diethylpyro-carbonate

DFF	DNA fragmentation factor
dH_2O	Distilled water
Dim	Homo-dimerisation domain
DISC	Death-inducing signaling complex
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide
DSL	Delta-Serrate-Lag2
Ebf	Early B cell factor
E. coli	*Escherichia coli*
EBER	Epstein-Barr virus encoded RNA
EBNA	EBV nuclear antigen
EBNA-LP	EBV nuclear antigen leader protein
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDAC	*N*-Ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERE	EBNA2-responsive element
ER/EBNA2	Estrogen responsive EBNA2
EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FASL	Fas ligand
FBS	Foetal bovine serum
FEVR	Familial exudative vitreoretinopathy
FR	Family of repeats
FSC	Forward light scatter
G	Growth phase of cell cycle
G418	Geneticin

Gal	Galactosidase
GC	Germinal Centre
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR
Gly	Glycine
Gly-Ala	Glycine Alanine
Gp	Glycoprotein
GSK3β	Glycogen synthase kinase 3beta
HAT	Histone acetyltransferases
HES	Hairy/Enhancer of Split-Complex
HD	Hodgkin's Disease
HDAC	Histone deacetylase
HD domain	Heterodimerisation domain
HDM2	Human double minute 2
HHV	Human herpes virus
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HNF4α	Hepatocyte nuclear factor-4α
HRP	Horse radish peroxidase
HRT	Hairy-related transcription factor
HSC	Hematopoietic stem cell
IAP	Inhibitor of Apoptosis Protein
IC	Intracellular
ICAD	Caspase activated Dnase inhibitor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal direct repeat
JAK	Janus kinase
JNK	c-Jun N-terminal kinase

kDa	kilo Daltons
LB	Luria-Bertani
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LDL	Low density lipoprotein
LEF1	Lymphoid enhancer-binding protein
LMP	Latent membrane protein
Luc	Luciferase
LNR	LIN12/Notch repeats
LRP5	LDL-receptor related protein 5
M	Mitosis
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MHC	Major histocompatability complex
MMP	Matrix metalloproteinase
MMLV	Moloney Murine Leukemia virus
MOMP	Mitochondrial Outer Membrane Potential
MPP	Multi-potent progenitor
mRNA	Messenger Ribonucleic acid
miRNA	Micro Ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NEB	New England Biolabs
N^{EC}	Notch extracellular domain
N^{IC}	Notch Intracellular domain (activated Notch)
N^{TM}	Notch trans-membrane domain
NcoR	Nuclear receptor co-repressor
NF-κB	Nuclear Factor κB
NHL	Non-Hodgkin lymphomas
NIK	NF-κB-inducing kinase
NK	Natural Killer

NLS	Nuclear localisation signals
NPC	Nasopharyngeal carcinoma
N-terminal	Amino terminal
O.D.	Optical density
OHL	Oral hairy leukoplakia
OMM	Outer Mitochondrial Membrane
ONPG	*o*-nitrophenyl-*β*-D-galactopuranoside
ORF	Open reading frame
OriP	Origin of Replication
OriLyt	Lytic Origin of replication
PAC	Puromycin N-acetyl-transferase
PAGE	Polyacrylamide gel electrophoresis
PARP-1	Poly(ADP-ribose) polymerase 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PES	Phenazine ethosulphate
pH	Power of the hydrogen ion
PI	Propidium iodide
PI3K	Phosphatidylinositol 3'-OH kinase
PMSF	Phenylmethanesulphonylfluoride
pRb	Retinoblastoma protein
PS	Phosphatidylserine
PTLD	Post transplant lymphoproliferative disease
Qp	Q promoter
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
$RbCl_2$	Rubidium chloride
RBP-Jκ	Jκ-recombinant-binding protein
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease

ROX	6-carboxyl-X-rhodamine
Rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
Runx	Runt related gene family
RV	Rhadinovirus
S	Synthetic phase of cell cycle
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sFasL	Soluble Fas lignad
SHM	Somatic hypermutation
shRNA	Short hairpin RNA
SIL	Scl interrupting locus
SKIP	Ski-interacting protein
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
SP domain	Serine/proline rich domain
SSC	Side scatter
STAT	Signal transducers and activators of transcription
STIL	Scl/Tal1 interrupting locus
SV40	Simian Virus early promoter
+/- T	Plus/minus tetracycline
TAD	Transcriptional trans-activation domain
T-ALL	T-cell acute lymphoblastic leukemia
TBP	TATA Binding Protein
Tcf	T cell factor
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet	Tetracycline
TLE	Transducin-like enhancer of split
TLR	Toll like receptor
T_m	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor

TNFR	Tumour necrosis factor receptor
TNFSF	Tumour necrosis factor receptor super family
TPA	12-O-tetradecanoylphorbol 13-acetate
TR	Terminal repeat
TRADD	TNFR-associated death domain
TRAF	TNF-receptor associated factor
TRE	TPA-responsive element
tTA	Tetracycline-regulated trans-activator
upH_2O	Ultra pure water
U_L	Unique long region
U_S	Unique short region
UTR	Untranslated Region
UV	Ultra violet
VCA	Viral capsid antigen
v/v	Volume per volume
Wp	W promoter
Wt	Wild type
w/v	Weight per volume
Zp	Z promoter
β-gal	Beta-galactosidase

Units

%	Percentage
ºC	Degrees Celsius
bp	Base pairs
cm	Centimetre
g	Grams
H	hours
Kb	Kilobase pairs
kDa	Kilo Dalton
Kg	Kilogram
L	Litres
lb/in^2	Pounds per square inch
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 Farad
µg	Micrograms
µl	Microlitre
µM	Micromolar
µm	Micrometer/ micron

Publications in preparation

Manuscripts in preparation

Phelan, S., Loughran, S.T., Campion, E.M., Hayward, D. and Walls, D. Role and Regulation of Transducin-Like Enhancer of Split 1 (TLE1) expression in EBV infected B-Lymphocytes. In Preparation

Campion, E.M., Loughran, S.T., D'Souza, B.N., Phelan, S., Kempkes, B., Bornkamm, G., Hayward, D. and Walls, D. Downregulation of the Pro-apoptotic Nbk/Bik Gene During the EBV Growth Programme. Manuscript in submission.

Presentations: Posters

Phelan, S., Loughran, S.T., Campion, E.M., Hayward, D. and Walls, D. Transducin-Like Enhancer of Split 1 (TLE1) is a negative target of the Epstein-Barr virus in B lymphocytes. School of Biotechnology Research Day, Dublin City University. 25th January 2010

Phelan, S., Loughran, S.T., Campion, E.M., Hayward, D. and Walls, D. Regulation of B-Lymphocyte gene expression by the oncogenic Epstein-Barr virus. All Ireland Cancer Conference, Dublin, 1st-3rd December 2008

Campion, E.M., Loughran, S.T., D'Souza, B.N., Phelan, S., Kempkes, B., Bornkamm, G., Hayward, D. and Walls, D. Downregulation of the Pro-apoptotic Nbk/Bik Gene during the EBV Growth Programme. All Ireland Cancer Conference, Dublin, 1st-3rd December 2008

E. Campion, ST. Loughran, BN. D'Souza, S. Phelan, B. Kempkes, G. Bornkamm, SD. Hayward and D Walls. Downregulation of the pro-apoptotic bik/nbk gene during the EBV growth programme. Royal Academy of Medicine in Ireland, Winter meeting DCU, 11th January 2007

Eva Campion, Sinéad T. Loughran, Brendan N. D'Souza, Susan Phelan, Bettina Kempkes, Georg Bornkamm, S. Diane Hayward and Dermot Walls. The pro-apoptotic bik gene is a transcriptional target of the Epstein-Barr virus nuclear antigen 2. Poster at International Association for Epstein-Barr virus and Associated Diseases, Boston, MA, USA (July 8-12th, 2006)

Presentations: Oral

June 2008

"The Regulation of Cellular genes by the Epstein-Barr Virus." Transfer from M.Sc. to Ph.D.

November 2005

My studies were included in the talk: Pro- and anti-apoptotic cellular genes are regulated by Epstein-Barr virus. D. Walls. Invited Speaker, Viral Oncology Meeting. Johns Hopkins University, Baltimore MD, USA (November , 2005).

Chapter 1: *Introduction*

1.1 The Discovery of the Epstein Barr Virus (EBV)

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) (Burkitt 1958, Burkitt 1962a). Due to 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. These suspicions led Burkitt to map the 'lymphoma belt' in Africa, which identified the regions where significant numbers of BL malignancies were observed (Burkitt 1962b). Further to this geographical analysis Burkitt and colleague also tried to analyse Burkitt's lymphoma samples for the presence of a causative entity, but without success (Burkitt 1962b).

In 1964, research was carried out on cultured biopsies from Burkitt's patients by Anthony Epstein and his colleagues Yvonne Barr and Bert Achong. This study revealed, via electron microscopy, the presence of herpes viral particles in the lymphoma samples (Epstein and Barr 1964, Epstein, Achong and Barr 1964). Sera from BL patients were examined and subsequently found to have higher titres of anti-EBV antibody when compared to control patients (Henle, et al. 1969). Indeed, high antibody titres were also observed in a lymphoepithelial malignancy with a high incidence in south China, called nasopharyngeal carcinoma (NPC) (Henle, et al. 1970). These observations and further subsequent investigations led to the implication of EBV as an oncogenic virus, not only in Burkitt's lymphoma but also in a number of other malignancies, including undifferentiated nasopharyngeal carcinoma (Reviewed in Bornkamm 2009b). Remarkably, however, it became evident that EBV infection was widespread, indicating that EBV infection alone was not a causative agent for malignancy, and that other genetic and environmental factors were required for tumour development (Bornkamm 2009b). 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 has been established, but to date no defined functional role for malaria in EBV-led BL has been elucidated (Reviewed in Griffin and Rochford 2005).

1.2 Classification of EBV

Herpesviridae consist of a family of herpes viruses that infect birds, reptiles and mammals (Lacoste, et al. 2010). The herpes family of viruses can be divided into three sub-types according to their genome organisation, growth characteristics and cell tropism, namely α-herpesviruses, β-herpesviruses and γ-herpesviruses (Hardie 2010). The γ-herpesviruses are further divided into lymphocryptoviruses (LCV; also known as gamma1-herpes virus) and rhadinovirus (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 found in RVs. EBV [also known as human herpes virus 4 (HHV4)], is a γ-herpesviruses of the genus lymphocryptovirus that targets B lymphocytes (Lacoste, et al. 2010, Hardie 2010, Barozzi, et al. 2007). EBV was the first herpesviral genome to be completely sequenced (Baer, et al. 1984). Typical γ-herpesviruses replicate in epithelial cells and then establish a lifelong latent infection in the host lymphocytes (Hardie 2010, Kieff and Rickinson 2007). The EBV genome, like all herpesviruses has a signature structure of conserved genes divided by defined regions of genetic variation (Lacoste, et al. 2010). EBV, like all herpes viruses, 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).

1.3 Structure of EBV

Herpesviruses, including EBV, possess a toroid shaped DNA/protein associated core enclosed in a 162 capsomere nucleocapsid (Figure 1.1). The 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 and Rickinson 2007, Thompson and Kurzrock 2004).

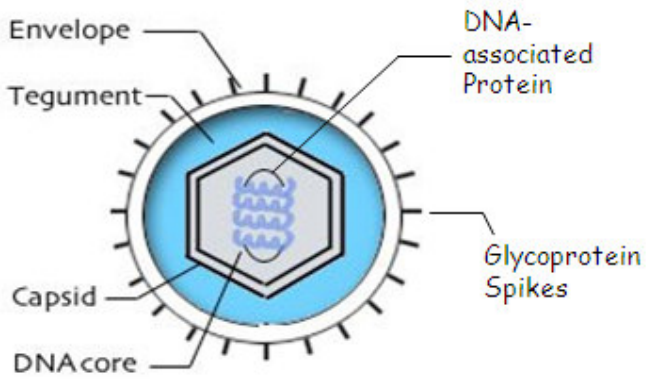


Figure 1.1: Schematic representation of herpes virus structure.

The EBV associated toriod protein core is illustrated within the icosahedral nucleocapsid. This capsid is itself covered by a protein tegument and enclosed in a glycoprotein covered envelope.

The double stranded EBV genome is 172 kbp long with a guanine/cytosine content of ~60% and encodes ~85 genes (Thompson and Kurzrock 2004, Kieff 1996). However, due to the splicing potential of the RNA transcripts the number of possible proteins far exceeds the number of genes present on the episome. The ends of the EBV genome contain a 500 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 kbp tandem internal direct repeat (IR1) that differentiates the unique short region (U_S) from the unique long region (U_L) which contains another three short tandem internal direct repeats (IR2-4) (Figure 1.2) (Lacoste, et al. 2010, Kieff and Rickinson 2007). Upon entry into the host cell, the linear

genome becomes circularised by the fusion of the two termini (Lindahl, et al. 1976).

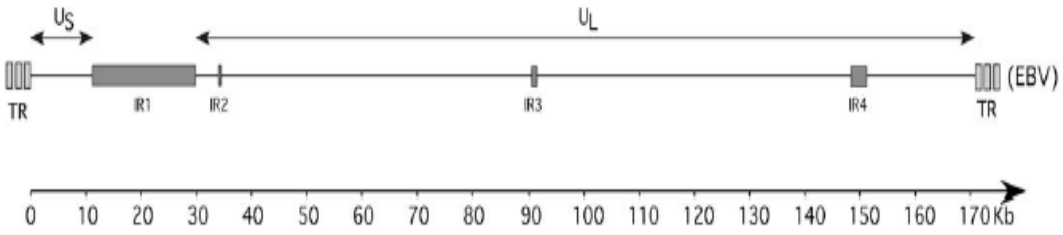


Figure 1.2: EBV Genome Schematic.

A schematic of the 172 kbp EBV genome including the internal repeats (IR) 1-4. The unique long arm (U_L) and the unique short arm (U_S) are indicated. Figure taken from Lacoste *et al* 2010.

1.4 Serotypes of EBV

Limited genetic variation has been found among the EBV isolates studied to date. Indeed, there are only two designated subtypes, designated type A and type B (also known 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 (EBNA2)-coding regions (Chang, et al. 2009) (EBNA2 functions as a transcription factor; see section 1.13.3). EBV type A was characterised from an EBV genome isolated from an infectious mononucleosis (IM) patient in North America, designated B95-8. AG987, a strain derived from an African BL case, was found to have a shorter EBNA2 sequence and was designated EBV type B. Remarkably, the EBNA2s of the two strains have only 55% homology at the protein level (Kieff and Rickinson 2001, Lucchesi, et al. 2008). Type A has been proven to be more competent at transforming human B cells into proliferating lymphoblastoid cell lines (LCLs) than type B (Rickinson, et al. 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. 2008). Although 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 sequence variation in other EBV genes such as the EBNA3 genes and EBNA-LP (Sample, et al. 1990)], the differences in the EBNA2 region have been proven to be the key behind the altered transformation efficiencies and capabilities of the two strains. In 1989, Cohen *et al* proved that the distinction between the immortalising capabilities of the two strains was EBNA2-dependent. A recombinant type B EBV strain was developed in which the type B EBNA2 coding region was substituted by the coding sequence for type A EBNA2. This resulted in an increased transforming efficiency in the type B strain on a par with the type A variant (Lucchesi, et al. 2008, Cohen, et al. 1989).

The diminished transformation proficiency of the type B EBNA2 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 EBNA2s on B cell immortalisation. In 1989 Cohen *et al* found that the EBV *Latent Membrane Protein 1* gene (*LMP1*), an EBV EBNA2 target, showed similar expression levels in LCLs proliferating due to either EBV type A or B. It was recently discovered however, that there is a transient difference in LMP1 levels immediately following B cell infection by the two types of EBV (Lucchesi, et al. 2008, Cohen, et al. 1989). The initial induction of LMP1 by EBNA2 type A is a significantly earlier and more intense response than the induction initiated by EBNA2 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 (Lucchesi, et al. 2008). A small number of cellular genes were also highlighted as differentially regulated by the two forms of EBV. EBV type A has been found to *trans*-activate the cellular target genes listed in Table 1.1 more potently that EBV type B:

Table 1.1: EBNA2 target genes that are differentially regulated by EBV type A and type B

Gene	Function
Runx3	Positively affects B cell proliferation (Spender, et al. 2005)
MARCKS	Regulates membrane ruffling and cell spreading (Myat, et al. 1997)
IL-1β	Role in lymphocyte proliferation (Krauer, et al. 1998)
ADAMDEC1	A metalloprotease expressed in lymphocytes. Function unknown (Bates, et al. 2002)
CXCR7	Required for LCL proliferation or survival (Lucchesi, et al. 2008)

1.5 EBV Epidemiology

Epidemiological evidence points to a key role for EBV in some malignancies such as African endemic BL. Indeed, EBV is present in approximately 95% of African BL tumours, in comparison to a 10-20% EBV-positivity in sporadic cases of BL found worldwide (Bornkamm 2009a). Epidemiological studies have also further pointed to an aetiological role for EBV in the western world in the development of IM, and for its 100% association with undifferentiated nasopharyngeal carcinoma tumours in southern Asia, especially the Canton region (de Thé 2005, Klein 2005). However, to date no definitive link has been established between EBV genotype and disease distribution.

1.6 Primary EBV Infection

Upon initial infection via exposure to EBV-positive saliva, EBV infects the stratified epithelium of the oropharnyx where it replicates, then migrates and establishes latent infection in the B lymphocyte constituency (Sixbey, et al. 1984, Murray and Young 2002). Through its viral proteins, the actions of which mimic several growth factors, transcription factors, and anti-apoptotic factors, EBV subverts control of the cellular pathways that regulate diverse homeostatic cellular functions (Thompson and Kurzrock 2004). Humans are

the only natural host of EBV. Infection usually occurs at a young age and is asymptomatic; however, due to the hygienic conditions in more developed countries, EBV infection is often postponed until adolescence or adulthood. When infection occurs after childhood, the primary infection can lead to IM. This lymphoproliferative symptomatic infection is self limiting, and characterised by a sore throat, fever, malaise, lymphadenopathy and mild hepatitis (Thompson and Kurzrock 2004, Baumforth, et al. 1999). However, once active infection is complete, EBV establishes a lifelong latent infection of the memory B cell compartment. During latent infection only a limited number of viral genes are expressed. The panel of eleven characterised EBV latently expressed genes are, six EBV Nuclear Antigens EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C 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). Throughout the life of the host, intermittent and silent activation of latently infected cells will allow sporadic shedding of EBV virions into the saliva (Hardie 2010). Throughout the host's life the viral load is maintained at a fixed level with, on average, approximately one EBV positive cell detected in every million peripheral blood lymphocytes. However, although each host maintains a fixed level of infection throughout their lifespan, the load level among individuals has been found to vary (Miyashita, et al. 1995).

The EBV infection process begins by exposure of the host to the virus via salivary contact. Once in the host's system, EBV infects the epithelial cells of the oropharnyx. This, however, is a poorly defined process (Young and Rickinson 2004). EBV establishes a transient lytic growth programme in this target site to produce more EBV virons. These pass into the lymphatic system where the viral surface glycoprotein (gp) gp350 binds to the B lymphocyte surface marker CD21, the receptor required to bind the C3d component of complement (Fingeroth, et al. 1984, Nemerow, et al. 1985). A second viral glycoprotein, gp42, binds to the human leukocyte antigen (HLA) class II molecule of both naive and memory B cells (Nemerow, et al. 1987, Borza and Hutt-Fletcher 2002). After antigen binding, EBV is endocytosed

into the cell via CD21-derived cytoplasmic vesicles and the capsids are released into the cytoplasm (Carel, et al. 1990, Nemerow and Cooper 1984). This instigates mRNA synthesis, B cell blast formation, homotypic cell adhesion, surface CD23 expression and interleukin (IL)-6 production (Thompson and Kurzrock 2004). Within 12 hours the linear viral genome moves into the nucleus and becomes a circular episome (Reviewed in Kieff and Rickinson 2007) with a variable number of terminal repeats (TR) which gives rise to a 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 EBNA2 (Thompson and Kurzrock 2004). An EBNA2- induced shift from the EBV latent Wp promoter to the alternative viral Cp promoter occurs at approximately 24-48 hours post-infection, and leads to an increase in EBNA mRNA expression (Kieff 1996, Takacs, et al. 2010). By 32 hours post-infection, all of the EBV latent proteins (six EBNAs and three LMPs) are expressed; however, significant EBV-encoded non-coding RNA (EBERs 1 and 2) expression is not detected until 70 hours post-infection (Kieff 1996, Murray and Young 2002). These EBV proteins quickly begin to subvert normal cell function instigating B cell activation and proliferation. This occurs due to EBV-led manipulation of normal B cell activation pathways such as (i) CD40 signaling, (ii) B cell receptor (BCR) signaling and (iii) Toll like receptor (TLR) signaling. LMP1, the only EBV latent membrane protein essential for B cell immortalisation, functions as a ligand-independent constitutively active tumour necrosis factor (TNF)-receptor. LMP1 mimics activated CD40 and therefore confers the survival signals to the EBV-infected cell that can be provided by CD40. LMP2A, while not an essential protein for immortalisation, provides an important survival signal *in vivo* by mimicking BCR activation. EBV also commandeers the Toll signaling pathway to enhance the chances of a successful infection. EBV activates Toll signaling through TLR7, taking advantage of the proliferative effects of stimulated TLR7. Together these three events are required for optimal B cell activation. The success of EBV

as a B cell transforming agent is therefore due to its adept nature, by which it utilises advantageous cell pathways such as CD40, BCR and Toll signaling and suppresses limiting factors associated with these pathways. An example of this is the EBV-led modification of downstream TLR7 signaling. TLR7 up-regulates interferon regulatory factor 5 (IRF5) which possess both anti-viral and tumour-suppressive properties. EBV stimulates TLR7, benefitting from the proliferative effects but then limits the negative effect of the TLR7 target IRF5, by inducing the expression of a dominant negative IRF5 splice variant (Martin, et al. 2007). Cytotoxic T cells (CTL) detect many of these freshly infected B lymphocytes by identifying and eradicating cells expressing EBV latency proteins (Hislop, et al. 2007). Enough infected cells evade detection, however, to allow EBV to establish a latent infection in the memory B cell population. These B cells elude the vigorous CTL response by a poorly-understood mechanism which leads to the repression of many or all of the EBV latency-associated genes (Uchida, et al. 1999).

1.7 EBV Latency Programmes

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 established from LCLs and tumour biopies, and have been characterised as latency Type 0, I, II and III, and the locations of their transcripts on the EBV genome are depicted in Fig 1.3. The distinguishing EBV latent gene expression pattern between each form of latent infection is defined in Table 1.2. Some controversy surrounds the designation of latency Type 0. In this 'persistence' latency, no EBV latent proteins are detected (Shah and Young 2009). Type I latency is characterised by Burkitt's lymphoma; the only latent genes expressed are EBNA1 from a third latent *EBNA* promoter, the Qp promoter, along with the non-coding groups of transcripts known as EBERs and BARTs. In Type II latency, EBNA1 and three latent membrane proteins (LMP1, LMP2A and LMP2B) are expressed, along with the non-coding EBERs and BART trancripts. EBNA1 expression

again originates from the Qp promoter. Nasopharyngeal carcinoma is characterised by type II latent gene expression. All known latent viral genes are expressed in Type III latency -EBNA1, -2, -3A, -3B, -3C and EBNA-LP along with LMP1, -2A and -2B and the non-coding EBERs and BARTs. However, unlike in Type I and II latency, EBNA1 expression is initiated from the Wp/Cp promoter. Initially the Wp promoter drives EBNA expression; however, as EBNA-LP and EBNA2 expression increases, transcription switches predominantly to the Cp promoter. Type III latency is characterised by the EBV expression profile found in LCLs; also, some forms of Burkitt's lymphoma can be classified as Type III latency (Reviewed in Kieff and Rickinson 2007, Thompson and Kurzrock 2004, Shah and Young 2009, Lopes, Young and Murray 2003). These latent expression programmes are not a definitive profile of EBV activity within a cell, as recent evidence has highlighted significant variations in the level of EBV encoded microRNA expression. The function and effect of these microRNAs is still under scrutiny (Reviewed in Shah and Young 2009).

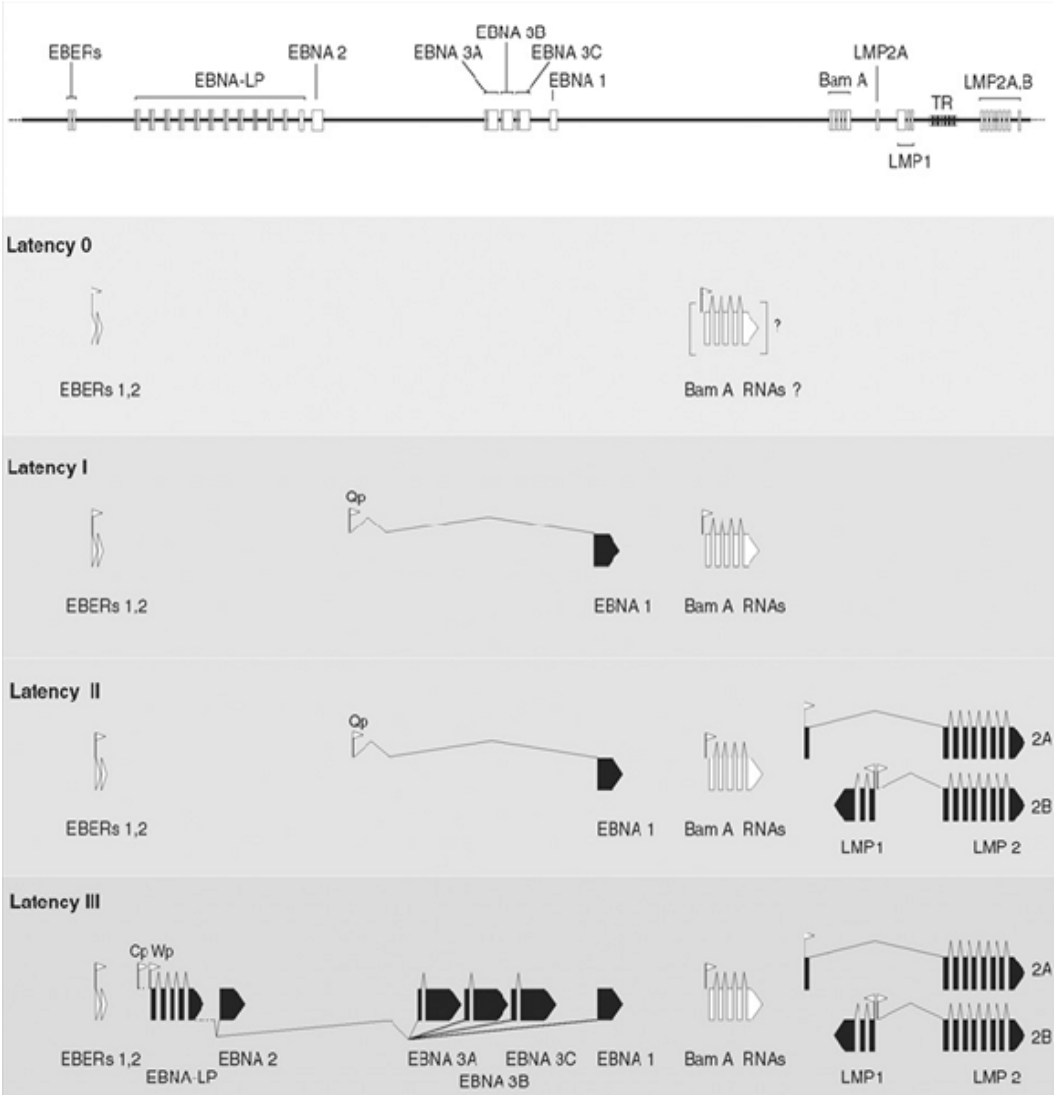


Figure 1.3: Pattern of EBV latency transcripts.

The transcriptional profile of EBV in each type of latency. The direction of transcription relative to each gene is indicated. White illustrations indicate non-coding RNA, black illustrations indicate mRNA (Adapted from (Kieff and Rickinson 2007)).

The EBV latency programme found in a given cell can be influenced by the immune status of the environment. In non-malignant EBV associated diseases such as IM, the cells have a Type III latency programme. However, in these non-malignant cases the infection is not silent; there is a self limiting and symptomatic response observed in the host as the immune system detects and targets the EBV assault. In EBV associated malignancies, Type III latency is generally found only in immunosuppressed patients, such as in

tumours from transplant and AIDS patients. In immunocompetent hosts, the EBV latency programme Type I or Type II is usually observed. These latency types express less EBV viral proteins reducing the likelihood of detection by the immunosurveillance system of a healthy host, allowing EBV to be sustained as an asymptomatic infection (Dolcetti and Masucci 2003).

Table 1.2: EBV gene expression in EBV-associated malignancies

Type of Latency	Viral Genes Expressed	Associated Malignancies
Type 0	None Detectable	Sustained asymptomatic infection
Type I	EBNA1, EBERs, BARTs	Burkitts Lymphoma, Gastric Carcinoma
Type II	EBNA1, EBERs, BARTs, BARF0 LMP1,LMP2A, LMP2B	Hodgkins Disease, Nasopharyngeal Carcinoma, Peripheral T/NK lymphoma
Type III	All EBV Latent Genes	AIDS-associated lymphomas PTLD, IM
Other	EBNA1, 2, LMP1	T cell Lymphomas
Other	EBERs, EBNA1,2	Smooth Muscle tumors
Other	EBNA1, 2, LP, LMP1	Oral Hairy leucoplakia (OHL)

1.8 EBV Associated Diseases

EBV infection has been linked to a number of B, T and NK lymphocyte malignancies such as Burkitt 's lymphoma, Hodgkin's lymphoma, peripheral T cell lymphoma and nasal T/NK lymphomas, that arise in both immunosuppressed and immunocompetent hosts. EBV has also been associated with a host of non-lymphoid malignancies including nasopharyngeal carcinoma, smooth muscle tumours in immunocompromised patients (Jenson, et al. 1999) and a selection of gastric carcinomas (Reviewed in Dolcetti and Masucci 2003). It has been observed from a

diverse panel of tumours that high grade tumours are three times more likely to be EBV positive than low grade tumours (Hummel, et al. 1995). A list of EBV associated diseases and their features are given in Table 1.2.

Table 1.3: EBV Associated Diseases

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

To date it is generally accepted that immunocompromised patients frequently succumb to EBV related diseases due to the absence of cytotoxic T cells that would normally eradicate a large population of the EBV-infected B cells.

Indeed, the immunosurveillance of the cytotoxic T cells pressurises the EBV infected cells into sustaining a latent state (Maeda, et al. 2009). There are many ways in which a person can become immunocompromised and is therefore more likely to develop an EBV-associated disease. Immunodeficiency can arise from a number of sources including anti-rejection drugs administered to transplant patients, patients with Wiskott-Aldrich syndrome or X-linked lymphoproliferative syndrome, Ataxia telangiectasia, acute lymphoblastic leukemia or AIDS (Cohen 2005).

It is not yet clear how EBV-associated diseases become established in immunocompetent hosts, although a number of plausible theories have been presented. Tumourgenesis of epithelial cells is thought to arise when latently infected memory B cells migrate to peripheral tissues and progress into plasma cells. These EBV infected plasma cells enter the lytic cycle exposing the epithelial cells to EBV virions. The events leading to lymphoid malignancies depend on the incorrect passage of EBV-infected B cells through the germinal centre (GC). It has been considered that EBV-associated BL arises due to a combination of a chromosomal translocation leading to the activation of the oncogenic *c-myc* and the passage of the infected cells into the GC where EBV drives proliferation of the B cell. C-myc subverts natural anti-proliferative cell signals thus enabling continuous proliferation of the EBV-stimulated cells. EBV-related Hodgkin's lymphoma is thought to occur in a similar manner. EBV- infected B cells move into the GC, but cellular mutations prevent their exit, causing the cells to be blocked at the GC. EBV continues to drive the proliferation of these cells resulting in a neoplasm of B cells that enlarges the lymph node in a manner that is characteristic of classical Hodgkin's lymphoma (Reviewed in Maeda, et al. 2009; Pattle and Farrell 2006; Tao, et al. 2006).

In vivo, EBV-infected BL cells usually exhibit type I latency, expressing only EBNA1, EBER and BARTs. However, some do exist as type III latency BLs. When brought to cell culture type I BLs can maintain type I latency but also have the potential to drift to express the type III latency pattern. Interestingly, *in vivo* type III BLs explanted to culture always maintain their EBV episome,

sustaining type III latency in culture. Indicating that *in vivo* type III BLs have evolved with EBV and require the virus for survival (Reviewed in Kieff and Rickinson 2007) (see Figure 1.4).

1.9 Established EBV Infection *in vivo*

In its normal life cycle, a naïve B cell that comes into contact with a recognisable antigen will migrate to the GC, there to be transformed into a memory B or plasma cell. The naive cell does this by undergoing somatic hypermutation (SHM) to increase B cell receptor recognition for the antigen. Within the GC, the cell also undergoes class-switch recombination (CSR) to alter the class of antibody expressed (Cader, et al. 2010). EBV-infected naïve B cells undergo the same processes as their naturally-stimulated counterparts, migrating into the GC and undergoing SHM and the CSR immunoglobulin rearrangements, and then passing out of the GC as latently EBV infected memory B cells which persist silently in the host.

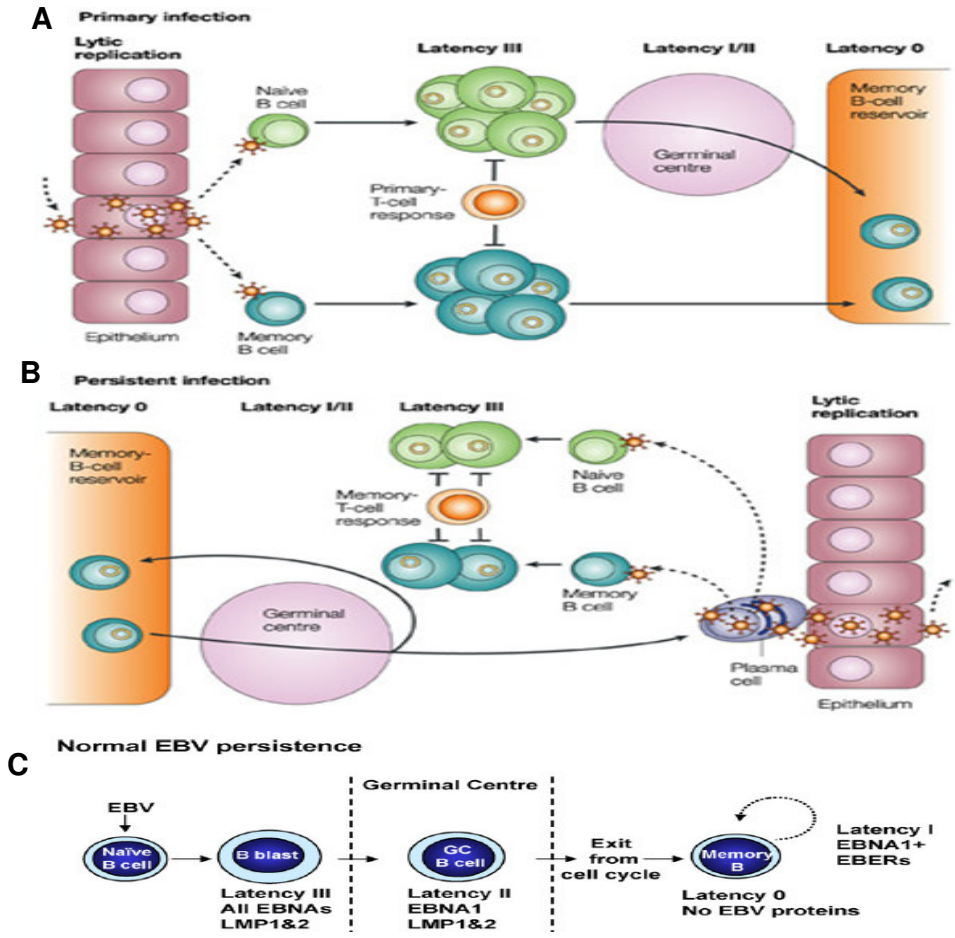


Figure 1.4: Schematic of EBV primary and persistent infection events.

(A) Primary infection involves the passage of EBV into the epithelial cells of the oropharnyx; from here it passes into the lymphatic system infecting naive and memory B cells, driving them into a proliferative state. The majority of these are eliminated by CTLs. Within the GC, the infected cell mimics natural physiological processes such as SHM and CSR to emerge as a long-lived, undetected EBV infected cell that establishes the reservoir of EBV infected cells (Type 1 or Type 0 viral gene expression) for persistent lifelong host infection in the memory B cell compartment; **(B)** Once EBV infection has been established within the host, sporadic shedding of virus occurs following the submission of the EBV positive memory B cells to the host's physiological control. The immune system can cause migration of the memory B cells back into the GC, which can lead to further differentiation of the memory cell into a plasma cell. These terminally differentiated cells will leave the GC and may move into the mucosal sites initiating the lytic cycle, producing viral progeny that can be shed into the oropharnyx (Adpated from Young and Rickinson 2004). **(C)** An illustration of EBV persistence *in vivo*. EBV utilises the normal B cell development pathway to establish long-term persistence. After naïve B cell infection, EBV expresses all latent genes (latency III); however, to emulate an activated naïve B cell and to evade immune detection, the infected cell moves into the GC and a switch to latency II occurs. This facilitates minor proliferation of the cells in the GC where the cell is concealed from immunosurveillance. Once expelled from the GC the infected cells switch to either latency I or latency 0 to maintain infection without instigating an immune response (Figure adapted from Allday 2009).

Evidence is building which indicates that EBV infection of resting B cells mimics the mechanisms behind physiological B lymphocyte activation via antigen stimulation and T cell association. Once EBV-transformed, human B cells gain cellular characteristics common to naturally activated B lymphocytes, such as blast morphology, expression of B cell activation markers and production of IgM (Dolcetti and Masucci 2003). One way in which EBV can pass by host immune detection is to present itself as a functioning memory B cell. Antigen presenting B cells present the CD40 protein on their cell surface. CD40 interacts with the CD40 ligand on an activated T cell to create a cell survival signal to the immune system (Kooten and Banchereau 1997). EBV infected cells mimic this survival mechanism by expressing both CD40 antigen and the CD40 ligand on the B cell surface. This method of EBV stimulated cell survival signaling has been shown to be a cell survival mechanism for B cell transformation (Imadome, et al. 2003), NK/T lymphoma (Imadome, et al. 2005) and gastric carcinoma-derived epithelial cells (Imadome, et al. 2009).

The mechanism of EBV internalisation into B lymphocytes has been elucidated; however, the ability of EBV to infect T/NK and epithelial cells is less apparent. T/NK cells show variable CD21expression, leading to the possibility of another potential mode of entry for the virus (Dolcetti and Masucci 2003, Tsoukas and Lambris 1993). Many studies have shown that CD21 is not expressed by epithelial cells (Speck, et al. 2000, Burgos and Vera-Sempere 2000), and this is corroborated by the inability of EBV to infect epithelial cells unaided *in vitro.* However co-cultivation of epithelial cells with EBV infected B lymphocytes can effectively enable EBV infection of epithelial cells (Imai, et al. 1998), suggesting that the mechanism for epithelial EBV infection requires infected B cell contact. When a global picture is considered, EBV is responsible for causing a surprisingly small amount of pathology. Taking into account that approximately 95% of the world's population are EBV carriers, only a small portion of these hosts will actually develop an EBV-related malignancy (Middeldorp and Pegtel 2008).

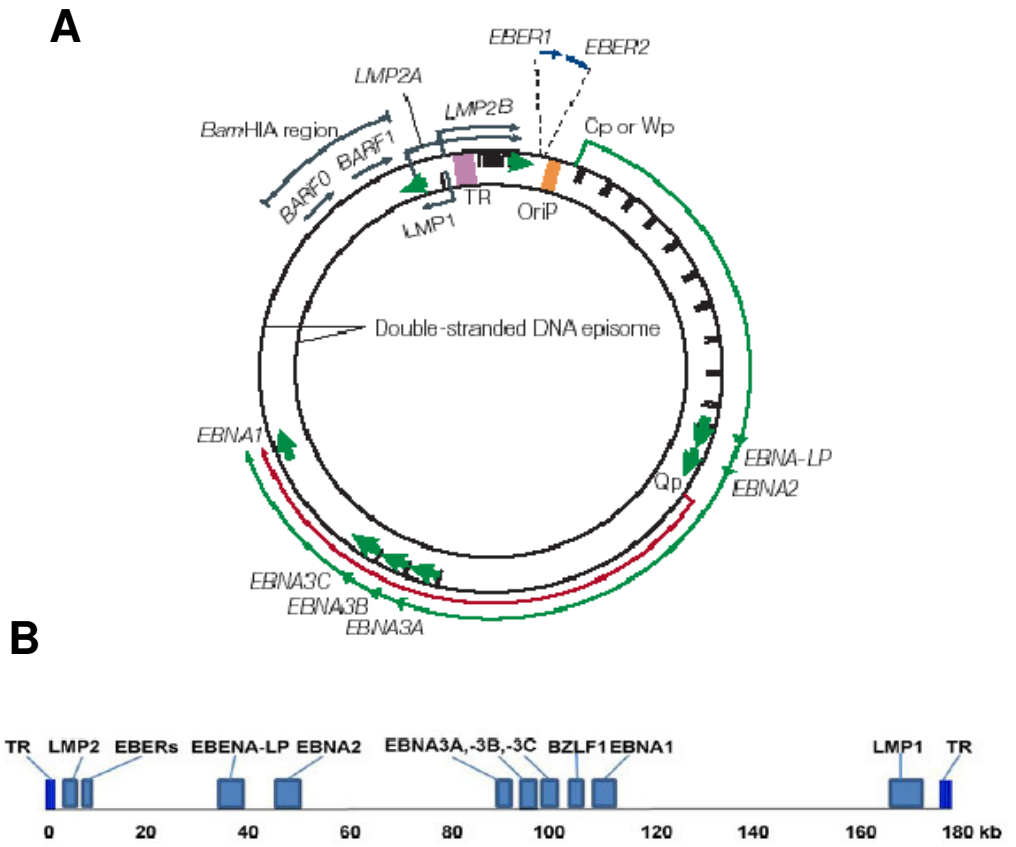


Figure 1.5: Schematic of the EBV Latent Genes.

(A). Diagram of the double stranded EBV episome with the EBV latent gene transcription sites marked. 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 Cp/Wp promoter during type III latency. The EBNA latency III primary transcript is alternatively spliced to give rise to the EBV nuclear antigen-coing mRNAs. The Qp promoter is active during latency I and II and gives rise to the expression of EBNA1, as highlighted by the red arrow. **(B).** Diagram illustrating the locations of the ORFs of EBV latent genes (EBNA1, 2, 3A, 3B, 3C, EBNA-LP, LMP1, LMP2A and LMP2B) and the EBV encoded RNA's EBER1 and EBER2. The location of the ORF encoding the key immediate early gene BZLF1 is also indicated (Figure adapted from Chang, et al. 2009).

1.10 The Lytic and Latent Cycles of EBV

EBV has two replicative phases, a virus production phase and a latent persistence phase. Infection with EBV involves both programmes, as the lytic cycle permits assembly of EBV virions whilst the latent cycle guarantees maintenance of a constant reservoir of virus within the host to ensure continuity of the infection. The viral lytic cycle is an explosion of viral replication that harms the host and in turn reduces the sustainability of the virus. This method of replication is not "in the best interests of the virus" as it will either itself be eliminated by the host immune system or it shall eliminate the host, which is vital for the existence of the virus (Shen and Shenk 1995). The two life cycles use two different origins of DNA replication, the lytic cycle uses OriLyt (Hammerschmidt and Sugden 1988), whilst the latent cycle uses OriP (Yates, et al. 1984, Yates, et al. 1985) (Reviewed in Takacs, et al. 2010).

EBV has co-evolved with its host by modifying its existence so as not to threaten the host and activate the immune system, sustaining this symbiotic relationship (Middeldorp and Pegtel 2008). EBV lytic infection is a significant disturbance within the host that presents many markers leading to recognition and elimination of EBV lytic activity by the body's immune system and this cycle can be used as a target for drug treatment. Survival of EBV is dependent on the virus persisting long enough to infect new hosts. Cell death caused by the lytic cycle alerts the immune system to the presence of EBV virions; however, by establishing a latent infection EBV can hide from the host's surveillance and maintain an equilibrium whereby EBV can persist in memory B cells and sporadically infect new hosts following intermittent activation of EBV replication in cells that become terminally differentiated. EBV lytic infection can be controlled by administration of the anti-viral drug acyclovir. This inhibits viral DNA polymerase-led replication. Acyclovir, however, has no effect on latent infection due to the absence of this viral enzyme. During latent infection, replication of the genome takes place by subversion of cellular processes, and utilisation of the cell's own DNA polymerase to replicate the genome (Reviewed in Kutok and Wang 2006).

LMP2 inhibits viral reactivation in an attempt to protect latently infected B lymphocytes from immune detection (Longnecker 2000).

1.11 EBV Lytic Gene Expression

The EBV lytic cycle involves the expression of approximately 80 viral proteins including transcription activators, DNA replication factors and structural proteins such as viral capsid components (Kutok and Wang 2006). During the EBV lytic cycle, genes are expressed that delay cell death long enough to allow viral replication to be completed (de Oliveira, et al. 2010). Lytic gene expression follows a sequential order (Amon and Farrell 2005). The immediate early genes include BZLF1, BRLF1 and BILF4 and the products of these genes *trans*-activate the early lytic genes (Reviewed in Young and Rickinson 2004). The lytic cycle begins directly after infection and the immediate early gene *BZLF1* is expressed just 1.5 hours after infection (Reviewed in Chang, et al. 2009). BZLF1 alone can activate the lytic cycle; it is a master regulator of lytic genes required for replication (Amon and Farrell 2005). BRLF1 is the second immediate early gene and is a key mediator of viral lytic replication and along with BZLF1 is the earliest indicator of lytic infection. BZLF1 and BRLF1 together can activate the lytic cascade in the infected cell. The early lytic viral genes are expressed soon after the immediate early genes and approximately 30 different EBV RNA transcripts have been classified as early lytic products (Hummel and Kieff 1982). Of these early gene products the most abundant are BALF2 and BHRF1. BALF2 is important for DNA replication, while *BHRF1* is a partial homologue of the human *bcl-2* gene and contributes apoptotic resistance to the cell (Young and Rickinson 2004). The late lytic genes encode structural proteins or glycoproteins that permit enveloping of the viral package. The late genes BCLF1, BNRF1 and BXRF1 encode the major nucleocapsid protein, the major external non-glycoprotein of the viron and the basic core protein respectively. EBV also encodes a glycoprotein, BCRF1, which shows 84% sequence homology to human IL-10 (Vieira, et al. 1991). IL-10 is an anti-inflammatory cytokine and thus BCRF1 serves to aid evasion of the infected

cell from the immune response. After replication the linear EBV genomes are cleaved at their terminal repeats (TR) and packaged into their viral envelopes before being released from the sacrificed cell (Reviewed in Kutok and Wang 2006).

1.12 EBV Latent Gene Expression

No infectious virus particles are generated during the EBV latent infection programme. The panel of latently expressed EBV genes is highly restricted and, as mentioned already, is confined *EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2A, LMP2B, EBER1* and *EBER2*. Of these eleven latently expressed proteins, only six are required for B cell immortalisation -EBNA1, EBNA2, EBNA3A, EBNA3C and EBNA-LP along with LMP1. These genes subvert normal B cell mechanisms to establish a long term sustained silent infection. EBV maintains its circular viral genome by usurping the natural cell division processes, utilising cellular DNA polymerase when the cell divides and distributing its episomal copies equally among the daughter cells (Kutok and Wang 2006).

1.13 The EBV Latent Gene Products

1.13.1 Epstein-Barr Virus Nuclear Antigen 1 (EBNA 1)

EBV nuclear antigen 1 (EBNA1) is a sequence-specific DNA binding phosphoprotein that has a number of critical roles in the EBV infected cell including activating replication of the episome, maintenance of the genome and maintaining the latent EBV Infection (Reviewed in Thompson and Kurzrock 2004). EBNA1 is a 73 kDa protein comprising 641 amino acids (aa) and is the only EBV protein detectably expressed in all EBV-associated tumours and EBV-proliferating cells in healthy carriers. EBNA1 has also been found to be a stable protein with a long half life, which has been attributed to its Glycine-Glycine-Alanine repeat sequence shown in Fig. 1.6 (Kirchmaier and Sugden 1997).

The EBNA1 protein consists of a short amino terminal region, a stabilising 20-40 kDa Glycine-Glycine-Alanine (Gly-Gly-Ala) repetitive sequence flanked by Arginine rich regions and a highly charged acidic carboxy terminal sequence (Hennessy and Kieff 1985). The gly-gly-ala repeat sequence has been implicated in down-regulating the host's EBV-induced immune response by preventing antigen presentation by the host cell (Levitskaya, et al. 1995); however, it is now accepted that the main role for this repeat sequence is the stabilisation of the active protein through prevention of proteasomal degradation.

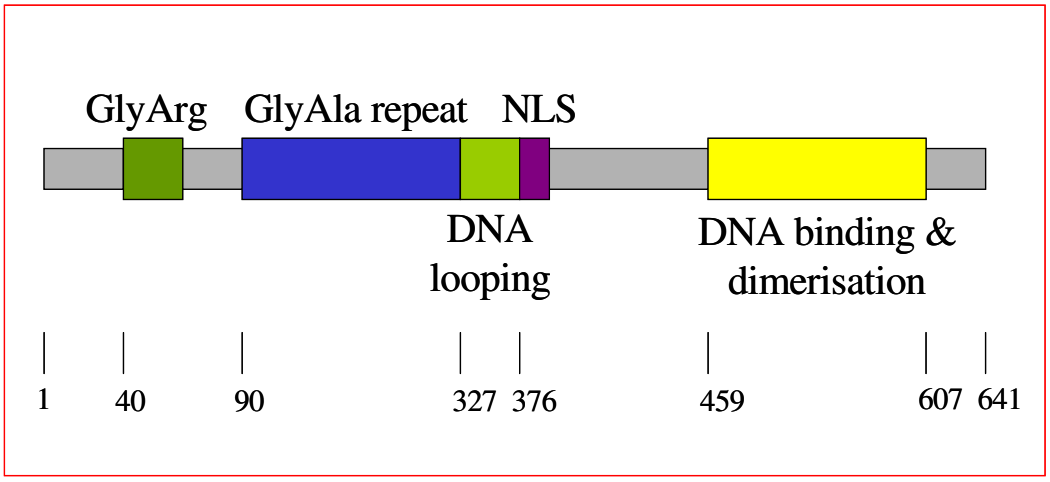


Figure 1.6: Functional domains of the 641 aa EBV nuclear antigen 1 (EBNA1).

The GlyAla box highlighted in blue is a repetitive region of a 20-40 kDa glycine-alanine repetitive sequence; the length of this region varies between viral strains. The Nuclear localization sequence (NLS), DNA binding domain and a protein dimerisation domain have also been highlighted on the above protein schematic (Figure adapted from Avolio-Hunter and Frappier 1998).

1.13.2 EBNA1 Mechanism and Function

EBNA1 plays a crucial role in EBV persistence as the protein responsible for both maintenance of the infection and promoter-dependant activation of the other *EBNAs*. The EBV genome does not usually integrate into the host genome but persists as a circularised episome (Humme, et al. 2003). EBNA1 plays a critical role in maintaining EBV persistence by ensuring minimal loss

of EBV latently infected cells during cellular replication. It achieves this by binding in a sequence specific manner to the OriP of the plasmid, activating replication of the genome and dictating equal partitioning of the EBV genome to the daughter cells once during every cellular S phase (Aiyar, et al. 1998). EBNA1 has been shown to possess anti-apoptotic function by directly antagonising p53 function. This is thought to occur due to sequestering of the de-ubiquitination enzyme, HAUSP/USP7, which leads to p53 destabilisation (Reviewed in Bornkamm 2009a).

EBNA1 also regulates the transcription of its own gene. EBNA1 homo-dimerises and binds to the specific consensus site 5' G(A/G)TATCAT-ATGCTA(C/T) 3'. This motif has been identified in two regions on the EBV genome: in the *EBNA* Q promoter and also at two sites in the OriP, upstream of the *EBNA* Cp. These sites have been designated the *dyad symmetry* (DS) and *the family of repeats* (FR). Activation of the Cp promoter requires the binding of EBNA1 to the FR-responsive element upstream of the Cp. The FR is composed of 20 repeat sequences, and binding by EBNA1 leads to the activation of the Cp and expression of the EBNA proteins (Almqvist 2005; Sugden and Warren 1989). EBNA1 expression is regulated in a latency-dependent manner by the Cp and Qp (Altiok, et al. 1992). During latency III, EBNA1 is expressed through the Cp and positively self-regulates its expression through the FR binding site of the Cp. Conversely, during latency I and II, EBNA1 is expressed through the Qp and this binding results in negative self-regulation (Reviewed in Almqvist, et al. 2005). A consequence of this is lower EBNA1 levels expressed in type I and II latency than those in type III latency cells.

1.13.3 Epstein-Barr Virus Nuclear Antigen 2 (EBNA 2)

EBNA2 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 the cell cycle progression from G_0 to G_1. Together with EBNA-LP, EBNA2 is the first EBV latent gene expressed upon infection of a B cell (Reviewed in Middeldorp, et al. 2003).

EBNA2 can be nucleoplasm-, chromatin- and nuclear-matrix associated and localises to large nuclear granules (Hennessy and Kieff 1985; Petti, et al. 1990). A crucial role for EBNA2 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 found a deletion encompassing the EBNA2 open reading frame (ORF) and 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 EBNA2 to the genome. This implicated EBNA2 as a vital EBV latent protein in the process of EBV-led B cell immortalisation (Reviewed in Thompson and Kurzrock 2004, Young, et al. 2000). Further studies have shown that EBNA2 is also essential for maintaining immortalised B cells (Kempkes, et al. 1995b). As described previously in section 1.4, EBNA2 has two known variants, EBNA2A and EBNA2B, that share only approximately 55% protein homology and are found in the two isoforms of EBV (type A/1 and type B/2 respectively) (Aitken, et al. 1994, Adldinger, et al. 1985).

1.13.4 Structure of EBNA2

EBNA2A is 483 aa long whilst EBNA2B is composed of 455 aa. Both contain a negatively charged highly homologous amino terminal region that facilitates homo-dimerisation (Figure 1.7). This is followed by a proline-rich domain which can contain from 10-40 successive prolines. The homology between the two types of EBNA2 differs most significantly in the “diversity” region which then leads into the domain responsible for binding the 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 carboxy terminus is responsible for nuclear localisation (Palermo, et al. 2008; Zimber-Strobl and Strobl 2001). Three fundamentally required aa regions of the EBNA2 protein 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; Cohen and Kieff 1991; Ling, et al. 1993).

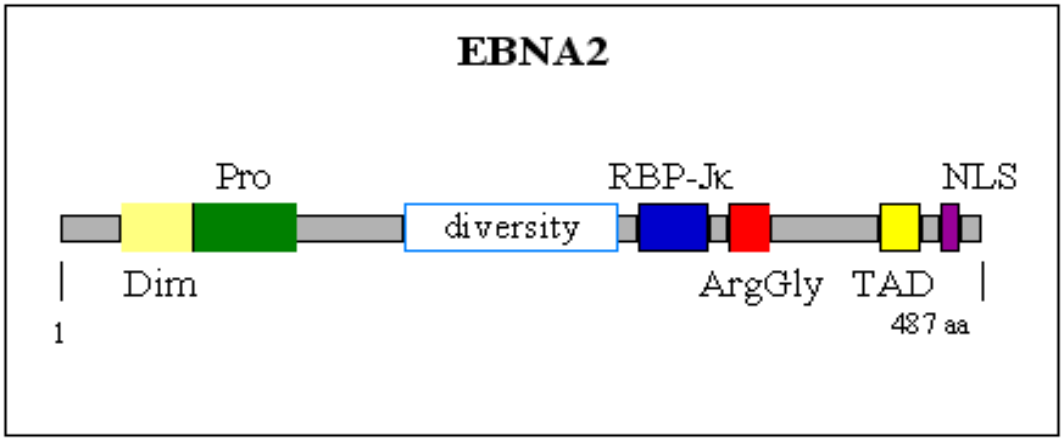


Figure 1.7: Illustration of the Structural domains of Epstein-Barr virus nuclear antigen 2.

EBNA2 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 EBNA2A and EBNA2B, a domain responsible for interaction with RBP-Jk/CBF1 (RBP-Jκ), an arginine-glycine rich stretch (ArgGly) and a negatively charged carboxy terminus region, which harbors a trans-activation domain (TAD) and nuclear localization signal (NLS) (adapted from Zimber-Strobl and Strobl 2001).

1.13.5 EBNA2 transcriptional regulation by EBNA2

EBNA2 does not directly bind DNA; rather, it utilises a number of cellular transcription factors to achieve specific promoter activation including (i) CBF1/RBP-Jκ, (ii) the ETS family protein Spi-1/Pu.1, (iii) ATF/CRE, (iv) the CREB-binding protein CBP and (v) the chromatin remodelling complex SWI/SNF (Henkel, et al. 1994; Ling, et al. 1994; Johannsen, et al. 1995; Pegman, et al. 2006; Zimber-Strobl, et al. 1993; Laux, et al. 1994; Waltzer, et al. 1994; Wu, et al. 1996; Sjoblom, et al. 1998; Wang, et al. 2000; Wu, et al. 2000). EBNA2 has two principal mechanisms by which it activates EBNA2-responsive genes: (i) it associates with its target genes to alleviate the repressive effect elicited by bound CBF1/RBP-Jκ (detailed in Fig. 1.8) and this is followed by (ii) strong transcriptional promotion of the target gene,

depicted in Fig 1.8 (Palermo, et al. 2008). EBNA2 can also interact with members of the basal transcription machinery (BTM) through its acidic *trans*-activation domain, including TFIIH, TFIIE, TFIIB, TAF40 and RPA70 (Palermo, et al. 2008; Tong, et al. 1995a; Tong, et al. 1995b; Tong, et al. 1995c). Further to these interactions, EBNA2 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 EBNA2 for specific promoter regulation, of particular interest is the cellular DNA binding protein, Cp binding factor 1 (CBF-1) which binds to the cognate DNA sequence 5'CGT GGG AA 3' (Zimber-Strobl, et al. 1993). CBF-1 is also known as recombination signal-binding protein 1 for Jκ (RBP-Jκ). CBF1/RBP-Jκ is a ubiquitously expressed transcription factor that has been highly conserved during evolution (Zimber-Strobl and Strobl 2001). CBF-1 directs EBNA2 to its target genes, allowing regulation of the genes by EBNA2. EBNA2 drives the EBV Cp promoter via an upstream EBNA2 responsive element (called the EBNA2-responsive enhancer (E2RE) by alleviating CBF1/ RBP-Jκ mediated repression (Jin and Speck 1992; Sung, et al. 1991). In the absence of EBNA2, CBF1/ RBP-Jκ acts as a co-repressor in the cell bound by a large multi-protein complex of cellular proteins comprising 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. EBNA2 mediates trans-activation of genes by competing with the SMRT-HDAC co-repressor complex, thus relieving the target promoter of this repressor complex (Figure 1.8).

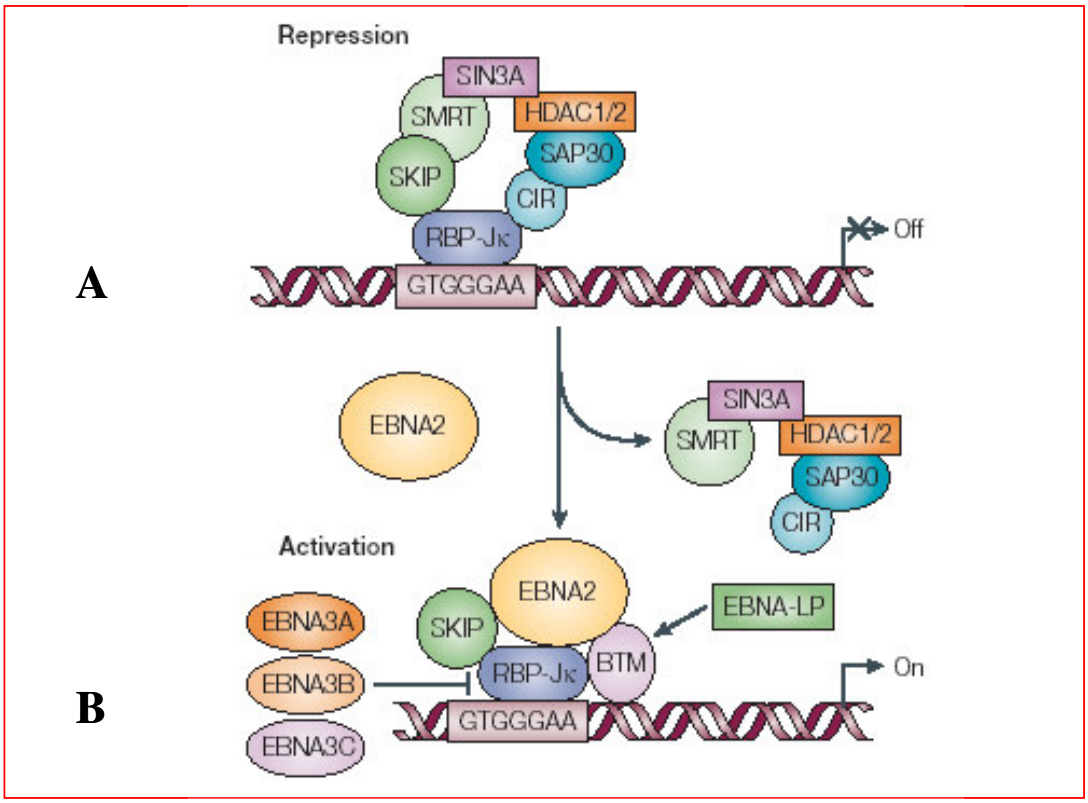


Figure 1.8: Schematic for EBNA2-mediated promoter activation.

(A) EBNA2 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) EBNA2 abolishes CBF1-mediated repression by competing with the SMRT-HDAC co-repressor for binding to both CBF1 and SKIP. The EBNA2 acidic domain recruits the BTM, TFIIB, TFIIH and p300 to drive transcriptional activation. (Figure adapted from Young and Rickinson 2004).

Once repression is relieved, EBNA2 brings strong transcriptional activation to the promoter (Hsieh and Hayward 1995). To instigate this, EBNA2 binds histone acetyltransferases (HATs) and chromatin remodelers such as SWI/SNF to alter chromatin structure, rendering the gene accessible for transcription. EBV utilises cellular RNA polymerase II (RNA pol II) for transcription of target genes. The activity of RNA pol II is tightly controlled and requires phosphorylation of the RNA pol II c-terminal domain (CTD) subunit (Palancade and Bensaude 2003), shown in Figure 1.9. EBNA2 interacts with general transcription factors such as TFIIH and TFIIE along

with the cyclin dependent 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 EBNA2 requires CDK9 recruitment for efficient transcriptional activation (Bark-Jones, et al. 2005). The CTD Ser-5 phosphorylation has also been shown to fluctuate relative to EBNA2 expression levels, demonstrating that Ser-5 phosphorylation may be an EBNA2-dependent 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 EBNA2 for the phosphorylation of Ser-5, thus activating the CTD and RNA pol II activity (Palermo, et al. 2008; Ramanathan, et al. 2001). EBNA2 interaction with TFIIH aids phosphorylation of the CTD, leading to strong sustained transcriptional up-regulation of the target gene (Palermo, et al. 2008).

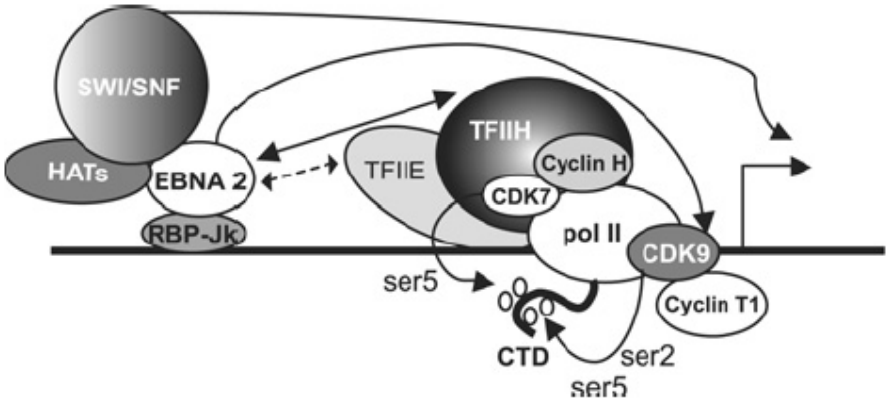


Figure 1.9: Mechanism for EBNA2 led transcriptional activation.

Association with the target promoter leads to binding of EBNA2 and the chromatin remodelers SWI/SNF along with HATs, to make the gene accessible for transcription. EBNA2 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 EBNA2 has a task in this process (Figure adapted from Palermo, et al. 2008).

EBNA2 functions in two ways, (i) to alleviate repression to allow up-regulation of its target genes: it binds to the repressor domain, as described in figure 1.9, alleviating the repression complex; (ii) EBNA2 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). EBNA2 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 latent membrane proteins LMP1, LMP2A and LMP2B and the EBNA Cp promoter (Ling, et al. 1994; Johannsen, et al. 1995; Pegman, et al. 2006; Burgstahler, et al. 1995; Spender, et al. 2002) (Reviewed in Kieff and Rickinson 2007). EBNA2 has also been shown to elicit negative transcriptional regulation. Evidence from LCL studies has exposed EBNA2 as a repressive influence on Nurr77-mediated apoptosis. This inhibition mechanism involves the interaction of the EBNA2 transcriptional complex with Nurr77 protein, sequestering Nurr77 within the nucleus and preventing signaling to the mitochondria that would lead to cytochrome *c* release and stimulation of apoptosis (Lee, et al. 2002, Li, et al. 2000). To date EBNA2 is known to transcriptionally repress two cellular genes, the immunoglobulin heavy chain locus (IgM) (Jochner, et al. 1996) and the pro-apoptotic *bik* gene (Campion, 2008).

1.13.6 EBV latent promoter switching by EBNA2

It is accepted that transcription through the EBV W promoter is an inefficient process that leads only to the amplification of the promoter proximal genes, EBNA-LP and EBNA2 (Palermo, et al. 2008; Bell, et al. 1998), (see Figure 1.5 for Wp coordinates relative to the EBNAs). The Wp is located in the internal repeat region 1 (IR1) and thus there are multiple copies of the Wp present on the genome, see Figure 1.5. This is thought to facilitate the production of abundant EBNA2 transcripts which are required to switch

transcription from the Wp to the Cp (Palermo, et al. 2008). As discussed previously, phosphorylation of the CTD subunit of RNA pol II is required for transcriptional initiation and is also critical for efficient elongation and RNA processing of transcripts (Palancade and Bensaude 2003). The Wp itself does not appear to competently maintain transcription to allow the remaining *EBNAs* to become fully transcribed. The Wp, however, produces sufficient *EBNA2* mRNA such that the EBNA2 protein made stimulates CDK9 dependent phosphorylation and recruits general transcription factors that activate and maintain the RNA pol II function from the Cp. The C promoter contains an E2RE, allowing successful transcriptional initiation, promoter clearance, elongation and RNA processing of the product of interest. This yields an approximately 120 kb primary transcript encoding the full range of EBNAs. Due to the inefficiency of the Wp activity, the switch to EBNA2-led Cp transcription does not occur until approximately 48 hours after infection (Reviewed in Palermo, et al. 2008).

1.13.7 EBNA2 Hijacks the cellular Notch Signaling pathway

In recent years, the discovery of the ability of EBNA2 to exert its function using overlapping effector proteins involved in the Notch signaling cascade has led to the hypothesis that EBNA2 may be a viral functional analogue of the activated Notch signaling protein (Reviewed in Kempkes, et al. 2005). EBNA2 hijacks and manipulates B cell function, by usurping the mechanisms and diverse role of Notch signaling in normal B cell physiology as well as B lymphocyte malignancy.

1.13.7.1 The Notch Pathway

The Notch signaling pathway is a highly conserved, fundamental multitasking cell signaling pathway that plays a critical role in diverse systems such as the nervous system, somitogenesis, cardiovascular and endocrine development. Notch regulates these systems by modulating cell mechanisms such as cell fate specification, differentiation, proliferation, apoptosis and adhesion (Bolos, et al. 2007).

The Notch signaling pathway is one of the key cellular pathways that is regulated by EBV. This regulation is elicited by EBNA2 which may be considered as a functional homologue of the activated Notch1 receptor (N^{IC}). Notch belongs to a conserved family of trans-membrane receptors that regulate cell fate. All of the members of this family are single-pass *trans*-membrane proteins that respond to the ligands of the Delta-Serrate-Lag2 (DSL) family (Struhl and Adachi 1998; Miele 2006). The Notch signaling pathway was originally discovered through genetic studies in the fruit fly *Drosophila melanogaster*, and the name derives from the effect of some Notch alleles to lead to notched wings upon mutation (Hansson, et al. 2004). To date, there have been four mammalian Notch genes identified: Notch 1, Notch 2, Notch 3 and Notch 4. These are *trans*-membrane spanning proteins and possess three major domains, a large external ligand binding region and an internal signal transducing region along with a *trans*-membrane domain (see Figure 1.10 below). It is understood that the extracellular ligands (Delta-like-1, Delta-like-3 and Delta-like-4, also Jagged-1 and Jagged-2) interact with Notch receptors of adjacent cells activating the Notch cell signaling pathway. Notch signaling is regulated post-translationally by events such as glycosylation of the receptor or ligand (Reviewed by Bolos, et al. 2007).

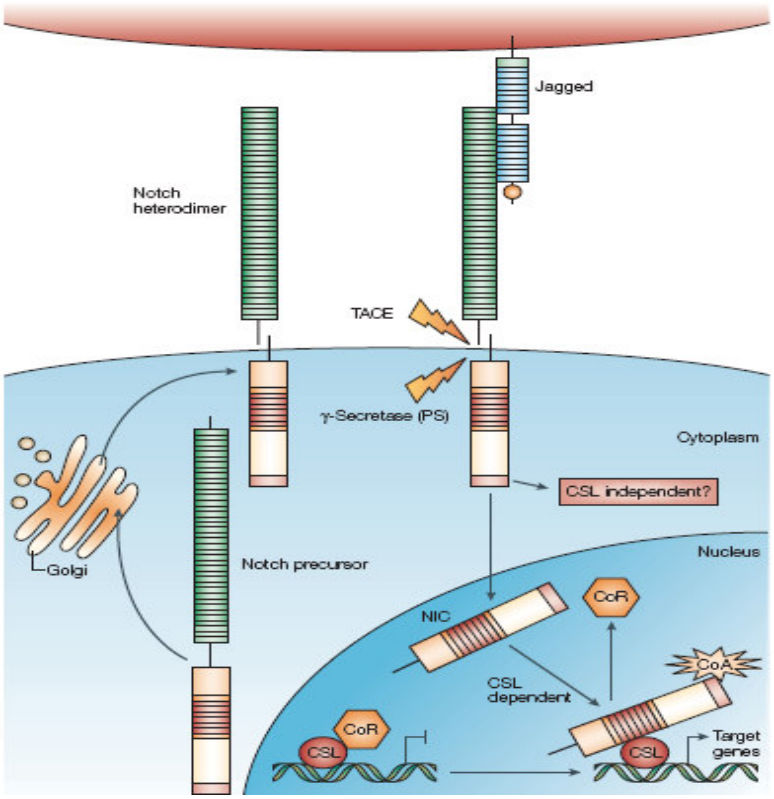


Figure 1.10. Notch Signaling.

Notch proteins are synthesised in the golgi prior to transportation to the cell surface. Interaction of the Notch extracellular domain with Delta-like or Jagged ligands leads to a cascade of cleavage events described in the text. The CBF1 transcription factor is represented by CSL in the illustration. Evidence also points to a CBF-1 independent pathway; however, to date this pathway is poorly characterised (Figure adapted from Radtke and Raj 2003).

Once the Notch ligand binds to its receptor the notch signaling cascade is initiated. This leads to the dissociation of the notch receptor extracellular region (N^{EC}) from the notch receptor trans-membrane unit (N^{TM}) by metalloprotease-led cleavage via TNF-α-converting enzyme (TACE) (Brou, et al. 2000; Hartmann, et al. 2002). Further cleavage is then activated on the intracellular portion on the notch receptor by γ-secretase (Edbauer, et al. 2003). This liberates N^{IC} allowing translocation to the nucleus directed by the notch nuclear localisation signal (NLS) (Pinnix and Herlyn 2007). Within the nucleus the CBF1 transcription factor can be recruited by the activated N^{IC} to form a multi-protein transcriptional activator complex (Reviewed in Miele 2006). CBF1 is usually part of a repressor complex composed of SMRT, SKIP, CIR and histone deacetylases (Kao, et al. 1998; Taniguchi, et al. 1998;

Hsieh, et al. 1999; Zhou, et al. 2000; Oswald, et al. 2002). Cellular CBF1 binds to the recognition sequence 5' CGTGGGAA '3, repressing the associated Notch target gene. The introduction of activated Notch into the nucleus displaces CBF1 co-repressors and chromatin remodelling proteins, relieving this repression and allowing modulation of the Notch target genes via activated CBF1 (Fortini and Artavanis-Tsakonas 1994; Tamura, et al. 1995; Kato, et al. 1997; Tani, et al. 2001).

Notch signaling constitutes a cell-contact driven network of communication between cells. Notch signals regulate cellular events such as development, angiogenesis, differentiation and cell lineage commitment. Notch activates a diverse range of genes, for example the hairy enhancer of split complex related genes *Hes-1*, *Hes-5*, *Hey-1*, *Hey-2* and *Hey-L* (Maier and Gessler 2000; Jarriault, et al. 1995; Nishimura, et al. 1998). These transcription factors act as repressor proteins in the absence of Notch. *ERBB-2* (Chen, et al. 1997), also called *HER2*, encodes an epidermal growth factor (EGF) that, once activated, modulates cellular gene expression to increase cellular proliferation and promote resistance to apoptosis (Rusnak, et al. 2001). NF-κB2 forms a subunit of the NF-κB transcription factor (p52) which plays a role in apoptosis and inflammatory responses (Oswald, et al. 1998). The hairy-related transcription factor (Hey/*HRT*) (Nakagawa, et al. 2000) negatively regulates genes in the absence of Notch signaling and plays a particularly important role in the development of the cardiovascular system (Fischer, et al. 2005). Furthermore, evidence has shown that Notch regulation is context dependent. Notch signaling has dual functions within the body, regulating cell fate in either a positive or negative manner. This implicates Notch, in a framework-dependent manner, as both a tumour suppressor gene and as an oncogene. Notch plays an oncogenic role in some T cell and B cell malignancies, while the tumour suppressor activity of Notch has been shown in Keratinocyte-derived carcinoma and late stage cervical cancer (Bolos, et al. 2007; Radtke and Raj 2003).

1.13.7.2 Functional overlap of EBNA2 and Notch

As stated previously, EBNA2 function is elicited through the recruitment of cellular transcription factors such as CBF-1 which is a key effector of the activated Notch signaling pathway. This CBF-1-led gene regulation usually relies upon activation of the Notch signaling cascade, however, EBNA2 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 EBNA2 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 EBNA2 and N^{IC} proceed to enlist chromatin modifying molecules, some of which overlap. EBNA2 binds p300, PCAF and CBP (Wang, et al. 2000) and p100, a co-activator that stimulates TFIIE (Tong, et al. 1995b), while N^{IC} recruits GCN4, PCAF and p300. Indeed, unsurprisingly, EBNA2 and N^{IC} overlap in functional activities and target genes. Both EBNA2 and N^{IC} can inhibit Nur77-induced apoptosis by binding 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 EBNA2 targets *LMP1*, *CD23* and *c-myc* (Reviewed in Zimber-Strobl and Strobl 2001).

1.13.8 Epstein-Barr Virus Nuclear Antigen -3A, -3B and -3C (EBNA -3A, -3B and -3C)

The EBNA3 family comprises three nuclear proteins, EBNA -3A, -3B and -3C, encoded by three genes that lie adjacent to one another on the viral genome (Wensing and Farrell 2000). This family is expressed exclusively in B lymphocytes and all three interact with the CBF-1 isoform, RBP-2N (Krauer, et al. 1996). The NH_2-terminal regions of the three family members are conserved; however, the remaining two thirds of the proteins show diversity (Rowe 1999). Although their functions may overlap, the EBNA3s

have distinct roles and a contrasting spectrum of potential binding factors (Yenamandra, et al. 2009). This family produces strikingly stable hydrophilic proteins, a feature that may aid nuclear localisation (Kieff and Rickinson 2007). Their CBF-1 interaction implies a role in transcriptional regulation and associations with both the cellular Notch pathway and the activities of EBNA2. Also in parallel with EBNA2, the EBNA3 family shows sequence variation between type 1 and type 2 EBV strains. Type 1 EBNA3A, 3B and 3C are proteins of 944, 938 and 992 aa respectively. In contrast to this, the type 2 variants are 925, 946 and 1069 aa respectively. The EBNA3A variants share only 84% homology, which decreases to 80% homology for EBNA3B, and 72% for EBNA3C (Sample, et al. 1990). Despite the significant diversity between EBV types, there is as yet no evidence of a discernible effect of either variant in the role of B cell transformation *in vitro* (Kieff and Rickinson 2007; Tomkinson and Kieff 1992a). EBNA 3A and 3C are crucial for *in vitro* B cell immortalisation (Murray and Young 2002, Tomkinson and Kieff 1992a). *In vitro* EBNA3B appears to have a redundant function (Tomkinson and Kieff 1992b); however, it most likely does play a key role *in vivo* as EBNA3B is consistently present and induces a strong host recognition by cytotoxic T cells. If EBNA3B had no positive role *in vivo*, the immune pressure would have led to the negative selection and loss of the gene (Reviewed in Kieff and Rickinson 2007).

EBNA3A is vital for B cell immortalisation (Kempkes, et al. 1995) and has a range of cellular binding targets. The effect elicited by EBNA3A is concentration-dependent: at low concentrations it acts as a transcriptional repressor, however at higher expression levels it has a positive effect on transcriptional regulation (Cludts and Farrell 1998). When over expressed in an LCL EBNA3A binds CBF-1 to down-regulate *c-myc*, *CD21* and *CD23*, and leads to G_0/G_1 growth arrest. These studies indicated that EBNA3A is also crucial for LCL proliferation. Indeed, removal of EBNA3A expression from an LCL leads to cell death (Yenamandra, et al. 2009; Cooper, et al. 2003; Maruo, et al. 2003).

EBNA3C is to date the best studied of the EBNA3 family. It has been found to regulate both cellular *CD21* expression and that of the viral *LMP1* gene (Allday and Farrell 1994). *In vitro*, both EBNA3A and 3B have also been shown to activate *LMP1* transcription from its promoter in the presence of EBNA2 (Zhao, et al. 1996; Le Roux, et al. 1994). In fact, it has been shown that EBNA3C can activate or repress transcription in the presence or absence of EBNA2. This is perhaps due to the ability of EBNA3C to associate with transcription factors such as CtBP (binding protein to the C-terminal half of E1A) and NcoR (a nuclear receptor co-repressor) (Reviewed in Yenamandra, et al. 2009). Recent evidence by Kashuba et al. (2008) has also uncovered a new mechanism for EBNA3C that involves inhibiting the association of pRb with E2F1 via EBNA3C interactions with the pRb binding protein MRPS18-2, to facilitate entry of EBV-infected cells into S phase (Kashuba, et al. 2008).

1.13.9 Epstein-Barr Virus Nuclear Antigen – Leader Protein (EBNA-LP)

EBNA-LP, also known as EBNA5, is one of the first EBV proteins expressed in infected B lymphocytes (Kieff 1996). It is composed of amino-terminal repetitive segments, as it is encoded by a repeating sequence, followed by a unique carboxy-terminal sequence. This gives an EBNA-LP protein that varies in size from 20-130 kDa depending on the number of repetitive segments in the splice variant. EBNA-LP is not essential for B cell immortalisation; however, it potentiates the efficiency of the process (Hammerschmidt and Sugden 1989). In addition, loss of EBNA-LP has no diverse effect on immortalised cell lines (Allan, et al. 1992). EBNA-LP's functions within the EBV infected cell include associating with EBNA2 to drive the resting B lymphocytes into the G1 phase of the cell cycle by inducing Cyclin D2 and co-operating with EBNA2 to drive *LMP1* expression. In addition, recent studies have identified an independent role for EBNA-LP in the induction and repression of cellular genes (Reviewed in Peng, et al. 2005). EBNA-LP is mostly localised to the nucleus; mutants that cannot localise to the nucleus are incapable of co-activating EBNA2 targets (Ling, et al. 2009). Within the nucleus, EBNA-LP can associate with promyelocytic

leukemia nuclear bodies (PML NBs). Furthermore, the PML NB-associated protein Sp100A has been implicated as an important mediator for the co-activator function of EBNA-LP. Sp100A plays a role in chromatin remodelling (Reviewed in Ling, et al. 2009), and a recent study has suggested that histone deacetylase 4 (HDAC4) is sequestered in the cytoplasm by EBNA-LP; this is possibly a contributing factor in EBNA-LP co-activation (Portal, et al. 2006).

1.13.10 Latent Membrane Protein 1 (LMP1)

LMP1 is a 63 kDa integral membrane phosphoprotein that is a member of the tumour necrosis factor receptor (TNFR)/CD40 super-family (Figure 1.11). It functions as a constitutively active CD40 receptor (Gires, et al. 1997) and modulates immune responses within infected B cells (Middeldorp and Pegtel 2008). Reminiscent of an activated CD40 response, LMP1 induces adhesion molecule expression leading to the cell clumping typically observed in EBV-infected B cells with latency III (Soni, et al. 2007). LMP1 is also the only latent membrane protein required for B lymphocyte immortalisation. LMP1 expression has also been shown in NPC, EBV-positive HL and NK/T cell lymphoma, but no expression has been detected in BL (Tao, et al. 2006).

The LMP1 protein can be divided into three domains: (i) the first is a hydrophilic amino-terminal cytoplasmic tail (amino acids 1-23), which tethers LMP1 to the plasma membrane and orientates the protein; (ii) secondly, a *trans*-membrane region, consisting of six hydrophobic *trans*-membrane loops creating three membrane spanning domains, which are involved in self-aggregation and oligomerisation (amino acids 24-186); (iii) third, a long cytoplasmic carboxy terminus (amino acids 187-386), which possesses most of the signaling activity of the molecule (Young and Rickinson 2004, Middeldorp and Pegtel 2008). The carboxy terminal region contains two domains that interact with cellular proteins: these are the C-terminal Activation Region 1 (CTAR1) and Region 2 (CTAR2). These elicit LMP1-mediated regulation by activating up to four cell signaling pathways that lead to the activation of nuclear factor κB (NF-κB), the c-Jun NH_2 terminal kinase

(JNK), the p38/mitogen activated protein kinase (p38/MAPK) and the janus kinase/signal transducers and activators of transcription (JAK/STAT) (Murray and Young 2002).

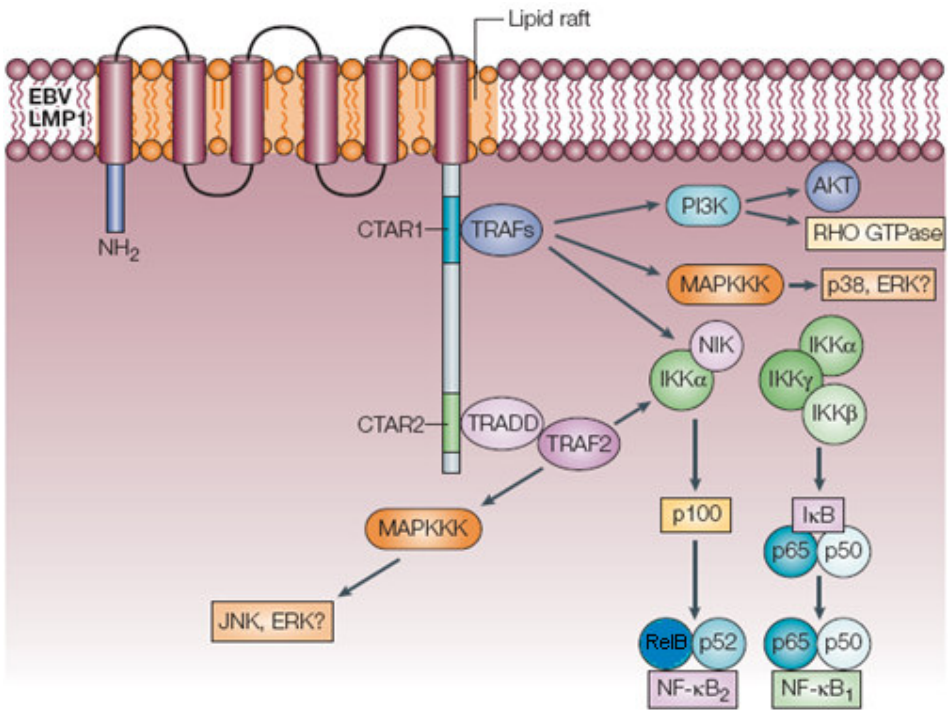


Figure 1.11: Schematic of the Structure of LMP1.

LMP1 elicits signals by association with a family of tumour necrosis factor receptor (TNFR)-associated factors (TRAFs) directly with CTAR1 or indirectly via TNF-receptor associated death domain (TRADD), with CTAR2. These factors then recruit a catalytic complex containing NF-κB-inducing kinase (NIK) and IκB kinases (IKKs) activating NF-κB signaling. The canonical NF-κB pathway involves an IκBα-dependent recruitment of the p50-p65 heterodimer while the alternative NF-κB pathway involves processing by p100 resulting in a p52-RelB heterodimer. The TRAFs can also recruit proteins to activate other signaling pathways such as the MAPK and PI3K pathways (Figure adapted from Young and Rickinson 2004).

LMP1 shares functional homology with cellular CD40, and both proteins can signal to activate NF-κB (Gires, et al. 1997; Lalmanach-Girard, et al. 1993). In turn, this up-regulation of NF-κB drives LMP1 expression in a positive feedback loop that sustains elevated NF-κB expression throughout some types of EBV latency (Johansson, et al. 2009). Normally, CD40 signaling involves a deactivated CD40 receptor on the cell surface that requires

interaction with a specific ligand presented by T cells, the CD40 ligand (CD40L), for activation. Once stimulated, CD40 activates NF-κB which has a positive effect on cell survival. This signaling is a tightly controlled process and dys-regulation can lead to malignancy. Studies have shown that when CD40 is rendered constitutively active, it drives lymphomagenesis (Hömig-Hölzel, et al. 2008). LMP1 acts as a constitutively active CD40 receptor; thus, regulation of LMP1 expression must be limited by some alternative mechanism to the receptor/ligand control seen in CD40 signaling, as otherwise LMP1 would instigate all EBV carriers to succumb to EBV-derived carcinogenesis (Kulwichit, et al. 1998; Shair, et al. 2007). Indeed, although CD40 and LMP1 signaling elicit similar eventual responses, divergent regulatory methods must be employed within the cell (Middeldorp and Pegtel 2008).

1.13.10.1 NF-κB activation

NF-κB activation has been found to be essential in the EBV life cycle and this transcription factor complex has also been shown to be constitutively active in certain EBV-associated malignancies. NF-κB is activated by a wide spectrum of stimuli and in turn, regulates a large number of diverse genes. This allows NF-κB to modulate a range of cellular processes such as inflammation, cell survival, proliferation and differentiation. NF-κB activation involves stimulation of certain cell surface receptors such as CD40, TNFSF and TLR, leading to IκB kinase complex (IKK) activation via signal transduction adaptor proteins such as TRAFs. IKK is composed of the sub-units IKKα, IKKβ and the regulatory sub-unit, IKKγ (also called NEMO). In the canonical NF-κB pathway, signaling to the IKK is elicited through (TGFβ)-activated kinase I (TAK-1); while, in an alternative pathway, NF-κB-inducing kinase (NIK) is employed (Figure 1.12). In the canonical pathway, this activated IKK complex is activated through the IKKβ subunit and leads to the phosphorylation and degradation of IκB from the IκB inhibitory complex, allowing the liberation of the NF-κB subunits, usually p65 and p50. In the non-canonical pathway, the p52 precursor protein, p100 is processed to yield

active p52. The two pathways converge in the dimerisation of NF-κB subunits followed by translocation to the nucleus to regulate NF-κB responsive genes in a sequence specific manner (Reviewed in de Oliveira, et al. 2010) (Hayden and Ghosh 2008).

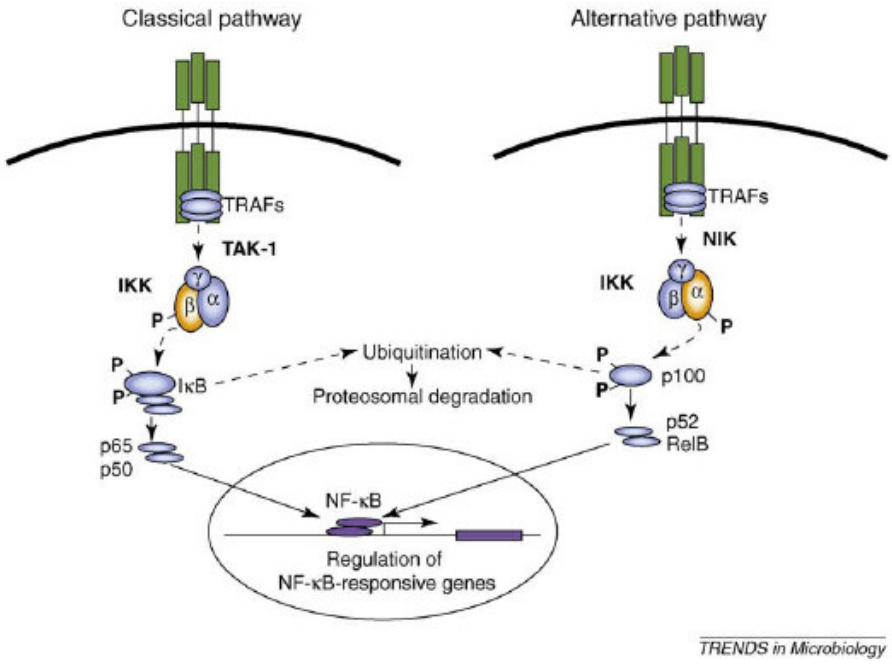


Figure 1.12: Schematic representation of NF-κB transduction.

This illustration describes a simplified version of the canonical and alternative NF-κB activation pathways. Binding of the internal domain, by factors such as the TRAF family members, results in activation and nuclear translocation of the heterodimers p65-p50 and p52-RelB respectively. IKKβ is essential for the canonical pathway, as it is responsible for the phosphorylation and eventual degradation of IκBα, a step which is required to liberate the NF-κB sub-unit, p65-p50. However, in the alternative pathway, the effector unit IKKα is required to phosphorylate p100, which initiates cleavage of the factor to generate p52. This dimerises with RelB and is translocated to the nucleus. This figure was taken from (de Oliveira, et al. 2010).

The NF-κB pathway involves activation of a cascade that leads to post-transcriptional modification and nuclear localisation of NF-κB and Rel family members. These, in turn, modify the expression of a diverse range of genes including those involved in: (i) cell survival (e.g. Cyclin D1 and Cyclin E); (ii) cell survival (e.g. cFLIP and IAPs); (iii) inflammation and angiogenesis (e.g. IL-1, IL-6, VEGF and ICAM-1); and (iv) invasion and metastasis (e.g. MMP2, MMP9 and ICAM-1) (Reviewed in de Oliveira, et al. 2010). The life cycle of

EBV involves characteristic steps such as: (i) cell binding and entry by the virus; (ii) the expression of latent viral genes; (iii) modulation of cellular genes to stimulate growth and suppress immune responses and (iv) transient lytic expression. NF-κB has been shown to play a role in each of these stages (Reviewed in de Oliveira, et al. 2010). During virus binding and internalisation, NF-κB is up-regulated by gp350/220 binding of the cell surface receptor, CD21. This activation of NF-κB facilitates binding to the NF-κB responsive elements in the EBV Wp and thus the expression of viral genes (Sugano, et al. 1997). In addition, NF-κB up-regulation drives the expression of *CD21*. As EBV infected cells can promote NF-κB activation in neighbouring cells via EBV dUTPase stimulation of TLR2, this may also serve to enhance the susceptibility of neighbouring cells to infection (Ariza, et al. 2009). During EBV latency, NF-κB activation is required to prevent a switch to the lytic cycle. NF-κB represses the lytic gene, *BZLF1*, preventing lytic re-activation (Morrison and Kenney 2004). However, unexpectedly, once viral lytic cycle is activated, NF-κB levels are once again elevated. This is believed to function as a survival mechanism to preserve the cell until viral replication is complete (de Oliveira, et al. 2010).

1.13.11 Role of LMP1

LMP1 displays a number of functions that are key to maintaining a sufficient reservoir of EBV-infected cells within the host. (i) EBV positive cells typically maintain the type I or type 0 latency programme *in vivo*. LMP1 has been shown to be transiently activated, reminiscent of type II latency, whilst the EBV infected cell intermittently passes through the GC. This drives proliferation of the B cell. Due to the occurrence of this propagation in the GC, LMP1-mediated proliferation is hidden from the immunesurveillance. Thus, LMP1 facilitates silent expansion of the EBV infected cell pool (Reviewed in Middeldorp and Pegtel 2008). (ii) LMP1, like EBNA1, can increase cellular resistance to p53 induced cell death in genetically damaged cells. This supports survival of the infected cell and could indicate one possible mechanism for EBV associated oncogenesis (Saridakis, et al. 2005;

Holowaty and Frappier 2004). The link between LMP1 expression and malignancy is one of the most robust associations with an EBV latent protein. The detectable expression of LMP1 in EBV-infected cells *in vivo* is relatively rare and the only cells exhibiting any LMP1 expression appear to be tonsilar cells, calculated at a LMP1-positive rate of 1:500,000 (Hudnall, et al. 2005). In contrast, immunocompromised patients who progress to develop lymphoproliferative disease display abundant LMP1 expression in tonsillar B cells. This not only relates LMP1 to oncogenesis but further reveals that the tonsils act as an *in vivo* reservoir for EBV infected B cells (Mowry, et al. 2008). LMP1 has been found to be highly expressed in a number of EBV-associated tumours including Post-Transplant Lymphoproliferative Disease (PTLD) (Liebowitz 1998), Hodgkins disease (Dukers, et al. 2000), and undifferentiated NPC (Khabir, et al. 2005). The expression of LMP1 has been associated with metastasis in NPC (Horikawa, et al. 2007). The oncogenic potential of LMP1 can be further substantiated by studies demonstrating morphological changes, altered adhesion, cell motility and exo-proteolytic activity in epithelial cells exposed to LMP1 expression (Dawson, et al. 2008).

LMP1 levels are regulated by diverse cellular mechanisms including ubiquitination (Aviel, et al. 2000), autophagy (Lee and Sugden 2008) and by micoRNA (miRNA) activity which blocks LMP1 activity (Lo, et al. 2007). In addition, LMP1 is regulated by viral EBNA2-dependent and independent mechanisms. When taken together, these indicate a possible area for targeted therapy to reduce oncogenesis in individuals with increased LMP1 expression (Middeldorp and Pegtel 2008).

1.13.12 Latent Membrane Protein 2A and 2B (LMP2A and LMP2B)

The latently expressed *LMP2* gene encodes two proteins known as LMP2A and LMP2B. Their transcripts consist of 9 exons which differ only in their first exon (Laux, et al. 1989; Sample, et al. 1989). Due to this, the structures of the two proteins have a high degree of similarity. Both proteins contain 12 trans-membrane domains and a 27 aa cytoplasmic C-Terminus. However, the first exon of the LMP2B sequence is non-coding (Kieff 1996). For this

reason investigations have been focused on the LMP2A transcript (Ikeda, et al. 2005). Structurally, LMP2A and LMP2B resemble signal modulators (Chen, et al. 2002). LMP2A is localised to the plasma membrane and cytosol (Longnecker 2000). The evidence indicates 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. A modulatory role for LMP2B in regulating LMP2A function has been suggested (Ikeda, et al. 2005; Scholle, et al. 2000).

LMP2A plays a major role in subverting normal B cell function. One of the best studied modulation capacities of LMP2A is thought to occur through hijacking B cell receptor (BCR) signals (Chen, et al. 2002). BCR signaling is a complex cellular communication system facilitating a variety of outcomes such as cell differentiation, survival, apoptosis and proliferation (Dal Porto, et al. 2004). LMP2A acts as a constitutively active receptor in the BCR pathway, activating inappropriate cell survival signals (Longnecker 2000, Caldwell, et al. 1998) and preventing BCR-mediated terminal differentiation (Chen, et al. 2002). When co-expressed with LMP1, LMP2A can inhibit plasma cell differentiation by impeding downstream BCR signaling factors required to complete B cell activation. These required factors include Lyn and consequently Syk, both adaptor molecules required for plasma B cell establishment. LMP2A-mediates proteosomal degradation of these factors, thus preventing plasma cell differentiation (Matskova, et al. 2007). B cell activation (plasma cell formation) induces the EBV lytic cycle in infected cells (Kieff and Rickinson 2001, Miller, et al. 1995), a vastly immunogenic process that exposes EBV existence (Rickinson and Kieff 2001). LMP2A modulation of the BCR cascade may serve to maintain EBV latency (Chen, et al. 2002) and therefore this protein may have a key role in sustaining EBV survival within the host. In addition to this function, *in vivo* studies have revealed that LMP2A promotes cell proliferation and survival; however, these mechanisms remain ill-defined (Longnecker 2000, Caldwell, et al. 1998). LMP2A function appears to be regulated by ubiquitination which leads to negative modulation of LMP2A signaling. LMP2A contains two repeat motifs (PPXY) that

associate with Nedd 4-family ubiquitin-protein ligases (E3s) to down-regulate LMP2A led activity (Reviewed in Ikeda and Longnecker 2009).

1.13.13 Epstein-Barr virus-encoded RNA's 1 and 2 (EBER1 and EBER2)

EBERs 1 and 2 are non-polyadenylated, uncapped, non-coding RNAs of 167 and 172 nucleotides respectively (Thompson and Kurzrock 2004). The EBERs are expressed in abundance in nearly all EBV-infected cells with the exception of oral hairy leukoplakia lesions from AIDS patients and some hepatocellular carcinomas (Sugawara, et al. 1999). EBERs have been identified as having a possible role in oncogenesis due to studies indicating a function for EBERs in maintenance of the BL cell malignant phenotype (Takada and Nanbo 2001). Upon reintroduction of EBER genes into the EBV negative BL-derived Akata cell line (Akata cells that were once EBV positive but lost their EBV genome) the cells regained the oncogenic potential possessed when Akata was EBV positive. However, recombinant EBV with EBER deletions can still transform B lymphocytes, leaving unclear the role of EBERs in B cell immortalisation. Conflicting interpretations for the role of EBERs with respect to interferon resistance exist. It appears that EBERs can protect the EBV-infected cell from interferon activity, but are not essential for EBV-led interferon resistance in infected cells. Furthermore, it has been reported that EBV-led B cell immortalisation efficiency is enhanced approximately 100-fold by EBER2 activity. Studies have identified a c-MYC binding site in the EBER1 promoter and it has been speculated that c-MYC may drive EBER expression (Reviewed in Bornkamm 2009a).

1.14 Wnt Signaling

Wnt is a key signaling pathway for cell maintenance and oncogenic control in adult differentiated cells. Wnt activity has been linked to cellular functions such as apoptosis, survival, proliferation and cell motility (Reviewed in Willert and Jones 2006). It has been determined that defects in the wnt canonical pathway contribute to the pathogenesis of a variety of malignant states

(Gelebart, et al. 2008). Wnts are secreted glycoproteins that interact with a dimeric cell surface receptor complex containing the proteins Frizzled and low-density-lipoprotein receptor 5 or 6 (Mikels and Nusse 2006).

In the canonical Wnt signaling pathway (Figure 1.13), Wnt receptor activation stimulates the stabilisation of the protein β-catenin within the cell by preventing phosphorylation. This allows nuclear translocation of β-catenin, facilitating binding to the T cell factor (*Tcf*) and lymphoid enhancer-binding proteins (LEFs) (He, et al. 2004). This β-catenin and Tcf/LEF nuclear complex drives Tcf/LEF transcription factors as transcriptional co-activators for Wnt target genes. In addition, the transcriptional repressor, *transducin like enhancer of split 1* (*TLE1;* see chapters 4 and 5*),* appears to play a role in the Wnt pathway. This role involves the binding of the Wnt-associated proteins Tcf/LEF, by TLE1, negatively regulating Wnt targets (Daniels and Weis 2005; Logan and Nusse 2004). This β-catenin–Tcf/Lef mediated regulation modifies the expression of a number of genes pertinent to the study of tumourgenesis and EBV cell control including *c-myc* (He, et al. 1998), *Cyclin D1* (Shtutman, et al. 1999; Tetsu and McCormick 1999) *MMP7* (Crawford, et al. 1999) and WISP proteins (Pennica, et al. 1998). The Wnt effector protein, β-catenin, is a multi-functional protein found in the cell membrane, cytoplasm, and nucleus. Due to this wide distribution of β-catenin, the cell has developed several methods of regulating β-catenin levels, such as forming mutually exclusive repressor complexes with adenomatous polyposis coli **(**APC) (Figure 1.13) and E-Cadherin (Orsulic, et al. 1999). Increasing evidence suggests that EBV modulates the *Wnt* signalling pathway. However, conflicting studies have been published, some proposing that this effect is mediated by LMP1, while others have indicated that LMP1 expression has a minimal effect on the E-cadherin/β-catenin interaction (Reviewed in Webb, et al. 2008). In epithelial cells it has been observed that LMP2A can modulate Wnt signaling by inducting β-catenin (Morrison, et al. 2003).

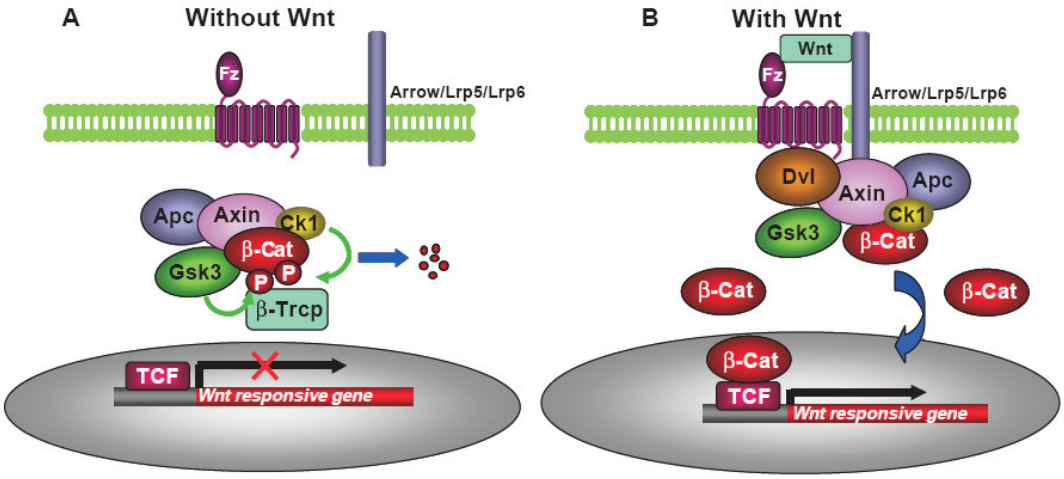


Figure 1.13: The Wnt signalling pathway. (A) In the absence of Wnt, Axin, the scaffolding protein assembles a β-catenin repressor complex containing Apc, Gsk3, Ck1 and β-catenin. Ck1 and Gsk3 sequentially phosphorylate β-catenin. Trcp, a component of an ubiquitin-ligase complex, recognises the phosphorylated β-catenin. Trcp conjugates β-catenin with Ubiquitiņ flagging β-catenin for proteosomal degradation. **(B)** Wnt signals are thought to be received through frizzle (Fz)-Lrp5/Lrp6 co-receptor complex. The Lrp5/Lrp6 complex recruits Axin to the plasma membrane, causing dissociation of the β-catenin repressor complex. This prevents phosphorylation and thus degradation of β-catenin, allowing accumulation of β-catenin in the cytoplasm from which it is translocated to the nucleus. Once in the nucleus it forms a complex with the DNA bound TCF/LEF transcription factor. This results in activation of Wnt-responsive genes (He, et al. 2004).

1.15 Cell cycle progression and EBV

Cells have a defined division cycle that progresses through a number of phases to create two daughter cells. Quiescent cells are non-dividing or resting cells designated G_0. The cell cycle is regulated in four discrete 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. Cell Division occurs during M-phase. 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. In the absence of cyclin dependent kinase inhibitors (CDKIs), D-type cyclins are catalytically activated by either cdk4 or cdk6. Loss of CDKIs correlates with the induction of D-type cyclins. 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/cdk4/6 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 (Figure 1.13) (Brennan 2001).

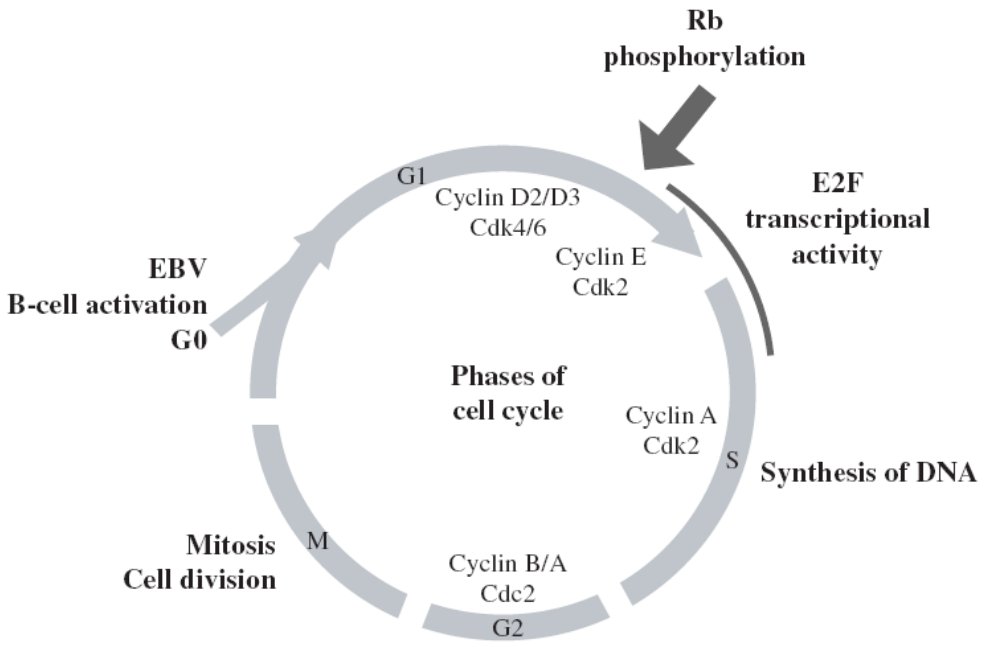


Figure 1.14: Phases of lymphocyte cell cycle.

Quiescent cells are in resting or G_0 phase, the stimulated cells move into the growth phase (G_0/G_1) in which the cell enlarges. Once G_1 checkpoints are met, the cell passes into a DNA synthesis phase (S) before returning to a second growth phase (G_2) prior to mitotic phase (M), in which the cell is divided into two daughter cells in an orderly fashion. Once this cycle is completed the resulting cells return to the resting state (G_0) (Flaitz and Hicks, 1998). (Figure adapted from Brennan 2001).

B lymphocytes are found generally in a quiescent state in the peripheral blood circulation. B cell activation due to a normal immune response results in rapid stimulation of many genes but of particular note are the four key genes *Cdc-2, Cyclin E, CD23* and *Cyclin D2* (Hollyoake, et al. 1995). EBV-led stimulation of quiescent B cells results in the activation of the same genes, thus indicating that EBV exploits the normal B cell programme to activate B cell cycle progression. Following EBV infection, the first cell cycle protein to be activated is Cyclin D2 (Spender, et al. 1999) and this is regulated by EBNA2 and EBNA-LP (Sinclair, et al. 1994). This Cyclin D2 induction is accompanied by a loss of the cyclin-dependent kinase inhibitor (CDKI), p27kip1. Approximately 6 hours later, the pocket protein, pRB is

phosphorylated and thus inactivated, facilitating progression of the cell into G_1 phase. EBNA3C has been found to be capable of negatively regulating the activity of pRB (Knight, et al. 2005). EBNA3C is a vital EBV latent protein required for B cell immortalisation (Kieff and Rickinson 2001), and it has been demonstrated that loss of EBNA3C results in a reduction of S and G_2/M phase cells. This is thought to be due to the EBNA3C-associated loss of pRb activity (Maruo, et al. 2006). This EBV-elicited response mimics exactly the events that occur during normal B cell stimulated proliferation. This implies that EBV exploits the normal cell pathway to subvert normal cellular progression regulatory factors such as pRb.

The EBV proteins EBNA2 and LMP1 have also been implicated in the regulation of another key cell cycle transcription factor, p53 (Chen and Cooper 1996; Li, et al. 2008). LMP1 has been shown to regulate p53 at both a transcriptional and translational level (Li, et al. 2008, Li, et al. 2007). p53 is a tumour suppressor gene that induces cell cycle arrest, apoptosis, DNA repair or senescence in response to genotoxic stimuli (Li, et al. 2008, Cannell, et al. 1998). Mutations in p53 are one of the most frequently detected molecular events in carcinogenesis (Hollstein, et al. 1991). p53 expression has been found to increase after EBV infection. This is surprising as EBV drives the infected cell to divide, causing indefinite cell proliferation (Cannell, et al. 1998, Rickinson and Kieff 1996). Evidence suggests that EBV appears to keep p53 at basal expression levels that are below the cell cycle arrest or apoptotic threshold but can still easily respond to genotoxic stimuli. Alternatively, the p53 transcription factor may have an unknown function that somehow supports EBV infection (Li, et al. 2008; Cannell, et al. 1998).

1.16 Apoptosis and EBV

In mammals, homeostasis is controlled by a fine balance between cell proliferation and cell death. Cell death can be divided according to three morphologies: apoptosis, necrosis and cell death associated with autophagy (Krysko, et al. 2008). During the establishment of latent infection, EBV targets the molecular machinery in the host B cell to promote long term survival of the cell by inhibiting apoptotic pathways. The EBV strategy of apoptotic resistance may be instrumental as a contributing factor in the development of EBV-associated malignant disease.

Apoptosis is a genetically pre-programmed form of cell suicide that functions to eliminate redundant or superfluous cells, thereby maintaining homeostasis (Figure 1.14). Apoptotic cells exhibit dramatic morphological changes including cell shrinkage, nuclear re-organisation, blebbing and eventual fragmentation of the cell into membrane bound apoptotic bodies (Kuroda and Taniwaki 2009). This is a multistage process dependent upon a tightly regulated and well defined programme for its initiation and execution (Fink and Cookson 2005).

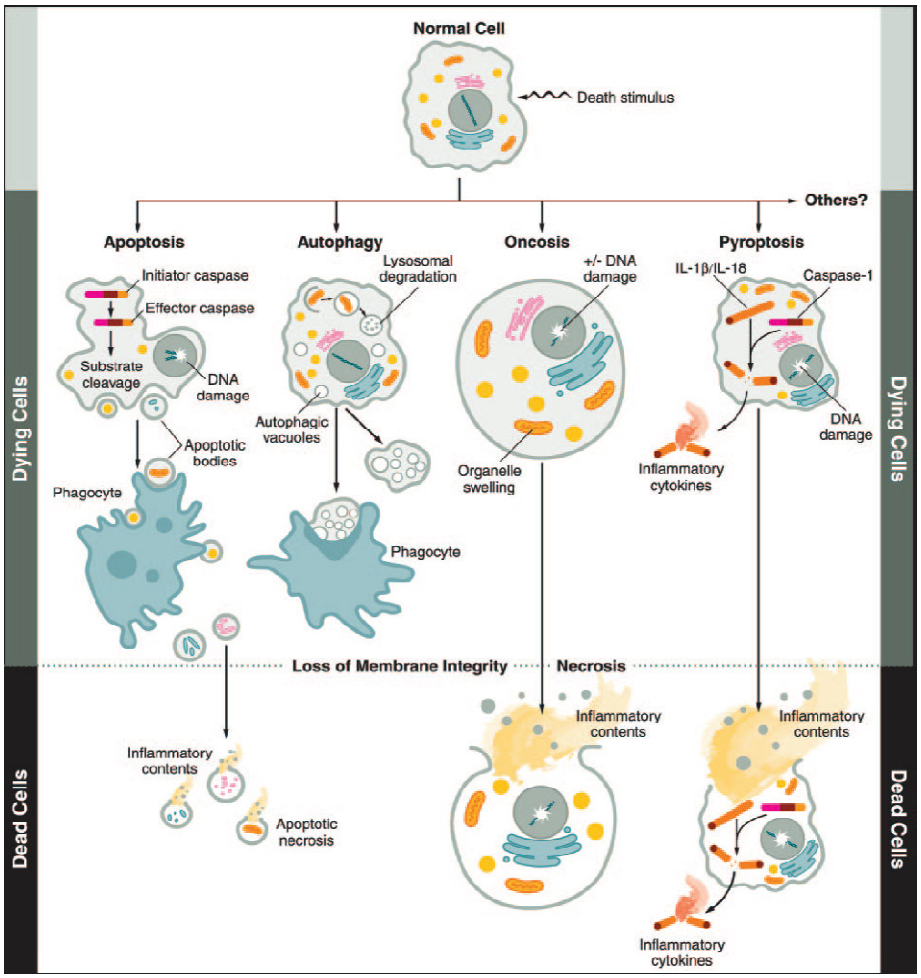


Figure 1.15: The process and outcome of the apoptotic and necrotic cell death pathways.

Apoptotic cells send signals to the immune system to alert phagocytes. If apoptotic bodies are not recovered, and thus neutralised by the immunesurveillance, they will in time result in the slow release of inflammatory materials. In addition, both necrotic cascades conclude in a sizeable release of pro-inflammatory substances. Consequently, the death inducing pathway chosen greatly influences other cells in the environment (Fink and Cookson 2005).

Apoptosis has numerous crucial roles: during embryogenesis, apoptosis eliminates excess cells to shape organs; it maintains tissue homeostasis within the developed organism, and it also functions as a safety mechanism within the body by eradicating cells that show aberrant growth due to the de-regulation of oncogenes as a response to radiation damage or due to viral infection. In the case of virus-infected cells, the induction of early cell death would severely limit virus production and reduce or eliminate spread of virus progeny in the host. In order to maximize their replicative capacity many

viruses, including EBV, have evolved mechanisms that suppress the apoptotic programme (Shen and Shenk 1995; Lin, et al. 2000). A failure in the ability to elicit apoptosis in a redundant or aberrant cell can lead to the cell gaining an extended life span in which it can survive genetic mutations and continue to proliferate inappropriately. The increased resistance to apoptosis is a feature of cancer cells that allows them to function outside the normal control parameters of cellular regulation and contributes to their resistance to chemotherapeutic drugs and treatments. The ability to induce apoptosis is a critical attributing factor in the success or failure of chemotherapy. Indeed, identifying apoptotic targets could reduce the chemotherapeutic load, diminishing the short and long term side effects of treatment. Thus, extending the knowledge of apoptosis and the mechanisms of apoptotic regulators (such Bcl-2 family proteins) could aid in the development of more targeted cancer therapies (Reviewed in Kuroda and Taniwaki 2009).

The defining morphology and biochemical markers of apoptosis distinguish this form of cell death from the others (Krysko, et al. 2008). These morphological features include blebbing of the plasma membrane, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. The defining biochemical markers of apoptosis are externalisation of phosphatidylserine, a change in the permeability of the mitochondrial membrane, release of signature mitochondrial proteins, caspase dependent activation and DNA cleavage due to nuclear localisation of a caspase activated DNase (Reviewed in Krysko, et al. 2008) (Sprick and Walczak 2004). The characteristic events of apoptosis are regulated by a family of related caspases. Caspase induction is initiated by one of two pathways, the extrinsic or ‘death receptor’ pathway and intrinsic or ‘mitochondrial’ pathway (See Figure 1.16). The extrinsic pathway involves the stimulation of the TNF receptor super family, by their corresponding ligands (TNF, Fas or TRAIL), leading to the activation of caspase-8. The intrinsic pathway is known to be controlled by Bcl-2 family proteins (described below); if the pro-apoptotic family members tip the balance, the mitochondrial membrane becomes permeable, releasing pro-apoptotic factors such as cytochrome *c*, AIF or

Diablo/Smac, triggering the activation of caspase dependent or independent cell dismantling (Reviewed in Kuroda and Taniwaki 2009, Sprick and Walczak 2004).

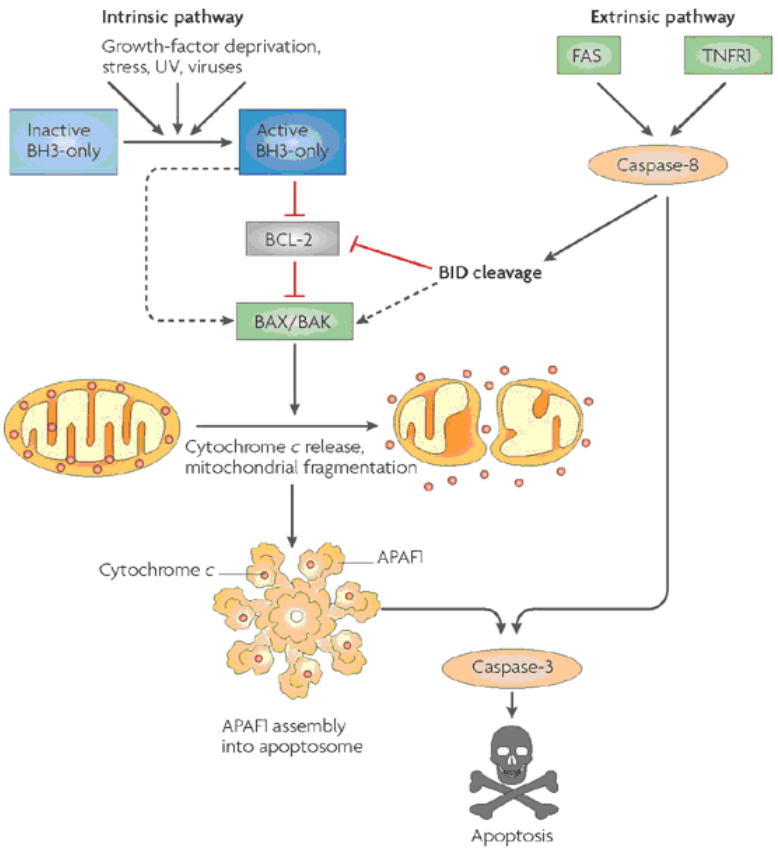


Figure 1.16: The Intrinsic and extrinsic apoptotic pathways.

Extrinsic apoptosis instigation requires activation of factors such as FAS and TNF to stimulate Caspase-8 activity. Intrinsic stimuli include cellular stress such as growth factor deprivation or toxic agents such as viruses. The intrinsic pathway involves either the alleviation of anti-apoptotic Bcl-2 family member led Bax and Bak inhibition, due to BH3-only protein expression or post-translational activation, or via direct activation of Bax and/or Bak (as shown by the dotted line) by pro-apoptotic members such as Bim, Bid or Puma (Kuroda and Taniwaki 2009). Once 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. The caspases in turn cleave a series of substrates, activating DNases resulting in the destruction of the cell. The extrinsic pathway can bypass the mitochondrial step and activate caspase-8 directly, ultimately leading to cell dismantling (Adapted from Youle and Strasser 2008).

The fate of a cell is a fine balance dictated by the interactions between the anti-apoptotic and pro-apoptotic members of the Bcl-2 family. The Bcl-2 family regulates the integrity of the mitochondrial membrane; disruption of

this membrane by some of the Bcl-2 proteins can lead to cellular dismantling (Kuroda and Taniwaki 2009). The Bcl-2 family consists of about 20 homologues of pro- and anti-apoptotic regulators (Kirkin, et al. 2004). These share between one and four conserved Bcl-2 homology (BH) domains (BH1, BH2, BH3 and BH4). The anti-apoptotic members of this family include Bcl2, Bcl-X_L, Boo and Bfl-1. The pro-apoptotic members are further divided into the BH3-only proteins (Bik, Bim, Bad, Bid, Hrk, PUMA, Noxa and Bmf) and the pro-apoptotic members (Bax, Bak, Bok and Bcl-X_s) (Kuroda and Taniwaki 2009, Sprick and Walczak 2004). Pro-apoptotic members can neutralise the anti-apoptotic members to stimulate apoptosis within the cell (Kuroda and Taniwaki 2009). The Bcl-2 family respond to cues from within the cell and its environment e.g. cellular stress, DNA damage, deprivation of necessary factors, to determine whether or not a cell will undergo programmed cell death.

In a healthy cell, the pro-apoptotic Bcl-2 proteins Bax and Bak are bound and inactivated by the anti-apoptotic Bcl-2 family members. Bax is located in the cytosol or loosely associated with membranes, while Bak is localised to the outer membrane of the mitochondria and endoplasmic reticulum (ER). The generally accepted model for the Bcl-2-led apoptotic mechanism indicates that anti-apoptotic Bcl-2 family members bind Bax and Bak to prevent oligomerisation of the two on the mitochondrial outer membrane (Huang and Strasser 2000; Czabotar, et al. 2007; Puthalakath and Strasser 2002). Upon the presentation of death signals to the cell, BH3-only proteins are activated transcriptionally or post-transcriptionally. BH3-only proteins have a stronger affinity to the anti-apoptotic Bcl-2 proteins than either Bax or Bak. The BH3-only proteins displace Bax and Bak, liberating them and allowing them to form oligomers on the mitochondrial membrane, stimulating mitochondrial outer membrane permeabilisation, resulting in the release of the apoptotic factors into the cell (Kuroda and Taniwaki 2009) (Figure 1.17). Importantly, EBV can modulate both the pro and anti-apoptotic Bcl-2 family members during infection to prolong the life of the host cell.

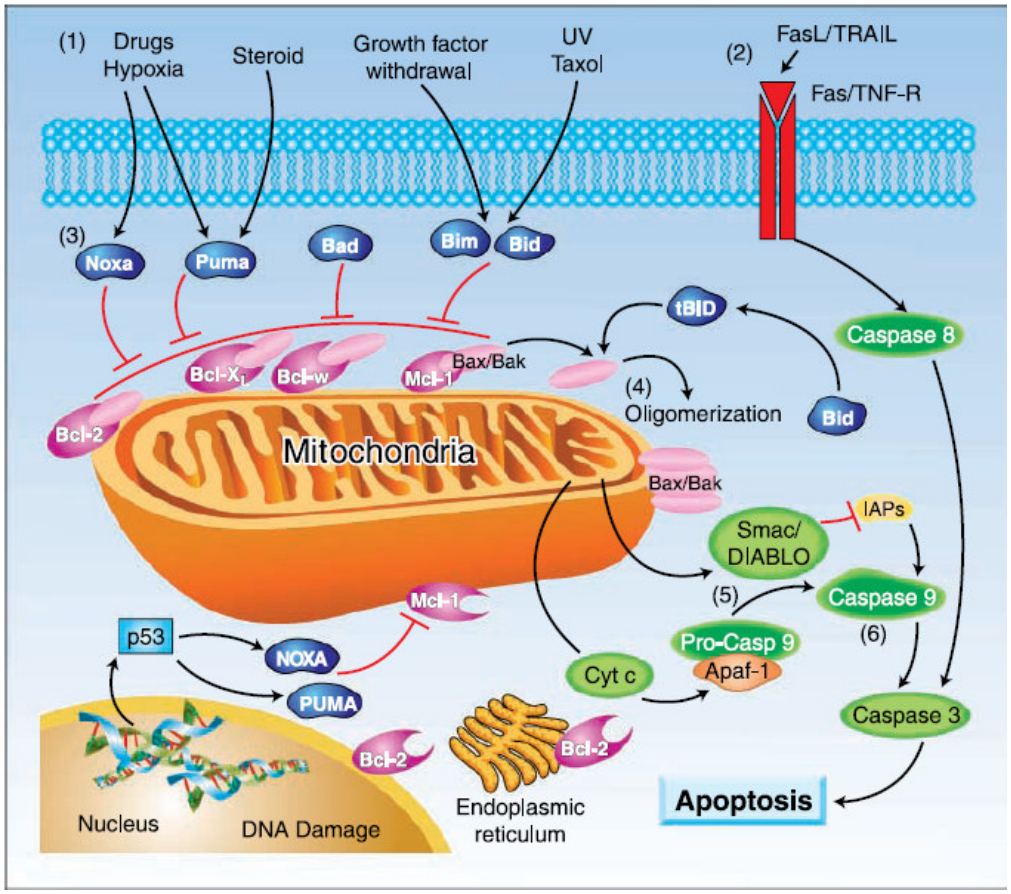


Figure 1.17: Bcl-2 family mediated apoptosis pathway.

(1) The intrinsic apoptotic pathway is mediated by the Bcl-2 family. This pathway is activated by extracellular stress factors such as cytotoxic drugs, hypoxia, growth factor withdrawal and DNA damage. **(2)** The extrinsic pathway is Bcl-2 family independent and involves activation of caspase 8 via death receptors such as Fas and TRAIL. **(3)** In the intrinsic pathway, anti-apoptotic family members such as Bcl-2, Bcl-Xl and Mcl-1 bind the pro-apoptotic factors, Bax and Bak within the cell, sequestering these proteins and preventing apoptosis activation. The pro-apoptotic BH3-only family members, Bim, Bid, Bad, Noxa and Puma directly interact with the anti-apoptotic family members liberating Bax and Bak. **(4)** The activated Bax and Bak proteins can homo-oligomerise in the mitochondrial outer membrane, inducing permeability of the membrane. **(5)** Permeabilisation facilitates the release of cytochrome c and/or Smac/DIABLO from the mitochondria. Once in the cytoplasm, cytochrome c associates with apoptosis protease-activating factor 1 (APAF-1) and pro-caspase 9, allowing the activation of caspase 9 which in turn stimulates caspase 3, which initiates a caspase cascade resulting in cellular dismantling. Smac/DIABLO antagonises inhibitors of apoptosis (IAPs) aiding apoptotic progression (Figure taken from Kang and Reynolds 2009).

1.17 EBV regulation of apoptosis

The ability of EBV to establish and maintain life-long *in vivo* latency is due in part to its ability to regulate apoptosis within the cell. The EBV LMP1 has been implicated in a multitude of apoptotic regulatory functions, such as the up-regulation of *A20*, which encodes an anti-apoptotic protein that inhibits p53-mediated apoptosis (Fries, et al. 1996). LMP1 has a particularly strong role in Bcl-2 family modulation. LMP1 induces the expression of anti-apoptotic Bcl-2 family members which sequester Bax and Bak, thus preventing heterodimerisation of the two at the mitochondrial membrane and inhibiting the activation of apoptosis (Mei, et al. 2007). The interactions between pro and anti-apoptotic Bcl-2 family members are selective. Noxa, the pro-apoptotic BH-3 only protein binds to Mcl-1 but not to the other anti-apoptotic multi-domain proteins such as Bcl-2 and Bcl-x_L (Mei, et al. 2007). LMP1 interferes with this balance by up-regulating Mcl-1, thus hindering Noxa-induced apoptosis (Reviewed in Le Clorennec, et al. 2008). The pro-apoptotic protein, Bad, interacts only with Bcl-2 and Bcl-x_L (Mei, et al. 2007). The EBV latent protein LMP2A has been associated with increased Bcl-xL levels, and the inhibition of this up-regulation in LMP2A transgenic mice resulted in increased apoptosis (Portis and Longnecker 2004). In addition, LMP1 has been shown to induce *Bcl-2* (Reviewed in Le Clorennec, et al. 2008). Both Puma and Bim can interact with all known anti-apoptotic Bcl-2 family members, and the co-operation of two EBV latent proteins, EBNA3A and EBNA3C, results in the transcriptional down-regulation of *Bim* (Anderton, et al. 2007). EBV also modulates the pro-apoptotic *Bcl-2* gene, *bfl-1*. Bfl-1 (also known as A1) can inhibit the pro-apoptotic Bid protein (both full length and truncated) preventing activation of Bax and Bak. Bfl-1 also plays a negative role in p53-mediated apoptosis. It has been demonstrated that *bfl-1* is a direct transcriptional target of both LMP1 (D'Souza, et al. 2004) and EBNA2 (Pegman, et al. 2006). EBNA2 has also been shown to bind to and repress the activity of the pro-apoptotic factor Nurr77, an orphan receptor that responds to apoptotic stimuli by trans-locating from the nucleus to the mitochondria and inducing cytochrome c release (Lee, et al. 2002).

1.18 Aims of this study

Previous work in our laboratory has shown that EBV regulates cellular genes, including *bfl-1. Bfl-1* plays a crucial role in regulating the survival of EBV-infected B cells (D'Souza, et al. 2000). Furthermore, it has been demonstrated that this regulation is elicited by two different EBV latent proteins utilising two different pathways: LMP1 regulates *bfl-1* via NF-κB (D'Souza, et al. 2004) while EBNA2 modulates *bfl-1* by recruiting a Notch transcription factor, CBF1 (Pegman, et al. 2006). In this study (Chapter 3), aspects of the mechanism of *bfl-1* regulation in EBV-infected cells are elucidated.

The primary focus of this study was to identify novel cellular genes that are targetted, following B cell infection, by EBV. Untouched Naive B cells were first infected with a recombinant Green Fluorescent Protein (GFP)-expressing strain of EBV. The expression of selected cellular genes was then compared before and after infection by real-time semi-quantitative PCR (RT-qPCR) using an RT Profiler PCR SuperArray. This experiment led to the identification of several novel cellular genes that are candidate targets of EBV. Several genes showing the most promise in that regard were further investigated using a cell culture model of EBV infection, known as ER/EB2.5, in which EBV latent gene expression could be activated/inactivated using estrogen. One interesting cellular gene, namely *TLE1*, was determined to be a negative target of EBV, and the role and regulation of *TLE1* was investigated in more detail (Chapters 4 and 5). The study also led to the identification of a small number of other genes showing potential as EBV targets following infection (Chapter 6 and 7).

EBV subverts a wide variety of responses within the cell to alter the phenotype, survival and proliferation status of infected cells, but the mechanisms by which these modifications are achieved have not all been defined. Identifying cellular genes that are deregulated by EBV may lead to the identification of novel host-virus interactions that present promise as therapeutic targets for EBV-associated diseases. The main objectives of this

study were, therefore, to identify and determine the role of cellular genes that are regulated by EBV.

Chapter 2: *Materials and Methods*

2.1 Materials

2.1.1 Biological Materials

2.1.1.1 Cell lines

Table 2.1 Cell lines used in this study

Cell Line	EBV Status	Classification	Description
AG876	+	Type III BL	Type III BL cell line expressing all of the EBV latent genes.
Akata 4E3	-	EBV Negative BL	During cultivation of the Epstein-Barr virus (EBV)-positive Burkitt's lymphoma (BL) line Akata, EBV DNA was lost from some of the cells and EBV-negative clones were isolated (Shimizu, et al. 1994).
Akata BX1	+	EBV Positive BL	EBV negative BL (Akata 4E3) infected with a recombinant GFP tagged strain of EBV. In this EBV strain, the non-essential thymidine kinase encoded by the BXLF1 ORF was disrupted by a cassette expressing neomycin resistance and a modified green fluorescence protein (GFP) (Molesworth, et al. 2000).
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).
BL41P3HR1-ER/EBNA2 (9A)	+	EBV Positive BL (Stable transfectant)	EBV negative BL infected with the P3HR1 strain of EBV and subsequently transfected with an EBNA2 fusion protein where EBNA2 activation is dependent on the presence of estrogen as above (Kempkes, et al. 1996).
BL41-ER/EBNA2 (K3)	+	BL (stable Transfectant)	EBV negative BL cell line stably transfected with an EBNA2 expression plasmid in which the EBNA2 activation domain is fused to the estrogen binding domain, rendering the activation of EBNA2 dependent on the presence of estrogen (Kempkes, et al. 1996).
DG75	-	EBV Negative BL	Lymphoid B cell line derived from an Israeli Burkitt's-like lymphoma case (Ben-bassats, et al. 1977).

DG75 tTA EBNA2	-	Stable transfectant	Tetracycline regulated system whereby the expression of EBNA2 can be induced by the removal of tetracycline from the growth media (Floettmann, et al. 1996).
EREB2.5	+	LCL	An LCL established by co-infecting B-cells with a 28kb mini-EBV plasmid that only expresses estrogen-responsive EBNA2, together with the EBV P3HR1 strain in which its own EBNA2 gene is deleted (Kempkes, et al. 1995).
IB4	+	LCL	An LCL established by infection of fetal cord blood lymphocytes with the EBV strain B95.8 (King, et al. 1980).
P493-6	+	LCL	An EREB 2-5-derived cell line, P493-6, in which c-Myc is expressed under the control of a tetracycline-regulated promoter (Pajic *et al*, 2000).
Ramos	-	Type I BL	EBV negative B Lymphocyte cell line derived from an American Burkitt's Lymphoma patient (DSMZ).

All BL cell lines and LCLs were obtained from Professor Martin Rowe, University of Wales, Cardiff, Wales. The estrogen-responsive cell line EREB 2.5 was a gift from Dr. Ursula Zimber-Strobl, GSF-National Research Centre for Environment and Health, Munich, Germany. The EREB 2.5-derived cell line, P493-6 was a gift from Prof. Dr. med. Georg W. Bornkamm, Institute of Clinical Molecular Biology and Tumor Genetics Munich, Germany. The estrogen responsive cell lines BL41K3 and BL419A were gifts from Dr. Bettina Kempkes GSF-National Research Centre for Environment and Health, Munich, Germany. Akata-4E3 was received from Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.

2.1.1.2 Antibodies used in this study

Table 2.2 Antibodies used in this study

Antibody	Name	Description	Supplier
Mouse Anti-EBNA2	PE2	A monoclonal antibody that reacts with the latent gene product EBNA2.	Professor Martin Rowe, University of Cardiff, Wales, and Dako.
Goat anti-mouse IgG, Alkaline Phosphatase (AP) Conjugate	Anti-mouse IgG, AP conjugate	A goat anti-mouse antibody that reacts with mouse IgG, and is conjugated to AP.	Promega
Rabbit Anti-TLE1	TLE1 Antibody	Rabbit polyclonal reactive to synthetic peptide conjugated to KLH derived from within residues 200 - 300 of Human Tle1.	Abcam
Goat Anti-rabbit IgG, Horse Radish Peroxidase (HRP) Conjugate	Anti-Rabbit IgG, HRP Conjugate	A goat anti-rabbit antibody that reacts with rabbit IgG, and is conjugated to HRP.	Santa Cruz
Mouse Anti- β - Actin Clone AC-15	Anti- β – Actin A1978	Monoclonal IgG Raised against β-cytoplasmic actin N-terminal peptide.	Sigma

2.1.1.3 Bacterial Strains used in this study

Table 2.3 Bacterial Strains used in this 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 + , lacI ^{q} ZΔM15]
***E. coli* XL1-Blue**	*end*A1, *rec*A1, *gyr*A96, *thi-1*, *hsd*R17, *rel*A1, *sup*E44, lac [F´, *pro*AB, lacI ^{q} ZΔM15, Tn10 (Tet^r)]

2.1.1.4 Bioinformatics Software

Table 2.4 Web based bioinformatics tools used in this study

Tool	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.
MatInspector	http://www.genomatix.de/products/MatInspector/MatInspector1.html	Nucleic Acid Sequence Searches, to identify putative transcription factor binding sites.
Multialin	http://multalin.toulouse.inra.fr/multalin/multalin.html	Comparison and alignment of nucleotide sequences.
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php	Identification of restriction enzymes 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-dimers/hairpins formation.
RTB Primer	http://medgen.ugent.be/rtprimerdb	Real time primer database
Translate tool	http://www.expasy.org/tools/dna.html	Translation of a nucleotide (DNA/RNA) sequence to a protein sequence.

2.1.1.5 Plasmids

Table 2.5 Expression and reporter constructs used in this study

Plasmid	Name	Source
pSG5 pSG5-EBNA2	Lindsey Spender, Ludwig Institute for Cancer Research, Imperial College School of Medicine, London.	pSG5-EBNA2 expresses the wild type B95.8 EBNA2 gene that has been cloned into pSG5 (Stratagene).
SV-EB2NA2	Baltimore.	SV-EB2NA2 expresses the B variant EBNA2 gene that has been cloned into pSG5 (Stratagene).
pSG5 hHey1	Dr. Borja Belandia, Institute of Reproductive and Developmental Biology1 and Department of Histopathology, Imperial College London, London, United Kingdom.	The complete open reading frames of full-length human Hey1 were amplified by PCR and sub-cloned into pSG5.
pRTS-1	Professor Dr. Georg W. Bornkamm, GSF-Research Centre for Environment and Health, Institute of Clinical Molecular Biology and Tumour Genetics, Munich, Germany.	Novel tetracycline-inducible vector carrying all the elements for conditional gene expression including the gene of interest on one EBV-derived episomally replicating plasmid (Bornkamm *et al*., 2005).
pRTS-1 Hey1	This Study.	Generated by insertion of Hey1 ORF from pcDNA3-FLAG-*Hey1* into pRTS-1, generating a tetracycline regulatable vector capable of conditional expression of Hey1 protein.
3x enh κB luc	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U.K.	This reporter construct contains 3 κB elements upstream of a minimal conalbumin promoter linked to the firefly luciferase gene (Floettmann and Rowe, 1997).
pGA981-6	Dr. Bettina Kempkes, Institute of Clinical Molecular Biology and Tumour Genetics, GSF, Munich, Germany.	The pGa981-6 reporter construct (Minoguchi *et al*., 1997) was generated using a 50 bp oligonucleotide harbouring both CBF1 binding sites of the EBV *TP1* promoter, which was then ligated as a hexamer into plasmid pGa50-7 (Laux *et al*., 1994).
pCMV-LacZ	Clontech.	pCMV-LacZ contains *E.coli* β-galactosidase gene under the control of the CMV promoter-enhancer.
-1374/+81-Luc	Dr. Brendan D'Souza, School of Biotechnology, Dublin City University.	-1374/+81-Luc was generated by sub-cloning the *bfl-1* promoter sequence from a corresponding CAT reporter construct (Zong *et al*., 1999) into pGL2-

-1374/+81 mκB (-52)		Basic. -1374/+81 mκB (-52) contains a mutation at the NF-κB-like binding site at position -52 to -43 (D'Souza *et al*., 2004). -1374/+81 mκB (-833) contains a mutation at the NF-κB-like binding site at position –833. (D'Souza *et al*., 2004).
-1374/+81 mκB (-52) mCBF1 (-249)	This Study.	-1374/+81 mkB (-52) mCBF1 (-249) was generated by mutation to incorporate an Xba I restriction site at the CBF1 like binding site at position -251 to -259 of the -1374/+81 mkB (-52) single mutant vector.
pGL3-Basic	Promega.	The pGL3-Basic is a reporter construct containing the backbone of the pGL2-Basic with increased expression, and contains a modified coding region for firefly luciferase that has been optimised for monitoring transcriptional activity in transfected eukaryotic cells. pGL3-Basic lacks eukaryotic promoter and enhancer sequences.
pGL3 GATA4	Professor Manfred Gessler, Theodor Boveri Institute, Physiological Chemistry I, University of Wuerzburg, Wuerzburg, Germany.	A 5kb fragment of the 5' upstream GATA4 region was ligated into the *Sma*I site of the pGL3-basic vector (Promega).
pGL3 Hey1	Professor Manfred Gessler, Theodor Boveri Institute, Physiological Chemistry I, University of Wuerzburg, Wuerzburg, Germany.	Full length Human *Hey1* promoter fragment (3.9 kb) isolated from human BAC clone RP11-27N21, subcloned into pGL3 luciferase construct.
pcDNA3	Invitrogen.	The pcDNA3 Vector is a eukaryotic expression vector, harbouring the CMV promoter and the ampicillin and G418 resistance gene.
pcDNA3-TLE*1*	Professor Stefano Stifani, Center for Neuronal Survival, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada.	The complete open reading frame of full-length human TLE1, amplified by PCR and sub-cloned into pcDNA3.
sh205 sh206 sh207 sh208	Origene.	Sequence-verified TLE1 expression plasmids with gene-specific shRNA cassettes.
sh20003	Origene.	A sequence-verified plasmid without shRNA cassette insert.
sh30003	Origene.	A sequence-verified plasmid containing non-effective 29-mer scrambled shRNA cassette.

2.1.1.6 Oligonucleotides

Table 2.6 Oligonucleotides used in this study

Target	Primer Sequence	Annealing Temperature
AES	5' CCA CCT CTA GCT TTC TTC CTA 3' 5' G ACT GTC GGT TAC ACA TGT TC 3'	53ºC
Bfl-1 Promoter (Site Directed Mutagenesis)	5' GGG TTG TAC CAT TTC ACT CTA GAA CCA GCA ATC T 3' 5' AGA TTG CTG GTT CTA GAG TGA AAT GGT ACA ACC C 3'	62ºC
Bfl-1 (Real Time)	5' TTC ATA TTT TGT TGC GGA GTT 3' 5' AGC ATT TCA CAG ATC TTT CCT 3'	53ºC
CD23	5' GGA ATT GAA CGA GAG GAA CGA AG 3' 5' AAA GCC GCT GGA CAC CTG 3'	53ºC
CIDEB	5' AGG ATG GAA CTG CAG TGG AC 3' 5' AGG TCT CGA GGG TTT TGC TT 3'	53ºC
Cyclin D1	5' TAT TGC GCT GCT ACC GTT GA 3' 5' CCA ATA GCA GCA AAC AAT GTG AAA 3'	53ºC
Cyclin E	5' CTT CAC AGG GAG ACC TTT TAC 3' 5' CAT TCA GCC AGG ACA CAA TAG 3'	53ºC
E2A	5' TAGACACGCAGCCCAAGAA 3' 5' GGTACACCGAGGATGGAAGA 3'	55ºC
EBNA1	5' GAGCGGGGAGATAATGTACA 3' 5' TAAAAGATGGCCGGACAAGG 3'	55ºC
EBNA2	5' AGA GGA GGT GGT AAG CGG TTC 3' 5' TGA CGG GTT TCC AAG ACT ATC C 3'	60ºC
FAS	5' GGG GTG GCT TTG TCT TCT TCT TTT 3' 5' ACC TTG GTT TTC CTT TCT GTG CTT TCT 3'	58ºC
Fos	5' AAA AGG AGA ATC CGA AGG GAA A 3' 5' GTC TGT CTC CGC TTG GAG TGT AT 3'	60ºC
Hes-1	5' ACG TGC GAG GGC GTT AAT AC 3'	58ºC

	5' ATT GAT CTG GGT CAT GCA GTT G 3'	
hHey1 (pRTS-1 Cloning)	5' TCT ATG GGC CTC ACT GGC C AT GAA GCG AGC TCA CCC C 3' 5' AAT TCG CGG GCC TCA CTG GC C TTA AAA AGC TCC GAT CTC 3'	
Hey1	5' AGC CGA GAT CCT GCA GAT GA 3' 5' GCC GTA TGC AGC ATT TTC AG 3'	60ºC
Jun	5' ATG ACT GCA AAG ATG GAA ACG 3' 5' GCT CTC GGA CGG GAG GAA 3'	58ºC
LRP5	5' ACC TGG ACT TCG TGA TGG AC 3' 5' TGA ACA GCA AGA AGG TGG TG 3'	55ºC
MMP7	5' GGA TGG TAG CAG TCT AGG GAT TAA CT 3' 5' GGA ATG TCC CAT ACC CAA AGA A 3'	60ºC
NR4A2 (Nurr1)	5' CAA GGA ACC CAA GAG AGT GG 3' 5' TGT GTG CAA AGG GTA CGA AG 3'	53ºC
Pax5	5' TGGAGGATCCAAACCAAAGG 3' 5' GGCAAACATGGTGGGATTTT 3'	55ºC
Runx1	5' GATGGCACTCTGGTCACTGTGA 3' 5' CTTCATGGCTGCGGTAGCAT 3'	55ºC
Runx3	5' AGC GTG GGA GGC ACC TTT 3' 5' CAG GAT CAG GAA GGG TTA GTA ATC T 3'	53ºC
STIL	5' CCT TGC TTC ATC AAC CAT CA 3' 5' GTT TTG GGT TCT GGT GCA TT 3'	60ºC
TBP	5' CAC GAA CCA CGG CAC TGA TT 3' 5' TTT TCT TGC TGC CAG TCT GGA 3'	50-60ºC
TLE1 Stage 1	5' ACG GAG CCA GCT GTA TTG AC 3' 5' ACC TCC ACA TTG CTG CTC TC 3'	53ºC
TLE1 Stage 2	5' TC TTT CTC ATG GCC ACG GA 3' 5' T GCG TTT ATC TGT GCC TCT T 3'	53ºC
TLE2	5' C GAA GAC AAG AGT GAT TAC 3' 5' CAA GGA GGC TGG ACT GTC 3'	53ºC
TLE3	5' GAT ACG ACA GTG ATG GAG ACA 3' 5' ATT TTC AGG AGG GGA GTG T 3'	53ºC
TLE4	5' CCG TGG ACG ACA AAT ACA 3' 5' AAG GAT GAC AGA GCA AAG TG 3'	53ºC

TNFSF7	5' TAC GTA TCC ATC GTG ATG 3' 5' GTT GGT GCA GAG TGT GTC 3'	53ºC
TRAF4	5' ATC TCC TTG AGT GGC CCT TT 3' 5' CTG GTG GGA GAT GAA CTT GG 3'	53ºC

2.1.2 Chemical Materials

Table 2.7 Suppliers of chemical and biological materials used in this study

Supplier	Product/Code
Abcam	TLE1 antibody (ab15587).
Amersham	Rainbow Molecular Weight Markers (RPN800).
Amaxa GmBH	Cell Line Nucleofector Kit T (VCA-1002), Kit V (VCA-1003).
Applied Biosystems	AmpliTaq Gold DNA Polymerase (N8080240).
ASP	Presept Disinfectant Tablets (SPR25).
BD Biosciences	Annexin V-PE kit code 559763, FACS flow (342003).
BDH	Bromophenol blue (44385), EDTA (280254D), Glycine (444495D), Hydrochloric acid (28507BF), Isopropanol (296946H), Magnesium sulphate (29117), Methanol (29192BL), Nitrocellulose membrane (436107E), Potassium acetate (295814P), Sodium dihydrogen orthophosphate (30716), Sucrose (102745C), Tris(hydroxymethyl)methylamine (271195Y).
Biosciences	HiYield Gel/PCR DNA Fragment Extraction Kit (YDF100).
Digital Bio	Solution T/N Accustain, AccuChip2x Kit (AD2k-200).
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.
Finnzymes	Phusion (F-530S).
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), Trypsin (25090-028), Sodium Pyruvate (11360-039), SYBR Gold Nucleic Acid Gel Stain (S11494), SYBR Safe DNA Gel Stain (S33102).
Kodak	X-ray film (FS388).
Labkem	Nitrocellulose Membrane (436107E).
Labscan	Chloroform (A3505E).
Lennox Chemicals	IMS (B0982).
Merck	Calcium chloride (23821000), Glacial acetic acid (100632511), Magnesium chloride (1058321000), Potassium Hydroxide (50321000), Sodium carbonate (A654792), di-Sodium hydrogen phosphate (1065860500).
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).
New England Biolabs	Alkaline Phosphatase (CIP) & 10X dephosphorylation buffer (M0290S). T4 DNA Ligase & 10X ligation buffer (M0202L), Restriction enzymes.
Oxoid	Agar (L13), PBS tablets (BR14), Tryptone (L42), Yeast extract (L21), PBS (Br14A).
Pierce	SuperSignal® West Pico Chemiluminescent Substrate (34080).
Promega	dNTPs (U1330), Dual-Luciferase Reporter Assay System (E1910), Luciferase Assay System (E1501), Magnesium chloride 25mM (A3511), M-MLV reverse transcriptase & RT buffer (M1701), 5X Reporter lysis buffer (E3971), RNasin (N2111), Wizard® PCR Preps DNA purification system (A7170), Donkey Anti-Goat HRP V8051, Goat Anti-mouse Alkaline

	phosphatase conjugate S3721.
Roche	Taq polymerase & 10 X enzyme buffer (1146173), Hygromycin B (843555), Leupeptin (1017128).
Qiagen	Qiagen® Plasmid Purification Kit (12143), Quantitect ™ SYBR® Green PCR Kit (204143), UNG (1054568).
Santa Cruz Biotechnology	Anti-Rabbit IgG, HRP Conjugate (sc-2030).
Sigma-Aldrich Chemical Co.	3M sodium acetate, pH5.2 (S7899), Agarose (A5093), Ampicillin (A9518), Aprotinin (A4529), Ammonium Persulfate (215589), BCIP-NBT (B1911), Bovine serum albumin (A9647), Chlorofrom:isoamyl alcohol (24:1) (C0549), Colorburst electrophoresis markers (C4105), Con A (C7275), Coomassie Brilliant Blue R (B0149), Copper (II) Sulphate pentahydrate (31293), DEAE-dextran (D9855), DEPC (D5758), DMSO (D8779), Dnase I kit (D5307-1000UN), EDAC (E7750) β-estradiol (E8875), Ethidium bromide (E4391), Geneticin (G418) (A1720), Glucose (G7528), Glycerol (G5516), Hydrogen peroxide (H1009), Hydroquinone (H9003), L-glutamine (G7513), Manganese Chloride (M3634), MOPS (M3183), ONPG (N1127), Penicillin/streptomycin (P0781), Phenol:chloroform:isoamyl alcohol (25:24:1) (P3803), PMSF (P7626), Ponceau S (P7170), Potassium chloride (P4504), Puromycin (P8833), RedTaq (D4309), RNase A (R6513), Rubidium chloride (R2252), Lauryl Sulfate (L6026), Sodium Butyrate (B5887), Sodium chloride (S3014), Sodium hydroxide (S5881), α-Thioglycerol (M1753),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).
Stratagene	QuikChange Site Directed Mutagenesis Kit (200518).

2.2 Methods

2.2.1 DNA Manipulation

2.2.1.1 DNA Storage

DNA samples were stored in Tris-EDTA (TE) buffer (pH 8.0) at 4ºC. 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 4ºC and -20ºC.

2.2.1.2 Phenol/chloroform extraction and ethanol precipitation

Phenol/chloroform extraction followed by ethanol precipitation was performed to concentrate nucleic acid samples, remove proteins or change the buffers in which a sample was dissolved. 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 mins 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 mins at 13,000 x g at room temperature. Again the upper aqueous phase was removed to a fresh tube. One-tenth volume of 3M 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 -20ºC for a minimum of 20 mins. When dealing with very small quantities of DNA, samples were precipitated in ethanol at -20ºC overnight. The DNA samples were then centrifuged for 20 mins at 12,000 x g at 4ºC, the supernatant was removed and pellets were washed with 300µl 70% (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 mins at 10,000 x g, the supernatant was removed and pellets were air dried for approximately 10 mins. Pellets were re-suspended

in an appropriate volume of sterile TE (pH 8.0) or dH_2O and were stored at 4ºC or -20ºC respectively.

2.2.1.3 Restriction digestion of DNA

Restriction analysis was used as a means of DNA identification, for vector linearization or to excise specific DNA fragments from a plasmid. Restriction enzymes specifically 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 patterns were predicted for DNA sequences using the NEBCutter or the WebCutter 2.0 bioinformatics tool (Table 2.4). All restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). The addition of Bovine Serum Albumin (BSA) was required for certain enzymes to stabilize the enzyme during digestion, (working concentration 1X). DNA digestion reactions were performed according to manufacturer's instructions (New England Biolabs) and incubated for 3 hours at the optimum enzyme temperature (between 37ºC and 50ºC, usually 37ºC).

2.2.1.4 Dephosphorylation of linearised plasmid DNA

To minimize the occurrence of a linearised plasmid re-annealing to itself the vector is treated with Calf intestinal Phosphatase (CIP). Re-circularisation occurs when T4 DNA ligase catalyses the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA during a ligation reaction. The CIP enzyme catalyses the removal of 5´ phosphate groups from DNA. Since CIP-treated fragments lack the 5´ phosphoryl termini required by ligases, they cannot self-ligate, decreasing the vector background in cloning strategies.

Digested DNA (<100 ng/µl) was de-phosphorylated using CIP in a 100 µl volume (CIP was added 1 unit/100 pmoles for cohesive termini). The solution was mixed by pipetting, centrifuged briefly and incubated for 1 hour at 37ºC. This was followed by an enzyme denaturation step achieved by heating to 75ºC for 10 mins. The DNA was then purified by

phenol/chloroform extraction and ethanol precipitation, or gel purification. The purified linearised, CIP-treated DNA was stored at 4ºC until required for ligation.

2.2.1.5 Polymerase chain reaction (PCR)

PCR was used to amplify specific DNA through multiple cycles of denaturation, annealing and elongation. This involved the use of short synthetic oligonucleotides to bind to the sequences flanking the region of interest, one primer lies on the forward strand of DNA whilst the second primer anneals to the reverse strand. This allows the polymerization of both strands by the action of the thermostable enzyme *Taq* DNA polymerase. This method of exponential amplification can be used to generate target sequences of DNA for sub-cloning into linearised vectors, or as a method of identification. PCR reactions were set up by the addition of reagents in the following order:

Component	**Volume**
10x Reaction Buffer (+$MgCl_2$)	5µl
Template DNA (~500ng)	1µl
Forward primer (40pmole)	2.5µl
Reverse primer (40pmole)	2.5µl
dNTP mix (each at 10mM)	1µl
dH_2O to 50µl	37µl
Taq polymerase (5 U/µl)	1µl

2.2.1.6 Purification of PCR products

PCR products were purified from reaction contaminants such as primer dimers and amplification primers by one of two purification systems, the Promega Wizard® PCR Preps DNA Purification System or Bioscience

HiYield™ Gel/PCR DNA Extraction Kit according to the respective manufacturer's protocol. The purified DNA was stored at 4ºC until required for further use.

2.2.1.7 Ligation of DNA molecules

To join blunt or cohesive ended double-stranded DNA, the enzyme T4 DNA ligase was employed to catalyse the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini of the two. Cohesive end ligations were carried out in a 3:1 molar ratio of insert DNA to vector DNA. This was performed overnight at 16ºC or alternatively for 3 hours at 20-25ºC in a commercial ligation buffer (containing 5 mM ATP) with 10 units of T4 DNA ligase/ml in a total volume of 10 µl. After ligation, the samples were heated at 70ºC for 10 mins to inactivate the ligase and were transformed immediately or stored at –20ºC until required for transformation.

2.2.1.8 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. A JM109 *E.coli* strain was streaked from a glycerol stock on to a Luria-Bertani (LB) (Appendix) agar plate and incubated at 37ºC overnight. An isolated colony was then picked using a sterile inoculating loop and used to inoculate 5 ml of LB broth, which was incubated at 37ºC in a shaking incubator at 200 rpm overnight. Following this, 2.5 ml of 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 1L flask at 37ºC until the optical density (O.D.) of the culture was between 0.4 and 0.8, at 640 nm (approximately 4-5 hours). The cells were then transferred to two sterile 250 ml centrifuge tubes and collected by centrifugation at 4,500 x g, 4ºC for 5 mins. 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 4ºC for 5 mins followed by centrifugation at 4,500 x g at 4ºC for 5 mins. Cells were then gently re-suspended in 1/25 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 hour. Prior to storage the aliquoted cells were snap frozen in a dry ice/isopropanol bath. JM109 competent cells prepared by this method and stored at -80ºC are stable for 1 year.

2.2.1.9 Transformations

A 95 µl aliquot of competent cells was placed in a pre-chilled microfuge tube containing 5 µl DNA (~100 ng/10µl). The contents of the tube were mixed gently by flicking, and incubated on ice for 30 mins, during which time an aliquot of SOC medium (Appendix) was pre-heated to 37ºC. After 30 mins on ice the cells were heat-shocked in a water bath at 42ºC for 55-65 seconds, and were then removed immediately for incubation on ice for a further 2 mins. A 900 µl aliquot of preheated SOC medium was added to the cells which were then incubated at 37ºC in a shaking incubator for 1 hour. The cells were then pelleted by centrifugation for 3 minutes at 6,000 x g. Following centrifugation, 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 37ºC. 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.

2.2.1.10 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 overnight on a shaker (200 rpm) at 37ºC. A 1.5 ml aliquot of this culture was transferred to a sterile microfuge tube and centrifuged for 30 seconds at room temperature; the remainder was stored at 4ºC. The supernatant was removed carefully from the tube using a pipette, leaving the pellet as dry as possible. The pellet was re-suspended thoroughly using a pipette in 100 µl of buffer P1 (Appendix). 200 µl of fresh buffer P2 (Appendix) was added, and the tube contents were mixed by inverting the tube rapidly a number of times. 150 µl of ice-cold buffer P3 (Appendix) was added and the tubes were again mixed by inversion.

The lysate was centrifuged for 5 mins at 12,000 x g; the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1)

was added, mixed by vortexing and centrifuged for 5 mins at 12,000 x g. The upper aqueous phase was removed to a fresh tube, to which 2 volumes of 100% (v/v) ethanol were added; the solution was vortexed and centrifuged for 5 mins at 12,000 x g. The supernatant was discarded, the pellet was washed with 1 ml 70% (v/v) ethanol, centrifuged as before and the supernatant was removed. The pellet was air-dried, then re-suspended in 50 µl of TE (pH 8.0). 1 µl of DNase-free RNase A (20 µg/ml) was also added, and the mixture was vortexed, incubated at 37ºC for 1 hour and then stored at 4ºC. Glycerol stocks of all bacterial cultures were prepared at this stage by the adding 0.5 ml of a 50% (v/v) glycerol solution to 0.5 ml of the overnight bacterial culture of interest and storing at -80ºC.

2.2.1.11 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 -80ºC for future use.

2.2.1.12 Qiagen® plasmid DNA purification protocol (Midiprep)

Plasmid DNA was isolated and purified using the Qiagen® Plasmid Midi Kit. This process of plasmid amplification and isolation involves the lysis of the cells, by a treatment with lysozyme, followed by an SDS solution which denatures proteins after the lysis of the membranes. The precipitation of the SDS-protein complex with high-molarity salt specifically removes the chromosomal DNA from the supernatant, as 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 37ºC. 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 37ºC for 7-8 hours. 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 37ºC. 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 mins at 4ºC 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 mins. 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 mins. The mixture was then centrifuged for 1 hour 30 mins at 16,000 x g at 4ºC and the supernatant saved.

The Qiagen-tip 100 column 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 column and allowed to flow through the filter. 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 3.5 ml of room-temperature isopropanol. The DNA/isopropanol mixture was then aliquoted and centrifuged immediately at 13,000 x g for 45 mins at 4ºC and the supernatant was carefully removed using pipettes. The resulting pellet was washed with 70 % (v/v) ethanol (13,000 x g for 15 mins at 4ºC), allowed to air dry for 5 mins and re-dissolved in 30 µl of TE or dH_2O. DNA was then quantified by spectrophotometric analysis, and quality of the DNA was checked by Agarose gel electrophoresis (Adapted from the manufactures protocol Qiagen® Plasmid Purification Handbook, 2005).

2.2.1.13 Qiagen® EndoFree plasmid purification protocol (Maxiprep)

Endotoxins are frequent contaminants in plasmid DNA prepared from bacteria. These endotoxins may reduce the efficiency of transfection or induce non-specific effects within certain cell types that can inhibit reproducibility. 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 manufacturer's protocol (EndoFree® Plasmid Purification Handbook, 2005). Endotoxin free DNA was then quantified by spectrophotometric analysis, and quality of the DNA was checked by Agarose gel electrophoresis.

2.2.1.14 Determination of Nucleic Acid Sample Concentration.

The concentration of isolated nucleic acids can be determined spectrophotometrically. Nucleic acids absorbs UV light maximally at a wavelength of 260 nm; 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.

Shimadzu UV-160A Spectrophotometer

Absorbances were read on the Shimadzu UV-160A spectrophotometer using a quartz cuvette and nucleic acid concentrations were determined according to the following equations:

$$\text{Concentration of DNA (µg/µl)} = \frac{\text{Absorbance (260 nm)} \times 50 \times \text{dilution factor}}{1000}$$

$$\text{Concentration of RNA (µg/µl)} = \frac{\text{Absorbance (260 nm)} \times 40 \times \text{dilution factor}}{1000}$$

2.2.1.15 The NanoDrop® ND-1000 Spectrophotometer

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.

2.2.1.16 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 linear DNA fragments can be separated based on size by passing through a porous agarose matrix 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 (~60ºC) 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 1Kb or 100bp ladder, which was loaded as a size marker. The gel was run at constant voltage (5 V/cm, usually 100V), for 1 to 2 hours. After completion, the gel was stained in ethidium bromide (0.5 mg/ml) for 30 mins, destained in dH_2O for 15 mins and viewed under UV illumination.

2.2.1.17 Isolation of DNA from Agarose Gels.

Agarose gels were prepared in 1X TAE buffer as described previously in 2.2.1.16. Ethidium Bromide was added to the samples before electrophoresis so as to minimize manipulation with the gel. After electrophoresis, the gel was viewed in a dark-room under 70% UV illumination. The time of exposure to UV light was kept to a minimum, as overexposure to UV leads to DNA

damage. The DNA band of interest was excised from the gel using a clean scalpel blade, excess agarose was cut away (without disrupting the fragment) to minimise the size of the gel slice, which was then placed in a pre-weighed sterile microfuge tube.

2.2.1.18 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 4ºC or -20ºC.

2.2.1.19 Site Directed Mutagenesis

QuikChange site directed mutagenesis allows single or multiple specific base changes in a double stranded parental template vector. High fidelity *Pfu* turbo DNA polymerase replicates both strands of the plasmid utilising oligonucleotides, containing the desired mutations, complementary to each target strand. Successfully amplified mutant plasmid strands produced during the PCR will be nicked and un-methylated. This allows digestion of the un-mutated methylated parental vector by the enzyme Dpn I, an endonuclease specific for methylated or hemi-methylated DNA, permitting recovery of the successful mutant amplicons.

The plasmid DNA of interest was purified using a dam^+ E. coli host strain (JM109) and the midi prep DNA purification method described previously section 2.2.1.12. Oligonucleotides containing the desired mutations were designed ensuring the desired mutation anneals to the same sequence on the opposite strands of the plasmid and the base pair mismatch was flanked by ~10-15 bp of correct sequence on either side. Control and mutagenesis PCR reactions were set up by the addition of the reagents in the order listed in Table 2.8

Table 2.8 Components of PCR reactions

Control PCR		Sample PCR	
Component	**Volume**	**Component**	**Volume**
10x Reaction Buffer	5µl	10x Reaction Buffer	5µl
pWhitescript control (5ng/µl)	2µl	dsDNA template (50ng)	Xµl
Forward control primer (100ng/µl)	1.25µl	Forward primer (125ng)	Xµl
Reverse control primer (100ng/µl)	1.25µl	Reverse primer (125ng)	Xµl
dNTP mix	1µl	dNTP mix	1µl
dH_2O to 50µl	39.5µl	dH_2O to 50µl	Xµl
Pfu Turbo DNA polymerase	1µl	*Pfu Turbo* DNA polymerase	1µl

Control and Sample PCR reactions were carried out using the following cycling conditions listed in Table 2.9 below. Reduced cycle number decreases potential for random mutation generation.

Table 2.9 PCR Cycling Programme

Stage	Cycles	Temperature	Time
1	1	95°C	30 Seconds
2	16	95°C	31 Seconds
		X°C	1 minute
		68°C	7 minutes

The PCR reactions were then placed on ice for 2 mins. The reactions were incubated to 37ºC before addition of 1 µl of *Dpn I* restriction enzyme. These were incubated at 37ºC for 1 hour to allow digestion of the methylated parental dsDNA.

The mutated products were transformed into XL1-Blue supercompetent cells. The XL1-Blue cells were thawed gently on ice and aliquoted into 14 ml BD falcon polypropylene round bottom tubes. 1 µl of digested plasmid was added to each aliquot; pUC18 was used as a transformation control. The transformations were mixed gently and incubated on ice for 30 mins. Heat shock was then performed at 42ºC for 45 seconds. The reactions were then returned to ice for 2 mins. Following this, 0.5 ml of preheated NZY^+ broth (Appendix) was added to each reaction and incubated at 37ºC for 1 hour with

agitation on a shaker at 225-250 rpm. Then 250 µl of each sample was plated onto the appropriate agar plates. Mutagenesis and Transformation controls were spread onto LB^{amp} plates containing 80 µg/ml X-gal and 20 mM IPTG. Plates were incubated at 37ºC overnight.

2.2.1.20 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 2.2.1.12 was quantified (Section 2.2.1.14-15) and 15 µl of plasmid DNA at a concentration of 50-100 ng/µl was transferred to a sterile 1.5 ml eppendorf. Alternatively, for sequencing of PCR products, the DNA was purified as per section 2.2.1.6, quantified and 15 µl of product at a concentration of between 2-5 ng/µl was transferred to a sterile 1.5 ml eppendorf. The DNA was sent to MWG-Biotech, Ebersberg, Germany for sequencing. Sequencing forward and reverse primers were supplied by MWG-Biotech for most commercially available vectors. In the absence of existing sequencing primers (as in the case of the pRTS-1 vector or PCR products), a forward and/or reverse primer was designed and 15 µl at a concentration 2 pmol/µl (30 pmol total) of each primer was transferred to a sterile eppendorf and sent with each sequencing reaction to MWG-Biotech. Sequencing results were obtained electronically as linear nucleotide sequences.

2.2.3 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. All media compositions and media supplements are given in the Appendix.

2.2.3.1 Culture of cells in suspension

The cell lines AG876, Akata4E3, BJAB, BL41K3, BL419A, DG75, DG75-tTA-EBNA2, DG75-tTA-LMP1, EREB 2.5, IARC 290B, IB4, LCL327, Ramos were maintained in RPMI 1640 supplemented with 10% (v/v) 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 25cm^2 flasks and expanded in 75cm^2 flasks. P493-6 was maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 2% (v/v) L-glutamine, and 1% (v/v) Sodium Pyruvate. These cells were seeded at a density of 2 X 10^5 to 5 X 10^5 cells per ml in 25cm^2 flasks. Additional supplements were added to some culture media as described in Section 2.2.3.2. Cells were sub-cultured two or three times per week by harvesting the cells into a sterile centrifuge tube and centrifuging at 1000 rpm for 5 mins at room temperature. The cells were gently re-suspended in an appropriate volume of fresh supplemented medium and replaced into the tissue culture flask. All cell lines were incubated in a humid 5% CO_2 atmosphere at 37ºC in a Heraeus cell culture incubator.

2.2.3.2 Medium supplements

Certain cell lines require specific supplements to promote cellular maintenance and proliferation, activate/de-activate the function or expression of a gene of interest or to select cells containing transfected plasmids.

The DG75-tTA-EBNA2 and DG75-tTA-LMP1 cell lines were maintained in supplemented RPMI containing 1 µg/ml of tetracycline. Every three weeks the transfected cells were reselected by the addition of 500 µg/ml of hygromycin and 1,000 µg/ml of geneticin (G418) to DG75-tTA-EBNA 2 and 800 µg/ml of hygromycin and 2,000 µg/ml of G418 to DG75-tTA-LMPI. The stably transfected cell lines BL41K3 and BL419A were maintained under permanent selection, with the addition of 800 ug/ml or 1200 ug/ml of geneticin to BL41K3 and BL419A respectively. In experiments where EBNA2 activation was required, cells were washed twice in PBS and placed in fresh

supplemented RPMI with the appropriate antibiotics for selection. Estrogen was then added to a final concentration of 1 μM.

The estrogen responsive ER/EB2.5 cell line was maintained in supplemented medium containing 1 μM estrogen. To abolish functional EBNA2 activity, ER/EB2.5 cells were washed 4 times in PBS and re-suspended in estrogen-free supplemented medium. A further 2 washes were carried out 24 hours later and the cells were once again replaced in estrogen-free supplemented medium.

In the P493-6, functional EBNA2 activity was induced by culturing cells in supplemented medium containing 1 μM estrogen. To abolish functional EBNA2 activity, cells were washed 4 times in PBS, with a further 2 washes 60 hours later and replaced in estrogen-free medium. Expression of c-*myc*, was repressed by treatment with 0.1 μg/ml tetracycline was added to culture medium. For *myc* re-introduction, cells were washed 3 times with tetracycline-free culture medium and reseeded in tetracycline-free medium.

2.2.3.3 Trypan blue cell counts

Cell counts were carried out using an improved Neubauer haemocytometer slide. Cell viability was determined using trypan blue exclusion dye. A volume of 10 μl of trypan blue was added to 90 μl of a cell suspension and mixed. A sample 10 μl 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 X 10^4 cells/ml). Thus, cell counts were expressed as the number of cells per ml.

2.2.3.4 ADAM™ cell counter

Cell counts can also be made using an Advanced Detection and Accurate Measurement (ADAM™) cell counter which utilizes fluorescent cell staining to determine total and viable cell count in a give sample. Cells are mixed with two solutions one contains Propidium Iodide (PI), a fluorescent dye that will intercalate with DNA; however, PI cannot enter cells with intact membranes. Only non-viable cells will fluoresce with PI alone. The second solution contains PI and a detergent that will disrupt viable cell membranes allowing PI to stain both viable and non-viable cells. ADAM contains both laser optics and image analysis software that allows determination of both total and viable cell numbers in a sample from these two staining methods.

Two 14 µl aliquots of cells were taken, one aliquot was mixed with 14 µl of Solution T accustain (Digital Bio) the second with 14 µl solution N accustain (Digital bio). Following this, 20 µl of each cell mixture was then loaded into the appropriate labelled chamber in an AccuChip (Digital bio). Then 40-60 images are taken of the cell chambers. Average cell number and viability are calculated using the integrated image analysis software.

2.2.3.5 Cell storage and recovery

Mammalian cells can be stored indefinitely in liquid nitrogen; however, the correct protective medium is vital as the effects of freezing can be lethal. Formation of ice crystals or shifts in electrolyte concentrations can cause the loss of cell membrane integrity, leading to cell death. To reduce these effects significantly, DMSO, a cryoprotectant chemical which lowers the freezing point of the storage medium, must be added. FBS is a rich source of proteins and other growth factors. FBS is added to the freezing medium as a cryoprotective additive. The precise function of FBS is unclear; however, it has long been proven to enhance long term cryostability.

In order to prepare stocks of suspension cells for long-term storage, 1 X 10^7 cells in exponential phase were centrifuged, the excess medium was

discarded and the cells were re-suspended in 1 ml of freezing medium composed of 750 µl of supplemented RPMI, 150 µl of FBS and 10% (v/v) DMSO, mixed gently and transferred 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 37ºC and transferring to a sterile centrifuge tube containing 10 ml of pre-warmed supplemented medium. The cells were centrifuged at 100 x g for 5 mins, the cells were re-suspended in 5-10 ml of fresh supplemented medium, transferred to a culture flask and incubated at 37ºC in 5% CO_2.

2.2.3.6 Isolation of Naïve B cells

Naïve B cells were isolated from human peripheral blood mononuclear cells (PBMCs) using MACS naïve B-cell isolation kit II. This is an indirect magnetic labelling system for the isolation of untouched naïve B cells from human PBMCs. Non-B cells, i.e., T cells, NK cells, dendritic cells, monocytes, granulocytes, platelets, and erythroid cells, and non-naïve B cells, such as memory B cells, activated B cells, and plasma cells, are indirectly magnetically labelled using a cocktail of biotinylated CD2, CD14, CD16, CD27, CD36, CD43, and CD235a (glycophorin A) antibodies. These cells are subsequently magnetically labelled with Anti-Biotin MicroBeads for depletion. Highly pure naïve B cells are obtained by depletion of magnetically labelled cells. The purity of the sample was confirmed by FACS analyses of PBMC and the naive B-cell fraction stained with PE-labelled anti-CD27 (memory B-cell marker) and FITC-labelled anti-CD19 (pan-B-cell marker) antibodies.

2.2.3.7 EBV Infection

Recombinant GFP-tagged EBV was obtained from Akata BX-1 cells that had been re-suspended at a concentration of 4 x 10^6 cells/ml, and induced with 50 mg/ml of anti-human immunoglobulin G for 5 days (Molesworth, et al. 2000). The Virus was harvested and sterilised by passing through a 0.45-mm (pore-size) filter. Approximately 5 x 10^5 naïve B cells were incubated at 37ºC with 150 µl of dilutions of filtered recombinant GFP-tagged BX1 EBV virus. After 2 hours, the volume was brought to 3 ml with medium containing serum and the cells were re-incubated. Three days later the cells were harvested and analysed for expression of EBNA-2 by Western blotting.

2.2.3.8 Transient transfections

Transient transfection of cells was performed by electroporation, nucleofection or using the DEAE-dextran method. In all cases, cells were seeded at a density of 2-5 X 10^5 per ml of medium 24 hours prior to transfection. After this time in culture, cells were counted again. 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.

2.2.3.9 Electroporation of B lymphocytes

Transfection of AG876, IB4, BJAB and ER/EB 2.5 cell lines (Table 2.1) 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 nanometer-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 mins and the supernatant discarded. The cells were then washed in PBS, centrifuged at 100 x g for 5 mins and the supernatant again discarded. For each 1 X 10^7 cells, 220 µl of serum-free medium 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 mins. Each cell/DNA mix was then pulsed at 220-250 V (BJAB, 250 V; AG876, IB4 and EREB 2.5 220 V) with a capacitance of 960 µF (using a capacitance extender) and resistance set to infinity using a Bio-rad 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 cells were stored no longer than 10 mins after electroporation to avoid reduction in cell viability/gene transfer efficiency. Transfected cells were harvested 48 hours later.

2.2.3.10 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 and IB4 cells yielded higher percentage transfection efficiency, when compared to cells transfected using the electroporation technique.

The appropriate nucleofector solution to the particular cell line being transfected (Solution T/V for BJAB/IB4) was pre-warmed to room temperature and 50 ml of supplemented medium 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 37ºC/5% CO_2/humidified atmosphere. A total of ~7 µg DNA was prepared in sterile 1.5 ml eppendorfs

for each sample. For each transfection, 2.5 X 10^6 (IB4)/ 5 X 10^6 (BJAB) cells were collected by centrifugation at 90 x g for 10 mins 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 2.5 x 10^6 cells/100µl (IB4) or 5 X 10^6 /100µl (BJAB). Care was taken to ensure cells were stored in nucleofector solution no longer than 15 mins 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 (IB4 and BJAB). 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 37ºC/5% CO_2/humidified atmosphere until cells were harvested.

2.2.3.11 DEAE-Dextran-mediated transfection

Transfection of DG75 cells was performed using this method of transfection. During transfection, positively charged DEAE-dextran binds to the negatively charged phosphate groups of the DNA, forming aggregates. These complexes, when applied to cells, subsequently bind to the negatively charged plasma membrane. It is believed that cellular uptake of DNA is mediated by endocytosis, and further assisted by osmotic shock.

A maximum of 25 µg of DNA dissolved in TE buffer (pH 8.0) was used per transfection. Total DNA was prepared in a sterile microfuge tube and brought to a volume of 600 µl with TBS (Appendix). Following this, 600 µl of 1 mg/ml DEAE-dextran solution (Appendix) was mixed with the DNA solution. Meanwhile, 1 X 10^7 cells/transfection were centrifuged at 100 x g for 5 mins and the supernatant was discarded. The cells were then washed in PBS, centrifuged at 100 x g for 5 mins and the supernatant again discarded. The cells were then gently re-suspended in the DNA/DEAE-dextran mix. The transfection cocktails were incubated at 37ºC for 30 mins with gentle swirling every 5-10 mins to promote homogenisation. Transfections were terminated

by the addition of 10 ml supplemented medium to the tube and the cells were collected by centrifugation at 100 x g for 5 mins, washed in PBS as before and transferred to 6 well plates containing 10 ml supplemented medium for incubation. Cells were harvested at 48 hours post-transfection.

2.2.3.12 Microscopic analysis following gene transfer

Following selected transfections, gene transfer efficiency was monitored by fluorescence microscopy. Green fluorescent protein (GFP) expressing vectors were co-transfected, cells were harvested 4-72 hours post-transfection and positively transfected cells expressed this fluorescent protein. A volume of 500 µl of suspension cells was removed to a 1.5 ml sterile eppendorf and centrifuged at 100 x g for 5 mins. The supernatant was discarded and the cells were re-suspended in 20 µl of PBS/0.5 % (w/v) BSA and applied to a glass microscope slide. A coverslip was applied with care taken to avoid air bubbles and the slide was examined under bright field and fluorescent field microscopy using an Olympus DP-50 fluorescent microscope [excitation 450–500 nm, emission 515–565 nm] with a camera and StudioLife software (Olympus Optical Company).

2.2.3.13 Harvesting cells post-transfection for luciferase/β-gal assays

Cells were collected by centrifugation at 100 x g for 5 mins at room temperature. The cells were washed once in sterile PBS and centrifuged again at 100 x g for 5 mins. The supernatant was discarded, ensuring all traces of PBS were removed using pipettes. The cells were re-suspended in 50 µl of 1X reporter lysis buffer (diluted with dH_2O from a 5X stock, Promega) and the cell suspension transferred to a microfuge tube. The tubes were vortexed for 10-15 seconds. The lysates were clarified by centrifugation at 13,000 x g for 5 mins and the supernatant saved in a fresh tube. Samples were stored at –80ºC until luciferase/β-galactosidase assays were performed.

2.2.3.14 Luciferase assay

Following transfection, promoter activity was determined by means of the luciferase assay. Firefly luciferase from the firefly *Photinus pyralis*, a monomeric 61 kDa protein, catalyses luciferin oxidation using ATP-Mg^{++} as a co-substrate. In this luminescent reaction, light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined.

Luciferase assay reagent was prepared by reconstituting luciferase assay substrate with luciferase assay buffer (Promega) and stored in aliquots protected from light at –70ºC. Sufficient time was allocated to allow the luciferase detection reagent to equilibrate to room temperature. A volume of 20 µl of cell lysate was dispensed into individual wells in a white 96 well plate, along with 20 µl of 1X lysis buffer to act as a blank. Subsequently, in order to initiate enzyme activity, 100 µl of detection reagent was added to the lysate immediately prior to measuring, mixed by repetitive pipetting (3 times) ensuring no bubbles are produced. Then, light emission integrated over a period of 60 seconds, after a lag period of 10 seconds, was measured on a luminometer (Labsystems Luminoskan 391A). Luciferase activity levels were adjusted for transfection efficiencies, estimated using *β*-galactosidase assay.

2.2.3.15 β-Galactosidase assay

When measuring the effect of promoters or enhancers on gene expression, it is essential to include an internal control that will distinguish differences in the level of transcription from differences in the efficiency of transfection or in the preparation of extracts. This is best achieved by co-transfecting the cells with two plasmids: one that carries the construct under investigation, and another that constitutively expresses an activity that can be assayed in a separate experiment. An enzyme frequently used for this is *E. coli* β-galactosidase (β-gal). This assay is a convenient method for assaying β-gal

activity in lysates prepared from cells transfected with β-gal reporter vectors, in this case pCMV-LacZ.

The β-gal assay was performed using a sample from the same lysates assayed for luciferase activity. Cell extract (30 µl) was added to 3 µl 100X Mg solution, 66 µl 1X *o*-nitrophenyl-β-D-galactopuranoside (ONPG) and 201 µl 0.1M sodium phosphate (all described in Appendix) and incubated at 37ºC for >30 mins or until a faint yellow colour developed. This yellow colour development is the result of hydrolysis of ONPG by β-gal to form *o*-nitrophenyl. A reaction tube was included containing 1X lysis buffer instead of cell lysate in order to obtain a background reading. Reactions were terminated by addition of 500 µl 1M Na_2CO_3 (Appendix). Optical densities were read at 420 nm using a Shimadzu spectrophotometer over a linear range of 0.2-0.8.

2.2.3.16 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.

The term apoptosis was originally coined to describe a controlled type of cell suicide that involved a series of systematic characteristic cellular events (Kerr, et al. 1972). Within a healthy cell the phospholipid, phosphatidylserine (PS), can be found bound to the inner-leaflet of the cell membrane. However apoptotic caspase activity leads to the externalisation of PS, flagging the cell for phagocytic engulfment (Fadok, et al. 1992). The human anticoagulant, annexin V, is a 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. A fluorescent dye that binds to nucleotides and penetrates only damaged cellular membranes was used (vital dye). Intercalation complexes are formed by 7-Amino-actinomycin (7-AAD) with double-stranded DNA, which effect an amplification of the fluorescence.

The Annexin-PE Apoptosis Detection Kit (Becton Dickinson) contains recombinant annexin V conjugated to the fluorochrome, phycoerythrin (PE) and the vital dye 7-AAD. Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Cells that stain positive for Annexin V-PE and negative for 7-AAD are undergoing apoptosis. Cells that stain positive for both Annexin V-PE and 7-AAD 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 7-AAD are alive and not undergoing measurable apoptosis.

To perform the assay, cells were harvested and washed twice in ice-cold PBS. The cells were resuspended in 1X annexin-binding buffer at a concentration of ~1 X 10^6 cells/ml. A volume of 5µl of Annexin V-PE solution and 5 µl of 7-AAD solution were added to 100µl of cell suspension. The cells were incubated at room temperature for 15 mins in the dark and 400 µl of 1X annexin-binding buffer was then added. The samples were analysed within one hour by flow cytometry.

2.2.3.17 Stable Transfections

Stable cells lines were established by transfection (as in Sections 2.2.3.8-2.2.3.11) followed by selection with the appropriate drug 48 hours later. Within 2-4 weeks drug resistant cells grew out. In some cases, specific drug concentrations for selection in individual cell lines were known from previous publications/projects; otherwise, drug curves were prepared in advance of transfection to determine the minimum drug concentration to cause cell death. The pcDNA3 IB4 and pcDNA3 TLE1 IB4 stable cell pools were selected in 800 µg/ml G418.

2.2.3.18 Establishment of mammalian cell line sensitivity for cell selection

IB4 cells in exponential growth phase were seeded at a density of 1 x 10^5 cells/ml, in media containing a range of puromycin concentrations ranging from 0, 0.25, 0.5 and 1 µg/ml. These cells were cultured for 10 days under standard conditions. Medium was changed every 3 days and cell viability was measured every day by trypan blue exclusion. The minimum concentration of puromycin which killed >95% of the cell pool after 10 days was determined, and this concentration was used for subsequent selection procedures for the selection of puromycin resistant cells

2.2.3.19 Enrichment of live cell population using Ficoll

The process of electroporation damages a cell line dependent portion of the cell population beyond repair. Ficoll gradient separation discriminates between live cells and dead cells based on a cell density gradient (Boyum 1968), allowing enrichment for the live cell population post-transfection. Ficoll is a neutral, water soluble synthetic polymer of sucrose and epichlorohydrin possessing a high molecular weight. Ficoll has a density of 1.077 ± 0.001 g/ml, this can be overlaid with media containing lymphocytes, and after rapid centrifugation at room temperature, with no aided acceleration or

deceleration, the sample becomes segregated according to density. Healthy lymphocytes are not dense enough to penetrate the Ficoll layer. The viable lymphocytes concentrate at the interface of the growth medium and separation fluid; but dead cells, which are more dense, pass through the medium and gather below the Ficoll layer. Ficoll is commercially available as a ready to use separation medium called Lymphoprep™.

For each Ficoll purification, the transfected cells were collected by centrifugation at 100 x g for 5 mins and the supernatant was discarded completely. The cells were re-suspended in room-temperature supplemented RPMI to a final concentration of 1 x 10^7 cells/ 3 ml, before being transferred to a 12 ml centrifuge tube. The Lymphoprep™ was then carefully under-laid below the cell suspension, and the sample was centrifuged at 800 x g for 15 min at room temperature, ensuring that the brake and accelerator in the centrifuge have been disabled (use of a brake or accelerator can cause mixing at the interface). The culture medium remains above the Ficoll medium, the live lymphocytes gather at the interface and the non-viable cells and cellular debris are found either within the Ficoll medium or as a pellet at the bottom of the tube. The culture medium and lymphocyte layer above the Ficoll were gently transferred into a fresh container, using a pipette. The purified cells were collected by centrifugation at 100 x g for 5 mins and the supernatant was discarded completely. The cells were re-suspended in pre-warmed supplemented RPMI, transferred to a culture flask and incubated at 37ºC in 5% CO_2.

2.2.3.20 Enrichment of live cell population using Con A selection

Concanavalin A (Con A) is a plant derived lectin that binds specifically to the α-mannosyl glycoprotein present on the membrane of healthy B lymphocytes (Thøger-Andersen and Junker 1994). Panning a cell population with fixed Con A allows discriminative selection between live and dead lymphocytes. Equal volumes of 100 µg/ml Con A (Appendix) and the activating agent EDAC (75 mg/ml appendix) were combined and added to a cell culture grade dish to allow coating of the lectin to the surface. This was incubated for 2

hours at room temperature followed by 1.5 hours incubation with constant gentle agitation (30 rpm). The dish was then washed gently three times in PBS, and left under PBS to maintain hydration of the lectin. To sterilise the surface the dish was then subjected to UV irradiation for 20 mins. At this stage the dish can be stored under PBS for 3 days at 4ºC.

To begin panning, the Con A coated dish was blocked by washing with room temperature supplemented RPMI medium. The cells were then pipetted onto the dish, ensuring even distribution of the sample on the coated surface, and incubated for 20 mins at 37ºC in 5% CO_2, followed by 10 mins incubation at room temperature. The supernatant was then carefully removed and the dish was washed twice with PBS. To acquire the bound cells, pre-warmed medium was flushed gently against the Con A coated surface using a pipette. The purified cells were collected by centrifugation at 100 x g for 5 mins and the supernatant was discarded. The cells were re-suspended in pre-warmed supplemented RPMI, transferred to a culture flask and incubated at 37ºC in 5% CO_2.

2.2.3.21 Measurement of Cell Proliferation

The CellTiter 96 Aqueous One Solution cell proliferation assay is a colorimetric method for determining the activity of the mitochondria in active cells; this can be compared to control cells to determine relative cell proliferation. The assay is based on the addition of a reagent containing a novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron-coupling reagent, phenazine ethosulphate (PES) to cells in culture. The MTS tetrazolium compound is bio-reduced by cells into a coloured formazon product that is soluble in cell culture media. This conversion is thought to be facilitated by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan 1993). The quantity of formazon product, as measured by the amount of absorbance at a wavelength of 490 nm, is directly proportional to the number of living cells in culture.

To perform the assay, the CellTiter 96 Aqueous One Solution was thawed from frozen in a 37ºC water bath. Subsequently, 20 µl of reagent was added directly to 100 µl of cells in culture media (in triplicate) in 96-well plates. The plates were incubated at 37ºC/5 % CO_2 for 4 hour and absorbance at 495 nm was measured using a Tecan Saffire II 96-well plate reader. Triplicate control wells containing media alone were prepared to correct for background absorbance.

2.2.3.22 Immunostaining

This technique allows visualisation of the target protein of interest in situ using specific antibody/antigen interactions by either direct or indirect labelling. In this study, an un-labelled antibody specific for the target was used in co-ordination with a HRP-labelled secondary antibody against the primary. Immunostaining involves the fixation and permeabilsation of cells to a non-interfering solid support surface. Fixation is a key step ensuring the cells are stabilized on the support against further staining procedures, whilst preserving the cells in their native state. Fixation can be performed by using either aldehydes such as formalin to cause cross linkage of proteins or an alcohol solvent such as isopropanol to fix the mammalian cells by coagulating surface proteins. Each method binds the cells to the support surface.

The cells were pelleted by centrifugation at 100 x g for 5 mins, washed with 5 ml of PBS (Appendix) and centrifuged at 100 x g for 5 mins. The cell pellet was re-suspended in 20 µl of PBS and applied to a glass microscope slide, then fixed in ice-cold 100% (v/v) isopropanol for 5 mins, allowed to air dry, and subsequently rehydrated in PBS for 10 mins. The cells were incubated in 5% blocking buffer (Appendix) with constant gentle agitation for 1 hour at room temperature. The cells were then incubated in primary antibody again with gentle agitation for 2 hours at room temperature followed by overnight incubation at 4ºC. The slide was washed three times at 5 mins intervals with 1 ml PBS. The secondary HRP conjugated antibody was then incubated on the slide for 1 hour with gentle agitation at room temperature. The cells were

washed again as above before adding 3,3',5,5'-tetramethylbenzidine (TMB, Sigma), a substrate cleaved by HRP resulting in a coloured product. This allowed the probed slide to be examined under brightfield microscopy using an Olympus DP-50 with a camera and StudioLife software (Olympus Optical Company).

2.2.4 RNA Analysis

2.2.4.1 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 mins, and were then rinsed thoroughly in DEPC treated upH_2O and allowed to dry. As hands are a major source of RNase contamination, gloves were used at all times and changed frequently.

2.2.4.2 RNA analysis by gel electrophoresis

In order to examine the quality 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 Appendix. The RNA samples (1 µl of 1 ug/ul RNA) 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 65ºC for 10 mins prior to loading on the gel. The gel was run in 1X TAE as described in section 2.2.1.16. 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.

2.2.4.3 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 2.2.3.4.

2.2.4.4 RNA extraction using Tri-reagent

Cells grown in suspension were centrifuged at 100 x g for 5 mins, 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 X 10^7 cultured cells. The lysate was left at room temperature for 5 mins after which time the procedure could be halted by storing samples at –80ºC.

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 seconds until completely emulsified. Samples were incubated at room temperature for 15 mins. The resulting mixture was centrifuged at 13,000 x g for 20 mins at 4ºC. During centrifugation the mixture separated according to the density gradient 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 overnight at -20ºC, then centrifuged at 13,000 x g for 15 mins at 4ºC. 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 mins at 4ºC, and the 75% (v/v) ethanol was aspirated. Pellets were air dried and dissolved in DEPC treated upH_2O. The resulting RNA preparation was heated at 60ºC and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric (Section 2.2.1.15) and gel electrophoretic analysis (Section 2.2.1.16) and the remainder of the purified RNA was stored at –80ºC.

2.2.4.5 Total RNA isolation from cells using QIAgen RNeasy™ kit

RNA was extracted from small numbers of cultured cells (<5 X 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 2.2.1.15) and gel electrophoretic analysis (Section 2.2.1.16) prior to reverse transcription PCR (RT-PCR).

2.2.4.6 Quantification of mRNA from cultured cells by RT-qPCR

The quantification of mRNA purified from cultured cells was performed in a three-step procedure. In the first step, RNA was treated with DNase to remove any possible residual genomic or vector DNA, this process was stopped by heat inactivation of the DNase enzyme. The cDNA was then prepared from DNase treated RNA by RT-PCR with random hexamers serving as primers. During the third step, cDNA was amplified by real time quantitative PCR (qPCR). RT-qPCR is increasingly being adopted for RNA quantification based on its ability to monitor with a high degree of sensitivity, the amount of PCR product present at every cycle (i.e. in real time). This method shows accuracy over a wide linear range, as opposed to the endpoint detection by conventional PCR methods, thus allowing the real-time progress of the reaction to be viewed. Data is measured at the exponential phase of the PCR reaction, the real-time analysis method is based on the detection and quantification of a fluorescent reporter dye present in the reaction that gives a proportional response to the amount of product accumulated in a reaction.

2.2.4.7 Dnase treatment of mRNA

The RNA sample for analysis was digested with 1 µl 10x DNase Buffer and 1 µl RNase-free DNase I (Sigma-Aldrich) and incubated for 15 mins at 37ºC. 1 µl DNase stop solution (Sigma-Aldrich) was added to the reaction mixture and was incubated for a further 10 mins at 70ºC to denature the DNase enzyme. The RNA could then be synthesized to cDNA.

2.2.4.8 Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase

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 DNase treated RNA and the volume brought up to 10 µl with DEPC H_2O. The mixture was heated to 70ºC for 5 mins, to de-stabilise secondary mRNA structures, and then placed on ice for 5 mins. Then, the RT reactants were added in the order listed in Table 2.10.

Table 2.10 MMLV RT reactants

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

The reactions were placed in a thermocycler at 37ºC for 1 hour and 95ºC for 2 mins followed by storage at 4ºC.

2.2.4.9 Real time relative PCR (RT-qPCR)

cDNA generated by RT was quantified by real time PCR using QuantiTect® SYBR® Green PCR kit. In this study SYBR green was utilised as the method of choice as it is the most straightforward and least expensive method, making it the first choice for high throughput samples. SYBR green binds only to double stranded DNA by intercalating between two base pairs and only actively fluoresces upon binding to DNA. As SYBR green is not gene- or product-specific the assay must be optimised to ensure that only one product is being generated (dissociation analysis) and that it is the correct target amplicon. To normalise the optical system involved in real time analysis, ROX, a passive dye, is incorporated into the SYBR mastermix.

The QuantiTect® kit provided a SYBR® Green master mix that contained Taq polymerase, dNTPs, reaction buffer and ROX dye. Real time PCR reactions were set up as follows:

Table 2.11 Real Time PCR Reactants

Component	Volume
cDNA	2µl
Forward primer	1µl
Reverse primer	1µl
UNG	0.25µl
nuclease free H_2O	8.25µl
SYBR master mix	12.5µl
Total	25µl

Quantification of cDNA target was normalised for differences across experiments/samples using an endogenous control as an active reference (*TBP*). The PCR reaction mix was prepared for each sample (in triplicate) by addition of the reagents listed in Table 2.11 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. Samples were quantified using the ABI 7500 Prism detector (Applied Biosystems) under the following thermo-cycling conditions:

Table 2.12 Real Time PCR Cycling Parameters

Stage	Cycles	Temperature	Time
1	1	50ºC	2 mins
2	1	95ºC	15 mins
3	35	95ºC	20 Seconds
		XºC	30 Seconds
		72ºC	37 seconds
4	Dissociation	60ºC - 95ºC temperature rises 1ºC each step. Each step held for 5 seconds.	

During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analysing fluorescence emissions. The reporter dye (SYBR) signal was measured against the passive 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).

2.2.5 Protein Analysis

2.2.5.1 Preparation of cellular protein

Proteins were isolated from suspension cells for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by western blotting. Prior to isolation, cells were washed once in PBS. Suspension cells were centrifuged at 100 x g for 5 mins and the supernatant removed. Following this, 10 ml Ice-cold PBS was added; the cells were centrifuged again at 100 x g and all of the supernatant removed. 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 2 X 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 30 seconds at 6 second 40% Amp pulses (using a Vibra Cell or Branson Sonicator) to shear the DNA. The lysate was clarified by centrifugation at 13,000 x g for 10 mins at room temperature. The supernatant was aliquoted and stored at –20ºC until required for analysis.

2.2.5.2 SDS-polyacrylamide gel electrophoresis of proteins

SDS-polyacrylamide gel electrophoresis resolves cellular proteins based solely on their electrophoretic mobility. During polyacrylamide gel electrophoresis (PAGE), proteins are driven by an applied current through a mesh of cross-linked 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, denaturing the protein to its primary structure. Electrostatic repulsion between the bound SDS molecules causes the proteins to unfold into a similar rod-like shape, removing secondary and tertiary structure as a factor in separation. As 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.

2.2.5.3 Preparation of SDS-polyacrylamide gels

SDS-PAGE was performed using resolving gels between 7.5 - 10% (v/v) and 5% (v/v) stacking polyacrylamide gels prepared (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 mins. 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 cleared the bottom of the gel. When complete the plates were removed, separated and the gel was placed in transfer buffer (Appendix) prior to Western blotting.

2.2.5.4 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 HRP enzyme for detection.

2.2.5.5 Transfer of resolved proteins to nitrocellulose membrane

Following gel electrophoresis, gels were equilibrated in transfer buffer (Appendix) for at least 15 mins. 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 20 mins at 17 V.

2.2.5.6 Staining of proteins immobilized on nitrocellulose membranes

To visually confirm uniform transfer of protein to the nitrocellulose membrane Ponceau S stain was applied. 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. 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 mins 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.

2.2.5.7 Immunological probing method 1 – manual probing

Following Ponceau S staining, the membrane was incubated in 5% blocking buffer (Appendix) for 2.5 hours at room temperature followed by incubation with the appropriate primary antibody (diluted in blocking buffer, see Table 2.13) at 4ºC overnight.

Table 2.13 Incubation Conditions for Antibodies Used in Western Blotting (Manual Probing Method)

Primary Antibody	Name	Dilution In Blocking Buffer	Secondary Antibody	Dilution In Blocking Buffer
Anti-β-actin	Anti-actin AC-15	1/15,000	AP-conjugated Anti-mouse IgG	1/20,000
Anti-EBNA2	PE2	1/200	AP-Conjugated Anti-Mouse IgG	1/5,000
Anti-TLE1	Rbt. Poly. to TLE1: ab15587	1/200		

After overnight incubation, the membrane was washed three times in TBS-T (0.1% (v/v) Tween-20 in TBS [Appendix]) for 15 mins. The filter was then incubated with the appropriate secondary antibody (Table 2.13 for 1 hour 30 mins at room temperature, followed by three 15 min washes in TBS-T. All of the above incubations were carried out with constant agitation. The membrane was then placed in a clean container and colorimetric detection of the conjugated antibodies was performed by immersing the membrane in either 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT, Sigma) for AP conjugated antibodies or 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) for HRP antibody conjugates. When incubated with AP or HRP substrates, bound enzyme catalysed the production of a coloured product from the substrate that was easily observable. The membrane was then rinsed in distilled water to stop the reaction and photographed.

2.2.5.8 Immunological probing method 2 – SNAP i.d.™

Following Ponceau S staining, the membrane was applied to an incubation chamber according to manufacturer's guidelines. The SNAP incubation chambers were hydrated with dH_2O, and the nitrocellulose membrane was placed face down into the chamber, any air bubbles were removed by running a roller over the blot. A support was placed at the reverse of the membrane and the chamber was sealed; again, air bubbles were expelled

using the roller. This device was then placed within the SNAP i.d.™ system. The nitrocellulose support was then evenly covered with 0.2% blocking buffer (Appendix) ensuring the liquid was applied in an 's' shaped manner to prevent pressure against any one area of the blot, which may cause displacement of bound antibody. After blocking a vacuum was applied to the well pulling the blocking buffer through the membrane. The primary antibody was then added to the chamber and incubated for 10-15 mins at room temperature. The antibody concentrations used are as laid out below in Table 2.14.

Table 2.14 Incubation Conditions for Antibodies Used in Western Blotting SNAP i.d. Method

Primary Antibody	Incubation		Dilution In Blocking Buffer	Secondary Antibody	Incubation		Dilution In Blocking Buffer
	Time	Repeats			Time	Repeats	
Anti-β-actin	10mins	1	1/15,000	AP-conjugated Anti-mouse IgG	10mins	1	1/20,000
Anti-TLE1	15mins	2	1/500	Goat anti-rabbit IgG-HRP: sc-2004	10mins	1	1/500

After the indicated incubation period in Table 2.14, a vacuum was once again applied, clearing the well of the primary antibody by pulling it through the membrane. The membrane was then washed three times in 10 ml TBS-T (0.1 % (v/v) Tween-20 in TBS [Appendix]). The blot was then incubated for 10mins with the appropriate secondary antibody before applying the vacuum to the chamber to remove all liquid from the membrane. Again the membrane was washed three times in 10 ml TBS-T. The membrane was then placed in a clean container and developed using the colorimetric method described in section 2.5.7.

2.2.5.9 TLE1 Immunological dual probing method

To detect the nuclear repressor protein TLE1 a method combing classic manual probing and the utilisation of the SNAP i.d.™ system was used. The membrane was treated according to the manual method 2.5.7, with 2.5 hour incubation in 5% blocking buffer (Appendix). Primary antibody incubation was described in Table 2.14. After overnight incubation in primary antibody the membrane was fitted to the incubation well holder as described in 2.5.8 for probing using the SNAP i.d. method. The SNAP method was then followed repeating the primary antibody application and continuing with washings and secondary antibody probing as described in section 2.5.8. The membrane was then placed in a clean container and developed using the colorimetric method described in section 2.5.7.

Chapter 3: *Regulation of the Bcl-2 family member, Bfl-1, in EBV infected B Lymphocytes.*

3.1 Introduction

Type A EBV and type B EBV differ significantly in their *EBNA2* sequence. Whilst sequence variation in the *EBNA3*s and *EBNA-LP* exists, the difference in biological function between the two strains has been attributed to their differing EBNA2 isoforms (Cohen, et al. 1989). The two EBNA2 proteins share only 55% homology and the most conserved parts of the EBNA2 protein are the regions known to bind CBF1 and the activation domain (Lucchesi, et al. 2008). CBF1 is the key transcription factor associated with EBNA2-led gene regulation, although EBNA2 can also associate with Spi-1 and members of the Ets transcription family (Zimber-Strobl and Strobl 2001). It has been shown that the type A strain is far more efficient at B cell immortalisation than the type B strain (Rickinson, et al. 1987). To date it has been found that EBNA2A is a more efficient trans-activator of *CXCR7* and *Runx3*, both genes important in LCL proliferation (Lucchesi, et al. 2008).

Previous work in the laboratory established that the anti-apoptotic *bcl-2*-family member *bfl-1* is transcriptionally up-regulated by EBV and provides the cell with a survival advantage (D'Souza, et al. 2000). Bfl-1 (A1) is a Bcl-2 family member protein that is comprised of 175 aa. Bfl-1 is classed as an anti-apoptotic member of the Bcl-2 family, as it shares the Bcl-2 homology domains BH1, BH2 and BH3 (D'Sa-Eipper and Chinnadurai 1998). Functions of Bfl-1 include suppression of p53-mediated apoptosis, shielding cells from growth factor deprivation induced apoptosis and it also exhibits functionality in both cell proliferation and transforming processes (D'Souza, et al. 2000, D'Sa-Eipper and Chinnadurai 1998, D'Sa-Eipper, et al. 1996). The *bfl-1* gene has, in recent years, been acknowledged as a relevant therapeutic target due to molecular profiling studies that have identified *bfl-1* as up-regulated in a gene profile for a diffuse large B cell lymphoma subtype 'OxPhos' and in primary mediastinal large B cell lymphoma (Feuerhake, et al. 2005; Monti, et al. 2005; Dave, et al. 2006). *bfl-1* has been shown to be a direct nuclear factor-kappa B (NF-κB) target gene that can suppress apoptosis from a number of stimuli including TNFα, Anti-CD95 and chemotherapeutic drugs (Wang, et al. 1999; Werner, et al. 2002; Zong, et al. 1999). Brien *et al*

demonstrated that down-regulation of *bfl-1* in malignant B cells increases cell sensitivity to Anti-CD20 antibodies and to chemotherapeutic drugs such as doxorubicin, fludarabine and Cisplatin (Brien, et al. 2007). Previous studies have indicated that *bfl-1* is an EBV target that can be independently up-regulated by both EBNA2 and LMP1 (D'Souza, et al. 2004; D'Souza, et al. 2000).

D'Souza *et al* have previously determined, using a system in which the expression of LMP-1 was inducibly regulated by tetracycline, that LMP-1 expression alone can up-regulate transcription of the *bfl-1* gene in a B cell line. Further studies revealed that LMP-1 also *trans*-activates the *bfl-1* promoter and that the NF-κB signaling pathway may mediate this interaction (D'Souza, Rowe and Walls 2000). Furthermore, Pegman *et al* determined that *bfl-1* was independently *trans*-activated by EBNA2 and that this up-regulation involved the nuclear binding protein, CBF1. Removal of either of the EBV latent proteins LMP1 or EBNA2 did not lead to down-regulation of *bfl-1* expression; however, inhibition of both proteins from the cellular environment led to a significant reduction in *bfl-1* expression within the cell (Pegman, et al. 2006). Due to the body of evidence implicating EBNA2 variation as a key difference between the two strains, a comparative study of the functional activity of the two EBNA2 isoforms was performed in an attempt to determine if *bfl-1* regulation may be a consequence of the divergent biological functions of EBNA2A/EBNA2B. The *trans*-activation capabilities of EBNA2A and EBNA2B with respect to the *bfl-1* promoter were therefore directly examined. In addition, aspects of the mechanism of *bfl-1* promoter *trans*-activation by EBNA2 were also investigated.

In this chapter, results are presented that indicate the following; (i) the Notch responsive vector pGa981.6 is more responsive to EBNA2B expression than EBNA2A, (ii) both EBNA2 isolates *trans*-activate the *bfl-1* promoter to a similar degree, (iii) mutation of a consensus CBF1-like binding site in the *bfl-1* promoter consistently prevented EBNA2-*trans*-activation of the *bfl-1* promoter in EBV-positive BL and LCL backgrounds, (iv) mutating both the

CBF1-binding site and a NF-κB-like motif of the *bfl-1* promoter leads to almost complete inhibition of *bfl-1* promoter *trans*-activation in an EBV-positive cell line.

3.2 Results

3.2.1 Examination of potential functional differences in EBNA2A and EBNA2B isolates

It has been firmly established that type A EBNA2 can directly up-regulate *bfl-1* in B cell lines. Bearing in mind that *trans*-activation of *bfl-1* is such an important event in the B cell immortalisation process, this study then set out to investigate if there was a significant functional difference between the capabilities of EBNA2A and EBNA2B, with regards to the transcriptional regulation of *bfl-1*. The objectives in this section were: (i) To characterise two mammalian expression vectors containing the different isolates of EBNA2, and to confirm the expression of each EBNA2 isotype at the correct size and at a similar expression level by Western blotting, (ii) To determine whether the Notch-responsive promoter construct, pGa981.6, is preferentially regulated by either type of EBNA2 in an EBV-negative BL and (iii) to examine the *bfl-1* promoter activity in response to EBNA2A and EBNA2B expression, in an EBV-negative BL cell line.

Two different EBNA2 expression vectors containing either the EBNA2A open reading frame (ORF) (pSG5-EBNA2A) or the EBNA2B ORF (SV-EB2NA2) were obtained. pSG5-EBNA2 consists of wild type B95-8 EBNA2 coding sequence cloned into the pSG5 expression vector (Ling, et al. 1993) (Figure 3.1A). SV-pEB2NA2 consists of the EBNA2 coding region from the digested EBV *Bam*HI D1 fragment isolated from the AG876 cell line, sub-cloned into pSG5 (Wang, et al. 1990). For clarity, the EBNA2B expressing vector SV-EB2NA2 will be referred to as pSG5-EBNA2B in the remainder of this study. These constructs were characterised using restriction digest analysis (Figure 3.1B). In order to verify the expression of EBNA2A and EBNA2B protein from these constructs, the EBV-negative cell line DG75 was transiently transfected with pSG5-EBNA2A or pSG5-EBNA2B, or the corresponding empty vector (pSG5). Total protein was prepared 48 hours post-transfection and the presence of EBNA2A and EBNA2B at the expected size was

confirmed by Western blotting (Figure 3.1C). Protein extracts from cell lines known to express each EBNA2 isoform were used as positive controls. The LCL IB4 contains the type A EBV strain and expresses the EBNA2A protein; however, the EBV-positive BL, AG876, carries the type B strain and thus expresses the EBNA2B protein (For summary of cell lines see Table 2.1).

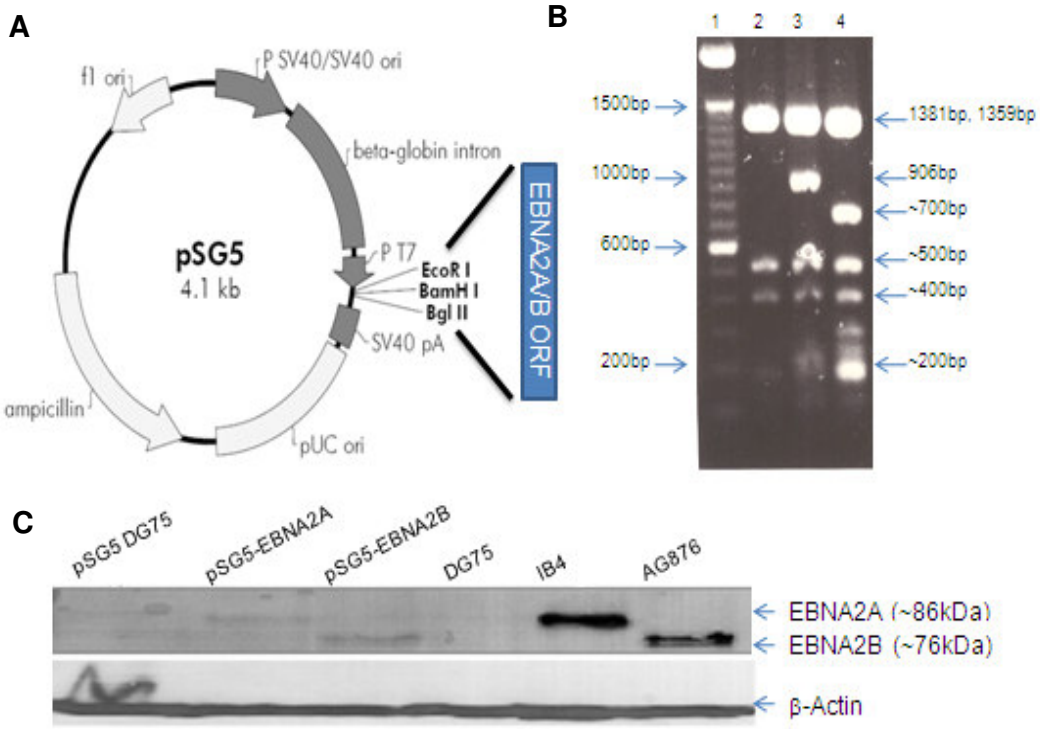


Figure 3.1: Characterisation of type A and B EBNA2 expression vectors.

(A) Schematic circular map of the pSG5 expression vector. Highlighted is the region into which the EBNA2A or EBNA2B ORF was sub-cloned. The human Simian Virus 40 immediate-early promoter (pSV40) and origin of replication (SV40 ori) are capable of supporting high-level expression in mammalian cells. The vector also contains the ampicillin resistance gene to facilitate selection in bacterial cells. **(B)** Restriction analysis of pSG5, pSG5-EBNA2A and pSG5-EBNA2B using *Hinf* I enzyme. pSG5 (lane 2) yields signature fragments at 1359bp, 1381bp, 517bp, 396bp and a number of very low molecular weight fragments upon digestion with *Hinf* I. pSG5-EBNA2A (lane 3) encompasses additional fragments at 906bp and ~200bp. pSG5-EBNA2B (lane 4) contains the key fragments of pSG5 in addition to fragments at ~700bp and ~200bp. A 1kb ladder is seen in lane 1. **(C)** The expression of EBNA2 from the constructs was confirmed by transient transfection in the EBV-negative cell line DG75. DG75 cells were transfected with either 7µg of pSG5-EBNA2A, or 7µg of pSG5-EBNA2B, samples were harvested 48 hours post-transfection and were detected in the upper panel, using PE2 (Dako, M7004) and normalised in the lower panel for the endogenous loading control β-Actin. The empty pSG5 expression vector was used as a control. Protein extracts from the cell lines IB4 and AG876 were used as positive controls for EBNA2A and EBNA2B respectively.

The two EBNA2-expressing plasmids were characterised by restriction digest analysis using *Hinf* I which cuts the construct at several sites, giving a distinct fragment pattern that can discriminate between the two EBNA2 expressing vectors (Figure 3.1B). The EBNA2A construct yields a unique band at 906bp which is not present in the empty vector or the EBNA2B expression vector. Likewise upon digestion the EBNA2B expression vector yields an identifying fragment at approximately 700bp. It can be seen that the EBNA2A and EBNA2B proteins were expressed at the anticipated sizes, EBNA2A (~86kDA) and EBNA2B (~76kDA) in accordance with the control for each protein (Figure 3.1C). Western blot analysis also revealed that both vectors expressed EBNA2 at the expected sizes; however, a slightly more intense band was observed for EBNA2B.

3.2.2 Differential effect of EBNA2A and EBNA2B isolates on Notch responsive promoter activity in the DG75 cell line.

To compare the *trans*-activation efficiency of the two types of EBNA2, a Notch responsive construct, pGa981.6 was employed. pGa981.6 is an established luciferase reporter vector that is sensitive to CBF1-driven activity. The EBV TP1 Promoter has two CBF1 binding sites that drive its activity. A 50bp oligonucleotide harbouring both CBF1 binding sites of the EBV *TP1* promoter had been sub-cloned as a hexamer into the plasmid pGa50-7 (Minoguchi, et al. 1997), and EBNA2 is a well established *trans*-activator of this Notch responsive reporter construct. To compare the *trans*-activation capabilities of EBNA2A and EBNA2B, DG75 was co-transfected with increasing quantities of either of the two EBNA2 expression plasmids, pSG5-EBNA2A and pSG5-EBNA2B (Table 2.5), together with 2 µg pGa981.6 and the pCMV-LacZ reporter construct. Forty-eight hours after transfection, cells were harvested (Section 2.2.3.13), and reporter enzyme activities (luciferase and β-galactosidase) were measured (Sections 2.2.3.14 and 2.2.3.15).

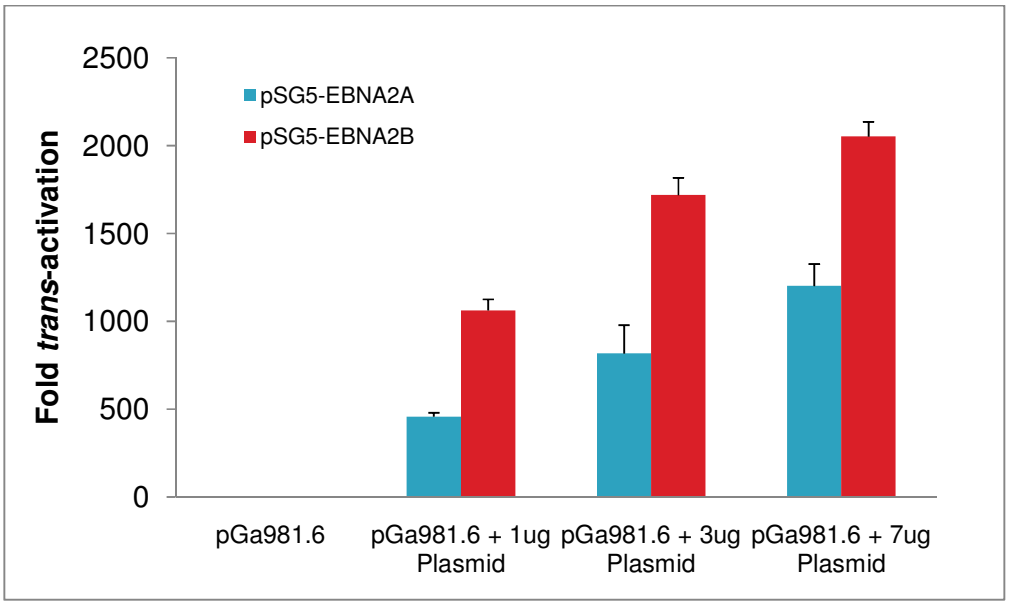


Figure 3.2: Both EBNA2 types *trans*-activate pGa981.6 in a dose dependent manner.

DG75 cells were co-transfected with 2µg pGa981.6 Luc and 2µg pCMV LacZ, along with increasing quantities of either the EBNA2A or EBNA2B expression vector. The effect of EBNA2 expression on the Notch responsive construct was monitored by luciferase assay and β-gal normalisation 48 hours post-transfection. Fold differences were calculated relative to pGa981.6 levels in triplicate experiments; arbitrarily assigned a value of 1. (Data are mean ±SD).

It can be seen that both EBNA2A and EBNA2B expression in DG75 results in a dose responsive *trans*-activation of the Notch receptive vector pGa981.6 (Figure 3.2). Furthermore, a more potent level of *trans*-activation was observed due to the expression of EBNA2B in contrast to the expression of EBNA2A.

3.2.3 *Trans*-activation of the *bfl-1* promoter by EBNA2A and EBNA2B in the DG75 cell line.

A similar strategy was used in order to investigate EBNA2A/EBNA2B trans-activation of the *bfl-1* promoter. DG75 cells were co-transfected with increasing concentrations of the individual EBNA2 expression plasmids, (Table 2.5), together with the reporter construct -1374/+81 *bfl-1* Luc (this plasmid contains the -1374 to +81 nucleotide sequence flanking the transcription start site of the *bfl-1* gene joined to the luciferase-coding gene). Cells were harvested 48 hours post-transfection and luciferase values were determined and normalised for β-galactosidase expression as described.

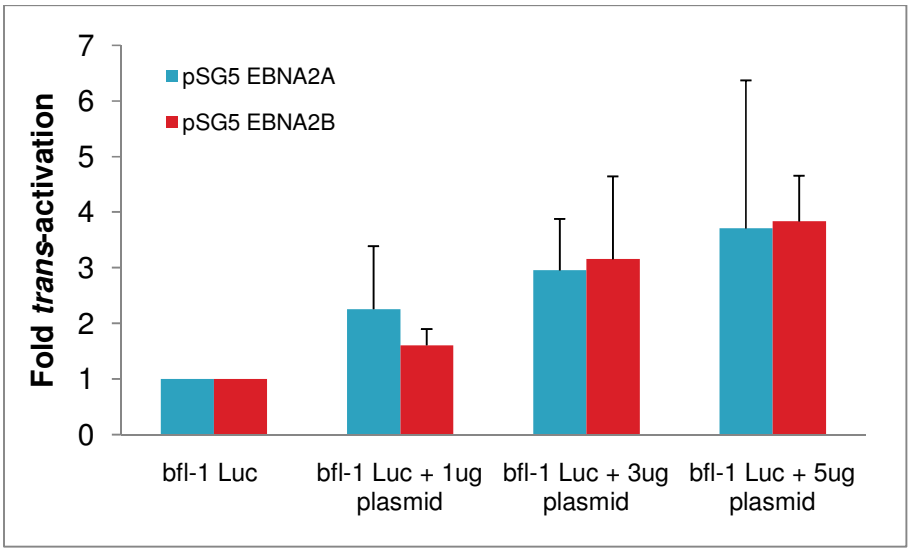


Figure 3.3: Both EBNA2A and EBNA2B *trans*-activate the *bfl-1* promoter in a dose dependent manner.

DG75 cells were co-transfected with 2 µg *bfl-1* Luc and 2µg pCMV LacZ, along with increasing quantities of either the EBNA2A or EBNA2B expression vector. The cells were harvested 48 hours post-transfection and the effect of EBNA2 expression on the *bfl-1* promoter construct was monitored by luciferase assay and β-gal normalisation. Fold differences were calculated relative to -1374/+81 *bfl-1* luc activity in triplicate experiments; arbitrarily assigned a value of 1. (Data are mean ±SD).

In this experiment it was found that co-transfection with increasing quantities of each individual EBNA2 expression vector led to dose-dependent increase in *bfl-1* driven luciferase activity. Furthermore, this *trans*-activation occurred to a similar degree for both EBNA2 isoforms (Figure 3.3).

3.2.4 Role of a consensus CBF1 binding site in the *bfl-1* promoter

It had been demonstrated previously in the laboratory that *bfl-1* is transcriptionally regulated by both LMP1 and EBNA2. In an LCL background, the loss of either LMP1 or EBNA2 did not result in decreased *bfl-1* expression; however the simultaneous loss of the two proteins resulted in a dramatic decrease in *bfl-1* mRNA levels (Pegman, et al. 2006). The use of NF-κB inhibitors revealed that LMP1-led up-regulation of *bfl-1* is NF-κB dependent. A putative NF-κB site at (-52 to -43) was identified on the *bfl-1* promoter and mutation of this site (reporter construct -1374/+81 mκB(-52)) resulted in the complete loss of LMP1-associated *trans*-activation (D'Souza, et al. 2004). In addition to this, a similar study had been performed to identify the mechanism behind EBNA2-led *trans*-activation of *bfl-1*. Inhibition of the NF-κB pathway did not affect the EBNA2-mediated activation of *bfl-1*, indicating that *trans*-activation of *bfl-1* by LMP1 and EBNA2 were mechanistically different. Co-expression with EBNA2 of a dominant negative CBF1 mutant revealed that CBF1 involvement was required for EBNA2 activation of *bfl-1*. A consensus CBF1 binding sequence was identified and, using a mutated *bfl-1* promoter reporter construct (-1374/+81 mCBF1(-249)), this site was confirmed as the essential motif required for its regulation by EBNA2 (Pegman, et al. 2006). The objective of this part of the study was (i) to generate a double mutant encompassing mutations at the NF-κB site (-52) and the CBF1 site (-249) and (ii) to determine how these combined mutations affected the transcriptional up-regulation of *bfl-1* in an EBV-infected cell line in which both EBNA2 and LMP1 are co-expressed.

3.2.4.1 Generation of two mutant *bfl-1* promoter constructs using site directed mutagenesis

Site-directed mutagenesis [QuikChange XL site-directed mutagenesis (Stratagene)] was used to generate two *bfl-1* promoter mutants (i) the parental *bfl-1* promoter-reporter vector, -1374/+81 *bfl-1 luc,* was mutated at the CBF1 motif to yield -1374/+81 mCBF1(-249) *luc* and (ii) the corresponding vector with its NF-κB-binding site already mutated, -1374/+81 mκB(-52), was further altered at the CBF1-binding site to generate the double mutant construct -1374/+81 mκB(-52) mCBF1(-249) (Figure 3.4).

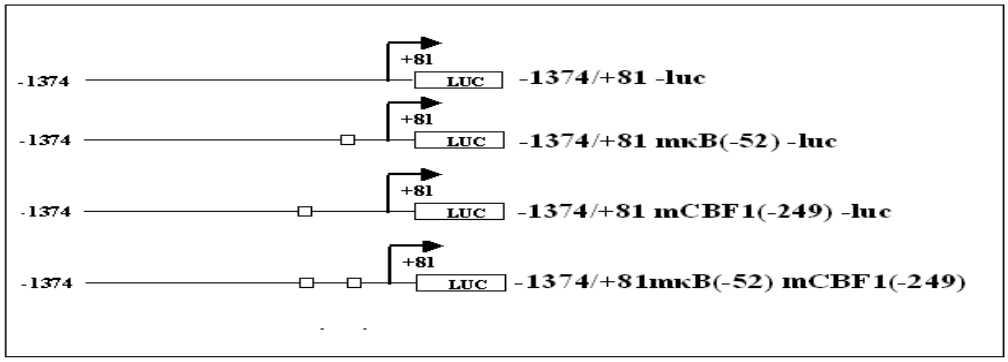


Figure 3.4: Schematic of the four *bfl-1* promoter constructs used for this study.

The promoter construct -1374/+81 *bfl-1 luc* was generated by amplification of the -1374/+81 region of the *bfl-1* promoter from a pCAT based vector, followed by sub-cloning into the *Xho* I and *Hind* III sites of the promoterless pGL2 basic reporter vector (D'Souza, et al. 2004). A mutation of the NF-κB motif (-52) was introduced into this promoter construct using site directed mutagenesis to generate -1374/+81 mκB(-52) (D'Souza, et al. 2004). In this study, site directed mutagenesis was employed to mutate the CBF1 binding site of the full length *bfl-1* promoter to generate -1374/+81 mCBF1(-249) *luc* and in the NF-κB mutated promoter to generate -1374/+81 mκB(-52) mCBF1(-249).

QuikChange XL site-directed mutagenesis allows mutation of methylated double-stranded plasmid DNA. This works by utilising a primer designed with significant similarity to the target region such that they can bind and amplify the target; however, point mutation/s incorporated into the primers allow amplification of a mutated product (Figure 3.5), facilitated by a high fidelity DNA polymerase, *pfuTurbo.* Amplification of the mutated product is followed

by digestion with the selective endonuclease, *Dpn*I, which digests only methylated and hemi-methylated DNA. The methylated parental DNA is digested by *Dpn*I allowing selection of the mutant plasmid. The resulting DNA can then be transformed into *E. coli* XL1-Blue cells, followed by purification and screening of the clones.

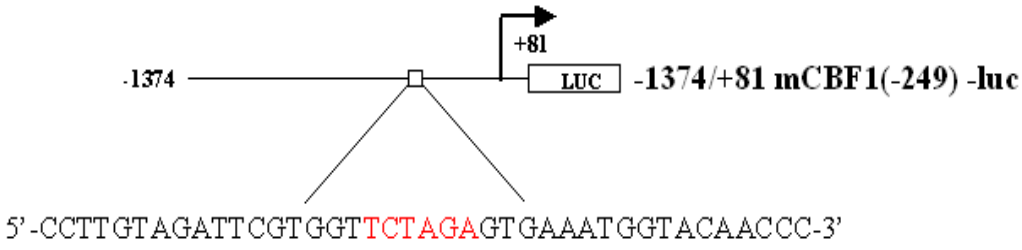


Figure 3.5: Oligonucleotide incorporating CBF1 Mutation.

The 40 base oligonucleotide is complementary to the *bfl-1* promoter CBF1 recognition motif. The highlighted centre region indicates the 6 base altered sequence for the CBF1 motif, shown in red. This mutation introduces a new restriction site for the enzyme *Xba* I (TCTAG), therefore allowing efficient screening of positive clones.

All purified clones were screened by restriction enzyme analysis with the enzyme *Xba* I. The pGL2 reporter construct already contains an *Xba* I restriction site; thus, the introduction of this second *Xba* I sites yields a DNA fragment of approximately 400bp upon digestion with *Xba* I. Digested clones were analysed by agarose gel electrophoresis, and positive clones were identified by the presence of an additional fragment at approximately 400bp.

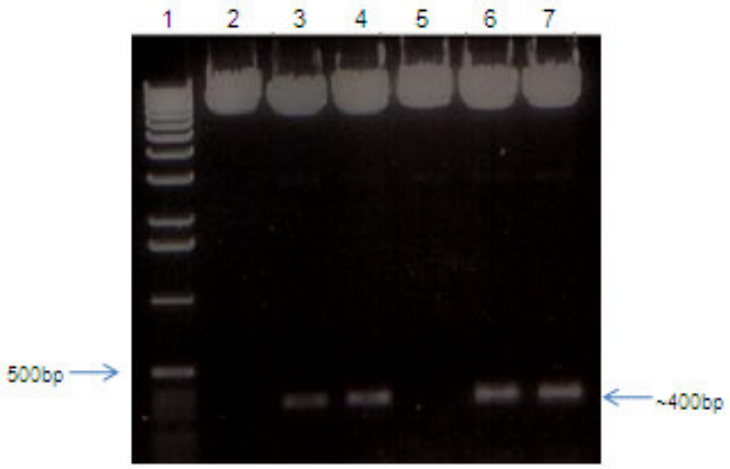


Figure 3.6: Screening of clones using *Xba* I restriction analysis.

All DNA was digested by *Xba* I and analysed using a 1% agarose-1X TAE gel and electrophoresis was carried out at 100V for 1 hour in 1X TAE. Lane 1 contains a 1 Kb ladder, lanes 2-7 are *Xba1* digests: lane 2 contains -1374/+81 *bfl-1 luc;* lanes 3 and 4 contain -1374/+81 mCBF1(-249) *luc* positive clones yielding the indicative fragment at ~400bp; lane 5 contains the -1374/+81 mκB(-52) plasmid, followed by the double-mutant clones -1374/+81 mκB(-52) mCBF1(-249) in lanes 6 and 7, also yielding the ~400bp fragment due to the incorporation of the second *Xba* I site.

The consensus CBF1 binding site was successfully altered in the template plasmids to generate the two new constructs -1374/+81 mCBF1(-249) *luc* and -1374/+81 mκB(-52) mCBF1(-249). To determine if simultaneous inactivation of the NF-κB-binding motif at (-52) and the CBF1-binding motif at (-249) altered the EBV-modulation of *bfl-1* activity, three different EBV-positive cell lines were transfected with these constructs and assayed for *bfl-1* promoter activity using the luciferase assay.

3.2.4.2 Regulation of *bfl-1* promoter constructs in the EBV-positive BL, AG876

The EBV-positive BL-derived cell line, AG876, was transiently transfected with the four *bfl-1* promoter constructs described previously (Figure 3.4). The full complement of EBV latent genes is expressed in AG876. Both EBNA2 and LMP1 are known trans-activators of *bfl-1* and as such, within this cell line, the *bfl-1* promoter activity can be driven by both EBV proteins. Cells were harvested 48 hours post-transfection and luciferase levels in the cells were quantified using a luminoskan 1400 Luminometer and normalised for β-galactosidase activity.

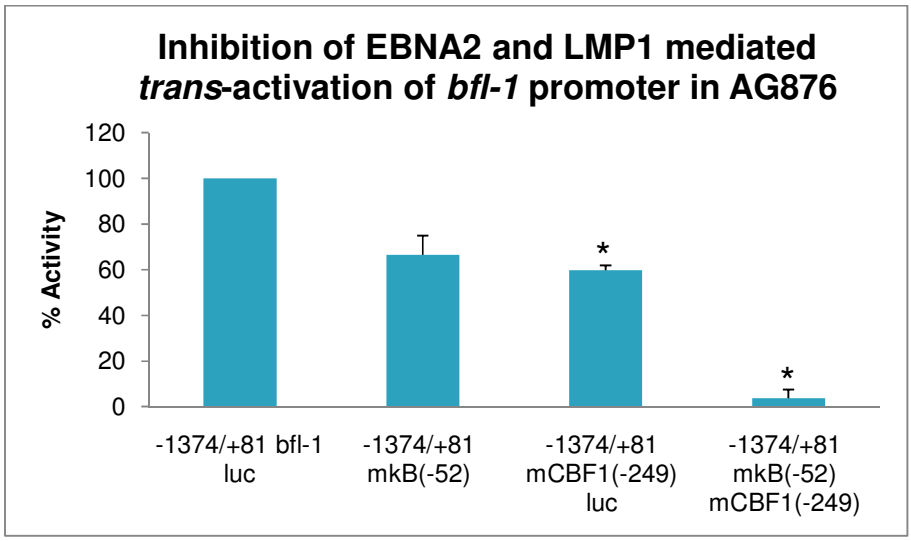


Figure 3.7: Mutation of NF-κB-like and CBF1-like binding sites on the *bfl-1* promoter inhibits *trans*-activation by EBV.

The AG876 cell line was transfected with 2 µg of each of the *bfl-1* promoter constructs (indicated underneath). Cells were harvested 48 hours post-transfection, and normalised luciferase values were expressed as percentage activity relative to that obtained from the unmutated *bfl-1* promoter on -1374/+81 *bfl-1 luc*, which was set at 100% activity. (Data are Mean ±SD, * P<0.05).

It can be seen that mutation of the NF-κB-like binding site alone [-1374/+81 mκB(-52)] resulted in approximately a 30% decrease in *bfl-1* promoter activity, while inactivation of the CBF1 binding site alone [-1374/+81 mCBF1(-249)], caused a 40% decline in *bfl-1* promoter activity. Furthermore, mutating both site [-1374/+81 mκB(-52) mCBF1(-249)] resulted in an almost complete loss of *bfl-1* promoter activity.

3.2.4.3 Regulation of the *bfl-1* promoter in the LCL, ER/EB2.5

ER/EB2.5 is an EBV-infected B cell line, as opposed to a BL-derived cell line. ER/EB2.5 cells were transiently transfected with the four *bfl-1* promoter constructs described above (Figure 3.4). The full complement of EBV latent genes is expressed in ER/EB2.5, in an LCL background. Cells were harvested 48 hours post-transfection and assayed for luciferase activity as previously described.

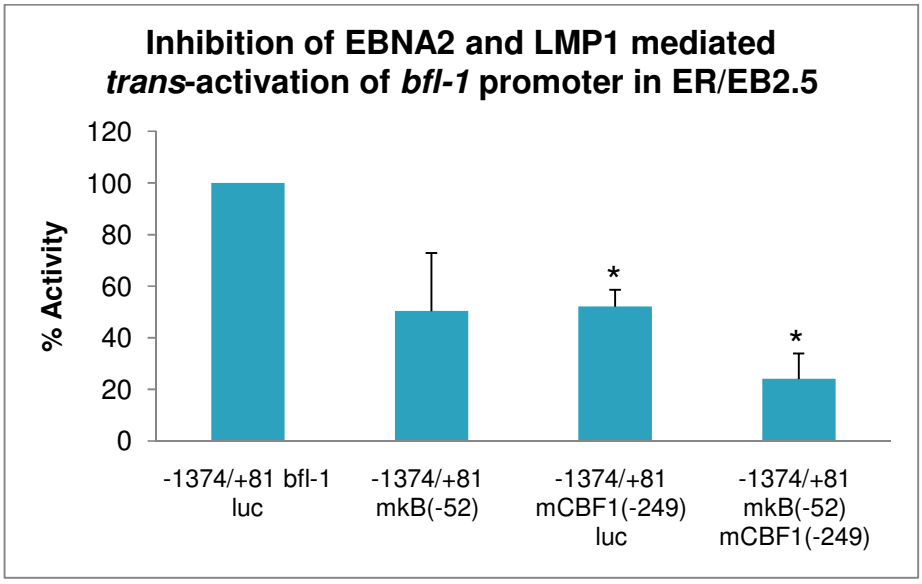


Figure 3.8: Mutation of NF-κB-like and CBF1-like binding sites on the *bfl-1* promoter reduces *trans*-activation by EBV.

The ER/EB2.5 cell line was transfected with 2 µg of each *bfl-1* promoter construct (indicated underneath each bar). Cells were harvested 48 hours post-transfection and normalised luciferase results were expressed as percentage activity relative to that obtained from -1374/+81 *bfl-1 luc*, which was set at 100% activity. (Data are Mean ±SD, * P<0.05).

As with the AG876 cell line, the wild type *bfl-1* promoter, -1374/+81 *bfl-1* luc was set as 100% promoter activity. The findings from this experiment indicate that transfection with -1374/+81 mκB(-52), resulted in approximately a 50% decrease in *bfl-1* promoter activity, while the -1374/+81 mCBF1(-249) *luc* construct resulted in approximately a 45% decrease in *bfl-1* promoter activity. Furthermore, as observed previously in AG876 (Section 3.2.4.2) mutating both the NF-κB-like binding site and the CBF1-like binding site, -

1374/+81 mκB(-52) mCBF1(-249), resulted in a further significant loss of *bfl-1* promoter activity; however in ER/EB2.5 this inhibition was not to the same extent as that seen in AG876. It is worth noting that ER/EB2.5 cells were also transfected with the empty pGL2 reporter vector, and this gave, on average, approximately 18% of the activity observed for the unmutated *bfl-1* promoter-reporter construct. This indicates the production of luciferase from the promoterless basal vector in this cell line.

3.2.4.4 Regulation of *bfl-1* promoter constructs in the EBV-positive LCL, IB4

The LCL IB4 was transiently transfected with the four *bfl-1* promoter constructs described above (Figure 3.4). Cells were harvested 48 hours post-transfection and assayed for luciferase activity as previously described.

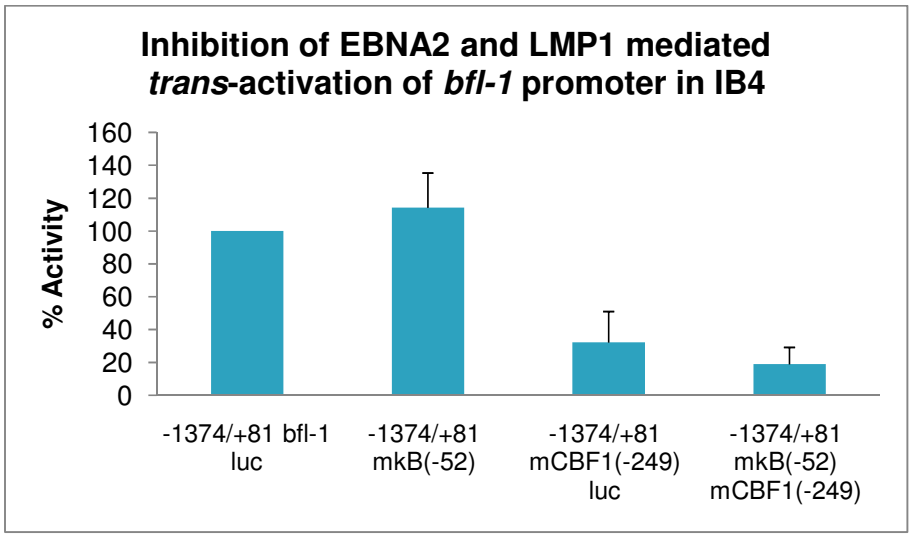


Figure 3.9: Mutation of NF-κB-like and CBF1-like binding sites on the *bfl-1* promoter inhibits *trans*-activation by EBV.

The IB4 cell line was transfected with 2 µg of each of the four *bfl-1* promoter constructs as indicated above. Cells were harvested 48 hours post-transfection, normalised luciferase results were expressed as percentage activity relative to that obtained from the wild type *bfl-1* promoter on -1374/+81 *bfl-1 luc*, which was set at 100% activity. (Data are Mean ±SD).

As with previous experiments in this study, the wild type *bfl-1* promoter, -1374/+81 *bfl-1* luc was set as 100% promoter activity. Surprisingly, this experiment indicated that transfection with -1374/+81 mκB(-52), had no effect on *bfl-1* promoter activity, while elimination of the consensus CBF1-binding site [-1374/+81 mCBF1(-249) *luc*] resulted in an approximately 68% decrease in *bfl-1* promoter activity. Furthermore, as observed above in AG876 and EREB2.5 cells (Sections 3.2.4.2 and 3.2.4.3), mutating both the NF-κB-binding site and the CBF1-binding site [-1374/+81 mκB(-52) mCBF1(-249)], resulted in a further decline in *bfl-1* promoter activity (cumulative loss

of 80% relative to the activity observed for the unmutated *bfl-1* promoter-reporter construct).

3.3 Discussion

The regulation of *bfl-1* by EBV has been demonstrated previously (Pegman, et al. 2006; D'Souza, et al. 2004; D'Souza, et al. 2000). In addition, an infection of naïve B cells with EBV was carried out in the present study (Described in chapter 4) and an analysis of RNA levels by RT-qPCR confirmed the up-regulation of *bfl-1* (134-fold increase) at 3 days post-infection (Table 4.1). The mechanism behind EBV-associated *bfl-1* up-regulation was further examined here. The evidence from this investigation indicates that there was no significant difference in the regulation of the *bfl-1* promoter in response to either EBNA2A or EBNA2B in an EBV-negative BL background. Bfl-1 expression from each vector was confirmed using Western blotting with the EBNA2 specific antibody, PE2, which has been confirmed to detect EBNA2A and EBNA2B with approximately equal efficiency (Lucchesi, et al. 2008). The expression level of ectopic EBNA2B was marginally higher than that of EBNA2A. Reporter studies indicated that EBNA2B *trans*-activated the CBF1 responsive vector pGa981.6 to a greater degree than EBNA2A, with approximately a 2-fold increase observed in the response to EBNA2B in comparison to EBNA2A (Section 3.2.2). The resulting difference in the potency of EBNA2A and EBNA2B mediated *trans*-activation of pGa981.6 was not statistically significant. Taking the relative ectopic levels of EBNA2A/2B into account, however (Figure 3.1), may account for the difference in promoter *trans*-activation observed. It is unlikely that there is any significant difference in the abilities of EBNA2A or EBNA2B to *trans*-activate the Notch responsive promoters.

Mutation at the LMP1-responsive consensus NF-κB site in the *bfl-1* promoter resulted in reduced *bfl-1* promoter *trans*-activation in AG876 and ER/EB2.5. However, mutation at the same site resulted in no detectable change in activity in the IB4 cell line. Furthermore, in the two cell lines in which modulation was observed for the NF-κB mutant the results were below the lower significance limit of $P<0.05$. It has been demonstrated that when LMP1 is expressed as the sole EBV protein in an EBV-negative BL, that the

presence of this NF-κB mutation in the *bfl-1* promoter construct prevents LMP1-mediated *trans*-activation (D'Souza, et al. 2004). However, it can be determined from published data, combined with the results from this experiment, that in these EBV positive cell lines, EBNA2 can compensate for the loss of LMP1 with respect to *bfl-1* regulation.

The CBF1 recognition site has been shown to be highly conserved, and the mutation introduced into the consensus CBF1 site in the *bfl-1* promoter was chosen because it was a published verified mutation sequence that has been shown, via electrophoretic mobility shift assay, to abrogate CBF1 binding (Tun, et al. 1994). The inactivation of the CBF1-binding site in the *bfl-1* promoter led to statistically significant down-regulation in *bfl-1* promoter activity in AG876 and EREB2.5 to a confidence interval of $P>0.05$. In IB4, a consistent reduction in *bfl-1* activity of between 40-80% was also observed; however the extent of this modulation varied to such a degree so as to not be statistically significant. The statistically significant loss of *bfl-1* activity from the single CBF1 site mutation in comparison to the single NF-κB site mutation in the same cell background indicates a more important role for EBNA2 here regarding *bfl-1 trans*-activation. Other published data from our laboratory showed that the loss of LMP-1 or EBNA2 resulted in little or no reduction in the steady state mRNA levels for *bfl-1* (Pegman, et al. 2006). Interestingly, in all three cell lines, the combined mutations at the CBF1- and NF-κB-binding sites resulted in a minimum of 80% inhibition in *bfl-1* promoter *trans*-activation, with almost complete loss of *bfl-1* activity in AG876. This implies that both sites are required for maximal EBV-mediated *bfl-1* regulation.

Chapter 4: *Regulation of cellular gene expression following EBV infection: Transducin-Like Enhancer of Split 1 (TLE1) is a negative target of EBV.*

4.1 Introduction

EBV is a ubiquitous human herpes virus that successfully establishes a lifelong infection in the host memory B cell compartment (Babcock, et al. 1998). The survival capacity of EBV is due to its strategy of subverting normal cellular signaling pathways, counterbalancing natural processes such as host immune detection and inherent cell fate for its own benefit (Middeldorp and Pegtel 2008). EBV has evolved many mechanisms to evade the host immune response and hematopoietic regulation. EBV latent gene products modulate B cell pathways to promote host cell survival, prevent differentiation into a plasma cell, and increase the threshold of resistance to apoptotic signals. A broad scope of cellular genes has been shown to be regulated either directly or indirectly by EBV.

EBV immortalised LCLs express all known latent viral gene products, including six nuclear antigens EBNA1, EBNA2, EBNA3A, -B, -C and EBNA-LP, three latent membrane proteins LMP1, LMP2A and LMP2B along with the non-polyadenylated RNAs, EBER1 and EBER2 (Kerr, et al. 1992). The proliferative capacity of these cells is due to EBV and studies with LCLs therefore facilitate the examination of the established type III latent infection state. In the immortalised LCL, however, EBV has already achieved cellular control by subversion of the relevant signaling pathways and regulation of EBV-specific cellular targets. It is important, therefore, to study the host-virus interactions that occur earlier during B cell activation and LCL outgrowth, and to rule out events that occur due to long-term culturing of infected cells.

In this chapter, the purification and infection of naïve B lymphocytes with a recombinant GFP-expressing EBV strain is described. In that experiment, RNA was prepared before and after infection (early during LCL outgrowth), and the levels of expression of selected cellular genes were investigated by reverse transcription semi-quantitative polymerase chain reaction (RT-qPCR). Novel genes modulated upon B cell infection were thus identified. One interesting negative target of EBV was discovered to be *TLE1*. Evidence is presented that EBV latency growth programme proteins repress the levels

of *TLE1* mRNA and protein in an LCL. The regulation of TLE family members in LCLs and BL cell lines was concluded to be complex.

4.2 Results

4.2.1 Identification of candidate genes whose expression is modulated following EBV infection of naïve B cells *in vitro*

In order to confirm the infectivity of the stock of GFP-tagged EBV (EBV BX1) (a gift of L. Hutt-Fletcher, Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, LA), an EBV-negative B cell line, Akata4E3, was first infected with this virus. A mock infection was also performed in tandem, in which no EBV was used. Twenty four hours after infection the cells were monitored for GFP expressed from the recombinant virus using FACS (Figure 4.1A). It can be seen that GFP-expressing cells were clearly evident following infection with EBV BX1, but not in the mock-infected cells (Figure 4.1A). In addition, expression of the early EBV latent gene, *EBNA2*, was confirmed in EBV BX1-infected cells using RT-PCR (Figure 4.1C). These experiments confirmed that the stock of EBV BX1 contained infectious virus particles.

Naïve B cells were then isolated from human PBMC by negative selection with a cocktail of magnetically labelled antibodies against various immune cell markers (MACS naive B-cell isolation kit II). Immune cells such as non-naïve B cells, T cells, NK cells, dendritic cells display a variety of distinct surface (markers not present on naïve B cells) and therefore bind labelled antibodies. One such marker is CD27, which is displayed on memory B cells but not naïve B cells. Passage of the labelled cell population through a magnetic field led to the depletion of all but unbound naïve B cells. Naïve B cells display CD19, the pan-B cell surface marker, but not CD27 (CD19-positive, CD27-negative). Naive B cell enrichment was verified by Flow Cytometry (FACS analysis) after dual staining with anti-CD19 and anti-CD27 antibodies (Figure 4.1B). It can be seen that, as expected, the PBMC population contained both naive B cells and memory B cells. Following enrichment by negative selection, however, naïve B cell enrichment was evident from the purified population of CD19-positive/CD27-negative cells observed.

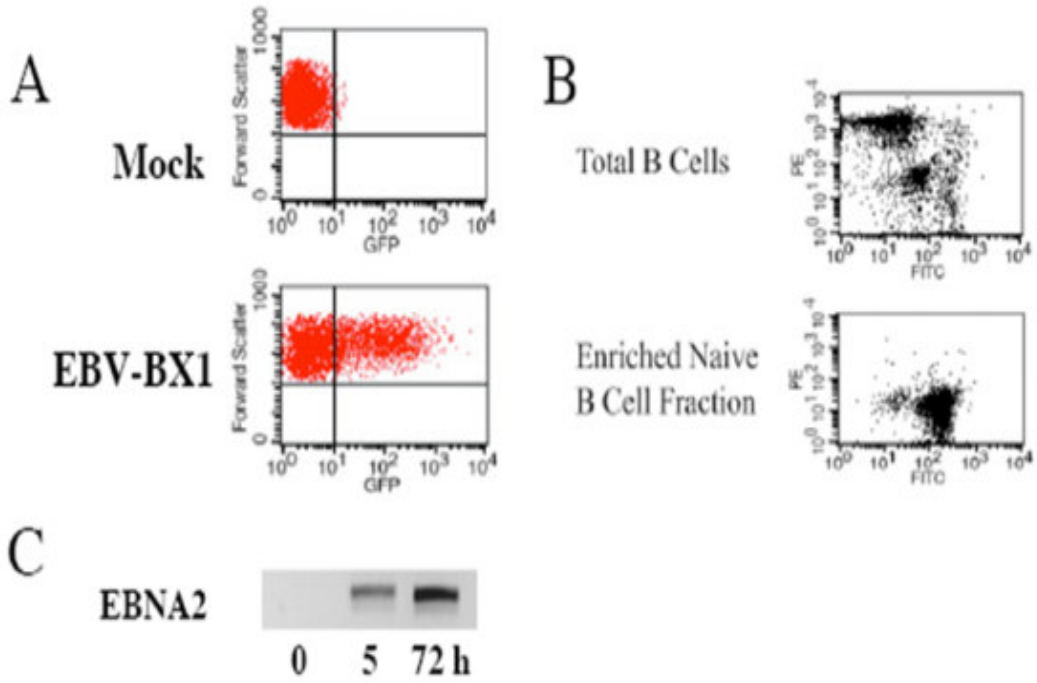


Figure 4.1: Preparation of recombinant EBV and isolation of untouched naïve human B cells.

(A) Akata 4E3 BL cells were infected with purified EBV-BXI, harvested at 24 hours and analysed by FACS. GFP expression levels were compared in mock-infected (upper) and untreated-EBV-infected (lower) cells. **(B)** Naïve B cells were separated from PBMC by negative selection. PBMC and the naïve B-cell fractions were stained with PE-labeled anti-CD27 antibody (memory B-cell marker) and FITC-labeled anti-CD19 antibody (pan-B-cell marker) and analysed by FACS. **(C)** RT-PCR detection of EBNA2 transcript at various time points following infection with GFP-tagged EBV.

EBV BX1 was then used to infect isolated naïve B cells (Section 2.2.3.7). Three days following infection, characteristic large blast-like cells were frequently in evidence, indicative of B cell growth transformation taking place (not shown). No such observations were to be made when mock-infected cells were examined.

Total RNA was then prepared from the infected cell population, and the relative expression levels of selected gene sets were evaluated using three PCR gene arrays: (i) the Human Apoptosis RT² Profiler™ PCR Array; this consisted of a panel of 84 key genes associated with apoptosis. The array included the TNF ligands and their receptors; members of the *bcl-2*, *TRAF* and *CIDE* families, (ii) the Human Notch Signaling Pathway RT² Profiler™

PCR Array, which included 84 genes involved in Notch signaling such as those encoding Notch-binding and receptor-processing proteins and several putative Notch target genes, and (iii) the Human Wnt Signaling Pathway RT² Profiler™ PCR Array which was composed of 84 genes related to Wnt-mediated signal transduction. Each array consisted of a 384-well plate and triplicate analysis was performed for each gene examined. Each array panel contained an additional five housekeeping genes for the normalisation of results, along with controls for genomic DNA contamination, RNA quality, and general PCR performance. In this experiment, analysis of the results obtained led to the compilation of a shortlist of fourteen cellular genes that showed the most interesting variations in expression in response to EBV infection (see table 4.1; other data from the PCR arrays are not the subject matter of this thesis). The potential candidates for EBV regulation were chosen based on a number of criteria including; (i) change in expression levels pre- and post-EBV infection; (ii) association with EBV targeted signaling pathways; (iii) structural/functional homology with known EBV targets and (iv) novelty with respect to published information on EBV regulation. The potential regulation by EBV of one of these selected genes, the *TLE1* gene, is the subject matter of the rest of this chapter; the other genes are discussed in subsequent chapters.

Gene Name	Description	N	3D	Up/Down Regulation
bfl-1	Bcl-2 related protein A1	33	26	134
CCNE1	Cyclin E1	32	24	286
CIDEB	Cell death-inducing DFFA-like effector b	34	15	502931
FAS	Fas (TNF receptor superfamily, member 6)	35	26	605
Fos	V-fos FBJ murine osteosarcoma viral oncogene homolog	20	29	648
Jun	V-jun sarcoma virus 17 oncogene homolog (avian)	23	31	201
LRP5	Low density lipoprotein receptor-related protein 5	32	27	57
		32	28	19
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	35	27	317
TNFSF7	Tumor necrosis factor (ligand) superfamily, member 7	35	26	626
TRAF4	TNF receptor-associated factor 4	35	28	133
NR4A2	Nuclear receptor subfamily 4, group A, member 2	26	29	14
STIL	SCL/TAL1 interrupting locus	31	24	129
Hey1	Hairy/enhancer of split related with YRPW motif 1	34	27	121
TLE1	Transducin-like enhancer of split 1	25	30	43
		24	30	44

Table 4.1: Fourteen selected genes showing altered regulation by EBV infection of Naive B cells.
Results obtained from the RT Profiler PCR SuperArray. The resulting fold change in mRNA expression levels from samples taken from negatively purified Naive B cells (N) against cells taken 3 days post-infection with EBV BX1 (3D). Fold regulation can be seen in the far right column. Changes highlighted in blue indicate down-regulated genes while entries denoted in red signify genes up-regulated after EBV infection. LRP5 and TLE1 have two results as they were present in more than one cell signaling pathway gene panel.

4.2.2 *Transducin-Like Enhancer of Split 1 (TLE1) is a candidate novel negative target of EBV*

TLE1 was identified as a potential cellular EBV target gene from the RT Profiler PCR SuperArray results presented in section 4.2.1. Three TLE family members were included on the RT Profiler PCR SuperArray: *TLE1, TLE2* and *AES*, as shown in table 4.2, of which only *TLE1* appeared to be modulated following EBV infection. Altered *TLE1* mRNA levels were obtained with comparable potency from two different RT Profiler PCR SuperArrays containing signature genes from the Notch and Wnt signaling cascades.

Gene Name	Description	N	3D	Up/Down Regulation
TLE1	Transducin-like enhancer of split 1	24.74	30.18	43.4
		24.15	29.6	43.7
TLE2	Transducin-like enhancer of split 2	31.4	31.1	-
AES	Amino-terminal enhancer of split	22.78	22.81	-

Table 4.2: Expression pattern of TLE family members in naïve B cells pre- and post-EBV infection.
Results obtained from the RT Profiler PCR SuperArray. The resulting fold change in mRNA expression levels from samples taken from negatively purified Naïve B cells (N) against cells taken 3 days post-infection with GFP-tagged EBV (3D). Fold regulation can be seen in the far right column. Changes highlighted in blue indicate down-regulated genes while entries denoted in yellow signify genes unaffected after EBV infection. Genes that have two results are present in more than one cell signaling gene panel.

There was an approximate 43-fold decrease in *TLE1* mRNA expression after the EBV infection of naïve B lymphocytes. This down-regulation was evident in each of the RT Profiler PCR SuperArray experiments. However, no changes were observed in the relative mRNA levels of the *TLE* family members, *TLE2* and *AES*.

4.2.3 *TLE1* is repressed by the EBV growth programme in the conditional LCL ER/EB2.5

The isolation and validation of a naïve B cell population is costly, and the continued use of untouched naïve B cells for analysis was not feasible for the duration of this project. Therefore, a well studied cell culture model capable of mimicking the B cell infection process was utilised to continue studies on the novel candidate EBV target genes. Initially, the gene expression changes observed in the freshly infected naïve B cells, due to EBV infection, were corroborated in the chosen cell culture infection model, ER/EB2.5.

The process of EBV-driven B cell proliferation requires the expression of EBNA2. ER/EB2.5 is a 'conditionally immortalised' LCL in which the EBV growth programme is dependent on the presence of β-estradiol (Kempkes, et al. 1995b). In this cell line, EBNA2 has been replaced with an ER/EBNA2 fusion protein whereby the EBV protein is joined to the hormone-binding domain of the β-estradiol receptor. It is well established that such fusions can cause the function of the individual partner protein to become hormone-dependent (Picard, et al. 1988; Eilers, et al. 1989). Thus, ER/EBNA2 protein is activated by the addition of β-estradiol to the growth medium, leading to EBV latency type III gene expression and cell proliferation. When ER/EB2.5 cells are cultured in the absence of β-estradiol they undergo cell cycle arrest and over time will not survive in culture, similar to un-infected resting B cells in culture. This permits the modulation of ER/EB2.5 between a resting state (-β-estradiol) and a cell cycling state (+β-estradiol).

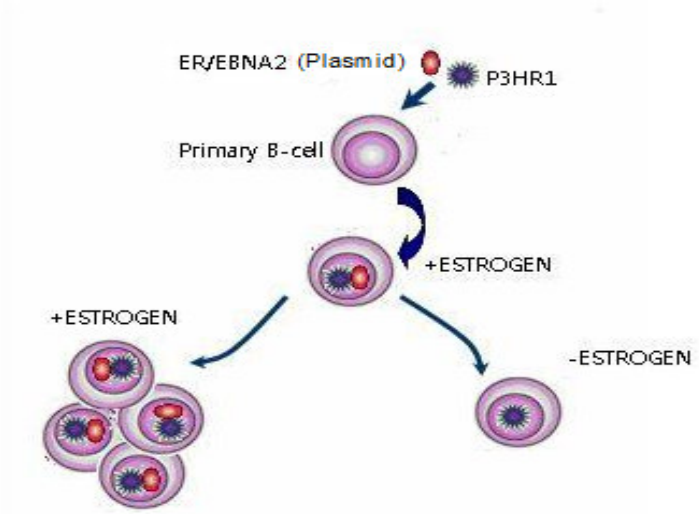


Figure 4.2: Schematic of ER/EB2.5 model.

ER/EB2.5 is an LCL that contains a mini-EBV plasmid expressing the chimeric EBNA2 protein as an autonomously replicating plasmid, plus the P3HR1 viral genome, thus complementing the EBNA2 deletion of the P3HR1 virus. EBNA2 expression and proliferation are dependent on the presence of estrogen (Campion 2008).

In this study, ER/EB2.5 cells were cultured in the absence and presence of β-estradiol in order to investigate the expression profiles for the panel of genes identified in the RT Profiler PCR SuperArray (Section 4.2.1). ER/EB2.5 cells were washed four times in PBS prior to re-seeding into β-estradiol-free supplemented RPMI medium. Twenty-four hours later the cells were washed twice in PBS and again cultured in β-estradiol-free medium for a further 48 hours so as to ensure complete inactivation of ER/EBNA2 and the EBV growth programme. To reactivate the EBV growth programme, 1 µM β-estradiol was added to the growth medium and cells were cultured for 48 hours. Total RNA was prepared from ER/EB2.5 cells at these different stages and samples were analysed by RT-qPCR using SYBR green-based PCR assays designed for each target gene shown in Table 4.1. In this way, the relative mRNA levels of each gene could be compared between proliferating ER/EB2.5 cells and those ER/EB2.5 cells in which the EBV growth programme has been inactivated.

To determine the effect of EBV driven growth on the expression levels of *TLE1*, RNA samples were harvested from ER/EB2.5 cells cultured as

described above in the presence and absence of β-estradiol. *TLE1* mRNA were assessed by Reverse-Transcription (RT) Real Time PCR (RT-qPCR). TATA Binding Protein (TBP) mRNA levels within the cells were also monitored in this fashion and allowed for the normalisation of *TLE1* expression levels between samples.

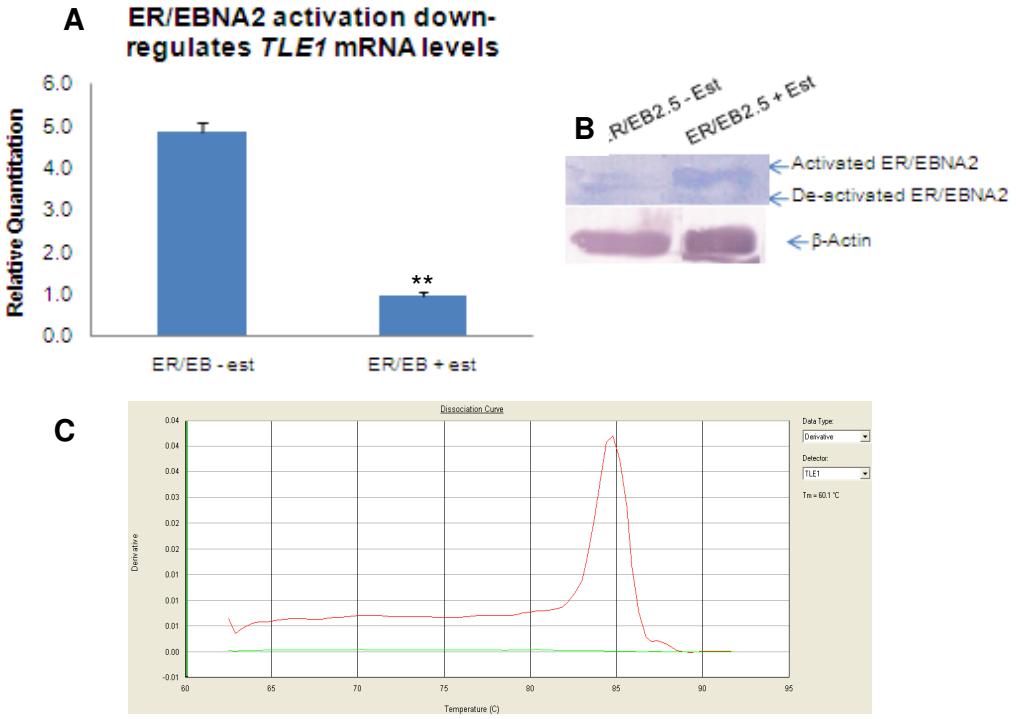


Figure 4.3: Induction of EBV driven proliferation in ER/EB2.5 leads to a decrease in the level of *TLE1* mRNA.

RT-qPCR analysis was carried out to determine the levels of *TLE1* expression in the cell culture model of resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). ER/EBNA2 was activated in the ER/EB2.5 cell line by the addition of 1 µM β-estradiol to previously β-estradiol-starved cells. RNA samples were taken before and after the addition of β-estradiol to the growth medium and reverse transcribed using random hexamers and MMLV reverse transcriptase. Amplification of cDNA was monitored in real time by detection of fluorescence intensity using SYBR green. **(A)** Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control being EBV cycling cells (ER/EB + est): arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01). **(B)** Western Blot showing activation of ER/EBNA2 in response to β-estradiol. Protein harvested from β-estradiol starved cells exhibit the lower ER/EBNA2 band indicating inactive ER/EBNA2. After 48 hours of β-estradiol supplementation, this band is detected at a higher molecular weight, indicative of active ER/EBNA2. All extracts were normalised to the loading control β-Actin. **(C)** The dissociation curve for the product of the *TLE1* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The results from this experiment correlated with the data obtained from the EBV-B cell infection experiment. *TLE1* mRNA levels were reduced five-fold following activation of the EBV growth programme in ER/EB2.5. In this experiment a characteristic shift was observed in the apparent molecular weight of chimeric EBNA2. This is thought to be due to either post-translational modifications or considerable changes in the tertiary structure of ER/EBNA2 (Figure 4.3B) (Kempkes, et al. 1995b). These data, when combined, support the negative regulation of *TLE1* by EBV at the transcriptional level.

Due to difficulties optimising a TLE1 protein detection assay, two Western blotting techniques were combined to detect TLE1. This combined method utilised classical immunological probing techniques and new SNAP i.d.™ technology; this method was described in detail in section 2.2.5.8.

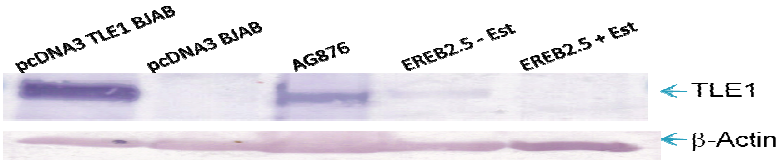


Figure 4.4: TLE1 protein expression levels in ER/EB2.5.

Protein extracts were taken from ER/EB2.5 cells before and after ER/EBNA2 activation. TLE1 protein expression was investigated via western blot analysis. A positive TLE1 expression control for exogenous (pcDNA3 TLE1 BJAB) and endogenous (AG876) TLE1 expression was included in the blot, along with a negative control (pcDNA3 BJAB). The upper panel illustrates the level of TLE1 protein detected using a rabbit polyclonal IgG (Abcam, ab15587) and normalised for the endogenous loading control β-Actin, using a mouse monoclonal IgG, depicted in the lower panel.

Down-regulation of TLE1 expression at the protein level by β-estradiol induction in ER/EB2.5 was confirmed by SDS PAGE/western blotting (Figure 4.4). As a control for antibody specificity, the EBV-negative BJAB cell line was transfected with a *TLE1* expression vector (pcDNA3 TLE1). The empty expression vector, pcDNA3 was also used to transfect the same cell line as a negative control. The BL cell line, AG876 demonstrates high expression levels of TLE1 and was a positive control for endogenous TLE1. The cellular protein β-Actin was probed to ensure equal loading for every sample. β-

estradiol-deficient ER/EB2.5 cells display a clear and discrete band in the correct region when probed with the TLE1 antibody. After activation of the EBV growth programme this band is no longer present, implying the loss of TLE1 protein expression in these cells (Figure 4.1). These results show that *TLE1* is a negative target of the EBV growth programme in an LCL.

To further examine whether the modulation of *TLE1* expression in ER/EB2.5 is EBV dependent, an β-estradiol starvation time course experiment was performed (Figure 4.5). ER/EB2.5 cells previously cycling in the presence of β-estradiol were washed as described in section 4.2.3. These cells were then seeded into T75 flasks at a density of 5 X 10^5 cells/ml (ER/EB – Est 0 hr) and passaged every 48 hours. Total mRNA samples were harvested from cells at three intervals for the duration of the β-estradiol withdrawal period (Indicated in Figure 4.5). Relative *TLE1* mRNA expression levels were determined using RT-qPCR. It can be seen that as ER/EBNA2 is de-activated in the ER/EB2.5 cell line, the *TLE1* mRNA levels increase significantly (Figure 4.5A). The de-activation of the ER/EBNA2 was verified by Western blotting (Figure 4.5B).

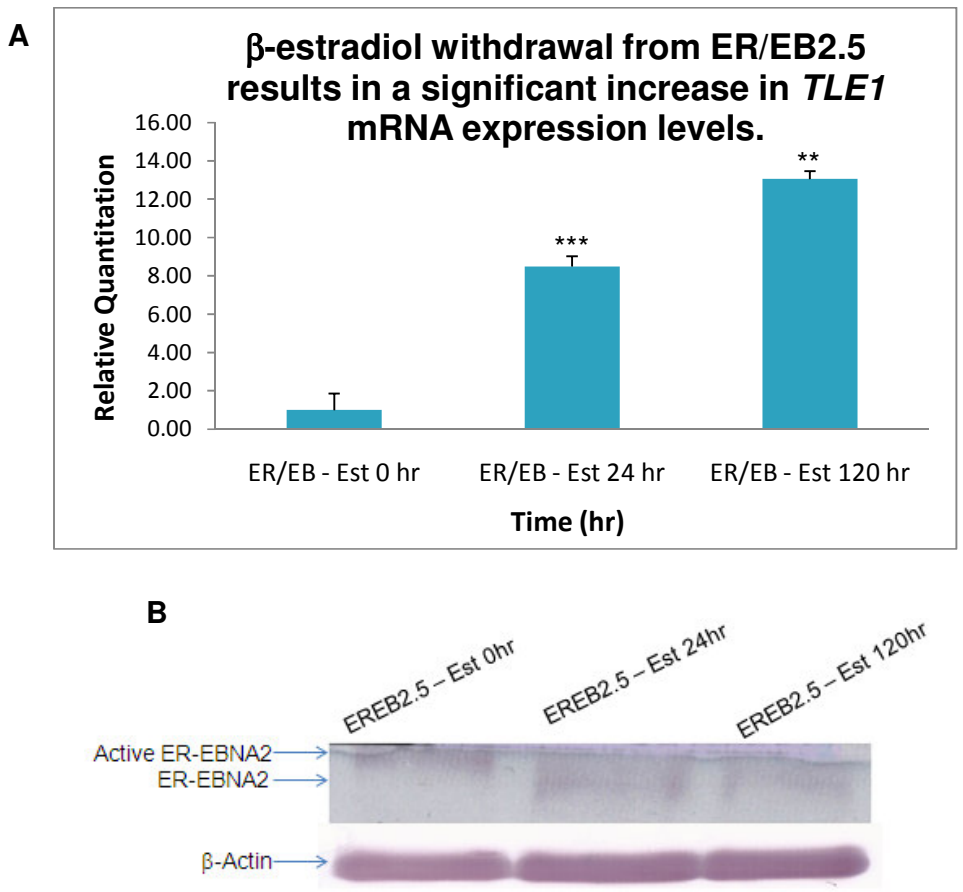


Figure 4.5: De-activation of ER-EBNA2 by withdrawal of the β-estradiol results in a significant increase in *TLE1* mRNA levels.

(A) RT-qPCR was performed to determine the effect on *TLE1* mRNA in response to ER/EBNA2 de-activation (β-estradiol withdrawal). RNA samples were prepared at the post-withdrawal time points indicated on the graph. Assay and normalisation of results (relative to *TBP*) was performed as described in the legend to Figure 4.3. In this experiment, the control was designated as ER/EB – Est 0 hr; arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01, *** P<0.001). **(B)** Western Blot showing de-activation of ER/EBNA2 in response to β-estradiol withdrawal. Protein harvested from cells cultured from β-estradiol medium (ER/EB2.5 –Est 0 hour) exhibit the active 120 kDa ER/EBNA2 protein. After 24 hours, this band is detected at a lower molecular weight, indicative of inactive ER/EBNA2, due to the loss of β-estradiol. All extracts were normalised to the loading control β-Actin.

In an additional experiment with further time points, the modulation of *TLE1* mRNA levels in response to EBV-driven growth was examined (Figure 4.6). Comparative *TLE1* mRNA levels were monitored at various time points in ER/EB2.5 cells (i) as β-estradiol was withdrawn from ER/EB2.5 cells and (ii)

after the cell line was once again induced to proliferate due to EBV through β-estradiol induction. Proliferating ER/EB2.5 cells cycling continuously in the presence of β-estradiol were washed and re-cultured in β-estradiol free medium to de-activate the EBV programme. *TLE1* mRNA expression peaked 48 hours following β-estradiol withdrawal. Basal expression for *TLE1* appears to reach a steady state at a level lower than that observed at the 48 hour time point (ER/EB2.5 –Est 48 hours). The removal of β-estradiol from ER/EB2.5 cells halts EBV driven proliferation and causes cellular growth arrest. However, even in a resting state, basal *TLE1* expression levels are elevated significantly in comparison to cycling ER/EB2.5. After 120 hours of β-estradiol depletion, 1μM β-estradiol was re-introduced to the growth medium and a decrease in *TLE1* mRNA expression is once again noted. These results show that *TLE1* down-regulation coincides with EBV latent gene expression in the ER/EB2.5 conditional LCL.

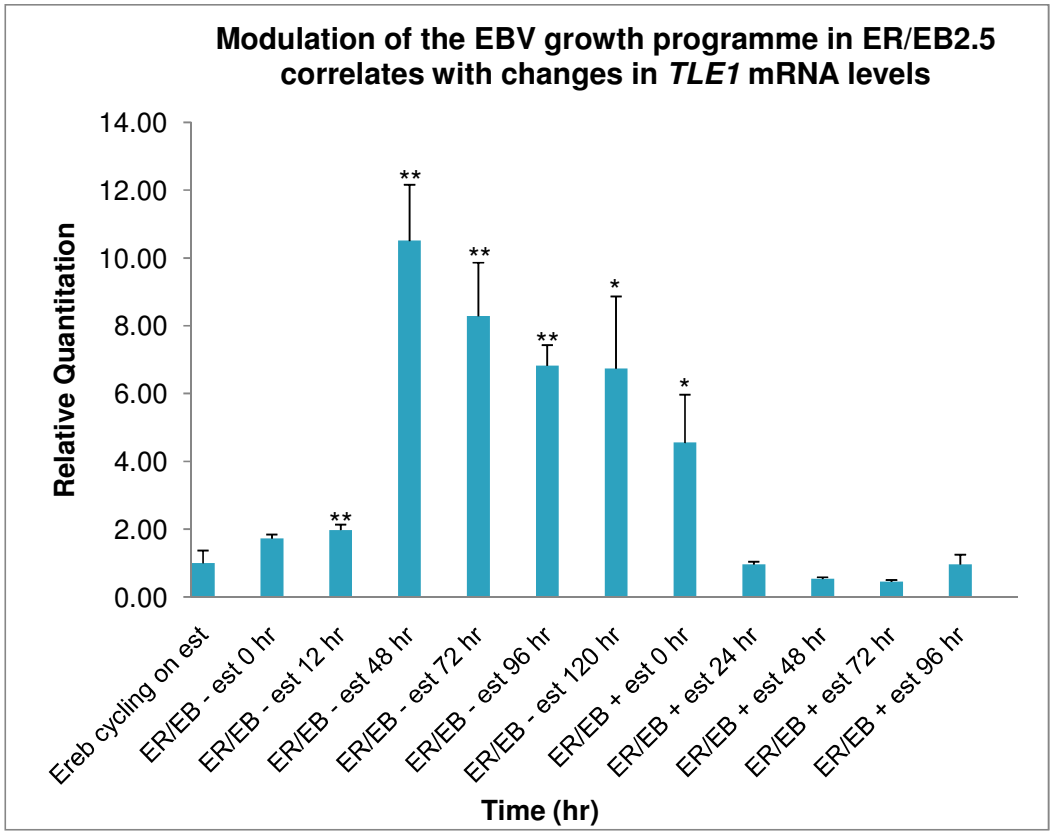


Figure 4.6: Modulation of the EBV growth programme in ER/EB2.5 correlates with changes in *TLE1* mRNA levels.

To monitor the modulated effect of *TLE1* mRNA levels in response to activation and de-activation of EBV driven proliferation, an expanded time course of ER/EB2.5 cycling cells was performed. This involved taking samples from EBV proliferating cells, re-culturing these cells in β-estradiol deficient medium (resting B cell) and re-induction of the EBV growth programme. ER/EB2.5 cycling continuously on the EBV growth programme were washed 4 times in PBS and were then re-suspended in β-estradiol free medium (ER/EB – est 0 hr). Twenty four hours later the cells were washed twice in PBS and again the cells were maintained in β-estradiol free medium. Samples were taken at the time points indicated while the ER/EB2.5 cell line was cultured in β-estradiol deficient medium and β-estradiol supplemented medium. RNA samples were monitored and normalised using *TBP* as described in the legend to Figure 4.3. In this experiment, the control was designated as ER/EB cycling on β-estradiol; arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$, ** $P<0.01$).

4.2.4 EBNA2 *trans*-activates *TLE1* in the conditional BL derived cell line BL41-ER/EBNA2 (BL41-K3)

Burkitt's Lymphoma (BL) cell lines arise from human malignant lymphoma cells biopsied from BL patients. Proliferation in these cell lines is driven by *C-Myc* (Gruhne, et al. 2009). BL41-K3 is an established EBV-negative cell line that is capable of constitutively expressing an β-estradiol receptor-EBNA2 fusion protein (ER/EBNA2) whose function is dependent on β-estradiol. BL41-K3 was derived by stably transfecting an EBV negative BL cell line, BL41 with an ER/EBNA2 expression plasmid. ER/EBNA2 can enhance its own transcription from the plasmid (Kempkes, et al. 1996), leading to an increase in its own expression. BL41-K3, is therefore, an EBV-negative BL cell line that can be induced to express ER/EBNA2 but no other latent viral proteins.

In order to assess the effect of EBNA2, when expressed as the sole EBV protein, on *TLE1* expression levels, BL41-K3 cells were prepared for EBNA2 activation by washing twice in PBS prior to the addition of β-estradiol to a final concentration of 1 µM in supplemented RPMI medium.

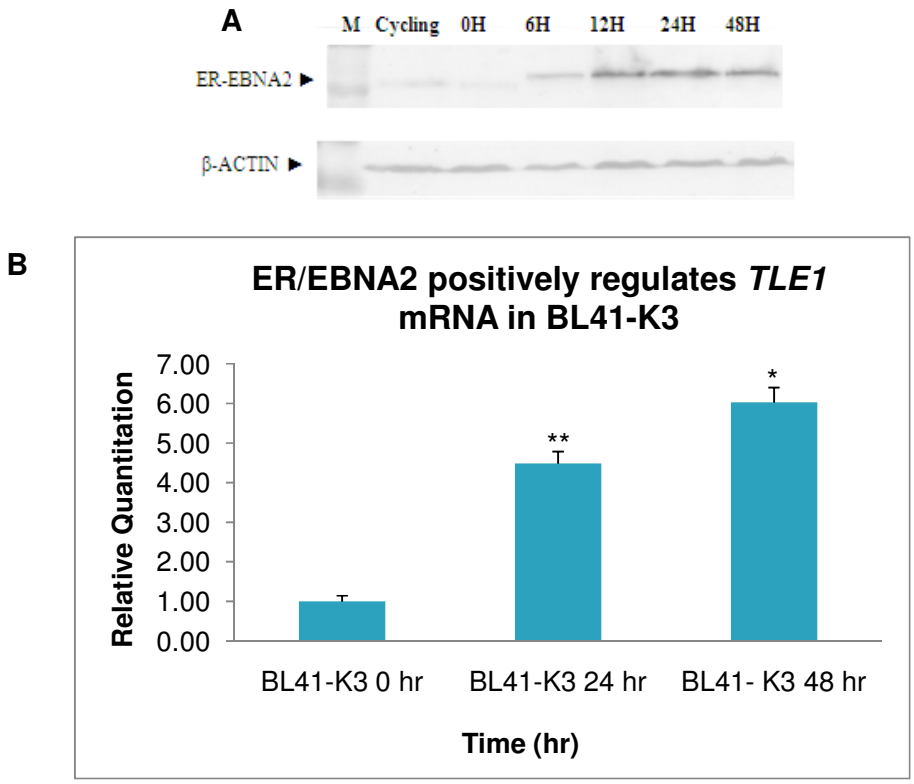


Figure 4.7: ER/EBNA2 positively regulates *TLE1* mRNA in BL41-K3.

ER/EBNA2 was activated in BL41-K3 by the addition of 1μM β-estradiol to the growth medium. Protein and total RNA extracts were prepared at the times indicated following activation of ER/EBNA2 in BL41-K3. **(A)** Western Blot analysis showing activation and up-regulation of chimeric EBNA2 in response to β-estradiol addition (upper panel). The lower panel shows the levels of the internal loading control β-Actin (in collaboration with E. Campion). **(B)** RT-qPCR analysis of *TLE1* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to Figure 4.3, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as BL41-K3 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$, ** $P<0.01$).

Western blot analysis confirmed ER/EBNA2 activation as before (Figure 4.7A). In this experiment it can be seen that the steady-state level of *TLE1* mRNA increased in response to activation of ER/EBNA2 (Figure 4.7B). Bearing in mind the previous data generated in this study regarding *TLE1* regulation in an LCL background, it was surprising to observe that in a BL background, ER/EBNA2 activation coincided with significant up-regulation of *TLE1* mRNA levels. *TLE1* expression was up-regulated after 24 hours, with a 4.5-fold increase observed for *TLE1* mRNA levels in response to EBNA2.

The solitary EBV latent protein present in the BL41-K3 cell line is EBNA2; this implies that in a BL background *TLE1* is not repressed by EBNA2, but in fact is up-regulated by this EBV protein in a BL background.

4.2.5 EBNA2 does not *trans*-activate *TLE1* in a BL-derived cell line (BL41-9A) which expresses other EBV latent proteins

BL41-9A is an EBV-positive EBNA2-deletion containing cell line stably transfected with an ER/EBNA2 expressing vector. BL41-9A is, therefore, similar to BL41-K3 described previously; however, in response to ER/EBNA2 activation with β-estradiol, BL41-9A goes on to express the full complement of EBV latent genes (except LMP1), whereas BL41-K3 expresses only activated ER/EBNA2. BL41-9A was generated by infection of the parental EBV-negative BL41 cell line with the EBNA2-deficient non-immortalising P3HR1 EBV virus strain (Kempkes, et al. 1996), followed by stable transfection with an ER/EBNA2 containing recombinant mini-EBV genome. Upon activation, ER/EBNA2 *trans*-activates the full repertoire of EBV latent genes. The transcriptional up-regulation of LMP1 is ordinarily a consequence of EBNA2 expression; yet, in the BL41-9A cell line, LMP-1 expression never transpires and the molecular basis for this is not yet understood. Thus, BL41-9A is an EBV-positive BL derived from the EBV negative BL41 and serves as a useful model for the examination of EBV latent gene activity in a BL cell background.

In order to investigate the ability of EBV to regulate *TLE1* in BL41-9A, an ER/EBNA2 activation time course was undertaken. BL41-9A cells were primed for ER/EBNA2 activation by washing cells twice in PBS prior to the addition of β-estradiol to a final concentration of 1 μM in supplemented RPMI medium. Total mRNA samples were harvested as before and relative *TLE1* mRNA levels were determined using RT-qPCR.

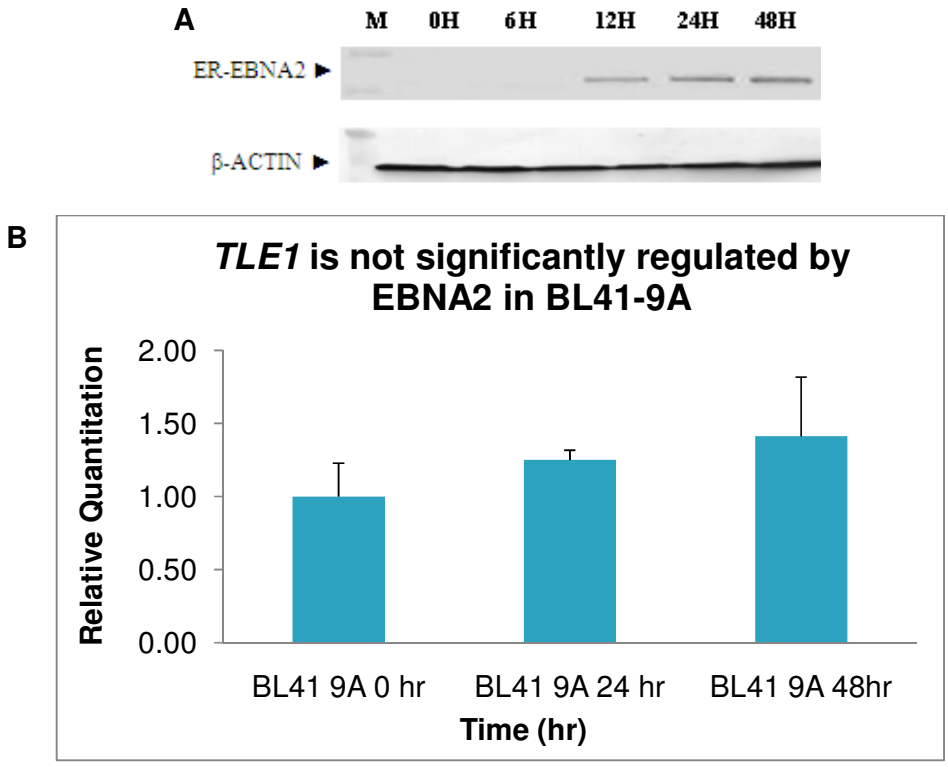


Figure 4.8: *TLE1* is not significantly regulated by EBNA2 in BL41-9A.

ER/EBNA2 was activated in BL41-9A by the addition of 1 μM β-estradiol to the growth medium. **(A)** Western blot analysis confirmed activation of chimeric EBNA2 in response to β-estradiol induction (upper panel). The lower panel is the β-Actin loading control. **(B)** RNA samples were prepared at the time points indicated post-induction. RT-qPCR analysis of *TLE1* mRNA levels in response to ER/EBNA2 activation was performed as described in the legend to figure 4.3; results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as BL41-9A 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD).

In this experiment, activation of ER/EBNA2 was again observed by Western blot analysis, (Figure 4.8A). Surprisingly, addition of β-estradiol to BL41-9A cells did not lead to a significant change in *TLE1* mRNA levels over the time period. In the associated cell line BL41-K3, it was shown that ER/EBNA2 activation *trans*-activated *TLE1* expression. In BL41-9A, one or more of the other EBV latent gene products, or their cellular targets, may therefore impair *trans*-activation of *TLE1* by ER/EBNA2.

4.2.6 Induction of EBNA2 in the EBV-negative BL cell line, DG75-tTA-EBNA2, has no significant effect on *TLE1* mRNA levels

The cell line DG75-tTA-EBNA2 was used to further investigate a potential role for EBNA2 in *TLE1* regulation. DG75-tTA-EBNA2 is a stable transfectant in which the induction/repression of EBNA2 may be achieved using a non-toxic concentration of tetracycline in the growth medium (Floettmann, et al. 1996). DG75-tTA-EBNA2 was previously generated by stably transfecting the EBV-negative BL cell line DG75 with two expression vectors, pJEF-3 and pJEF-31. Stable integration of these plasmids was achieved by selection using hygromycin, geneticin and tetracycline. The effector vector, pJEF-3 contains a constitutively expressed tetracycline regulated *trans*-activator (tTA). The responsive construct, pJEF-31, can express EBNA2; however, the inducible promoter that drives this EBNA2 expression contains several binding sites for the tTA. In the presence of tetracycline, the expressed tTA is bound by the drug and thus cannot activate the expression of the EBNA2 promoter. Upon removal of tetracycline from the medium, tTA binds to the promoter activating the transcription of EBNA2 (Floettmann, et al. 1996). DG75-tTA-EBNA2 cells are maintained in supplemented RPMI medium containing 1 µg/ml of tetracycline. The induction of EBNA2 was initiated by washing the cells 4 times in PBS followed by re-seeding them in tetracycline-free culture medium.

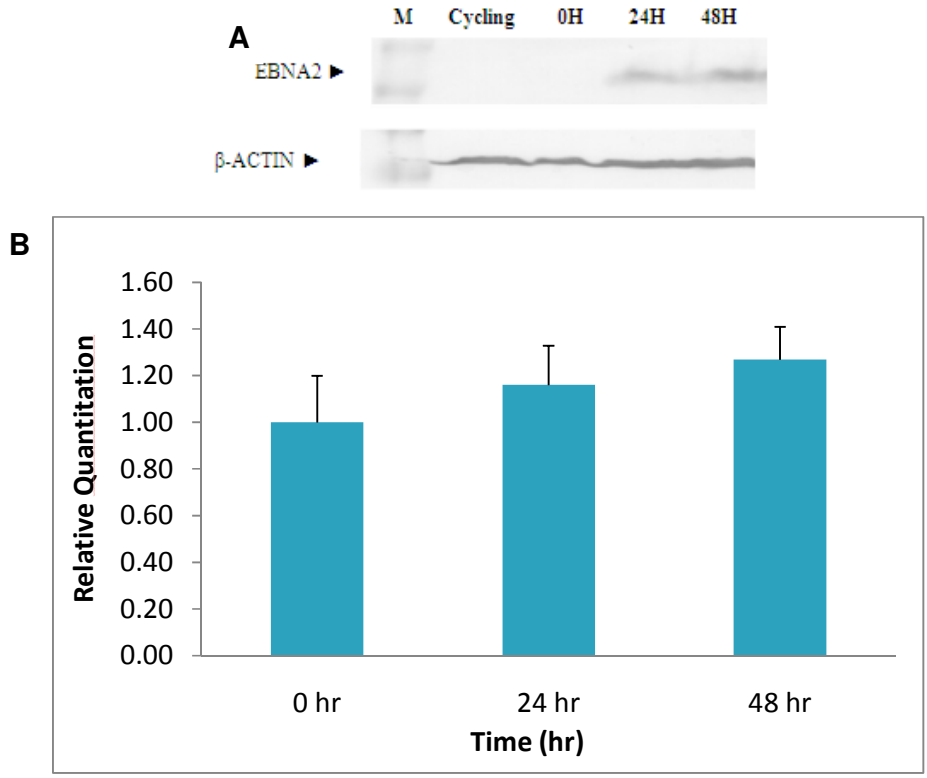


Figure 4.9: EBNA2 induction did not significantly regulate *TLE1* in DG75-tTA-EBNA2.

EBNA2 expression was induced in DG75-tTA-EBNA2 by the removal of tetracycline from the supplemented RPMI medium. **(A)** Western blot analysis confirmed induction of EBNA2 expression in response to tetracycline removal. Cells were harvested and analysed for EBNA2 expression at the time points indicated (upper panel). The lower panel shows the β-Actin loading control. **(B)** RT-qPCR analysis of *TLE1* mRNA levels in DG75-tTA-EBNA2 in response to EBNA2 induction. RNA samples were prepared at the time points indicated post-induction. RT-qPCR analysis was performed as described in the legend to Figure 4.3; results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as DG75-tTA-EBNA2 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD).

The induction of EBNA2 in the experiment was confirmed by Western blotting (figure 4.9A). Contrary to the previous result obtained with BL41-K3, no significant modulation of *TLE1* mRNA was seen in response to EBNA2 induction in DG75-tTA-EBNA2. Although these experiments both involve expressing EBNA2 as sole EBV protein in a BL cell background, the results obtained are not in agreement. This discrepancy may be due to the different induction systems used in each cell line or as a result of acquired genetic

differences between BL41 and DG75. It is also possible that there may be a potential effect due to the difference in the properties of ER/EBNA2 (BL41-K3) versus wild-type EBNA2 (DG75-tTA-EBNA2). The results of these experiments, therefore, do not concur and it cannot be concluded whether or not EBNA2 alone can transcriptionally regulate *TLE1*.

4.2.7 Relative *TLE1* mRNA levels are not repressed due to C-Myc driven growth in the conditional LCL P493-6

The conditional LCL P493-6 is a stably transfected derivative of the LCL ER/EB2.5 (discussed previously in section 4.2.3) in which the expression of ectopic C-Myc may be regulated. In LCLs, EBNA2 drives the expression of *C-Myc* and it has been demonstrated that constitutive *C-Myc* expression can substitute for EBNA2 to maintain proliferation in ER/EB2.5 (Pajic, et al. 2000). A tetracycline-negatively regulated C-Myc expression construct was introduced into the established stable cell line ER/EB2.5. This vector, pMyc-tet, possesses the ability to express both C-Myc and the tetracycline regulated *trans*-activator, tTA, from two different promoters (Gossen and Bujard 1992). The tTA molecule is constitutively expressed from a CMV promoter; however, C-Myc is under the control of the TP-tetO7 promoter, whose activation is dependent upon the binding of tTA (Pajic, et al. 2001). When present, tetracycline binds the free tTA preventing binding to the TP-tetO7 thus repressing ectopic C-Myc expression (Figure 4.10). *Hence P493.6 when starved of β-estradiol represents a resting B-cell that can be stimulated to proliferate either in response to (i) C-Myc in the combined absence of tetracycline and β-estradiol (simulating BL cell growth) or (ii) EBNA2 in the presence of both β-estradiol and tetracycline (simulating an EBV-driven LCL)* (Pajic, et al. 2000; Schuhmacher, et al. 2001). It was therefore of interest in this study to utilise the duality of P493.6 to determine if *TLE1* repression occurred in B cells driven to proliferate due to *C-Myc* and/or EBV.

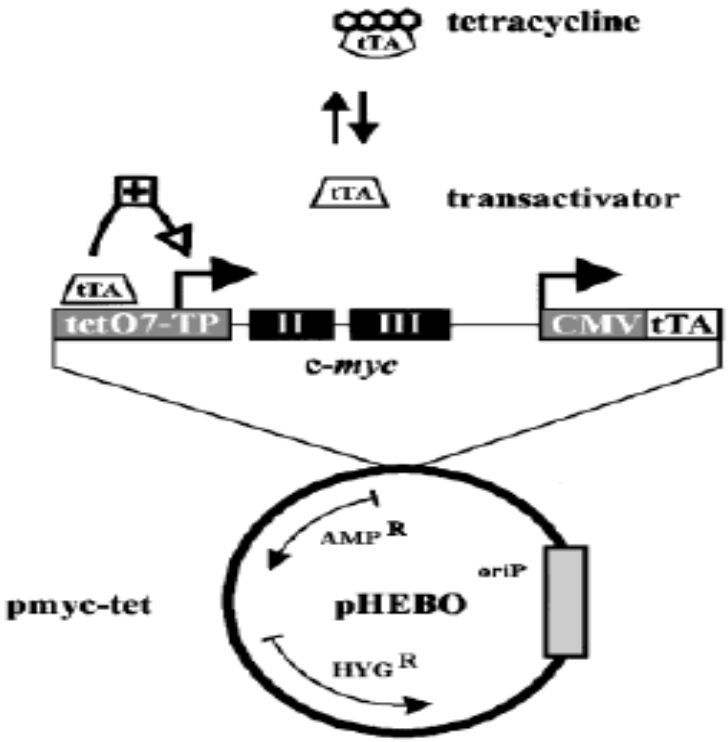


Figure 4.10: Conditional expression in P493-6.

Schematic illustrating pMyc-tet, the C-Myc expression construct. The two coding exons of C-Myc (depicted by black boxes II and III) were cloned under the control of the promoter TP-tetO7. The construct also includes an expression cassette for the *trans*-activator tTA regulated by the CMV promoter (CMV-tTA). Tetracycline inhibits TP-tetO7 *trans*-activation by sequestering tTA, rendering it unavailable for promoter binding. pMyc-tet is based on the EBV-derived vector pHEBO. Selection marker genes for ampicillin and hygromycin are illustrated along with the origin for latent replication by oriP (adapted from Pajic, et al. 2001). To activate ER/EBNA2, β-estradiol is added to the culture medium to a final concentration of 1 µM. Expression of ectopic C-Myc is repressed by treatment with 0.1 µg/ml tetracycline as described previously (Pajic, et al. 2000; Pajic, et al. 2001).

A time course experiment was carried out in which the P493.6 cell line was cultured on the EBV growth programme (+ β-estradiol, + Tet; reminiscent of parental ER/EB2.5) or the C-Myc growth programme (- β-estradiol, - Tet; reminiscent of an EBV-negative BL cell line). In the latter case, P493-6 cells were washed twice in PBS and cultivated in the absence of tetracycline and β-estradiol for a period of 72 hours.

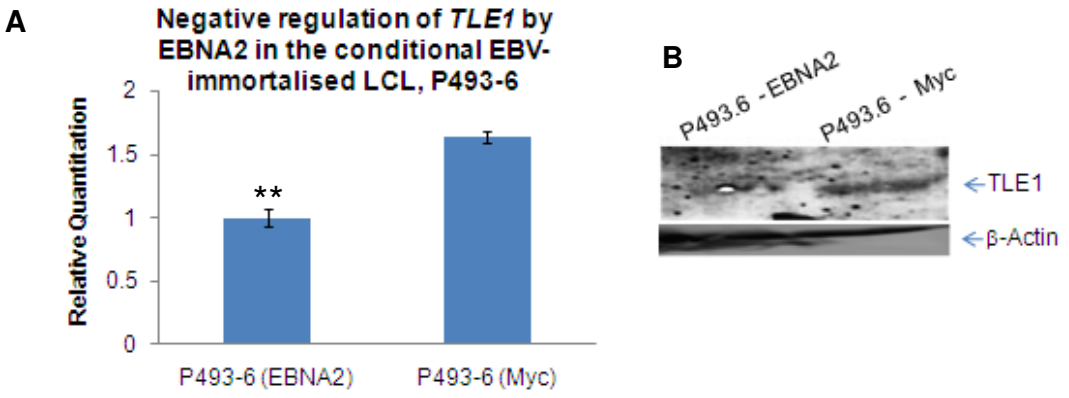


Figure 4.11: Negative regulation of *TLE1* by EBNA2 in the conditional EBV-immortalised LCL, P493-6.

The steady state levels of *TLE1* mRNA and protein were significantly higher in P493-6 cells proliferating due to C-Myc than in their EBV-driven counterparts. P493-6 cells were divided and separately cultured to permit cycling on the EBV growth programme (+ β-estradiol, + Tet) or C-Myc programme (- β-estradiol, - Tet). Total protein and RNA samples were taken during each growth programme. RT-qPCR and Western blotting were performed to determine the relative levels of *TLE1* expression in the p493-6 cells proliferating due to EBV (P493-6 EBNA2) relative to *C-Myc* driven cells (P493-6 Myc). **(A)** At the cycling programme indicated, RT-qPCR was performed as described in the legend to Figure 4.3. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment the control set was P493-6 EBNA2 cells, arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01). **(B)** Western blot analysis confirmed higher TLE1 protein expression levels in the P493.6 Myc cells in comparison to the EBV cycling cells.

It was determined from the RT-qPCR analysis that the steady state level of *TLE1* mRNA was significantly lower in cells proliferating due to EBV (+ β-estradiol, + Tet) in contrast to the cells proliferating due to C-Myc (- β-estradiol, - Tet) (see Figure 4.11A). This result was corroborated by Western blot analysis (Figure 4.11B), confirming that cells proliferating due to the C-Myc programme display a higher quantity of TLE1 protein relative to the same cell line cycling due to EBV. EBV-driven P493-6 cells are like the parental cell line ER/EB2.5. Thus these results show once again that, when the EBV-driven growth programme is switched off in ER/EB2.5 cells, the level of TLE1 increases. As P493-6 is a clonal derivative of ER/EB2.5, the C-Myc driven growth programme can be uncoupled from the EBV growth programme in the same isogenic background. These results show increased *TLE1* expression when the P493.6 cell line is proliferating due to the C-Myc programme relative to the EBV programme. It must be noted that in LCLs,

EBNA2 positively *trans*-activates *C-Myc*; thus, the EBNA2 proliferating P493.6 cells also express endogenous C-Myc expression. However, in the absence of EBNA2 this expression is lost, hence the requirement for ectopic C-Myc to drive proliferation. Therefore C-Myc activity is present in both cell lines but at different levels. In P493.6 cells cycling due to C-Myc, *TLE1* is up-regulated by the loss of EBV-driven proliferation in ER/EB2.5. Consequently the increase in *TLE1* expression in the C-Myc-maintained cells may be due to alleviation of the negative modulation derived from EBV latent proteins rather than attributable to positive regulation by C-Myc. However a positive C-Myc effect on TLE1 expression cannot be discounted in this model.

4.2.8 *TLE1* mRNA expression levels in a panel of BLs and LCLs

In this study, two types of B cell lines were utilised, BLs and LCLs. Primary B lymphocytes proliferate during only a very limited time in culture (Hahn 2002). However if these cells are infected with EBV *in vitro* it leads to the establishment of continuously proliferating LCLs. LCLs display the full complement of EBV latent genes, and induce changes that include (i) the formation of cell clumps in culture, (ii) display of cell surface adhesion and (iii) activation markers and resilience against apoptosis (Bornkamm 2009b). In addition to growth transformation, sustained EBV activity is required to maintain an immortalised LCL. Thus LCLs exhibit growth and expression profile characteristics directed by EBV not only for the initial process of immortalisation but also on an ongoing basis. Burkitt's lymphoma, on the other hand, is a B cell malignancy in which the cells contain characteristic chromosomal translocations of the long arm of chromosome 8 along with the *Ig* heavy or κ and λ light chain loci on chromosome 14, 2 or 22 respectively, bringing *C-Myc* regulation under the *Ig* transcriptional control (Bornkamm 2009b). BLs exhibit significant shared characteristics although they do display cell line to cell line variation in characteristics such as EBV status and the precise chromosomal breakpoint translocation. Generally BLs grow in single cell suspension as they display no cell adhesion molecules. In addition they lack B cell activation markers, and EBNA1 is the only EBV latent gene

expressed (EBV positive BL only) and BL cells are sensitive to apoptosis arising from adverse growth conditions and toxic agents (Bornkamm 2009a). These features of the BL phenotype are present during *in vitro* culture of all BL cells from biopsies. However over time some of these have been shown to 'drift' towards type II and type III latency (group-II and group-III phenotypes respectively) (Reviewed by Bornkamm 2009a). The group-III BL cell lines are phenotypically altered compared to the group-I cells due to the presence of the EBV growth programme proteins. Group-III BLs, reminiscent of LCLs, express the complete EBV repertoire of latent genes including six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP) three virus-encoded latent membrane proteins (LMP1, LMP2A, LMP2B) and the EBERs (Middeldorp, et al. 2003). Group-III BLs also display enhanced cell survival. These two phenotypically differing types of cell lines permit the study of EBV in two situations, one in which EBV is vital to the initiation and maintenance of the cell line (LCLs), and in the other wherein it may provide advantages to the cell but was not a key factor that led to the immortalisation of the cell (BL).

In this part of the study *TLE1* expression in a panel of B cell lines was evaluated. This panel consisted of cell lines with group-I phenotype (Akata4E3, DG75, Ramos and BJAB) along with EBV-positive cell lines displaying the group-III phenotype (AG876 and IB4). DG75 and Ramos are both EBV-negative BLs. The cell line Akata4E3 was an EBV-positive BL that spontaneously lost the EBV genome to give rise to an EBV-negative BL cell line (Shimizu, et al. 1994). BJAB is an EBV-negative non-Burkitt, B lymphoma cell line (Menezes, et al. 1975; Yamamoto, et al. 2000). IB4 is an EBV-immortalised LCL generated by infecting cord blood with the B95.8 strain of EBV (King, et al. 1980). The EBV-positive BL AG876 exhibits an activated B cell phenotype expressing the latency III growth programme (group-III phenotype). All cell lines were maintained in the appropriate growth medium and passaged every 48 hours. Protein and total RNA samples were harvested during the exponential phase of growth for each cell line (approximately 24 hours post-passaging once the cell population had almost completely doubled).

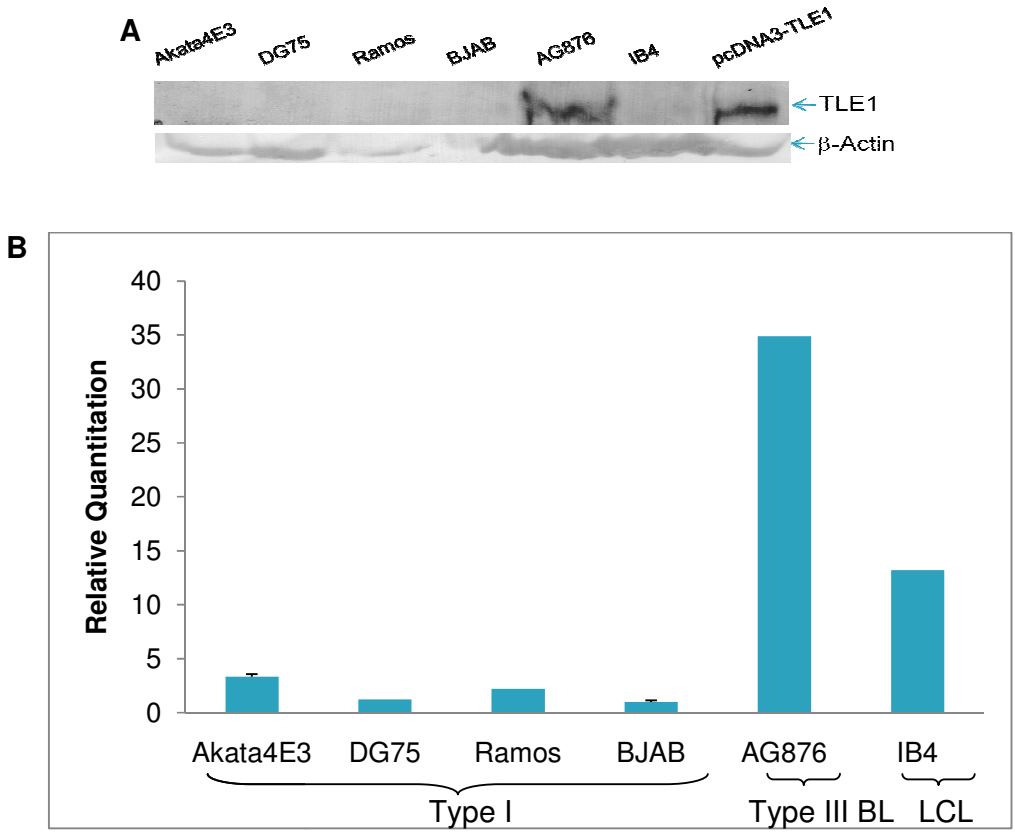


Figure 4.12: Comparative *TLE1* mRNA and Protein levels in B cell lines.

Western blotting and RT-qPCR were used to monitor *TLE1* levels in a range of B cell lines including the EBV-negative B cell lines DG75, Ramos, BJAB and Akata4E3, the EBV-positive AG876 (group-III BL cell line), and the EBV-immortalised LCL IB4. **(A)** Western blot analysis of TLE1 in protein extracts from BL-derived cell lines (Type I and Type III as indicated) and an LCL. TLE1 is shown in the upper panel. The lower panel shows β-Actin expression as a loading control. **(B)** RNA samples were prepared from each cell line in the same time frame, and RT-qPCR analysis of *TLE1* mRNA levels was performed as described in the legend to Figure 4.3. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment BJAB cells were set as control cells, arbitrarily assigned a value of 1. (Data are Mean ±SD).

TLE1 was seen to be expressed at lower levels in the EBV-negative BLs relative to the EBV-positive cell lines analysed (Figure 4.12B). AG876 showed the highest *TLE1* mRNA and protein levels of the B cell line panel analysed (Figure 4.12A). The expression levels obtained for AG876 were in the region of 32-fold higher for *TLE1* expression than those obtained in the latency I cell panel. However, relative to IB4, the expression levels of TLE1 in AG876 are only approximately 3-fold higher than in IB4.

4.2.9 Expression of other TLE family genes in ER/EB2.5

A recent report concerning the Runx transcription factor included an analysis of *TLE* family gene expression in a range of B cell lines (Brady, et al. 2008). In agreement with the results presented here, it was shown that *TLE1* is expressed at a higher level in LCLs in contrast to BL type I cells. Additionally, this group demonstrated results indicating that *TLE2* was not detectably expressed in a range of B cell lines, and that both *TLE3* and *TLE4* were expressed in high levels in both LCLs and BLs. This prompted an analysis of *TLE* family gene expression in the present study to determine if *TLE1* was differentially-regulated by EBV relative to the other *TLE* family members. The RT Profiler PCR SuperArray results presented earlier (Section 4.2.1) indicated that *TLE1* was repressed by EBV during B cell infection, and that *AES* and *TLE2* mRNA levels remained unchanged. *TLE* family members are quite closely related, and for this reason a discriminatory SYBR green assay for each family member was designed in this study. Primers were designed with high specificity to their target but with significant discrepancy to the other family members so as to avoid detection of the latter in the absence of the primary target. Each assay was verified to produce a single PCR product following melt curve analysis and agarose gel electrophoresis of the RT-qPCR product generated. Each product was then sequenced to confirm the specific identity of the target (data not shown).

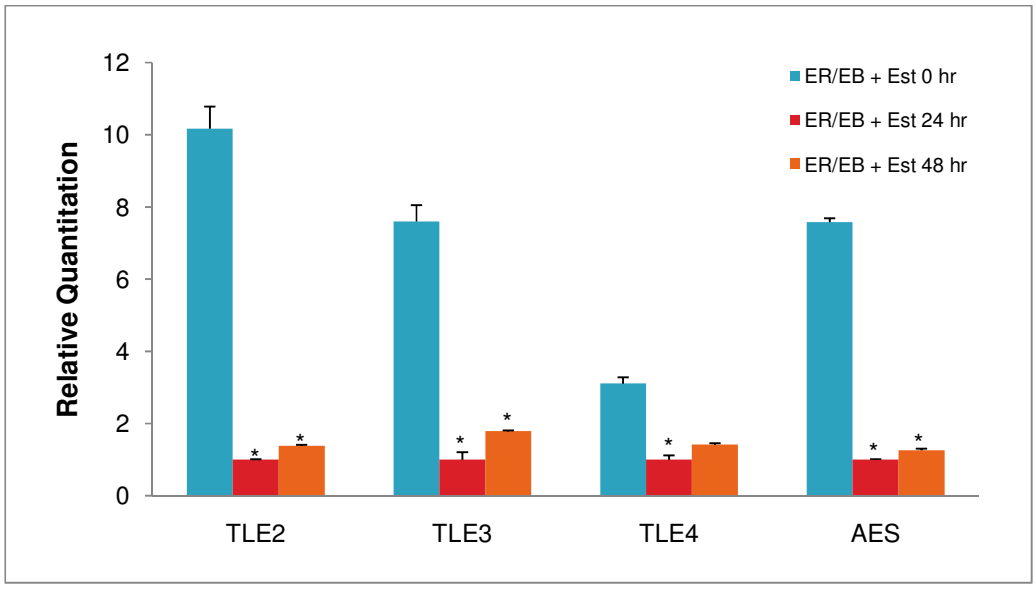


Figure 4.13: Effect of the EBV growth programme on the *TLE* family gene expression.

A β-estradiol induction experiment was carried out using ER/EB2.5 to assess the effect of ER/EBNA2 activation on the mRNA levels of the *TLE* family members. RNA samples were taken during β-estradiol induction at the time points indicated. RT-qPCR analysis was performed as described in the legend to Figure 4.3, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as ER/EB + Est 24 hr; arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$).

The results generated from the *TLE* family study following activation of the EBV growth programme in ER/EB2.5 indicated that all *TLE* family members were to some degree negatively regulated by EBV. This was an unexpected observation bearing in mind the results from the RT Profiler PCR SuperArray which indicated no change in expression level for *AES* or *TLE2* post-infection. This may represent a shortcoming of the ER/EB2.5 cell culture model as a true representation of an infected naïve B cell. It was shown previously in this study that *TLE1* undergoes significant transcriptional repression and de-repression following ER/EBNA2 activation and inactivation respectively in ER/EB2.5 (Section 4.2.3). In a second experiment in which EBV-driven growth was de-activated following deprivation of β-estradiol, total RNA samples were again taken and analysed for mRNA levels from *TLE* family genes.

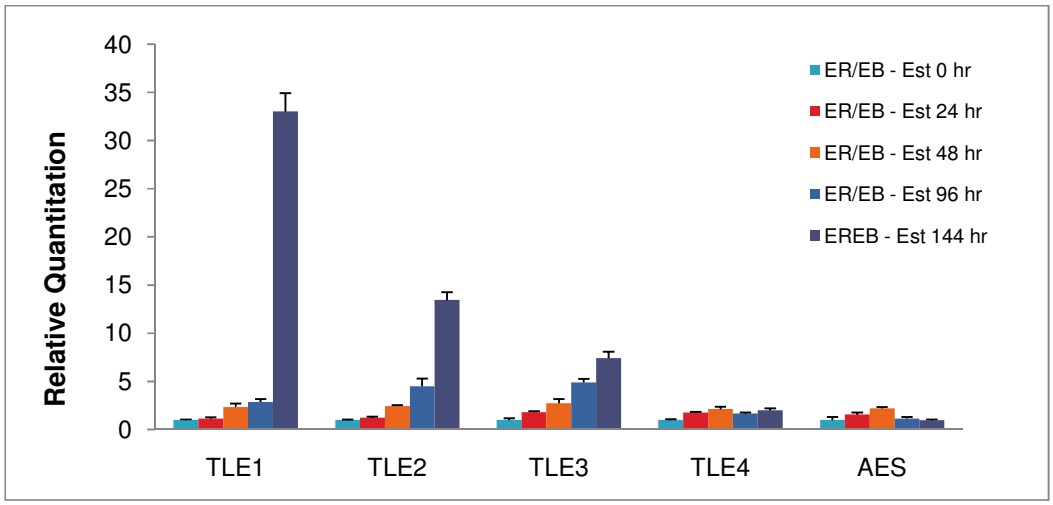


Figure 4.14: Withdrawal of the EBV growth programme positively regulates several *TLE* family members.

ER/EBNA2 function was deactivated in the ER/EB2.5 cell line by withdrawal of β-estradiol from the culture medium. RNA samples were prepared at the time points indicated post-deactivation. RT-qPCR analysis was performed as described in the legend to Figure 4.3, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as ER/EB –est 0 hr; arbitrarily assigned a value of 1. (Data are Mean ±SD).

It can be seen from this experiment that relief of transcriptional suppression was most potent for *TLE1,* relative to the other family members, in response to EBV growth programme inhibition. The *TLE1* mRNA level increased 32-fold as a result of ER/EBNA2 deactivation. In agreement with the results from the induction of EBV-driven proliferation in ER/EB2.5 (Figure 4.13), *TLE2* and *TLE3* exhibited greater transcriptional de-repression than *TLE4* and *AES*. Neither *TLE4* nor *AES* displayed any de-repression after the de-activation of ER/EBNA2, although both appeared to be repressed by the activation of ER/EBNA2. These results confirm that EBV transcriptionally modulates a number of *TLE* family members in ER/EB2.5; however, *TLE1* appears to be the most potently regulated and the de-activation of β-estradiol elicited no significant modulation of *AES* or *TLE4* family members over the time period of the experiment.

4.2.10 Regulation of *TLE* family members in the P493-6 cell line

The results of this investigation have so far indicated that EBV differentially regulates members of the *TLE* family to some extent in an LCL background. Activation of EBV-driven proliferation in the LCL, ER/EB2.5, appears to repress the transcriptional expression of each *TLE* family member. The P493-6 cell line, described previously in section 4.2.7, was next employed to further examine the modulation of the *TLE* family in response to EBV-driven growth in an LCL. The P493-6 cell line can be induced to undergo, (i) *EBV* driven proliferation [due to the induction of ER/EBNA2 by β-estradiol and the repression of ectopic C-Myc expression by the presence of tetracycline (+β-estradiol, + Tet)], reminiscent of the parental cell line ER/EB2.5 when β-estradiol induced, or (ii) *C-Myc driven growth* [stimulated by the absence of β-estradiol and tetracycline (-β-estradiol, - Tet)]. P493.6 therefore allows a comparative analysis of the EBV and C-Myc growth programmes in an isogenic cell background.

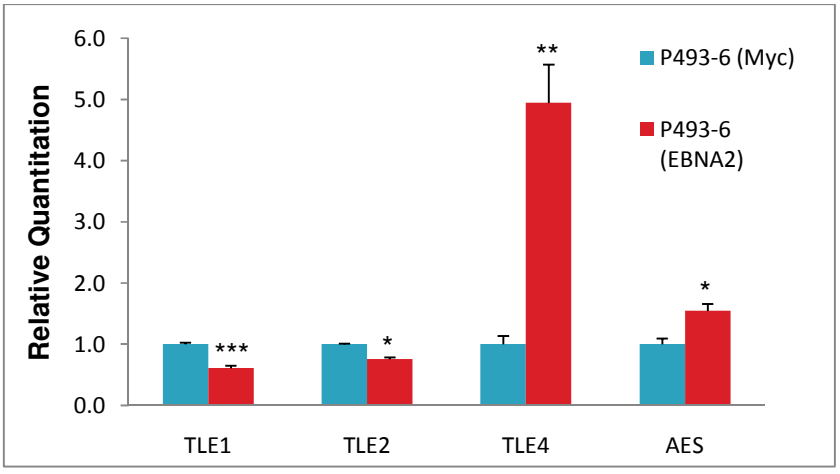


Figure 4.15: Steady state *TLE* mRNA levels in P493.6 cells driven to proliferate on the EBV growth programme (P493.6 EBNA2) or due to ectopic C-Myc (P493.6 Myc).

RT-qPCR was performed to determine *TLE* family mRNA levels in the p493-6 cell line proliferating due to *C-Myc* (P493.6 Myc) relative to cells proliferating due to EBV (P493.6 EBNA2). At the cycling programme indicated, RT-qPCR was performed as described in the legend to figure 4.13. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment the control was set at P493-6 Myc cells; arbitrarily assigned a value of 1. (Data are Mean ±SD, *P<0.05, ** P<0.01, *** P<0.001).

It was observed that there were significant variations in the steady-state levels of mRNA from *TLE* family members in P493.6 cycling on the EBV versus C-Myc growth programmes. The mRNA levels of both *TLE1* and *TLE2* were higher in cells cycling due to C-Myc in contrast to cells proliferating due to EBV. In contrast to this, the steady state levels of *AES* and *TLE4* were lower in C-Myc-driven cells. Although readily detectable in the parental ER/EB2.5, *TLE3* mRNA could not be detected at all in P493.6. This unanticipated problem remains to be explained and it was not, therefore, possible to conclude on *TLE3* expression in this case. Taking into consideration the results obtained from the P493.6 and ER/EB2.5, EBV-driven growth appears to have a stronger negative impact on the transcriptional activity of both *TLE1* and *TLE2* in comparison to the remaining TLE family members, AES and TLE4.

Figure 4.15 shows that *TLE4* and *AES* mRNA levels are higher in P493.6 cells driven to proliferate due to EBV in comparison to those cycling on the C-Myc growth programme. Interestingly, while it has been shown that activation the ER/EBNA2 by β-estradiol in ER/EB2.5 leads to the repression of *AES* and *TLE4* at the transcriptional level, inhibition of ER/EBNA2 by withdrawal of β-estradiol from the growth medium led to little change in the mRNA levels of *TLE4* and *AES*. In contrast to this, the TLE family members *TLE1*, *TLE2* and *TLE3* are modulated by both the β-estradiol-dependent activation and deactivation of the EBV growth programme in ER/EB2.5.

4.2.11 Expression of *TLE* genes in a panel of BL and LCL cell lines

Previously in this study a trend was discerned with respect to *TLE1* expression in B cell lines available within the laboratory (Section 4.2.8). *TLE1* mRNA levels were generally considerably lower in EBV-negative BLs than in the EBV positive BLs and LCLs. To determine if any such trend exists with respect to regulation of the other TLE family members in the same panel of B cell lines, total RNA was extracted as before from each cell line 24 hours after passaging, and RT-qPCR was employed to examine the relative mRNA levels of each TLE family member.

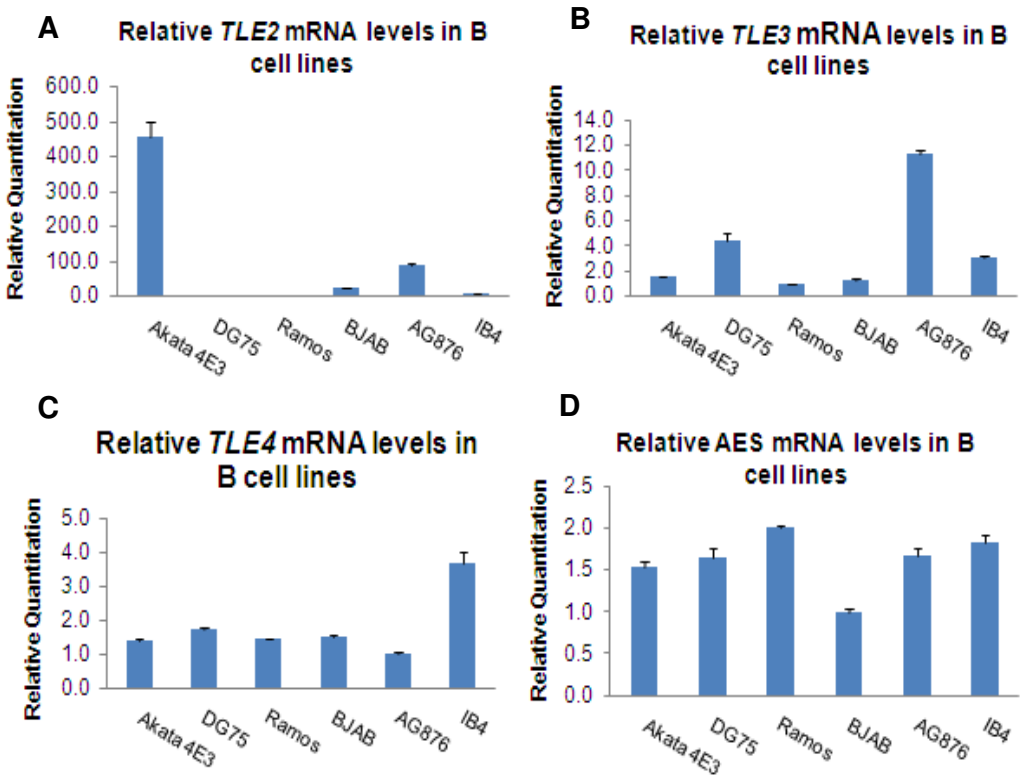


Figure 4.16: RT-qPCR analysis of the relative mRNA levels of TLE family members in a range of EBV-associated B cell lines.

RT-qPCR was used to monitor the expression of the *TLE* family in a range of B cell lines including EBV-negative B cell line extracts for DG75, Ramos, BJAB and Akata4E3, EBV-positive BL AG876, and the EBV-immortalised LCL, IB4. RNA samples were prepared from each cell line in the same time frame, and RT-qPCR analysis of *TLE1* mRNA levels was performed as described in the legend to Figure 4.3. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. The Control cell line was arbitrarily assigned a value of 1. (Data are Mean ±SD).

No correlation was observed for any of these genes between the different B cell lines (Figure 4.16).

4.3 Discussion

In the search to elucidate the mechanisms behind EBV infection, a number of large-scale studies have been performed using technology such as DNA microarrays, and RT-QPCR arrays. These experiments give rise to large volumes of data that can simultaneous identify genes associated with the spectrum of EBV products or with individual products. However, many of these published studies have utilised inducible cell culture systems in which selected or complete EBV expression can be controlled in an immortalised cell background. The present study was unique as it was performed using primary untouched naïve B cells for comparison. This ensured that any irreversible changes that occur in the naive B cell post-infection could be identified in addition to reversible gene modulation. This method facilitated the analysis of EBV infection from the initial contact through to the EBV immortalised cell lines, which are so intensively studied. A number of previously unidentified genes such as *TLE1*, *STIL* and *Nurr1* were implicated as being modulated in response to EBV infection, in untouched naïve B cells, using the RT Profiler PCR SuperArray.

In this study, a naïve B cell pool was infected with EBV in an attempt to identify novel cellular gene targets of this virus. A selection of differentially regulated candidate cellular genes was then examined in a cell culture model (ER/EB2.5) that permits activation/inactivation of EBV latent gene expression with β-estradiol. Like any LCL, ER/EB2.5 cells proliferate due to the effects of EBV latent gene products, and ER/EBNA2 inactivation (in the absence of β-estradiol) leads to a cessation of the EBV growth programme. The ER/EB2.5 system may have shortcomings, however, in terms of its ability to 'mimic' what happens during EBV infection and B cell immortalisation. Firstly, there is evidence that ER/EB2.5 cells, in the absence of β-estradiol, mimic a resting B cell but not a true naïve B cell. Once a naïve B cell has come into contact with antigen, irreversible changes occur within the cell to generate a memory B cell. When the naïve B cell associates with its cognate antigen the BCR becomes endocytosed allowing the internalisation of the antigen. The antigen is then degraded and formed into complexes with major

histocompatibility complex (MHC) II to facilitate presentation by the B cell. Once infected by EBV, naïve B cells proceed to differentiate just as the uninfected cells. Secondly, unlike naïve B cells, ER/EB2.5 cells may be epigenetically programmed, thus restricting the potential to undergo changes in gene expression (Gordadze, et al. 2004). Thirdly, β-estradiol has been found to activate PI3K signaling from the endoplasmic reticulum, which in turn may complicate the analysis of some genes (Spender, et al. 2006). Despite these inconsistencies, ER/EB2.5 is a widely used cell culture model that facilitates analyses of the roles of EBV latent genes in infected B cells.

Regulation of TLE1 by EBV

The discovery of new cellular genes that are EBV targets was of particular interest in this study. Traditionally, emphasis has been placed on transcriptional activation of cellular genes; however, over time it has become clear that gene repression plays an equally important role in cell fate (Cinnamon and Paroush 2008a). One noteworthy gene that displayed potential as a novel EBV negatively-regulated target in the naïve B cell infection RT Profiler PCR SuperArray study was *TLE1*. In conditional LCLs the negative effect of EBV on *TLE1* expression was also consistently demonstrated; thus, it can be surmised from the evidence available that EBV down-regulates *TLE1* in an LCL. The results from the RT Profiler PCR SuperArray and the conditional LCL ER/EB2.5 were in agreement and suggest that *TLE1* is a novel negative target of EBV. Conflicting results were obtained, however, for the regulation of two other *TLE* family members, *AES* and *TLE2*. In the RT Profiler PCR SuperArray the mRNA levels for *AES* and *TLE2* did not change following the infection of naïve B cells with EBV; however, activation of ER/EBNA2 in ER/EB2.5 resulted in down-regulation of both family members (Figure 4.13). Furthermore, the removal of β-estradiol from the growth medium resulted in the up-regulation of *TLE2*, although *AES* expression remained relatively unchanged after ER/EBNA2 de-activation (Figure 4.14). These inconsistencies may highlight the inadequacy of comparing a growth-arrested ER/EB2.5 cell to a true resting naïve B cell.

Interestingly, EBNA2 *trans*-activated *TLE1* when activated (in the form of ER/EBNA2) in one BL cell line, but this effect was not corroborated in a second such cell line in which EBNA2 expression was controlled from a tetracycline-regulated promoter (Section 4.2.6). In BL41-9A, a conditional cell line in which an almost complete repertoire of EBV latent genes is expressed in response to activated ER/EBNA2, *TLE1* mRNA levels remained unchanged throughout ER/EBNA2 activation. However in the isogenic cell line, BL41-K3, in which EBNA2 is the sole EBV protein, *TLE1* mRNA levels increased significantly following ER/EBNA2 activation. The EBNA3 family negatively regulate EBNA2 targets by competitively binding to CBF1, sequestering the transcription factor and preventing its utilisation by EBNA2 (Waltzer, et al. 1996). It may, therefore, be the case that the co-expression of the EBNA3 family in the conditional BL tempers the ability of EBNA2 to *trans*-activate *TLE1*. The lack of LMP1 induction following EBNA2 activation in BL41-9A may also imply a role for LMP1 in *TLE1* repression.

Fundamental differences between BL-derived cancer cells and LCLs may account for the discord observed regarding *TLE1* regulation in response to EBV proteins in these two cell types. LCLs are immortalised cell lines that were transformed by infection of naïve B cells by EBV. Cancerous cell lines occur due to immortalisation arising from a different source or sources. This malignant transformation is generally due to a collection of aberrant events that allow cell survival and proliferation. As such LCLs and tumour derived cell lines are quite different in origin but can display many of the same pro-survival characteristics. From the experiments performed in this study it can be concluded that in an LCL, *TLE1* expression is down-regulated following EBV infection and latent gene expression. This is in agreement with the observations of *TLE* repression in haematological oncogenic cells. The basis of cell line transformation of the two cell sources used here, malignant BLs and non-malignant LCLs, could be the causative factor for the lower levels of *TLE1* expression observed in EBV-negative B cell lines when compared to LCLs.

TLE family proteins

TLE1 is a member of the human transducin-like enhancer of split (TLE) family. The TLE family is composed of five members; *amino terminal enhancer of split* (*AES)*, *TLE1*, *TLE2*, *TLE3* and *TLE4*. This family contains the first characterised vertebrate homologues of the well studied *Drosophila* gene known as *groucho* (Stifani, et al. 1992). In 1968, *groucho* was the first metazoan co-repressor identified and was so named due to a viable mutation in the gene resulting in supernumerary bristles growing above the eyes of adult Drosophila. This growth was reminiscent of the thick eyebrows of Groucho Marx and the gene took its name from there (Buscarlet and Stifani 2007). Groucho was mischaracterised when initially identified as a 'transducin-like enhancer of split', as it was mapped to the *enhancer of split* chromosomal region, and although it did not show significant relation to known proteins it contained a 330 amino acid stretch that shared likeness with the β subunit of Transducin. However several years later this subunit was found to have no functional relation to the β subunit and Groucho was identified as a nuclear protein that mediated transcriptional repression. The impact of the mis-classification was to lead to the naming of the human homologues of *groucho* as *transducin-like enhancer of split 1, 2, 3* and *4* (Buscarlet and Stifani 2007).

Most TLE family members are approximately 80-95 kDa with the exception of AES, which is a shorter version at approximately 25 kDa. Thus, the TLE family can be divided into two subgroups according to their structure. TLE family members 1-4 share the same full length structural features (Figure 4.17). These include a five-domain structure characterised by two highly conserved N-and C-terminal regions, designated the Q and WDR domains respectively, and three less conserved internal domains denoted Gp, CcN and SP (Stifani, et al. 1992). The WDR is the most highly conserved region with 86% amino acid identity among species (Bajoghli 2007). The most divergent of the full length TLE family members is TLE2 (Stifani, et al. 1992). The second subgroup is composed of truncated versions of TLE1-4,

including AES, which lack either the WDR or Q domain (Zhang, et al. 2008). The highly conserved amino terminal Gln-rich (Q) and Trp-Asp-repeat (WDR) domains are essential for (i) the ability of Gro/TLE to homo/heterodimerise and (ii) to interact with a variety of DNA-binding proteins to mediate transcriptional repression (Buscarlet and Stifani 2007). As such these 'truncated' TLE members are thought to play a role in tandem with their full length counterparts.

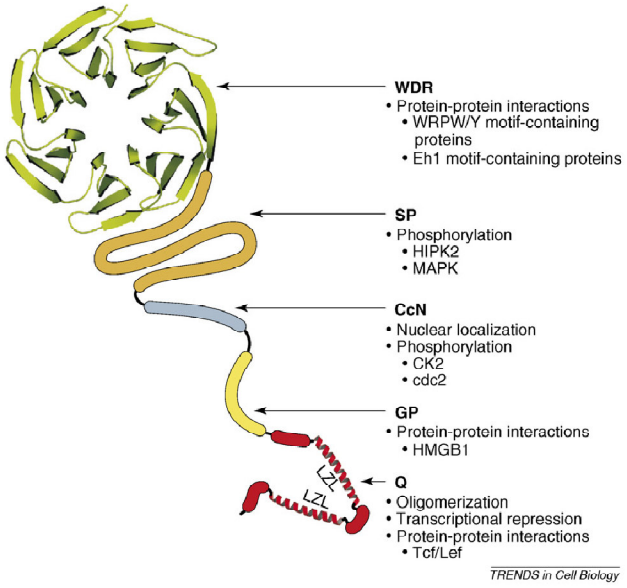


Figure 4.17: Structure of Gro/TLE.

Gro/TLE proteins have five evolutionarily conserved domains. These include: (i) the N-terminal Q domain (Red), which mediates Gro/TLE oligomerisation (Song, et al. 2004); (ii) The yellow GP region, that does not demonstrate significant conservation among the vertebrates or invertebrates; (iii) The blue CcN region, comprising of phosphorylation sites for CK2 and cdc2 (hence 'CcN'). These sites are adjacent to nuclear localisation signals; (iv) The brown SP domain, which is a serine/proline rich zone believed to be part of the repressor activity of Gro/TLE (Chen and Courey 2000), and (v) the WDR. This is the most substantially conserved region of the Gro/TLE protein (green domain). This mediates interactions with a variety of DNA-binding transcription factors that belong to either the WRPW or Eh1 motif family (Stifani, et al. 1992). Figure adapted from (Buscarlet and Stifani 2007).

AES retains only the Q and GP domains (Figure 4.18). The N-terminal region of AES is approximately 130 aa in size with 70-75% familial homology to the Q domain, followed by weak likeness to the GP region (See Figure 4.17). Due to a lack of a WDR domain, it cannot directly bind many TLE associated target proteins such as Hes and Pax. AES is believed to have two functions:

firstly to act with a similar function to other TLE members in certain contexts, and secondly to exist as a dominant/negative TLE family regulator by hetero-dimerising via the Q region of TLE and its Q domain-mediated targets (Buscarlet and Stifani 2007).

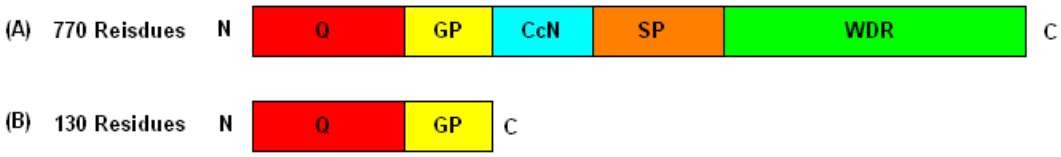


Figure 4.18: Schematic of TLE and AES structural Domains.

(A) The structure of full length TLE family members (TLE1-4) consists of 770 residues spanning five domains. The N terminal Q region, followed by the internal GP, CcN and SP domains, terminated at the WDR domain. **(B)** AES is a truncated family member composed of 130 residues. AES contains only the Q and GP domains.

TLE1 has been mapped to the chromosomal location 9q21.32 (Buscarlet and Stifani 2007), and *TLE4* has been mapped to 9q22.31, neighbouring *TLE1* (Hancock, et al. 2009). *AES* has been mapped to an apparent *TLE* gene cluster at chromosomal location 19p13.3. *TLE2* has been mapped in a tandem array with *AES* at this chromosomal location (Liu, et al. 1996). *TLE3* has been mapped to the chromosomal location 15q22 (Liu, et al. 1996).

The TLE proteins are near ubiquitous complex repressive nuclear factors that lack the ability to bind DNA. They can nonetheless interact with a diverse repertoire of transcription factors capable of binding DNA such as Hes, Runt domain proteins (Runx), LEF1/Tcf, Pax and C-Myc to elicit their response via specific gene regulatory sequences (Jennings and Ish-Horowicz 2008). TLE1-4 expression has been observed in a wide range of tissues; however, expression levels can vary significantly between each family member in the same tissue (Buscarlet and Stifani 2007; Jennings and Ish-Horowicz 2008). This variation in the expression levels of each TLE member in the same cell indicates differential regulation of each gene, thus implying a non-redundant function for each (Yao, et al. 1998). Gro/TLE family members appear to be highly conserved in both invertebrates and

vertebrates. It may be surmised that each individual TLE family member has a unique function which is cell and context dependent. The persistence of this highly conserved family signifies a crucial role for them in mammals (Buscarlet and Stifani 2007; Jennings and Ish-Horowicz 2008). TLE family members have been implicated as core repressor proteins in the absence of activated signaling in pathways such as Wnt and Notch. To ensure that targets of the Wnt and Notch signaling pathway are silenced in the absence of the appropriate signal, TLE family members bind to these targets as a repressor complex, thus preventing their transcription. This TLE-mediated repression is only alleviated in the presence of the activated effector protein. Evidence suggests that while TLE family members possess specialised roles in the cell, the remaining TLE family members may have the ability to elicit a similar response in the absence of the principal effector TLE in that cell type. This response may partially compensate for the loss of a family member but may not be sufficient for long-term or complete TLE-led repression. TLE2 interacts with the transcription factor Hes1 to stimulate repression; however, this repression is quantitatively weaker than the TLE1/Hes1 mediated effect (Grbavec and Stifani 1996; Fisher, et al. 1996; Grbavec, et al. 1998).

The regulation and function of TLE family proteins and association with malignant disease

EBV target genes are generally important players in signaling pathways that can influence the fate of the cell, such that EBV can promote survival and proliferation, whilst simultaneously averting cell death, terminal differentiation and detection by the immune surveillance. In this section the role of the *TLE* family will be discussed in an attempt to determine the possible advantages EBV could acquire by modulating the expression of the *TLE* family member, *TLE1*.

Due to the widespread expression of the TLE family, it was assumed that TLE activity was regulated by the distribution of their co-repressor partners. However, it has now emerged that TLE regulation is much more complex and it has been shown that TLEs can be regulated in a signal-induced

context dependent manner. To date, three main mechanisms by which TLE can regulate the expression of target genes have been identified: (i) availability of dependent co-repressors, (ii) competitive displacement of TLE by appropriate co-activators and (iii) post-translational modifications such as phosphorylation (Cinnamon and Paroush 2008b), (see Figure 4.19). In addition, although almost ubiquitously expressed, transcriptional regulation of *TLE* has also been observed in transcriptomic studies. A number of studies have demonstrated de-regulated *TLE* expression levels in malignant cells relative to their normal counterparts (Terry, et al. 2007, Allen, et al. 2006). Nevertheless, the regulators responsible for this transcriptional modulation have yet to be identified.

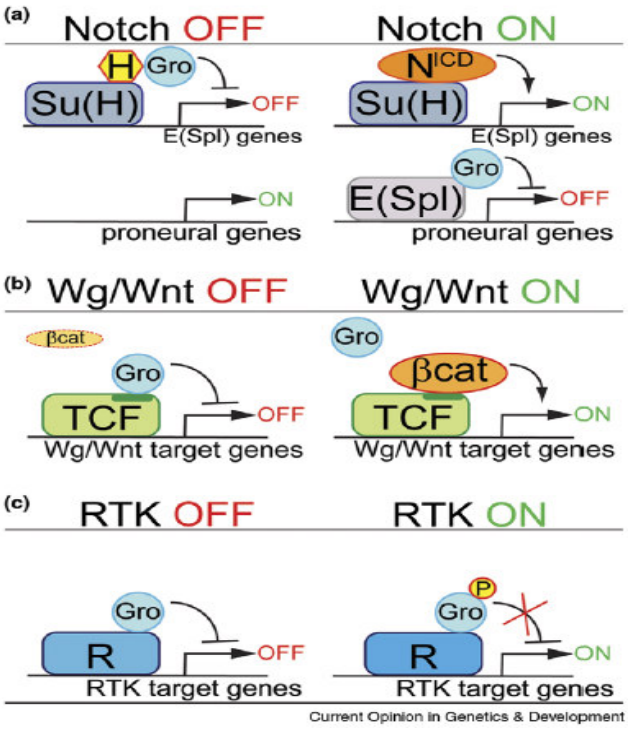


Figure 4.19: Mechanisms of TLE mediated repression.

Gro represents TLE1**; (A)** Co-repressor-dependent TLE activity. Notch activation stimulates the expression of TLE co-repressor proteins such as Hes/E(Spl). Hes can then recruit TLE and fulfil its role in Notch signaling. **(B)** Regulation in response to co-activator competition demonstrated in the Wnt signaling pathway. TCF and TLE co-operate to repress target genes in an un-stimulated cell. Wnt signaling activates the stabilisation of β-catenin; this co-activator can then displace TLE creating an activator complex that induces gene expression. **(C)** RTK signaling promotes the phosphorylation of TLE; this prevents TLE activity, thus activating RTK target gene expression. R represents a DNA binding repressor. Figure adapted from (Cinnamon and Paroush 2008a).

Evidence has shown that activity of TLE can be up or down-regulated by post-translational modifications such as residue specific phosphorylation (Jennings and Ish-Horowicz 2008). However, data have indicated that phosphorylation can have opposing effects on TLE1 function. Grg1, the murine TLE1 homologue, is found as part of a Hes1-associated protein repressor complex in rat neuronal cells. Phosphorylation of this complex leads to the dissociation of Grg1, leading to a decline in repression (Jennings and Ish-Horowicz 2008). Conversely, studies in Drosophila have shown that phosphorylation of the CcN region of TLE1 by CK2 kinase actually promotes the binding of transcription factors and chromatin, thereby in turn promoting repressor complex formation leading to increased repressional activity of TLE (Nuthall, et al. 2004). Thus, the effect of post-transcriptional regulation on TLE appears to be context-dependent.

Monitoring of the transcriptional and protein expression levels of these proteins has identified a link between altered TLE family expression and a number of malignant diseases (Jennings and Ish-Horowicz 2008). Indeed, de-regulated *TLE* expression has been directly associated with both cervical and colonic neoplasias. This aberrant expression of *TLE* appears to go hand in hand with elevated Notch expression levels (Liu, et al. 1996; Zagouras, et al. 1995). Elevated *TLE2* and *TLE3* expression has been shown to be a specific feature of higher grade meningiomas. Dys-regulated Notch expression is a key step for meningioma development and the evidence suggests that modulation of the Notch pathway by *TLE* expression signals a more malignant phenotype (Cuevas, et al. 2005). In addition to *TLE2* and *TLE3*, amplified expression of *TLE1* in mouse models has implicated *TLE1* as a causative factor in murine lung adenocarcinoma (Allen, et al. 2006). Furthermore, TLE1 protein expression alone has been highlighted as a signature marker for synovial sarcoma (Terry, et al. 2007). A considerable body of evidence, therefore, suggests that *TLE* expression may act as a promoter of oncogenesis. Many of the studies discussed so far recognise elevated *TLE* expression as a causative or sustaining factor in a number of malignancies; however, none of these studies have linked over expression of *TLE* to hematopoietic malignancy. Recent work utilising transcriptomic

microarray expression profiles comparing hematopoietic malignancies to healthy cells has revealed a very different scenario regarding *TLE* regulation and tumourgenesis in hematopoietic cells, *TLE* repression has been implicated as a key factor in cancer development such as AML (Dayyani, et al. 2008) and several other haematological malignancies (Fraga, et al. 2008). These findings suggest that *TLE* repression plays a role in the malignant transformation of hematopoietic cells or tumour maintenance thereof. These findings, therefore, lend support to the notion that EBV down-regulation of *TLE1* might elicit similar survival advantages in the EBV-infected cell.

Two TLE family members, *TLE1* and *TLE4* have been found to function as tumour suppressor genes in some translocation types of Acute Myeloid Leukemia (AML). The t(8:21) AML translocation is one of the most widely found genetic anomalies in acute myeloid leukemia. This translocation forms an AML-ETO fusion gene. Evidence has shown that this translocation alone cannot initiate leukemogenesis, implying that a second event must occur to stimulate the malignancy. Analysis of structural genomic stability in malignant AML cells indicated that the single most common genetic aberration in t(8:21) AML cells was deletion events concerning the long arm of chromosome 9, del(9q) on which both *TLE1* and *TLE4* have been mapped. This chromosomal abnormality, coupled with expression studies in t(8:21) AML cells, led to the identification of *TLE1* and *TLE4* as down-regulated targets occurring frequently in this subset of AML. Experimental analysis determined that complete knockdown of *TLE1* and *TLE4* using short hairpin RNA (shRNA) was sufficient to rescue these t(8:21) AML cells from apoptosis. Furthermore, *TLE1* and *TLE4* silencing led to increased proliferation of the AML cells in culture. In contrast to these results, the enforced expression of TLE1 and TLE4 in t(8:21) AML cells led to apoptosis and cell death (Dayyani, et al. 2008). These results implicate *TLE1* expression as a negative influence on the survival of a malignant T cell.

Further evidence from studies in a panel of B and T cell malignancies indicates that *TLE1* has a function in the acute myelogenous leukemia 1 (AML1) signaling pathway (Fraga, et al. 2008). To date it has emerged that,

in diffuse large B cell lymphoma, acute myeloid leukemia and other haematological malignancies, hypermethylation of the CpG island in the *TLE1* promoter is present, leading to silencing of the *TLE1* gene. Re-introduction of *TLE1* into these leukemia and lymphoma cells caused growth inhibition. This implies that the loss of TLE1 in these malignancies may have been a contributing factor in the instigation and/or maintenance of the oncogenic condition. Leukemia and lymphoma tumours in which the *TLE1* promoter remains un-methylated were also studied to determine the contribution of modulated *TLE1* expression on the malignant hematopoietic cell phenotype. shRNA knockdown was performed in these tumourgenic hematopoietic cells expressing TLE1, and this knockdown caused an increased growth rate in the malignant cells (Fraga, et al. 2008). The evidence available for the role of TLE1 in haematological malignancies indicates that in this context, TLE1 may drive a negative cell fate, hence acting as a tumour suppressor gene. Being a negative target of EBV, it may therefore be the case that *TLE1* down-regulation is an important host-virus interaction that contributes to B cell growth transformation.

The expression levels of TLE1 at both the mRNA and protein level were significantly lower in the EBV negative BLs when compared to the EBV positive cell lines (Figure 4.12). This was initially unexpected as it had previously been demonstrated that in an LCL, EBV activation resulted in down-regulation of TLE1 expression at both the mRNA and protein level. However, bearing in mind the published data from Fraga et al 2008, it can be seen that TLE1 silencing was found to be a feature of several types of malignancies of lymphoid origin. The results of the current study indicate that in addition to the malignancies studied by Fraga's group, a number of BL cell lines also appear to demonstrate significantly diminished levels of TLE1 expression. It would be highly beneficial in the future to determine if the down-regulation of TLE1 plays a role in the malignant state of these BL cell lines. The effect of re-introduction of TLE1 expression in these cell lines using an ectopic TLE1 expression vector could expose *TLE1* silencing as one of the mechanisms that enables cell proliferation in these cases. Although *c-myc* translocation is the characteristic feature of BL tumours, *c-*

myc translocation alone cannot cause BL. It has been shown that TLE1 has been silenced in a number of lymphatic malignancies due to promoter methylation (Fraga et al 2008). This could be pursued in these cell lines, simple treatment of these cell lines with methylation inhibitors such as 5-Aza-cytidine may result in TLE1 modulation.

Chapter 5: *Role of Transducin-Like Enhancer of Split 1 (TLE1) in an LCL*

5.1 Introduction

The infection of primary B cells by EBV induces cell activation and proliferation, an efficient process that leads to the outgrowth of permanently growing LCLs. Central to this B-cell immortalisation process is the modulation of host cellular gene expression by EBV viral gene products. The characterisation of these host-virus interactions has the potential to shed light on the mechanisms that drive cell transformation and the potential role of EBV in the development of EBV-associated malignancies. In this study, for the first time, the cellular gene *Transducin-Like Enhancer of Split 1 (TLE1)* has been identified as a candidate novel negative target of EBV. This study will now focus on identifying a role for *TLE1* in an LCL in an effort to determine the rationale for EBV-associated *TLE1* repression.

Studies have demonstrated that TLE family proteins are key negative regulators of several important cell signaling pathways including the Notch and wingless/Wnt signaling pathways (Reviewed in Buscarlet and Stifani 2007; Jennings and Ish-Horowicz 2008). TLE participates in these pathways, playing many critical roles in development, neurogenesis, and oncogenesis (Chen and Courey 2000, Dayyani, et al. 2008, Fraga, et al. 2008). However, contradictory evidence exists pertaining to the role of *TLE1*. Examples of *TLE1* inducing both a protective or destructive impact on cell fate have been shown. For instance, the *TLE* family member *AES* has been found to induce apoptosis in cerebellar granule neurons (CGNs). As a *TLE1* agonist, the apoptotic effect of *AES* in CGNs can be negated by the forced expression of *TLE1* (Zhang, et al. 2008). In the context of CGNs, *TLE1* asserts a protective role in the fate of the cell. Furthermore, substantiating the concept of *TLE1* as a pro-survival factor in cell fate, it has been shown that *TLE1* is up-regulated in 96% of synovial sarcoma tumours. TLE1 expression is now being signalled as a pathological marker for the identification of synovial sarcoma. The possibility of therapeutically targeting TLE1 in synovial sarcoma is also being investigated (Knosel, et al. 2010). In complete contrast to these findings are those discussed in Section 4.3, in which *TLE1* has been

found to possess a distinct negative role in the survival of hematopoietic cells compared to other cells types in which the *TLE* family has been studied. Thus *TLE1* functions in a context-dependant manner in cell survival and disease, and the potential role played by *TLE1* in an EBV-transformed haematopoietic cell line shall be examined further in this study.

Recruitment of TLEs to promoters by their DNA-binding associates induces repression of the bound target gene. An exact model has not yet been agreed, but repression is believed to occur by one of three possible routes: (i) TLE may act directly by preventing the 'activators' from binding to DNA, (ii) it could function by interruption of the basal transcriptional machinery, or (iii) it might act through its interaction with histone proteins. The models for TLEs' mode of action conflict with one another, leaving open the possibility that these proteins may utilise more than one distinct molecular mechanism to repress transcription (Jennings and Ish-Horowicz 2008). It has been speculated that TLE may encompass the intrinsic ability to bind, modulate and protect chromatin to facilitate transcriptional repression. This proposal was based on the known ability of the TLE family to exert their repressive effects by blocking the basal transcriptional machinery, recruiting HDAC's and interacting with the tails of core histones H3 and H4 (Sekiya and Zaret 2007).

TLE proteins have been shown to form homotetramers through interaction of the helices in their Q domains (Chen, et al. 1998). Studies in *Drosophila* have indicated that oligomerisation is required for TLE repression (Song, et al. 2004; Chen, et al. 1998). However, work recently carried out involving mutation of the Q domain (which is required for oligomerisation) indicated that oligomerisation occurs on a context-to-context basis and is not always necessary (Jennings and Ish-Horowicz 2008).

TLE1, like the other *TLE* family members has a role in both the Wnt and Notch signaling cascades. The overlapping expression of Notch and TLE proteins led to the suggestion that these proteins may play a co-operative role in cell maintenance (Liu, et al. 1996). Upon activation of the Notch

pathway, activated transcription complexes containing intracellular Notch (N^{IC}) and CBF1 initiate transcription of their cellular targets. The best characterised Notch targets are the members of the *Hes* family which encodes basic helix-loop-helix proteins (bHLH). TLE1 has been shown to act as a Notch antagonist by binding to the Notch co-repressors in the absence of Notch, thus silencing rather than activating the Notch target gene (Reviewed in Buscarlet and Stifani 2007). The Notch signaling pathway was described in detail in Chapter 1.

Wnt is a key signaling pathway for cell maintenance and oncogenic control in adult differentiated cells. Wnt activity has been linked to cellular functions such as apoptosis, survival, proliferation and cell motility (Reviewed in Willert and Jones 2006). It has been determined that defects in the wnt canonical pathway contribute to the pathogenesis of a variety of malignant states (Gelebart, et al. 2008) and also play a major role in osteoporosis (Patel and Karsenty 2002). The Wnt effector protein Tcf/Lef acts as a transcriptional repressor when bound to TLE1; however, once Wnt is activated β-Catenin directly displaces TLE1 from the Tcf/Lef repression complex, alleviating the TLE/Lef mediated repression. β-Catenin transforms the complex into an activator complex rather than a TLE-associated repressor complex. This confirms that TLE plays the role of a repressor in at least one key cellular pathway (Daniels and Weis 2005).

Here, evidence is presented that (i) TLE1 drives the activation of a CBF1-regulated promoter in an LCL, (ii) TLE1 does not significantly regulate the activity of an NF-κB responsive promoter in the same LCL, (iii) enforced expression of TLE1 in an LCL leads to a decrease in the rate of cell proliferation, (iv) ectopic TLE1 modulates expression of the key EBV latent gene *EBNA*2 and cellular Notch target genes such as *Cyclin D1*, *E2A, Pax5*, *Runx3,* and *CD23*, (v) knockdown of *TLE1* in an LCL using shRNA expression vectors leads to an increase in the rate of cell proliferation and (vi) *TLE1* knockdown also results in the up-regulation of the Notch target genes, *Cyclin D1*, *E2A*, *Pax5*, *Hes1* and *Hey1*.

5.2 Results

5.2.1 TLE1 *trans*-activates a CBF1-regulated promoter in an LCL

The pGa981.6 plasmid is a reporter vector that contains multimerised copies of a CBF1-binding sequence placed upstream of a minimal promoter that drives expression of a downstream *luciferase* gene (Minoguchi, et al. 1997). The transcription factor CBF1 is a primary nuclear effector of the Notch signaling pathway and expression of the reporter enzyme is therefore dependent on this Notch/EBNA2-regulated enhancer. TLE family proteins have been identified as repressor complex participants for Notch target genes (Fortini 2009).

To investigate a role for *TLE1* in the Notch signalling pathway in an LCL background, the IB4 cell line was co-transfected with pCMV2 FLAG *TLE1* (a *TLE1* expression vector) and pGa981.6. As before, and to allow for normalisation of results between individual transfections, a small quantity of β-galactosidase expression vector (pCMV LacZ) was also co-transfected in each case. The cells were harvested 48 hours post-transfection and luciferase levels expressed from the pGa981.6 luciferase reporter construct were quantified using a Luminoskan 1400 luminometer. These results were normalised against β-galactosidase values to compensate for variations in transfection efficiency.

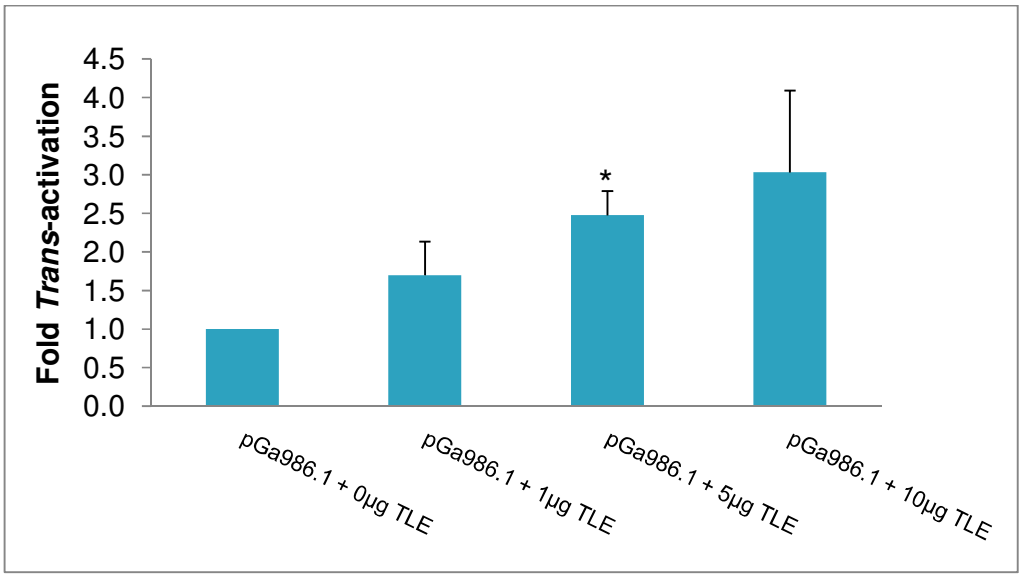


Figure 5.1: *TLE1* positively regulates a CBF1-dependent promoter.

IB4 cells were co-transfected with increasing quantities of pCMV2 FLAG *TLE1;* 0 µg, 1 µg, 5 µg and 10 µg, along with 2 µg of the luciferase reporter construct pGa981.6. Cells were harvested 48 hours post-transfection and assayed for luciferase activity. Luciferase values were evaluated based on the *β*-galactosidase activity measured from a co-transfected pCMV-LacZ reporter, which was included in all transfections. Normalised values were expressed as percentage *trans*-activation relative to the corresponding data obtained from co-transfection with the empty expression vector pCMV and pGa981.6 vector. Data are presented as the average of three independent experiments (Data are Mean ±SD, * $P<0.05$).

Transient transfection of the LCL IB4 showed that co-transfection of the pGa981.6 luciferase reporter plasmid with increasing quantities of pCMV2 FLAG *TLE1* expression vector led to a dose-dependent increase in promoter-driven luciferase values. *Trans*-activation was at its most potent, with an approximate 250% increase in activity upon co-transfection with 5 µg of the TLE1 effector plasmid. Although the extent to which TLE *trans*-activated expression of the luciferase reporter varied between experiments, these results nonetheless indicated that TLE1 could modulate the CBF1-regulated promoter in an LCL.

5.2.2 An NF-κB responsive promoter was not consistently regulated by TLE1 expression in an LCL

The NF-κB signaling cascade is triggered by a broad selection of stimuli and has been found previously to be modulated by EBV. In addition to driving EBV growth transformation and LCL proliferation, activated NF-κB plays a major role in the co-ordination of the immune and inflammatory responses (Tripathi and Aggarwal 2006). To investigate if TLE1 could also influence NF-κB-regulated gene expression, a co-transfection experiment with pCMV2 FLAG *TLE1* and an established NF-κB-responsive luciferase reporter construct, 3xEnh-Luc, was carried out. The 3xEnh-Luc vector contains 3 κB binding elements upstream of a minimal conalbumin promoter with a linked firefly luciferase gene. The LCL IB4 was co-transfected with 3xEnh-Luc and increasing quantities of the *TLE1* expression vector pCMV2 FLAG *TLE1*.

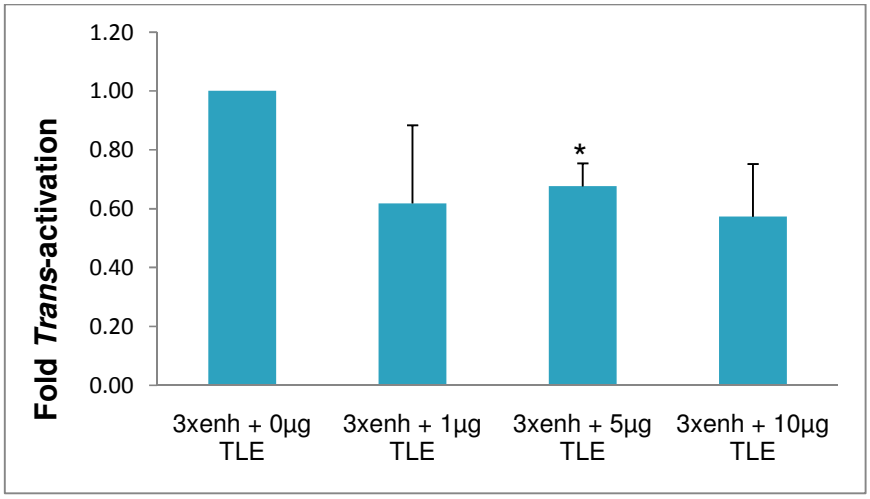


Figure 5.2: TLE1 does not consistently regulate an NF-κB-dependent promoter.

IB4 cells were co-transfected with increasing quantities of pCMV2 FLAG *TLE1;* 0 µg, 1 µg, 5 µg and 10 µg, along with 2 µg of the luciferase reporter construct 3xEnh-Luc. Cells were harvested 48 hours post-transfection and assayed for luciferase activity. Luciferase values were calculated as previously described and normalised values were expressed as percentage *trans*-activation relative to the corresponding data obtained from co-transfection with the empty expression vector pCMV and 3xEnh-Luc vector. Data are presented as the average of three independent experiments (Data are Mean ±SD, * $P<0.05$).

Co-transfection of 3xEnh-Luc with 5 µg pCMV2 FLAG *TLE1* gave a consistent 35% decrease in luciferase activity within the triplicate experiments performed in this study. It can be seen however that the effect was neither consistent between experiments nor dose-dependent for the regulation of the κB-dependant reporter due to TLE1 co-expression. Co-transfection with 1 µg and 10 µg of effector plasmid co-incided with inconsistent reporter enzyme activity readings over a number of experiments. In some experiments 1 ug of pCMV2 FLAG *TLE1* was sufficient to elicit a repressional effect on 3xEnh-Luc, but repeat experiments did not always concur. Given the inconsistencies and the modest scale of repression observed, a role of *TLE1* in the regulation of NF-κB target genes in LCLs was not pursued.

5.2.3 Expression of ectopic TLE1 in an LCL

The evidence presented so far in this study suggests that EBV down-regulates *TLE1* in an LCL. In order to investigate a role for this virus-host interaction, the pcDNA3-TLE1 plasmid was used to transfect the IB4 LCL and ectopically produce TLE1 protein. Transfected cell pools were established by transfecting IB4 with 5 µg of plasmid followed by drug selection to establish a stable pool of cells expressing exogenous TLE1. A similar pool of cells was simultaneously established with the corresponding empty vector, pcDNA3, to act as a negative control. The mammalian expression vector pcDNA3 possess the neomycin resistance gene (Figure 5.3A) that confers resistance to the antibiotic geneticin (G418). The ectopic expression of TLE1 in G418-selected cell pools was confirmed by Western blotting, immunostaining and RT-qPCR, and phenotypic properties were compared with those transfected with the empty vector control.

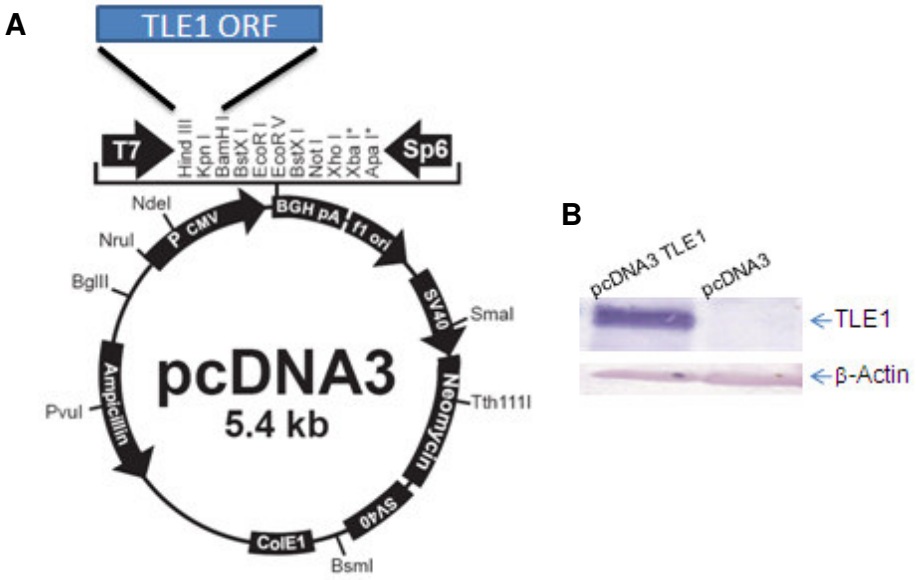


Figure 5.3: Expression of TLE1 from pcDNA3-TLE1.

(A) Schematic circular map of the pcDNA3-TLE1 expression vector. Highlighted is the region into which the TLE1 ORF, encompassing the DNA sequence for residues 1-770, was sub-cloned. The human cytomegalovirus immediate-early promoter (pCMV) is capable of supporting high-level expression in mammalian cells. The vector also contains the neomycin resistance gene to facilitate stable mammalian cell line selection. **(B)** The expression of ectopic TLE1 from transfected plasmid was confirmed by transient transfection in the low TLE1-expressing cell line BJAB, followed by Western blotting. TLE1 is shown in the upper panel. The lower panel shows β-Actin expression as a loading control. The empty pcDNA3 expression vector was used as control.

To confirm the expression of TLE1 from the pcDNA3-TLE1 vector, the BJAB cell line was transiently transfected with 5 µg pcDNA3-TLE1, along with a negative control transfection using 5 µg pcDNA3 empty vector. After 48 hours protein was harvested from the transfected cells and analysed by Western blotting (Figure 5.3B). To establish the stable cell pool, the IB4 cell line was transfected by electroporation with either 5 µg pcDNA3-TLE1 or 5 µg pcDNA3 expression vector. After 24 hours the cells were sub-cultured into fresh medium containing 800 µg/ml G418. Following three weeks selection cell pools were examined to determine the relative *TLE1* expression levels in each population.

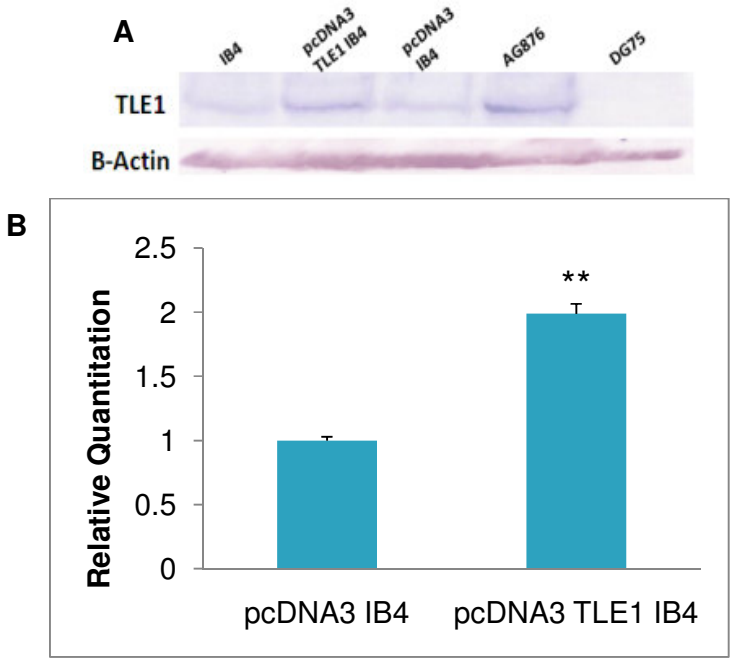


Figure 5.4: Enhanced TLE1 expression in pcDNA3-TLE1-IB4 cell pool.

Protein and total RNA were extracted from each cell pool and *TLE1* expression levels were verified using Western blot analysis and RT-qPCR respectively. **(A)** Western Blot showing TLE1 expression. The upper panel shows the levels of TLE1 protein in various cell extracts as indicated underneath the panel. All extracts were normalised to the loading control β-Actin (lower panel). TLE1 protein levels can be compared for the parental IB4 cell line, pcDNA3-TLE1-IB4 and pcDNA3-IB4. Controls included AG876 (positive control) and DG75 (negative control). **(B)** RNA samples from each pool were monitored by RT-qPCR and results were normalised as before (relative to *TBP)*. In this experiment, the control pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01).

Western blot analysis of protein extracts prepared from the two cell pools pcDNA3-TLE1-IB4 and pcDNA3-IB4, showed that TLE1 protein was expressed at a considerably higher level when the TLE1-expression vector was used. The parental IB4 cell line can be seen to express endogenous TLE1 at a lower level and equivalent to that seen in the pcDNA3-transfected cell pool. These observations were corroborated by RT-qPCR which demonstrates more than a two-fold increase in the level of *TLE1* mRNA when the TLE1 expression vector was used.

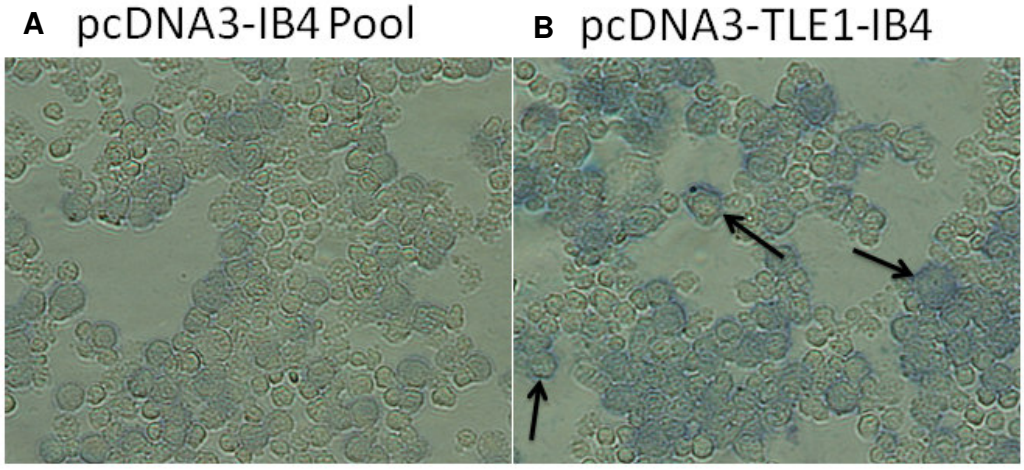


Figure 5.5: Increased immuno-staining for TLE1 in pcDNA3-TLE1-IB4 cell pool.

TLE1 staining of fixed cells sampled from the ectopic cell pool and the negative control at a magnification of 70x. TLE1 protein detected using a rabbit polyclonal IgG (Abcam, ab15587) at a dilution of 1:50 in conjunction with a secondary Goat Anti-rabbit IgG HRP Conjugate (Santa Cruz sc-2301) **(A)** pcDNA3-IB4 cells display low level staining for TLE1 **(B)** Exogenous TLE1 expression is detected by enhanced staining in the pcDNA3-TLE1-IB4 cells.

Immunostaining was then used to determine the relative TLE1 protein expression within each cell pool. Cells were fixed to a glass slide and incubated with a TLE1 specific antibody (Abcam) prior to probing with a HRP-conjugated secondary antibody (Santa Cruz). Images from figure 5.5 illustrate direct immuno-staining results for both the empty pcDNA3 IB4 cell pool and the ectopic TLE1-expressing pool. The results show that cells with elevated TLE1 (see arrows for an example) were present in high frequency in the pcDNA3 TLE1-transfected cell pool.

5.2.4 Ectopic TLE1 reduces LCL proliferation

In order to determine the consequence of increased TLE1 expression on LCL growth, cell counts and an MTS proliferation assay were performed on the IB4-derived cell pools, generated as described in section 5.2.3. Cell numbers were calculated using an Adam Counter, a desktop machine that determines total viable cell numbers and by utilising the vital cell dye, propidium iodide (PI). This was performed in tandem with the MTS-based cell titre proliferation assay. MTS is a tetrazolium salt that is reduced to purple formazan crystals by mitochondrial succinate dehydrogenase This method evaluates relative cell activity by quantifying the mitochondrial activity in a cell sample. Cells were seeded at 2 x 10^5 cells/ml and monitored over 48 hours according to manufacturer's recommendations.

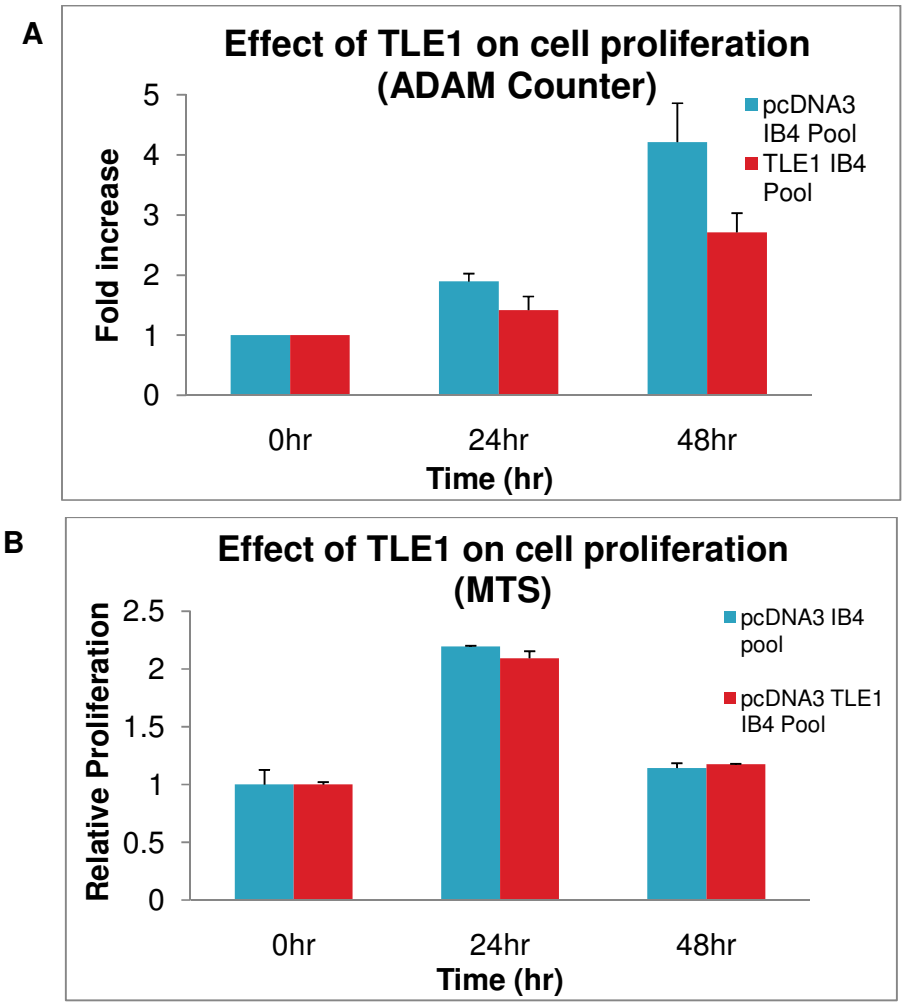


Figure 5.6: Effect of TLE1 on cell proliferation using MTS and ADAM counting methods.

Both cell pools were seeded at the same density and maintained under the same conditions. **(A)** cell numbers were determined using an Adam counter at three time points. This was performed in triplicate and the results were depicted as fold increase in cell number. The pcDNA3-TLE1-transfected pool showed lower cell population numbers than the empty vector control cell pool. **(B)** Cell pool mitochondrial activity was determined using the MTS assay at 24 hour intervals in triplicate. No difference could be seen in the overall rates of proliferation of the two cell pools by this method.

Direct viable cell counts performed using the ADAM™ counter repeatedly produced the same trend, showing higher cell numbers for the empty vector-transfected pool in comparison to the population expressing ectopic TLE1 (Figure 5.6A). However over time, although antibiotic selection was maintained, it became apparent in subsequent experiments that ectopic *TLE1* mRNA levels declined over time (Confirmed by RT-qPCR, data not

shown). This may have been due to the outgrowth of non-TLE1-expressing drug-resistant cells. The evidence therefore suggests that TLE1 expression had a negative effect on LCL cell growth. On the other hand, results from the MTS assay (Figure 5.6B), do not suggest any significant difference in the overall rates of mitochondrial activity between the two cell pools. The MTS assay measures total mitochondrial activity however and gives an indirect reflection of viable cell numbers. Limitations to the MTS assay have been widely accepted, and it is possible that the presence of G418 in the growth medium may have interfered with the efficacy of the assay (Wang, et al. 2010).

5.2.5 Ectopic TLE1 modulates the EBV *EBNA2* gene but has no effect on *EBNA1*

EBNA2, as described in detail in chapter one, is a master regulator that is critical for the initiation of EBV-associated B lymphocyte transformation. Furthermore, ongoing *EBNA2* expression is required to maintain LCL growth.

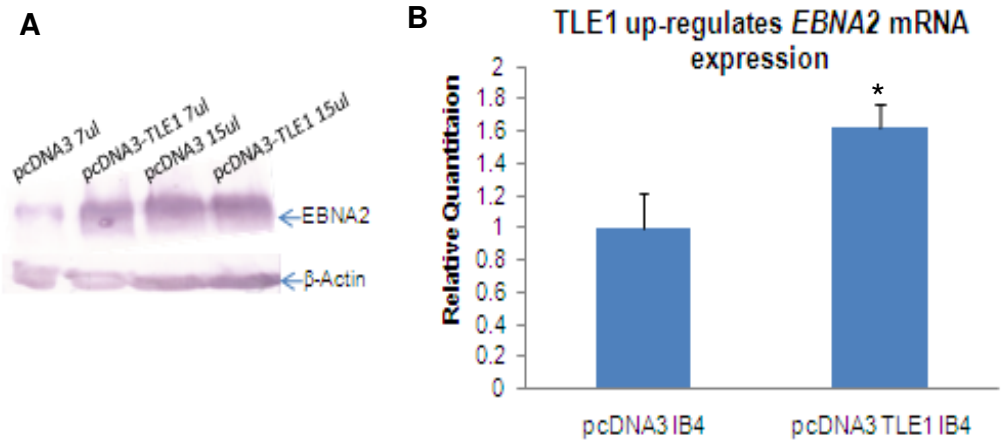


Figure 5.7: Ectopic TLE1 induces *EBNA2* expression.

Total RNA and protein was prepared from each cell pool, pcDNA3 IB4 and pcDNA3 TLE1 IB4 **(A)** EBNA2 protein expression was investigated via western blot analysis. The upper panel illustrates the level of EBNA2 and normalised for the endogenous loading control β-Actin. EBNA2 protein levels were strong at normal loading concentrations (15 ul). For this reason, a lower protein concentration was also used (7 ul) so that any subtle differences in protein levels can be clearly observed. **(B)** *EBNA2* expression levels were quantified using RT-qPCR. Results were normalised as before (relative to *TBP)*. Analysis was performed as previously described. In this experiment, the control pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$).

The LCL IB4 expresses wild type EBNA2 as part of the EBV latency III programme. However, Western blot analysis of the two cell pools pcDNA3-TLE1-IB4 and pcDNA3-IB4 showed that EBNA2 was expressed at a considerably higher level in response to ectopic TLE1 (Figure 5.7A). These results were confirmed by RT-qPCR (Figure 5.7B). In addition to *EBNA2*, *EBNA1* transcription was also examined in the two cell pools. However, no change was observed in the *EBNA1* mRNA level in response to ectopic TLE1 (Figure 5.8).

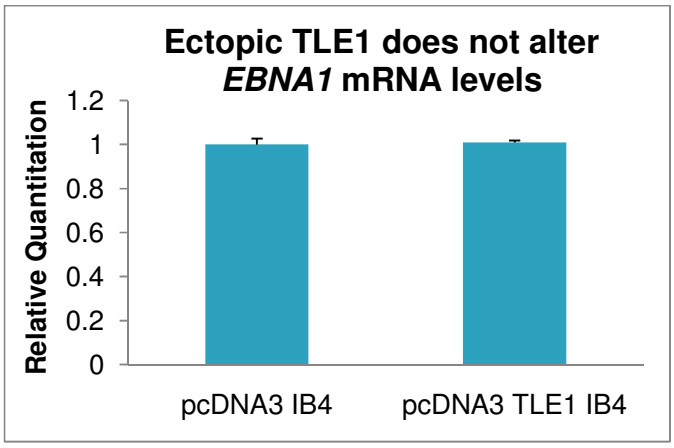


Figure 5.8: Ectopic TLE1 does not alter *EBNA1* mRNA levels.

Total RNA was prepared from the ectopic TLE1-expressing cell pool (pcDNA3 TLE1 IB4) and the negative control cell pool (pcDNA3 IB4). RT-qPCR was employed to monitor *EBNA1* mRNA levels. The analysis was performed as previously described for RT-qPCR experiments. The level of EBNA1 mRNA in the control pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD).

5.2.6 Ectopic TLE1 modulates a range of potential and known Notch target genes

To determine the rationale for EBV-led regulation of *TLE1*, several known Notch target genes were monitored after the modulation of TLE1 expression within an LCL. These Notch targets were chosen due to their known association with EBV, and their potential to interact with TLE1. Correlating changes in Notch target expression due to TLE1 or EBV activity may help to elucidate some of the mechanisms behind EBV B cell immortalisation.

5.2.6.1 Ectopic TLE1 modulates Cyclin D1

Cell cycle progression requires activation by a family of proteins called the cyclins. *Cyclin D1*, a member of this family, plays a fundamental role in cellular proliferation and as such is regulated by multiple cell signaling pathways including NF-κB, Notch and Wnt signaling. *Cyclin D1* must be activated for the cycling cell to progress from the G1 phase to the S phase. Conversely, *Cyclin D1* repression leads to terminal cell differentiation (Reviewed in Klein and Assoian 2008).

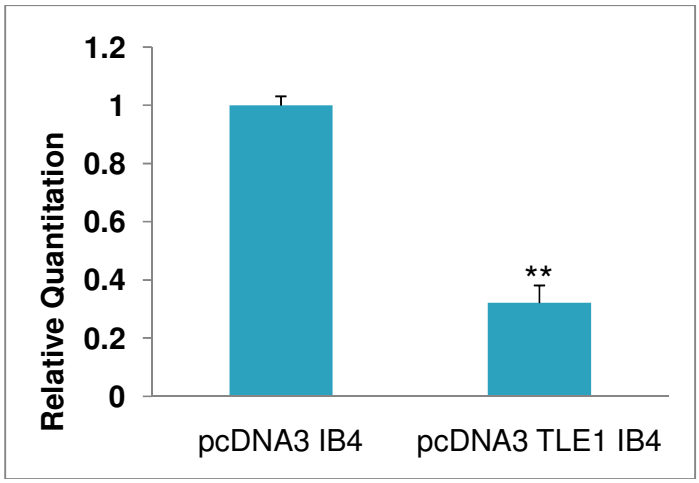


Figure 5.9: Ectopic TLE1 represses *Cyclin D1* expression.

Total RNA was extracted from each cell pool and *Cyclin D1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *CyclinD1* mRNA from pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, **P<0.01).

This experiment showed that *Cyclin D1* mRNA levels were reduced by approximately 70% in the cell pools that expressed ectopic TLE1 (Figure 5.9). This implies that TLE1 either directly or indirectly negatively regulates *Cyclin D1* in an LCL.

5.2.6.2 Ectopic TLE1 modulates E2A

E2A (also called TCF-3) is composed of two bHLH transcription factor proteins E12 and E47, arising from alternative splicing of the bHLH-coding exon. These proteins form homo- and heterodimers with other bHLH factors to modulate the expression of their target genes (Slattery, et al. 2008). It has been speculated that E2A proteins appear to be found more frequently in areas of rapid cell proliferation and differentiation (Rutherford and LeBrun 1998). E2A activity has been shown to be required for class-switch recombination after B cell activation (Portis and Longnecker 2003) and Notch signaling has been demonstrated to indirectly induce the degradation of E2A within T and B cells (Huang, et al. 2004b).

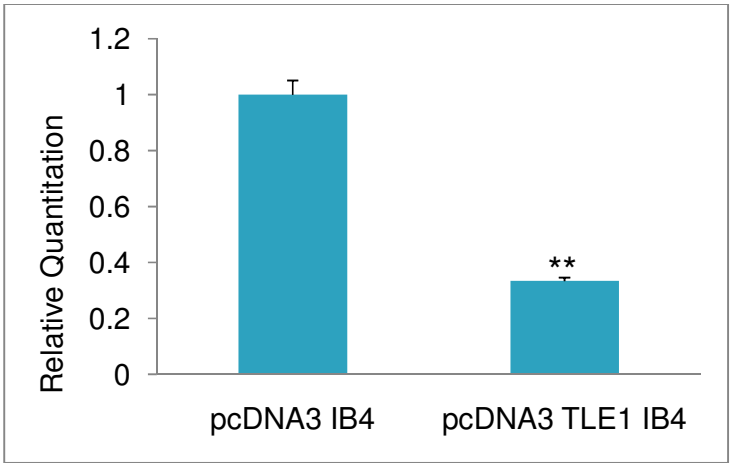


Figure 5.10: Ectopic TLE1 represses *E2A* at the transcriptional level.

Total RNA was extracted from each cell pool and *E2A* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *E2A* mRNA from pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, **P<0.01).

A significant down-regulation of approximately 70% was observed for *E2A* at the mRNA level in the ectopically-expressing TLE1 cell pool, in comparison to the negative control cell pool (Figure 5.10).

5.2.6.3 Ectopic TLE1 modulates Pax5

The expression of *Pax5* is found almost exclusively in B lymphocytes. Pax5 is a paired-domain transcription factor critical for both the initiation and maintenance of B cell lineage commitment (Decker, et al. 2009; Nutt and Kee 2007). The activity of Pax5 promotes key B cell features such as pre-BCR and BCR signaling, along with CD19 and CD21 stimulation (Reviewed in Cobaleda, et al. 2007). In addition to this, Pax5 activity has been shown to repress *Notch1* transcription in B cells (Souabni, et al. 2007). Pax5 has also been associated with EBV as it has been proven to be a crucial factor for Wp (*EBNA* promoter) activation (Tierney, et al. 2007) and a recent comparative study has indicated that *Pax5* expression, usually restricted to B cells, can be detected in non-B lymphocyte cases of PTLD in which EBV infection plays a role in pathogenesis (León, et al. 2009). These findings made Pax5 an interesting target for this study.

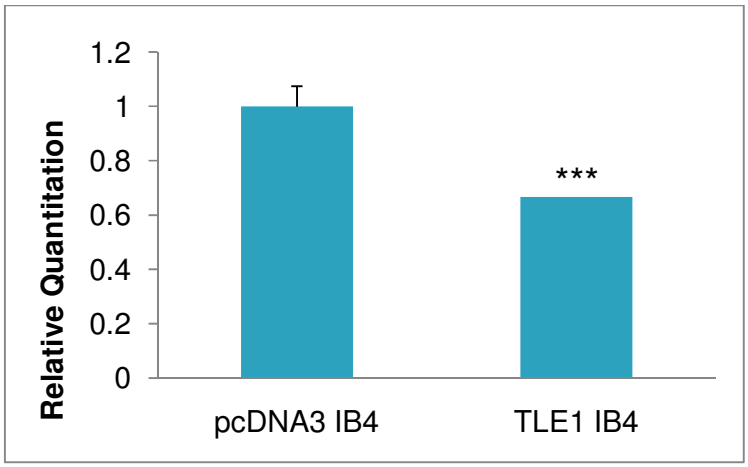


Figure 5.11: Ectopic TLE1 represses *Pax5* expression.

Total RNA was extracted from each cell pool and *Pax5* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Pax5* mRNA from pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, ***P<0.001).

In this experiment it can be seen that *Pax5* mRNA levels were significantly reduced (approximately 35% lower) in IB4 cells expressing ectopic TLE1 when compared to cells transfected with empty vector alone (Figure 5.11).

5.2.6.4 Ectopic TLE1 modulates Runx3

Runx3 is a transcription factor that can act as both a positive and negative mediator in a context dependent manner (Gao, et al. 2010). It belongs to the Runt family of transcription factors that includes Runx1 and Runx2 (Kania, et al. 1990). Human Runx proteins have a common structure comprising: (i) a crucial N-terminal Runt domain, which is key for the hetero-dimerisation function of Runx and mediates direct DNA binding to elicit regulation, (ii) followed by a *trans*-activation domain, and (iii) several repression linked domains, one of which is the TLE-linked recruitment motif, VWRPY, located in the C-terminal domain. TLE family members have been shown to bind the VWRPY region of Runx to act as a co-factor for Runx-led repression (Imai, et al. 1998; Levanon, et al. 1998; McLarren, et al. 2001). Runx proteins regulate cell differentiation and proliferation; furthermore, they possess a critical role in haematopoiesis (Gao, et al. 2010).

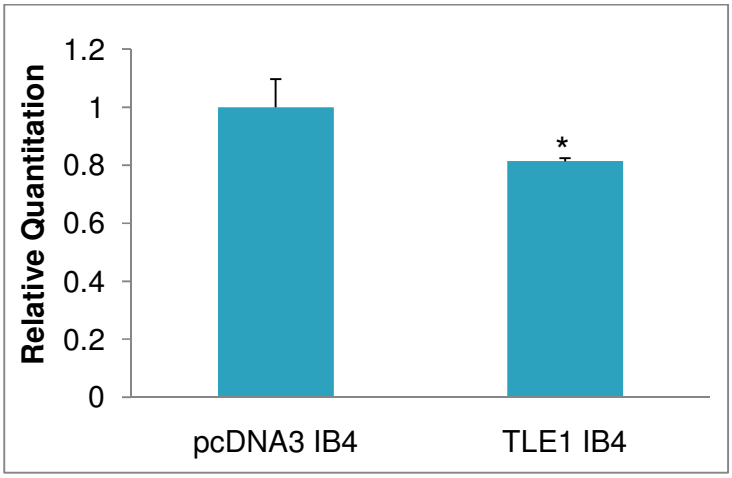


Figure 5.12: Ectopic TLE1 represses *Runx3* expression.

Total RNA was harvested from each cell pool as indicated. RNA samples were monitored by RT-qPCR and results were normalised as before (relative to *TBP)*. In this experiment, the level of *Runx3* mRNA from pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, *P<0.05).

It was observed that *Runx3* mRNA levels were down-regulated (approximately 20% lower) in the ectopic TLE1-expressing cell pool (Figure 5.12).

5.2.6.5 Ectopic TLE1 down-regulates CD23

CD23 is a low affinity IgE receptor; a *trans*-membrane protein of 46 kDa expressed on both B lymphocytes and cells of the myeloid compartment (Lemieux, et al. 2007). Human CD21 is the natural ligand for CD23 (Gorelik, et al. 2004), and both CD21 and CD23 are induced by EBV infection (Cordier, et al. 1990). *CD23* was first implicated as a direct EBV target in 1987 by Wang and colleagues (Wang, et al. 1987) and over time EBNA2 was found to be the EBV protein responsible for *CD23* induction.

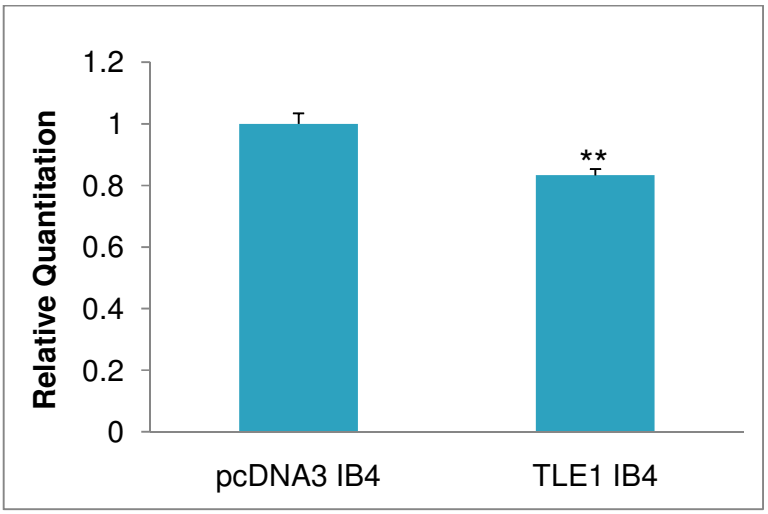


Figure 5.13: Ectopic TLE1 represses *CD23* expression.

Total RNA was extracted from each cell pool and *CD23* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *CD23* mRNA from pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD, **P<0.01).

In the experiment ectopic TLE1 was observed to exhibit a negative regulatory effect on *CD23* mRNA levels (approximately 17%; Figure 5.13). In an EBV infected cell, EBNA2 is the main driving force behind *CD23* expression. However in IB4, although TLE1 appears to positively regulate *EBNA2*, *CD23* –itself an *EBNA2* positive target- is modestly down-regulated in response to ectopic TLE1.

5.2.6.6 Ectopic TLE1 does not significantly modulate Hes1, Hey1 or Runx1

The mRNA levels of a number of prominent Notch target genes such as *Hey1*, *Runx1* and *Hes1* were not found to be significantly altered by the expression of ectopic *TLE1* in an LCL. *Hey1* is a key protein critical for managing embryonic development and differentiation (Steidl, et al. 2000). The Hey1 protein displays a carboxy-terminal YRPW motif that is similar to the TLE recognition site (Steidl, et al. 2000). *Runx1* was chosen as a target for investigation as it is a negative target of EBV and exists in B cells as a *Runx3* agonist.

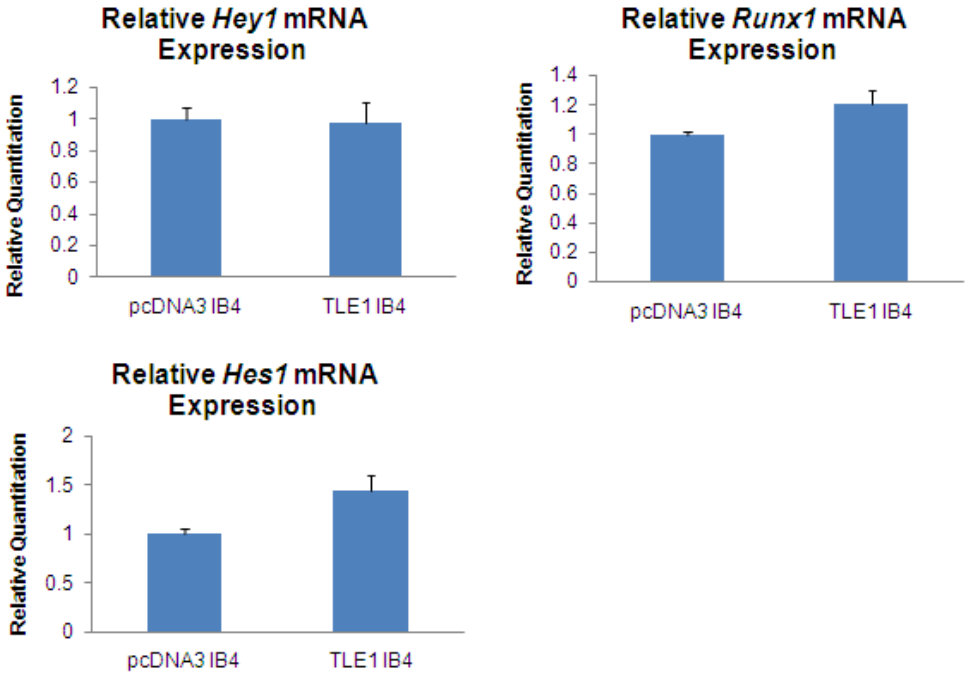


Figure 5.14: Ectopic TLE1 has no statistically significant transcriptional effect on *Hey1*, *Runx1* and *Hes1* expression in an LCL.

Total RNA was extracted from each cell pool and *Hes1, Hey1 and Runx1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Hes1, Hey1 and Runx1* mRNA from pcDNA3 IB4 was arbitrarily assigned a value of 1. (Data are Mean ±SD).

Endogenous TLE1 is produced at low levels in both of these cell pools and so it cannot be definitively established from this experiment if these genes are regulated by TLE1, as the threshold for an effective concentration of TLE1 may already be met. However, it can be concluded that ectopic TLE1 expression in an LCL does not modulate further the expression of *Hey1*, *Runx1* or *Hes1* at the transcriptional level.

5.2.7 shRNA knockdown of *TLE1* expression in the IB4 LCL

In this study, evidence of decreased cell proliferation was observed for an LCL cell pool expressing ectopic TLE1. Gene knockdown technology was next employed to determine if reducing the level of endogenous TLE1 might have a pro-proliferative effect in the same cell line. Plasmid vectors expressing short hairpin RNAs (shRNA) specific to *TLE1* mRNA were therefore obtained. A panel of such plasmids were first evaluated for efficacy of *TLE1* knockdown. An shRNA of scrambled sequence, expressed from the same parental vector was used as a negative control in every experiment. These plasmids were used to transfect IB4 and the growth/proliferation of transfected cells exhibiting decreased *TLE1* expression was then investigated using a number of assays including trypan exclusion cell counts, and the MTS proliferation assay.

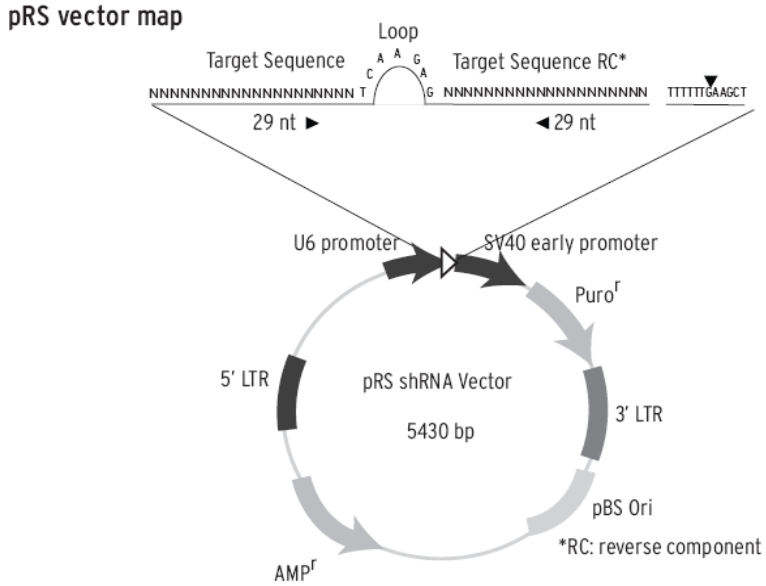


Figure 5.15: shRNA expression vectors.

Schematic illustrating the fundamental components of the shRNA vectors (Origene™). The amplified region of the construct indicates the shRNA-coding sequence in each plasmid. This region lies directly downstream of a U6 promoter and contains 29 nucleotides of target recognition sequence lying either side of a 7 nucleotide loop. The plasmids contain the ampicillin resistance gene for bacterial selection. The puromycin resistance gene to allow for selection of transfected mammalian cell lines is found downstream of an SV40 early promoter. shRNA vectors assigned the names sh205-sh208 denote vectors expressing differing, albeit functioning shRNAs against the same target, *TLE1*. All experiments were performed with an additional construct expressing scrambled shRNA as a control.

An experiment was performed to first determine the efficiency of the panel of shRNA expression vectors with regard to *TLE1* knockdown. The transfection efficiency of IB4 was determined to be approximately 15%. Due to this low overall efficiency, subtle differences in the effectiveness of the shRNA plasmids may have been masked by the remaining population of untransfected cells. To circumvent this issue a preliminary experiment was performed in which the TLE1 expression vector pcDNA3-*TLE1* was co-expressed with the shRNA vectors in IB4. Inclusion of the TLE1 expression vector would, therefore, give an enhanced level of TLE1 mRNA in the transfected cells, thereby making any knockdown effect of the co-expressed shRNA more obvious. IB4 cells were co-transfected with 2 µg of one of each shRNA vector along with 3 µg of pcDNA3-TLE1. Total RNA and protein are harvested from cells 48 hours post-transfection. *TLE1* mRNA and protein were monitored as before by RT-qPCR and Western blotting.

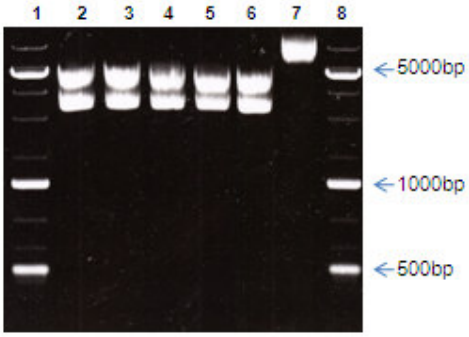


Figure 5.16: Topological analysis of shRNA vectors.

Agarose gel electrophoresis (1% agarose gel) was used to determine the topological condition of the shRNA vectors and pcDNA3-TLE1. Lane 1 and 8 contain 1 kb ladder (Fermentas). Lane 2-5 contains 500ng of the shRNA-expressing vectors sh205, sh206, sh207 and sh208 respectively. Lane 6 contains 500ng of the scrambled shRNA vector, sh30003 and Lane 7 contains 500ng of pcDNA3-TLE1.

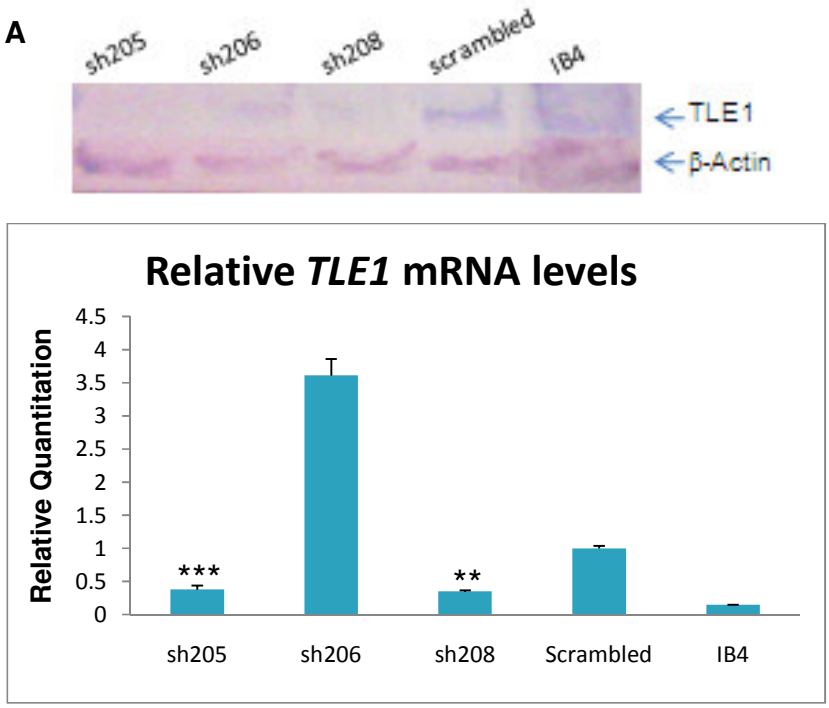


Figure 5.17: *TLE1* knockdown by shRNA vectors.

Preliminary analysis in IB4 cells to determine the efficacy of the shRNA expressing vectors co-transfected with pcDNA3-TLE1. The functioning shRNA vectors are designated sh205, sh206 and sh208. Results indicate that both sh205 and sh208 can reduce TLE1 mRNA levels. **(A)** Protein extracts taken 48 hours after transfection were analysed by Western blot analysis. The upper panel illustrates the level of TLE1 protein and normalised for the endogenous loading control β-Actin in the lower panel. **(B)** RNA samples were monitored for *TLE1* transcripts by RT-qPCR and results were normalised as before (relative to *TBP)*. In this experiment, the scrambled shRNA vector was appointed the control and arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01, *** P<0.001).

Co-expression of shRNA expressing vectors sh205 and sh208 showed significant knockdown of *TLE1* in comparison to the scrambled shRNA control, at both the mRNA and protein level (Figure 5.17). Sh206 co-expression did not lead to a decrease in TLE1 protein and in fact coincided with an increase in the level of *TLE1* mRNA. In a second experiment, IB4 was once again transfected with the full panel of shRNA vectors, but this time without the presence of the exogenous TLE1 expression vector pcDNA3-TLE1. Again IB4 cells were transfected with 2 µg of each shRNA vector by nucleofection. Total mRNA and protein were prepared from cells 48 hours post-transfection and *TLE1* expression was investigated as before by RT-qPCR and Western blotting.

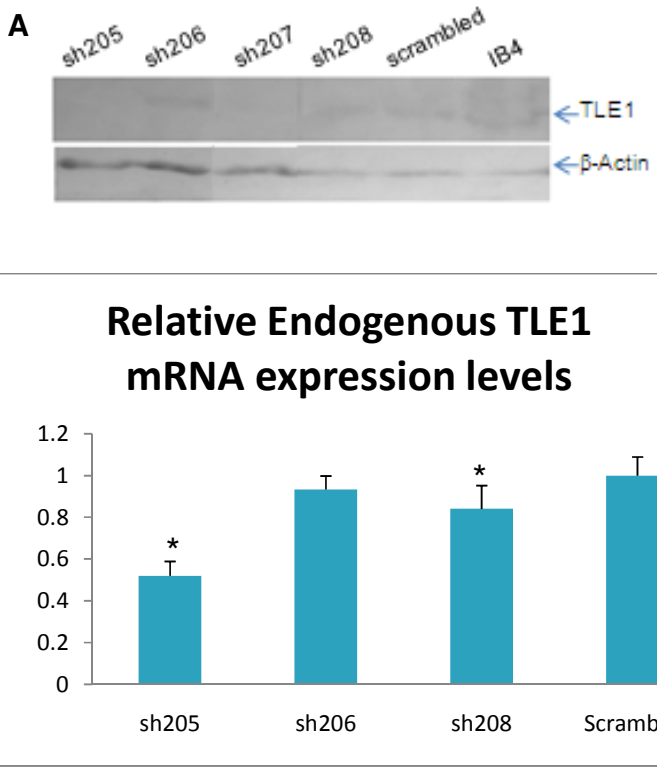


Figure 5.18: *TLE1* knockdown using shRNA expression vectors.

The IB4 cell line was transfected with the panel of shRNA vectors using electroporation to determine the most potent of the supplied shRNA vectors. The shRNA vectors designated were sh205, sh206, sh207 and sh208. **(A)** As previously described, protein extracts were taken 48 hours post-transfection and investigated through Western blot analysis. The level of TLE1 protein was detected as before and normalised to the loading control, β-Actin. **(B)** RNA samples were monitored for TLE1 transcripts by RT-qPCR and results were normalised as before (relative to *TBP)*. In this experiment, the scrambled shRNA vector was designated as the control; arbitrarily assigned a value of 1. (Data are Mean ±SD, * P<0.05).

In both experiments it was demonstrated that the shRNA vectors sh205 and sh208 were capable of reducing *TLE1* expression significantly in comparison the non-specific scrambled vector (Figure 5.18). For further studies the shRNA knockdown vectors, sh205 and sh208, were utilised for *TLE1* inhibition in conjunction with the scrambled shRNA expressing vector, as a control.

5.2.8 RT-qPCR analysis of mRNA in IB4 cells transfected with shRNA-expressing vectors

In order to determine the transcriptional effect of *TLE1* knockdown in an LCL, the IB4 cell line was transfected with 5 µg of one of three shRNA vectors; (i) scrambled shRNA, (ii) sh205 or (iii) sh208. Cells were purified by Ficoll gradient separation 24 hours post-transfection to remove unwanted dead cells and debris. Total RNA was harvested 72 hours post-transfection and RT-qPCR was used to determine the expression profile for a select panel of Notch target genes.

5.2.8.1 TLE1 knockdown modulates EBNA1

It was previously determined in this study that ectopic TLE1 did not alter the steady state levels of *EBNA1* in IB4 cells (Section 5.2.5). However, in this part of the study, *TLE1* knockdown had a negative effect on the levels of EBNA1 mRNA. It can be seen that reducing *TLE1* expression using shRNA led to a decrease in the level of *EBNA1* mRNA (Figure 5.19). Together, these results indicate that the endogenous level of TLE1 is sufficient to mediate EBNA1 regulation, but that ectopic TLE1 does not lead to further *EBNA1* modulation.

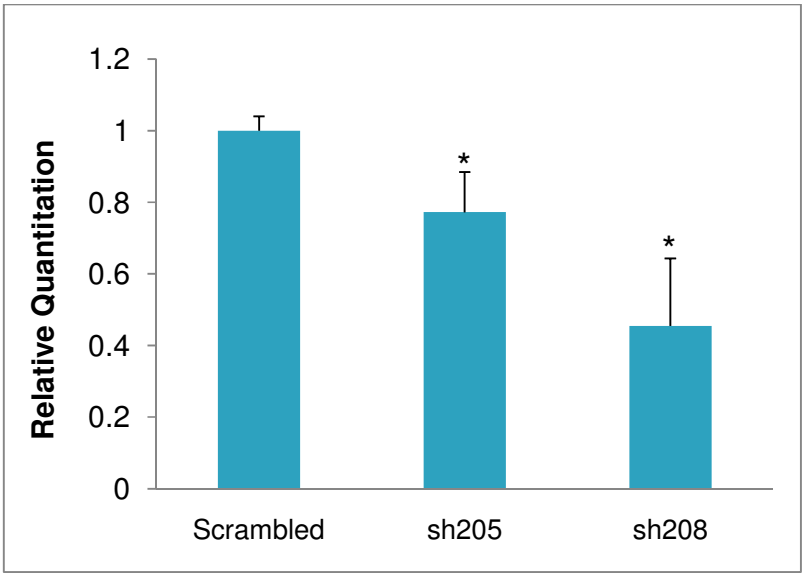


Figure 5.19: *TLE1* knockdown down-regulates *EBNA1* expression.

Total RNA was extracted from each shRNA-expressing cell pool 72 hours post-transfection and *EBNA1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *EBNA1* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD, *P<0.05).

5.2.8.2 TLE1 knockdown modulates Cyclin D1

It was previously determined in this study that ectopic TLE1 down-regulated *Cyclin D1* at the transcriptional level in IB4 cells (Section 5.2.6.1). In this part of the study, the effect of *TLE1* knockdown on *Cyclin D1* mRNA levels was examined. It can be seen that reducing *TLE1* expression using shRNA led to an increase in the level of *Cyclin D1* mRNA (Figure 5.20). This finding is in agreement with the results previously obtained in this study, and indicates that TLE1 negatively regulates *Cyclin D1.*

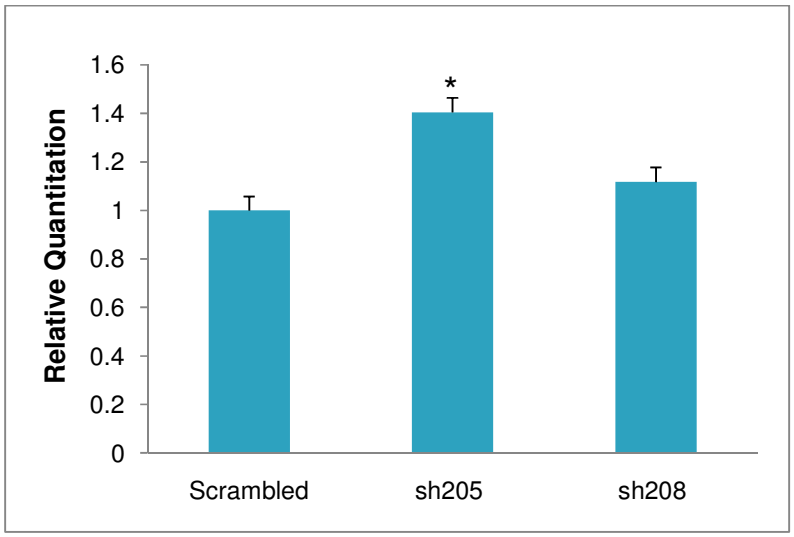


Figure 5.20: *TLE1* knockdown induces *Cyclin D1* expression.

Total RNA was extracted from each shRNA-expressing cell pool 72 hours post-transfection and *Cyclin D1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Cyclin D1* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD, *P<0.05).

5.2.8.3 TLE1 knockdown modulates E2A

It has been shown earlier in this study that ectopic TLE1 leads to significant down-regulation of *E2A* in IB4 cells (Section 5.2.6.2). Here, the effect of *TLE1* knockdown on *E2A* expression was next examined. It can be seen that *TLE1* knockdown using shRNAs led to an increase in *E2A* mRNA levels (Figure 5.21). These findings are in agreement with the results previously obtained in this study and indicate that TLE1 negatively regulates *E2A* at the transcriptional level.

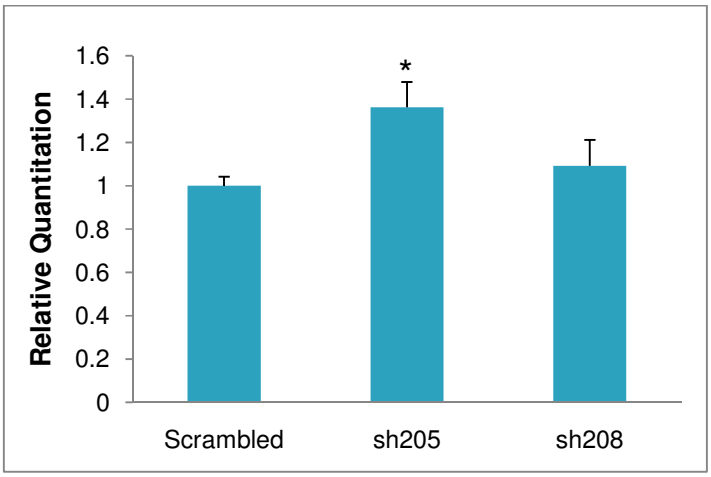


Figure 5.21: *TLE1* knockdown induces *E2A*.

Total RNA was extracted from each shRNA-expressing cell pool 72 hours post-transfection and *E2A* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *E2A* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD, *P<0.05).

5.2.8.4 TLE1 knockdown modulates Pax5

Results obtained earlier in this study showed that ectopic TLE1 led to *Pax5* down-regulation in IB4 cells (Section 5.2.6.3). In this part of the study, the effect of *TLE1* knockdown on *Pax5* mRNA levels was examined. It was determined that *TLE1* knockdown using shRNA led to an increase in *Pax5* mRNA levels (Figure 5.22). These findings are in agreement with the previously obtained results in this study, and indicate that TLE1 negatively regulates *Pax5* at the transcriptional level.

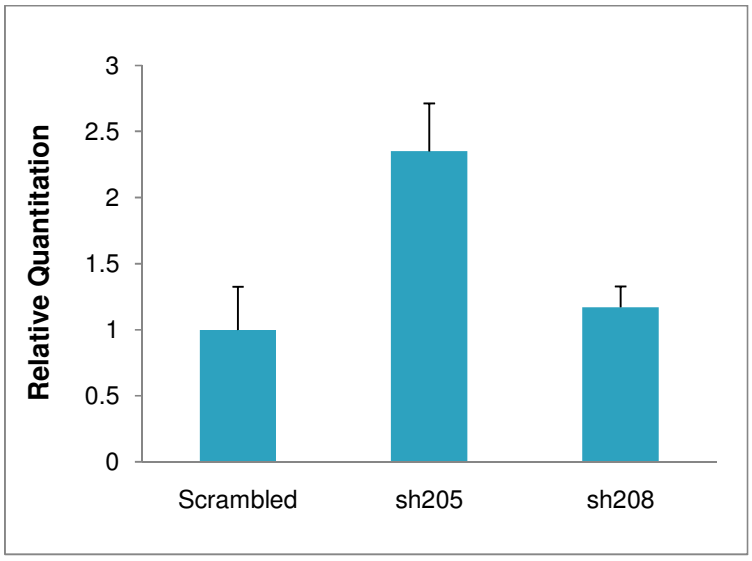


Figure 5.22: *TLE1* knockdown induces *Pax5*.

Total RNA was extracted from each shRNA-expressing cell pool at 72 hours post-transfection and *Pax5* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Pax5* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD).

5.2.8.5 TLE1 knockdown does not modulate Runx3

It was shown earlier in this study that ectopic TLE1 led to down-regulation of *Runx3* at the transcriptional level in IB4 cells (Section 5.2.6.4). Here, the effect of *TLE1* knockdown on *Runx3* mRNA levels was examined. It was determined that reducing *TLE1* expression using shRNA technology had no significant effect on the mRNA level from *Runx3* (Figure 5.23). Together, these results imply that although enforced TLE1 over-expression does repress *Runx3*, the endogenous level of TLE1 in IB4 appears not to have a regulatory effect on *Runx3.*

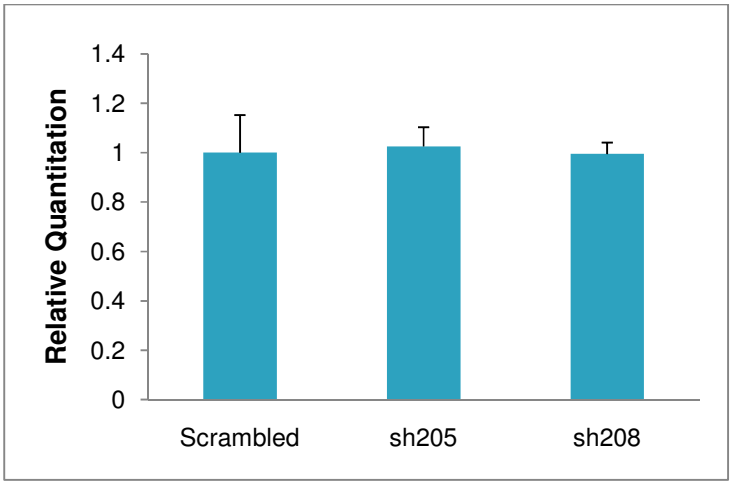


Figure 5.23: *TLE1* knockdown does not modulate *Runx3*.

Total RNA was extracted from each shRNA-expressing cell pool at 72 hours post-transfection and *Runx3* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Runx3* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD).

5.2.8.6 TLE1 knockdown does not modulate CD23

It was shown earlier in this study that ectopic TLE1 led to up-regulated *CD23* in IB4 cells (Section 5.2.6.5). Here, the effect of shRNA-mediated *TLE1* knockdown on *CD23* was next examined. No significant change in *CD23* mRNA levels was observed in response to *TLE1* knockdown (Figure 5.24). Together, these results imply that although enforced TLE1 over-expression does repress *CD23*, the endogenous level of TLE1 in IB4 appears not to have a regulatory effect on this gene.

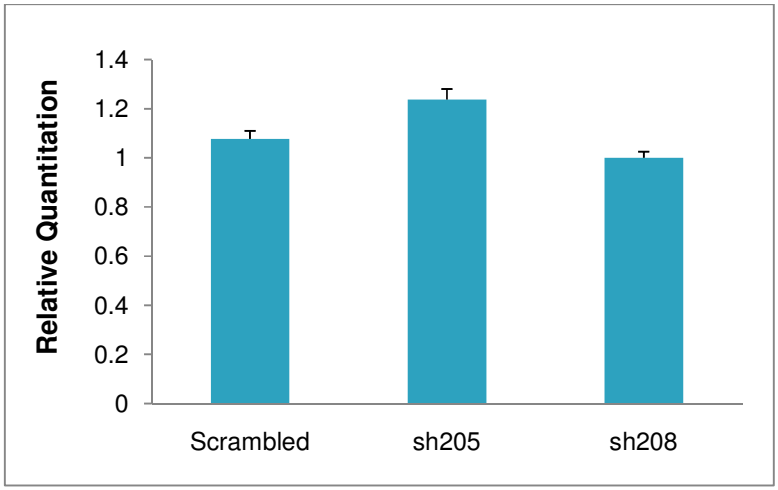


Figure 5.24: *TLE1* knockdown does not modulate *CD23*.

Total RNA was extracted from each shRNA-expressing cell pool at 72 hours post-transfection and *CD23* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *CD23* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD).

5.2.8.7 TLE1 knockdown modulates Hes1

It was shown earlier in this study that ectopic TLE1 had no effect on *Hes1* mRNA levels in IB4 (Section 5.2.6.6). Here however, *TLE1* knockdown was seen to correlate with increased *Hes1* mRNA levels (Figure 5.25). Together, these results imply that the endogenous level of TLE1 is sufficient to have a repressive effect on *Hes1* but that additional (ectopic) TLE1 does not lead to further *Hes 1* repression.

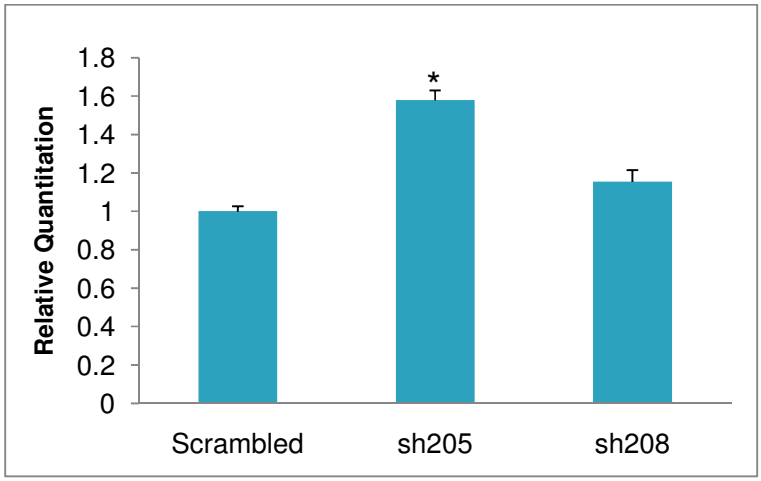


Figure 5.25: *TLE1* knockdown induces *Hes1*.

Total RNA was extracted from each shRNA-expressing cell pool at 72 hours post-transfection and *Hes1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Hes1* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD, **P<0.01).

5.2.8.8 TLE1 knockdown modulates Hey1

It was shown earlier that ectopic TLE1 had no effect on *Hey1* transcription in IB4 (Section 5.2.6.6), and so the effect of *TLE1* knockdown on *Hey1* mRNA levels was next examined. In this experiment, *Hey1* mRNA levels were seen to increase in response to *TLE1* knockdown (Figure 5.26). These findings imply that the endogenous level of TLE1 is sufficient to have a repressive effect on *Hey1* but that additional (ectopic) TLE1 does not lead to further repression of this gene.

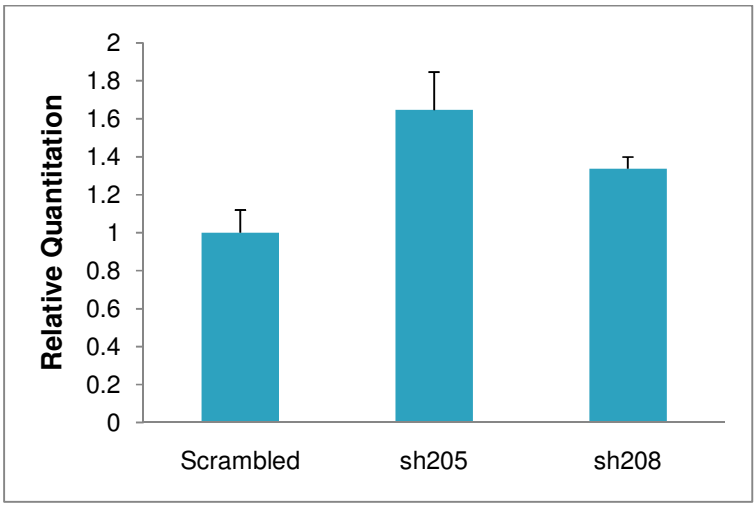


Figure 5.26: *TLE1* knockdown induces *Hey1*.

Total RNA was extracted from each shRNA-expressing cell pool at 72 hours post-transfection and *Hey1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Hey1* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD).

5.2.8.9 TLE1 knockdown does not modulate Runx1

It was shown earlier here that ectopic TLE1 had no effect on *Runx1* mRNA levels in IB4 (Section 5.2.6.6). Here, *TLE1* knockdown was observed not to have any significant effect on *Runx1* mRNA levels in IB4 (Figure 5.27).

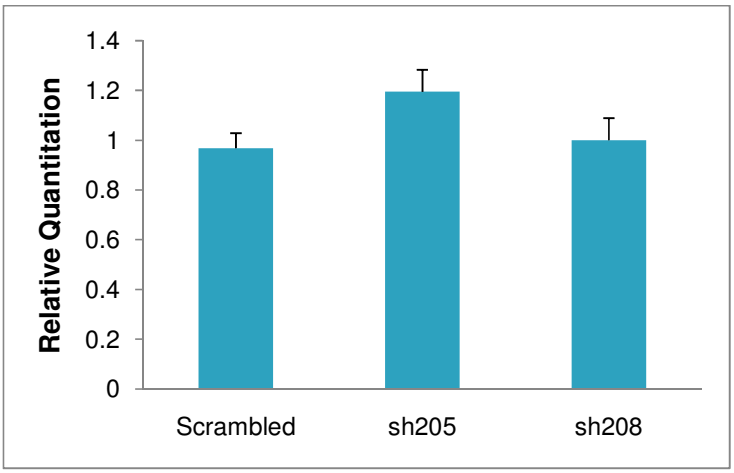


Figure 5.27: *TLE1* knockdown does not modulate *Runx1*.

Total RNA was extracted from each shRNA-expressing cell pool at 72 hours post-transfection and *Runx1* expression levels were verified using RT-qPCR. The results were normalised as before (relative to *TBP)*. In this experiment, the level of *Runx1* mRNA from the scrambled vector was arbitrarily assigned a value of 1. (Data are Mean ±SD).

5.2.9 Phenotypic investigations on IB4 cells transfected with shRNA-expressing vectors

Although transfection efficiencies of between 8-15% were achieved in IB4, the survival rate of puromycin-resistant transfectants was very low, making stable cell pool establishment using drug selection an ineffective process. While drug-resistant viable cells did remain following puromycin selection, cell numbers did not significantly increase so as to yield a workable population of cells. In cell lines in which the transfection efficiency is low, there is a rapid accumulation of dead cells following transfection, relative to the lower numbers of surviving drug-resistant cells. There are damaging effects associated with dead cell populations produced during the drug selection/transfection process, including increased pressure exerted on the resistant cells from degrading enzymes and necrotic factors secreted by the dying and dead cells. Furthermore, suspension cells proliferate optimally at specific cell densities at which the population generates sufficient levels of products such as growth factors. IB4 also displays an adhesive (clumping) morphology that may be hindered at low live cell densities. Reducing the culture volume to increase live cell density does not improve conditions for cell expansion as it has the side effect of also increasing the concentration of dead cells and enhancing the associated negative effects of secreted factors from the dead and dying population. In order to circumvent this problem, attempts were made to enrich the live cell population in transfected cell pools. A protocol was therefore first established in this study employing two different purification methods to extract live cells from cell pools with significant dead cell populations.

5.2.10 Stable knockdown of *TLE1* in IB4 using shRNA-coding plasmids

As the transfection efficiency of IB4 using the shRNA vectors was at such a low level, determining the effect of the repression of *TLE1* expression on cellular targets benefitted from an enrichment step for transfected cells. The shRNA expression vectors carry a puromycin resistance marker (Figure 5.15). Puromycin is a potent translational inhibitor and resistance is

conferred by the puromycin N-acetyl-transferase gene (*pac*), the product of which inactivates puromycin by acetylation of the drug. IB4 cells were first transfected with 5µg of each shRNA vector. After 24 hours, transfected cell pools were passaged in growth medium containing 1 µg/ml puromycin (concentration determined from a drug curve assay). Under these conditions, the majority of non-transfected cells are killed within 72 hours, after which the concentration of drug was reduced to 0.5 µg/ml. During the drug selection process, cells were passaged and sub-cultured into fresh medium every 2-3 days. However, a significant portion of dead cells remained in the population throughout selection. These cell pools were utilised (i) to demonstrate the transcriptional effect of *TLE1* knockdown on significant EBV-associated genes, and (ii) to determine if *TLE1* repression elicited any effect on the phenotype of the IB4.

5.2.11 Enrichment of live cell population via Lymphoprep™ and Concanavalin A (Con A) purification

The process of electroporation damages between 10-15% of the total IB4 cells beyond repair (determined from FACS analysis performed following transfections in this study). Piñero and colleagues determined that electroporation results in cell death arising from both necrosis and apoptosis to an equivalent degree (Piñero, et al. 1997) Apoptosis is a systematic controlled process that terminates the cell while endeavouring to bring forth negligible negative effects on neighbouring cells. However, necrosis is an unintentional form of cell death in which the cell unrestrictedly swells and bursts permitting the release of cellular components causing death signals that can be detected by adjacent cells (Fink and Cookson 2005).

Many studies use Ficoll gradient separation, following transfection of suspension cells, as an extra step that discriminates between live cells and dead cells based on their density (Guidicelli, et al. 2009; Pachernik, et al. 2002; Bénéteau, et al. 2007). Enhancement of the live cell population improves efficacy of results obtained in from transfected cells. Ficoll is a neutral, water soluble synthetic polymer of sucrose and epichlorohydrin

possessing a high molecular weight. Ficoll has a density of 1.077 ± 0.001g/ml, this can be overlaid with medium containing lymphocytes. After rapid centrifugation at room temperature the sample becomes segregated according to the densities of the components therein. Healthy lymphocytes are not dense enough to penetrate the Ficoll layer. Viable lymphocytes concentrate at the interface of the growth medium and separation fluid, but dead cells, which are more dense, pass through the medium and gather below the Ficoll layer (Boyum 1968). Ficoll is commercially available as a ready-to-use separation medium under a number of product names, the two most widely accepted for lymphocyte purification are Ficoll-Paque™ and Lymphoprep™. These products vary slightly in the aqueous component of the medium. However, Yeo et al performed a comparative study on the standard commercially available Ficoll-Paque™ and Lymphoprep™ confirming that the two solutions gave no significant difference in lymphocyte recovery, and showing that the condition of cells post-treatment was equivalent (Yeo, et al. 2009). For the duration of this study all Ficoll separations were performed using Lymphoprep™ (referred to henceforth as 'Ficoll').

IB4 cells were first transfected with 2 µg of pmaxGFP in order to express the GFP as a marker for successful transfection. After 24 hours the transfected cell pool was split into two, and one half of the pool was purified using Ficoll. Viabilies of purified and un-purified transfected cell populations were analysed by FACS. The distribution profile of the cell population is categorised by the analysis of overall size and granularity of the cells from each sample (Figure 5.28). Forward scatter (FSC), assigned to the X-axis, corresponds to approximate cell size and side scatter (SSC) is represented by the Y-axis, denoting cell granularity. The highlighted region (R1) represents the gated zone where a healthy population of IB4 cells is to be found. Cells situated to the left and below the R1 area are not morphologically representative of IB4 cells as they are smaller in size and possess less intracellular complexity. Cells outside the gated region are composed of dead and dying cells along with cellular debris. As can be observed in figure 5.28A and B, the Ficoll purified population showed

considerably less cells outside the gated region, thus representing an unmistakably healthier population in comparison the un-enriched transfected cells.

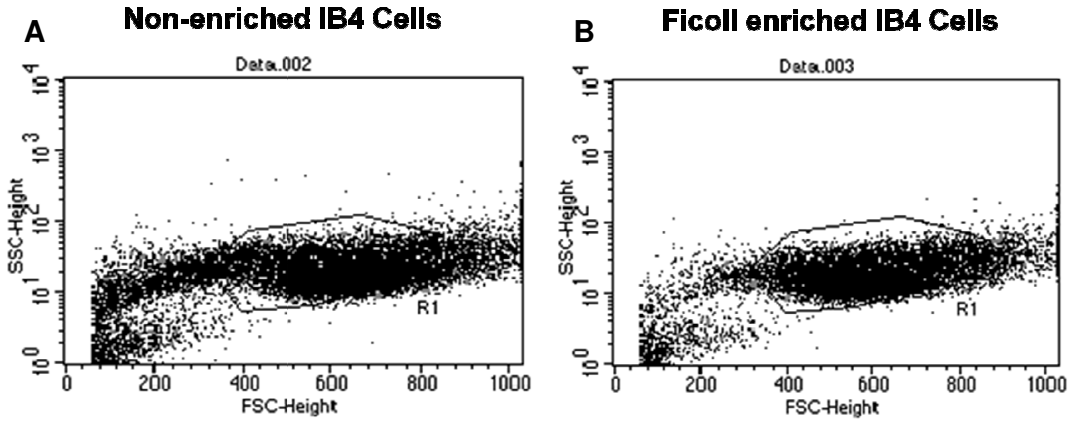


Figure 5.28: Purification of IB4 cells using Ficoll.

IB4 cells transfected with 2µg of pmaxGFP vector were divided into two equal populations at 24 hours following electroporation. One fraction remained unenriched while the second was purified by Ficoll gradient separation. Forward (x axis) and Side (y axis) scatter plots are shown for **(A)** untreated cells at 24 hours post-transfection displaying size and granularity distribution of the cell population, **(B)** an identical cell population subsequent to Ficoll enrichment**.**

The translocation of phosphatidylserine (PS) at the cell membrane is an early apoptotic event and can be detected using fluorescently conjugated Annexin V which binds PS with high affinity. As apoptosis progresses, cell membrane integrity becomes compromised, permitting the internalisation of material previously blockaded by the structurally sound membrane. At this point, late apoptotic cells can incorporate membrane-impermeant dyes such as 7-aminoactinomycin D (7-AAD) (McCarthy and Evan 1998). To determine the composition of live, early/late apoptotic cells in the above purified gated population, an apoptotic staining assay was performed that combined PE conjugated Annexin V staining (FACS FL-2 channel) and 7-AAD staining (FACS FL-3 channel). The use of dual staining allowed the identification of four sub-populations within the gated population: (i) healthy non-apoptotic cells that stained for neither dye, (ii) early apoptotic cells that incorporated only the Annexin V-PE conjugate, (iii) late apoptotic and necrotic cells that displayed double staining of Annevin V-PE and 7-AAD and (iv) necrotic cell cells and cellular debris staining only for 7-AAD (Reviewed in Brady 2004).

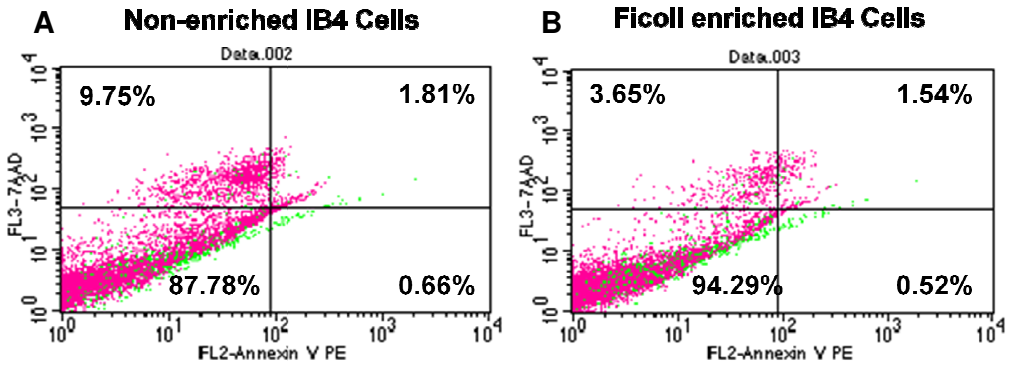


Figure 5.29: Removal of dead cells following Ficoll purification.

IB4 cells transfected with 2 µg pmaxGFP were split 24 hours after transfection. One fraction remained untouched while the second was purified using a Ficoll gradient. Both populations were stained with 7-AAD and Annexin V-PE to determine their survival/apoptotic profiles. Quadrant markers were based on healthy stained/unstained controls and the numbers shown are the percentage of cells in each quadrant. Green dots indicate GFP-expressing cells, pink dots indicates cells not-expressing GFP. **(A)** Un-enriched transfected cells, **(B)** an identical cell population following Ficoll enrichment**.**

Samples of input cells and Ficoll treated cells were stained with 7-AAD and Annexin V-PE. Ten thousand cells from the R1 region were analysed for their staining profile. From figure 5.29 it is shown, looking at the upper left quadrant, that Ficoll removed approximately 6% of the dead cells in the population. Thus the viable cell pool was enriched from 88% viable cells to 94%. In addition, no adverse effects were observed in the cell population post-Ficoll treatment (data not shown). Transfection efficiency (as determined from GFP-expressing cells) was determined to be consistently between 10-12%.

In the case of cell populations treated with puromycin, only a small percentage of cells contain the resistance marker to the drug, and this once again poses the problem of the production of a significant number of dying cells in the population. Although passaging cells removes cellular debris, a substantial portion of the dead population nonetheless remains. Ficoll separation is most efficient at high cell densities. After puromycin selection however, the fraction of live cells is considerably depleted, thus making a second round of Ficoll separation unattractive. A technique that could

exclusively extract the small live population was therefore required. A method based on the lectin Concanavalin A (Con A), which has been shown to exclusively bind to live B cells, was therefore investigated.

5.2.12 Selection with Con A as a tool for the purification of viable B cells following transfection

Con A is a plant-derived lectin that binds to the α-glucosyl and α-mannosyl residues presented by glycoproteins on mammalian cell membranes (Reviewed in Thøger-Andersen and Junker 1994; Sharon and Lis 1972). Lectins were first identified by their capacity to agglutinate red blood cells following binding to cell surface saccharides. The specificity of lectin recognition for their target saccharide allows discrimination between mammalian blood types. Con A binds exclusively to the mannose moiety of B cell surface glycoproteins (Thøger-Andersen and Junker 1994; Lei and Chang 2009). Along with agglutination, some lectins possess additional properties such as mitogenic activity. Con A has been widely used in lymphocyte research as a T cell mitogen, and although it exhibits an equal affinity for B lymphocytes, Con A fails to *trans*-activate the B cells into a proliferative state (Lei and Chang 2009; Stobo, et al. 1972). It has been found that Con A binds live B cells but cannot specifically associate with dead B lymphocytes (Thøger-Andersen and Junker 1994). The lectin/carbohydrate association is a reversible process that has enabled lectin utilisation for cell identification and enrichment (Lei and Chang 2009).

Here, the ability of Con A to selectively 'pull out' healthy B cells following transfection was assessed. IB4 cells were first electroporated with pcDNA3 plasmid. Electroporation is a mechanical process that introduces a temporary electric pulse across the cell membrane. This disturbs the phospholipid bilayer creating transient pores, allowing molecules such as DNA to permeate the cell. Once the voltage is removed, the membrane quickly reassembles to restore the viable cell. However, some pores may be too large to reform, and others may take in molecules that disrupt the ion balance within the cell, both resulting in ruptured non-viable cells (Weaver

1995). When using electroporation, equilibrium between transfection efficiency and cell viability must be maintained. Thus, the resulting transfected cell pool will contain both viable and non-viable cells. Here, panning of such cell populations involved first coating cell culture plates with Con A using coating buffer solution containing either 300 µg/ml Con A or 100 µg/ml Con A (performed as described in chapter 2, (Section 2.2.3.20). Cells were then allowed to adsorb to these plates, followed by elution/washing in fresh warmed medium with gentle mechanical pressure from a pipette (p1000). Cells thus eluted were then cultured in new six-well plates under standard conditions. Trypan blue staining was used to determine the numbers of viable/non-viable cells that were 'panned out' by this method.

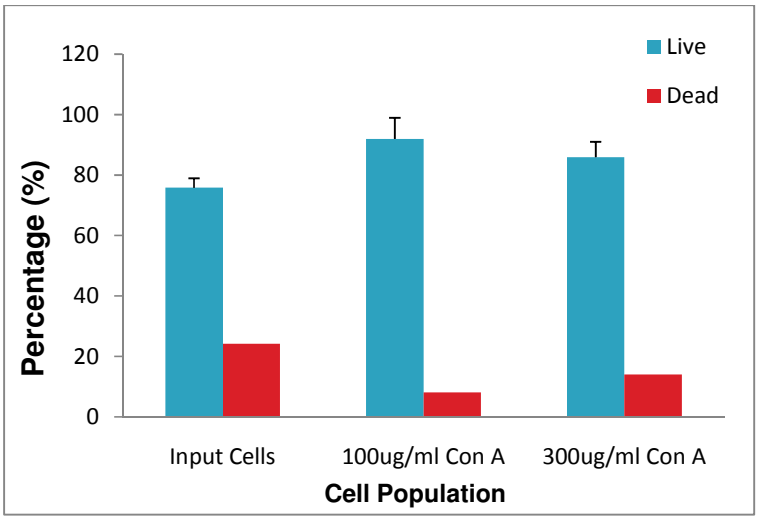


Figure 5.30: Live cell enrichment by Con A selection as determined by trypan blue staining.

Transfected IB4 cells were split 24 hours post-transfection. One cell fraction was maintained as an input control, another two samples were purified on plates that had been coated using two different concentrations of Con A (100 µg/ml and 300 µg/ml). Trypan blue exclusion was used to establish the live/dead cell numbers after panning with Con A**.**

Transfection by electroporation was seen to reduce the overall population viability to 75% (Figure 5.30). It can also be seen that panning with plates coated using the solution of 100 µg/ml Con A gave maximal live cell enrichment with the percentage of viable cells rising to 91%, in comparison to 86% viability when coating buffer with 300 µg/ml Con A was used. This shows that Con A-panning substantially enriched for viable cells. Initial

recoveries were low however (approximately 28% of the total live cell population). Examination of coated plate surfaces using microscopy following cell adsorption indicated that these were saturated with bound cells; thus, the limitation in this experiment was the lack of cell surface area for live cell adsorption. This was subsequently rectified by repeated panning of sample supernatants on the same plates or by coating plates with a larger surface area. These steps raised overall cell recoveries to approximately gross 50% of the live starting cell population.

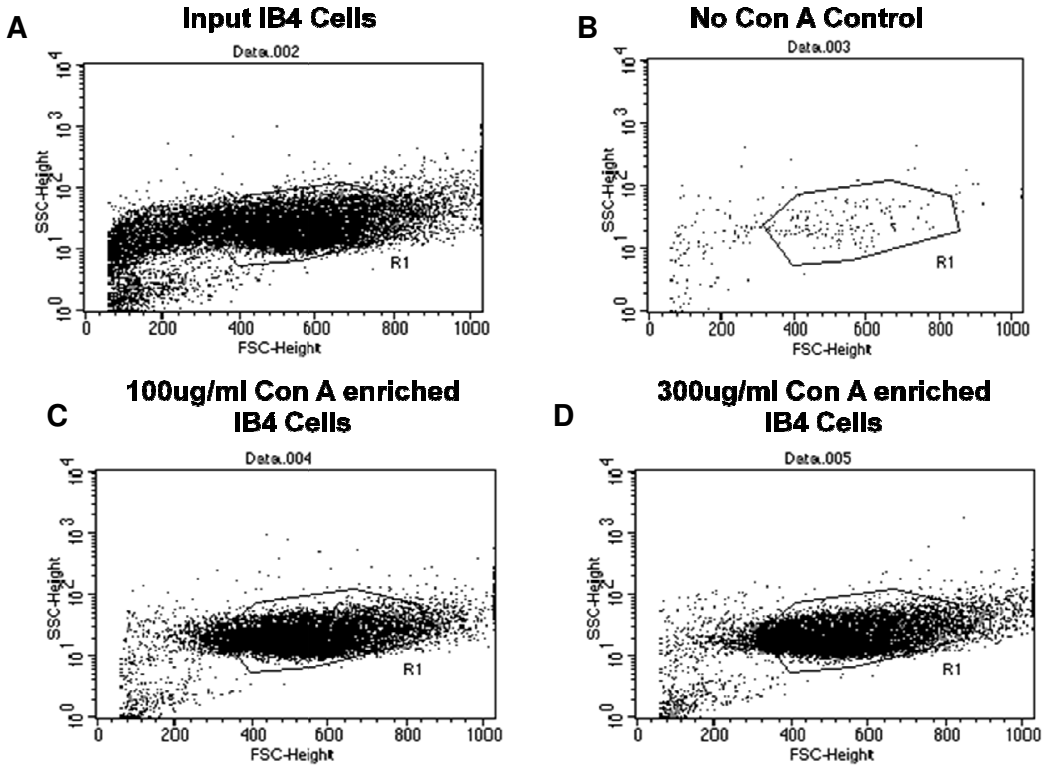


Figure 5.31: Live cell enrichment by Con A selection.

IB4 cells were transfected with an empty mammalian expression vector (pcDNA3) as per the standard protocol. 24 hours post-transfection the cell population was split and two fractions were purified in two different concentrations of the lectin, Con A. **(A)** Represents untreated cells 24 hours post-transfection **(B)** Illustrates cells selected in the absence of Con A to ensure specificity of the Con A selection. **(C)** Demonstrates the transfected cell population after panning in plates coated with 100µg/ml Con A. **(D)** Represents the transfected cell population after panning in plates coated with 300µg/ml Con A.

When FACS analysis was used, it was observed that a large portion (Approximately 35%) of the transfected cell population was to be found

outside the gated region R1 (Figure 5.31A). Panning with Con A however was seen to remove a significant portion of dead cells and debris by positively selecting for live B lymphocytes (compare figure 5.31, parts A, C and D). Con A selection resulted in a smaller population outside the gated region, reduced from 35% to approximately 17% and 19% when using plates coated with Con A at 100 µg/ml and 300 µg/ml respectively. The overall cell pool after Con A selection could now be seen to be composed of cells of typical viable IB4 profile (Figure 5.31C and D). The similar degrees of enrichment obtained at both coating concentrations of Con A indicated that saturation of the surface with lectin had probably been reached at 100 µg/ml. *N*-Ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), is a water soluble carbodiimide that activates Con A, facilitating the coating of cell culture grade plates via amine bonds. To verify that the result of selection was due to Con A binding, and not a non-specific effect of the activator compound, an EDAC-only treated plate was included in the experiment. It can be seen that negligible cell numbers were detected after panning with EDAC alone (Figure 5.31B). In addition, the EDAC-extracted population were of mixed morphology showing equal numbers of live and dead cells. These results showed that Con A could be used to successfully select viable B lymphocytes from transfected cell populations.

FACS analysis also showed clear evidence for the selective enrichment of live lymphocytes by Con A, corroborating the scatter plots and trypan blue exclusion assays. It can be seen that Con A-panning reduced the necrotic population from 15% to fewer than 2% of the total transfected cell pool (upper left quadrant, Figure 5.32).

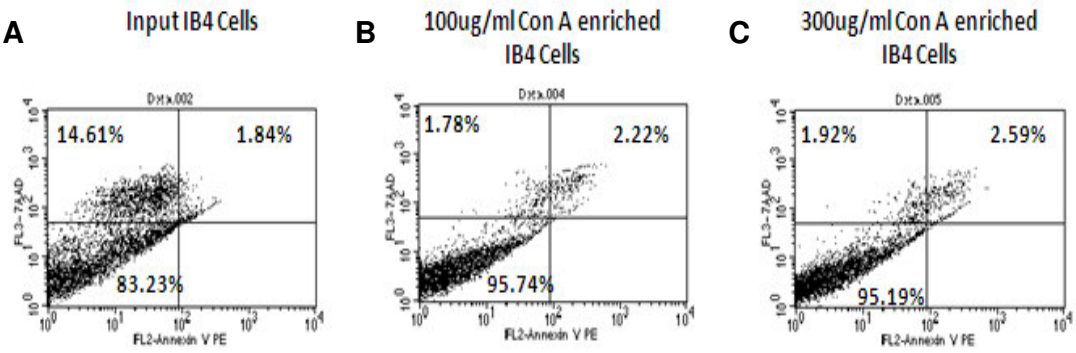


Figure 5.32: Con A-panning selects for viable cells following transfection of an LCL.

Twenty-four hours after transfection, IB4 cells were Con A-panned and their survival profile determined by 7 AAD/Annexin V PE staining. Quadrant markers were based on healthy stained/unstained controls and values reflect the percentage of cells in each quadrant. **(A)** Untreated cells; **(B)** and **(C)** Con A-selected cells.

After seven days, Con A-selected populations were again monitored by trypan blue staining and 7-AAD/Annexin V-PE staining and displayed no ill effects from the procedure (Figure 5.33).

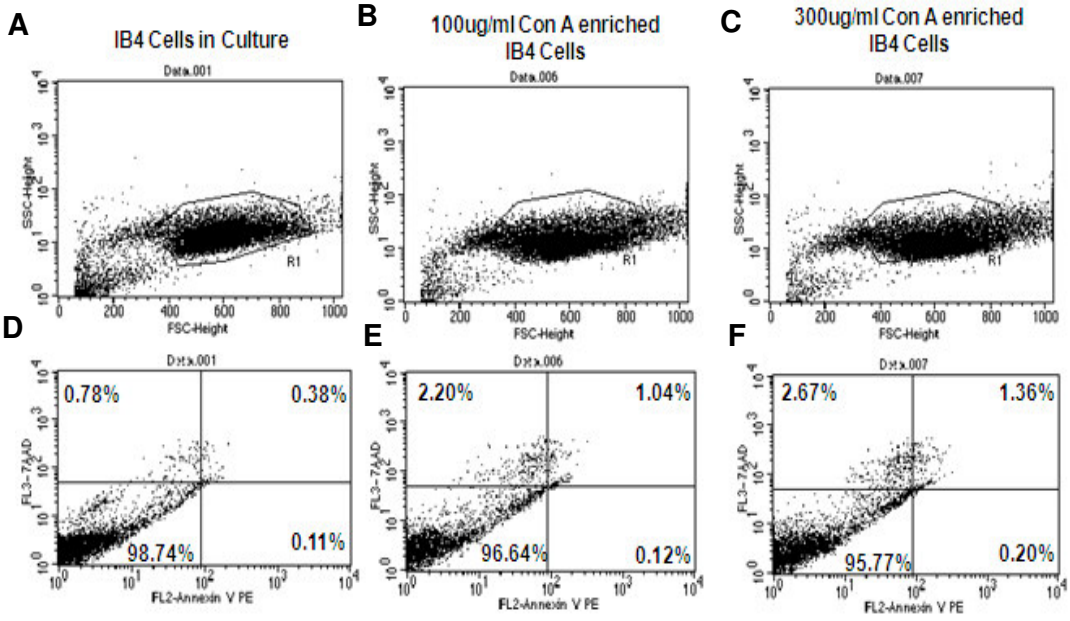


Figure 5.33: Con A selection displays no cytotoxic effects on enriched cell populations.

(A-C) Forward and Side scatter plots of cell populations as indicated above in each case. **(D-F)** 7 AAD/Annexin V PE staining of the corresponding cell populations (directly underneath the corresponding scatter plot). Quadrant markers were based on healthy stained/unstained controls and values reflect the percentage of cells in each quadrant.

5.2.13 The selection of cell pools expressing anti-TLE1 shRNAs: comparison of two live cell enrichment protocols

IB4 cells were next transfected with plasmids encoding the shRNAs sh205, sh208 and the scrambled control sh30003. In addition, 1 µg of pmaxGFP was incorporated into each transfection to permit monitoring of the transfected population. Following transfection, each cell pool was split into three and each fraction then treated in one of the following ways: (i) cells were allowed to recover for twenty four hours, followed by passaging in fresh medium containing 1 µg/ml puromycin (control; no additional live cell enrichment steps); (ii) a Ficoll purification step at 24 hours post-transfection, and a second Ficoll enrichment 3 days after drug selection in medium containing 1 µg/ml puromycin (Ficoll/Ficoll enrichment); (iii) Ficoll/Con A treatments consisting of two purification steps -Ficoll enrichment was performed 24 hours post-transfection followed by selection in 1 µg/ml puromycin; then Con A selection after 3 days. The relative success of each method was determined by FACS as before.

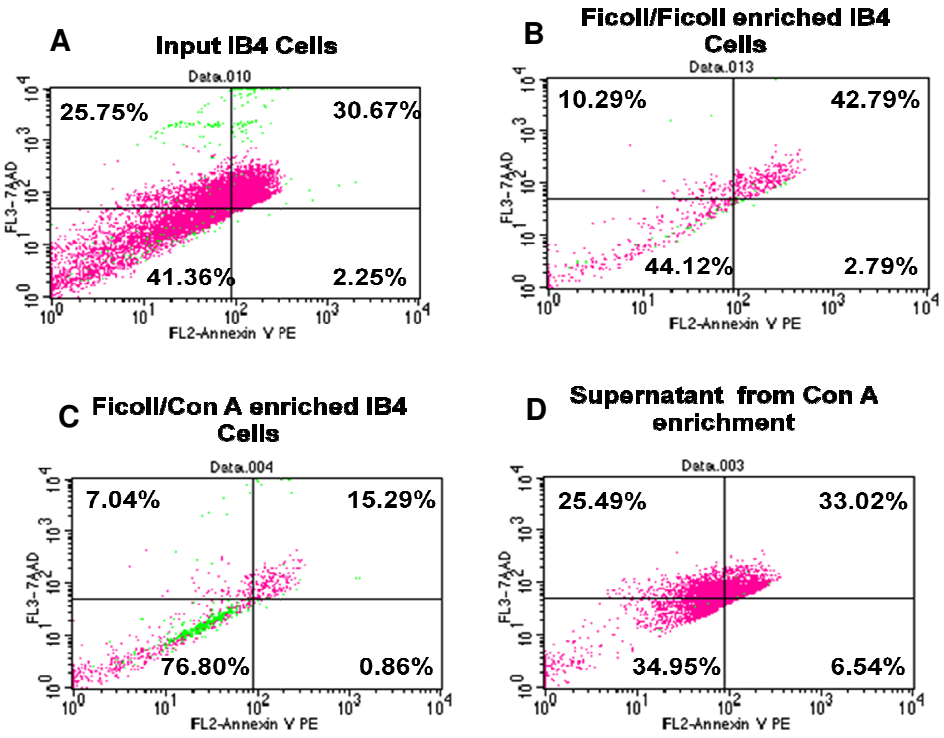


Figure 5.34: Survival profiles of shRNA-expressing cell pools.

IB4 cells were co-transfected with pmaxGFP and one shRNA-coding plasmid as described (only the results for shRNA205 are shown in the figure). Following selection (method named above each plot), survival profiles were determined by 7 AAD/Annexin V PE staining. Quadrant markers were based on healthy stained/unstained controls and values reflect the percentage of cells in each quadrant. Green dots represent GFP expressing cells. **(A)** un-enriched cells 3 days post-selection with 1µg/ml puromycin; **(B)** cells purified with Ficoll 24 hours after transfection and again at 3 days after puromycin selection; **(C)** the transfected cell population enriched 24 hours later using Ficoll followed by panning on Con A-coated plates following 3 days of selection with puromycin; **(D)** the cell population remaining in the supernatant following depletion with Con A.

As before, it can be seen that the combined Ficoll/ConA method selected a population with higher viability (76.8%; Figure 5.34C) relative to the Ficoll/Ficoll method (44%; Figure 5.34B). In addition, the GFP-expression population (and hence puromycin resistant) is largely seen to be viable with approximately 40% of the cells in this region expressing GFP (compare C with A and B, Figure 5.34). Moreover, the supernatant remaining following Con A-panning (see D, Figure 5.34) displays negligible numbers of GFP-expressing cells, suggesting that Con A-panning preferentially selected almost the entire viable GFP-expressing population.

When 7AAD-staining was plotted against GFP, it can be seen that the un-enriched and Ficoll-only enriched populations display a scattering of GFP-expressing cells across the top two quadrants, indicating both significant death and heterogeneity of GFP expression therein (Figure 5.35A and B). The unpurified cells showed the smallest transfected population (Figure 5.35A). It can be seen that Ficoll/Ficoll enrichment gave an increase in the fraction of transfected cells from 1.5% to 4% (Figure 5.35A and B). However, Ficoll/Con A enrichment gave by far the greatest yield of viable transfected cells, with 40% of cells expressing GFP therefore representing the transfected population. Finally, in the supernatant pool containing cells not bound during Con A selection (Figure 5.35D) only 0.05% of the total population were live GFP-expressing cells, indicating that Con A captured virtually the entire viable transfected cell population. The residual cells that did not bind to Con A were 84% dead cells, supporting the specificity of live selection by the lectin.

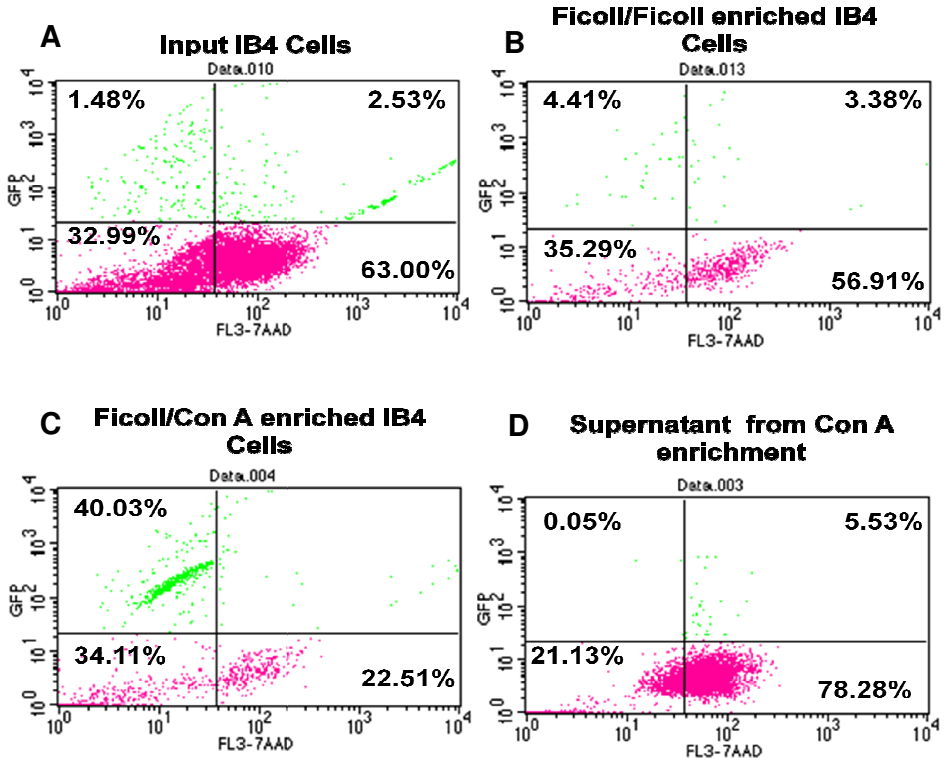


Figure 5.35: Con A selection enriched for the GFP expressing population.

IB4 cells were transfected with pmaxGFP and enriched using the methods shown above each plot. Transfected cell pools were stained with 7AAD to determine viability and plotted as GFP versus 7AAD. Green dots denote cells expressing GFP, pink dots represent cells that are not expressing GFP. Quadrant markers were based on non-transfected stained/unstained controls and values reflect the percentage of cells in each quadrant. The upper quadrants contain GFP-expressing cells while the lower quadrants denote non-GFP-expressing cells. Thus, unstained cells in the lower left quadrant are viable but do not express GFP, cells in the lower right quadrant are non-GFP expressing dead cells, cells in the upper right quadrant are dead GFP expressing cells and cells in the upper left quadrant are viable GFP expressing cells. The numbers in each quadrant correspond to the percentage of the total cell population found in that region. **(A)** This shows un-enriched cells 3 days post-selection with 1µg/ml puromycin. **(B)** Corresponds to cells purified with Ficoll 24 hours post-transfection and again 3 days after puromycin selection **(C)** This shows the transfected cell population enriched 24 hours after transfection with Ficoll, followed by Con A-panning following selection for 3 days with puromycin. **(D)** This shows the unselected cell population in the remaining supernatant following Con A selection.

IB4 cells were co-transfected with pmaxGFP and plasmid expressing either scrambled shRNA vector or sh205 and were then selected using the Ficoll/Con A method described above. A modest difference in apoptosis

between the two cell pools could be seen; the GFP-expressing population in the late apoptotic region was 24.0% (sh205) compared to 15.3% (scrambled shRNA control; Figure 5.36A). This may imply that knockdown of TLE1 leads to a reduced level of apoptosis when compared to the scrambled control; however, the distribution of the GFP populations is comparable in the two cell pools, indicating that there was no increased level of apoptosis in the positive transfectants (Figure 5.36 A-D). Cell populations are maintained by a fine balance of apoptosis and cell growth. When the proliferation rates of these two cell pools were compared, it was found that the sh205 transfected cell pool had a more rapid rate of cell growth in comparison to the scrambled cell pool. Therefore, taking into account these results, it is most likely that a decrease in TLE1 expression results in an increase in the proliferative capacity of an LCL.

Treatment of un-transfected IB4 cells with puromycin results in approximately 80% cell death after 72 hours. This implies that the majority of the live cells in the selected pools have retained the puromycin resistance conferred by the shRNA expressing vectors. The amount of healthy cells in both the sh205 and scrambled cell pool are similar; however, the sh205 population retained more GFP expressing cells than the scrambled cell pool. Therefore, it appears that the scrambled shRNA cell pool retained the drug resistance without the second plasmid expressing GFP. This may be a consequence of the growth advantage awarded to the LCL by reduced TLE1 expression.

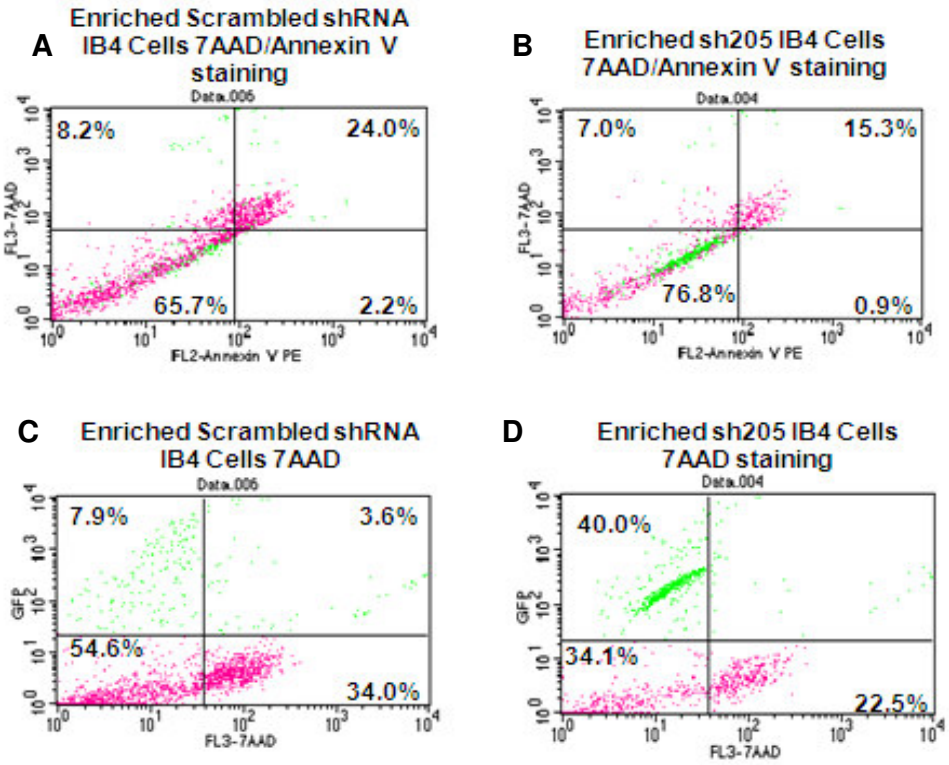


Figure 5.36: Comparison of scrambled and sh205 Con A enriched cell populations.

IB4 cells were co-transfected with pmaxGFP and either the scrambled shRNA vector or the sh205 vector. These cell pools were drug selected using puromycin and enriched using the Ficoll/Con A method. Transfected cell pools were analysed with either (i) by 7 AAD/Annexin V PE staining (Described in the legend to figure 5.34) or (ii) by using 7AAD staining to determine viability and plotted as GFP versus 7AAD. Green dots denote cells expressing GFP, pink dots represent cells that are not expressing GFP. Quadrant markers and staining described as per legend to figure 5.35. The numbers in each quadrant correspond to the percentage of the total cell population found in that region. **(A)** This shows the survival profile, determined by 7 AAD/Annexin V PE staining, of enriched scrambled shRNA cells 3 days post-selection with 1µg/ml puromycin. **(B)** Corresponds as per (A) for sh205 enriched cells. **(C)** This shows enriched scrambled shRNA cells stained with 7AAD to determine viability and plotted as GFP versus 7AAD. **(D)** Corresponds to sh205 enriched cells stained and plotted as per legend (C).

5.2.14 TLE1 knockdown increases the rate of IB4 cell proliferation

Trypan blue staining and MTS assays were next used to investigate the potential effect of shRNA-mediated *TLE1* knockdown on the proliferation of IB4 cells. In an attempt to minimize interference with the MTS assay, puromycin was withdrawn from the culture medium twenty four hours prior to

sampling. Cells were monitored over a 48 hour period and three independent experiments were performed in each case (Figure 5.37).

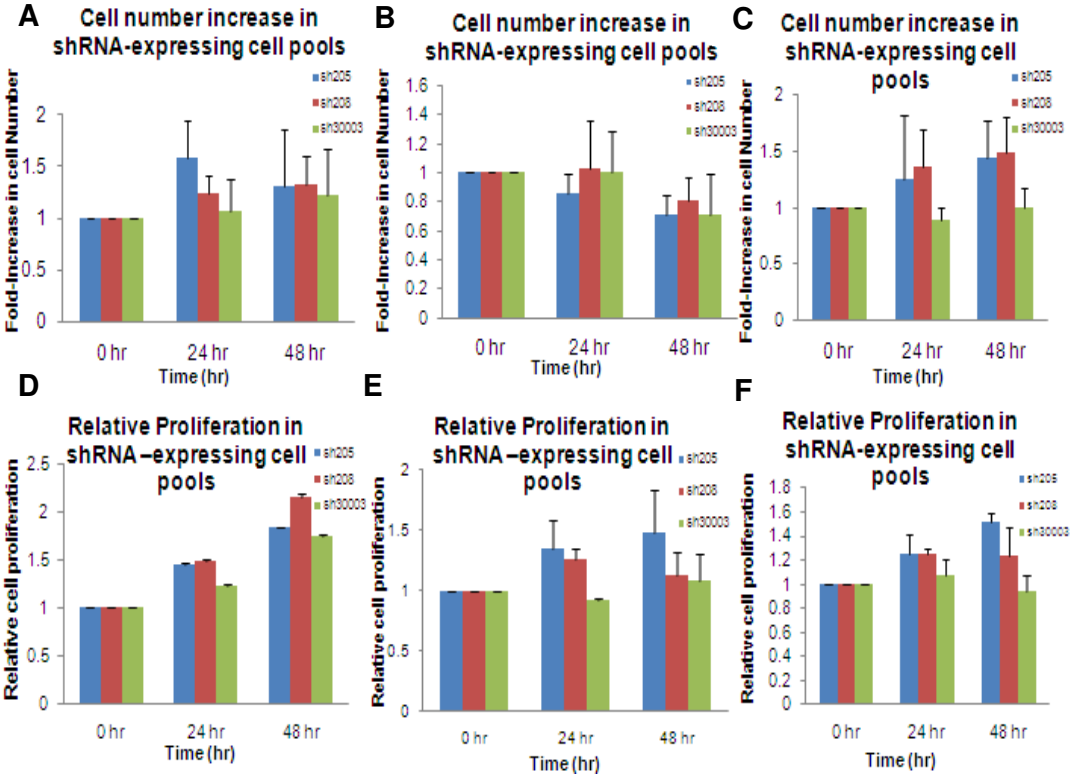


Figure 5.37: *TLE1* knockdown in IB4 cells correlates with increased proliferation.

(A-C) Cell numbers in each transfected cell pool were determined by trypan blue staining at three time points in three independent experiments. This was performed in triplicate and each individual experiment was plotted independently above, the results were represented as fold increase in cell number; **(D-F)** As per A-C except using MTS assay.

These experiments consistently showed higher cell numbers and rates of proliferation in the two cell pools expressing anti-*TLE1* shRNAs when compared to the control cell pool expressing scrambled shRNA (Figure 5.37 A-F). After 24 hours for instance the rate of proliferation, as determined by MTS assay, was 20% higher on average in the cases of TLE1 knockdown. These results imply that the inhibition of *TLE1* expression had a positive impact on the growth and proliferation of IB4 cells. Interestingly, they are in agreement with similar published studies for the knockdown of *TLE1* by shRNA in AML1-ETO myeloid leukemia cells (Dayyani, et al. 2008). Dayyani

and colleagues found that knockdown of *TLE1* in the Kasumi T cell line led to increased cell cycle progression and division. In addition, they demonstrated that, conversely, enforced expression of *TLE1* in that situation led to apoptotic cell death (Dayyani, et al. 2008). Here, enforced expression of *TLE1* in IB4 was not seen to have a significant apoptotic impact (Figure 5.37).

5.2.15 Analysis of *TLE1* knockdown cell pools by RT-qPCR

Unexpectedly, significant problems were encountered using RT-qPCR on RNA samples prepared from the puromycin selected shRNA knockdown cell pools. During each experiment, melt curve analysis is employed to confirm the amplification of a single product at the correct temperature. This involved a stepwise 1ºC incremental increase in temperature over a set range, and fluorescence exhibited by the sample is monitored during this time. SYBR green dye associates with double-stranded DNA; therefore, at the temperature threshold at which the generated PCR product dissociates, yielding single stranded product, there is a drop in the fluorescence activity. Thus, a sample with a single PCR product will demonstrate only one peak corresponding to the melting temperature of the product (Example in Figure 5.38A). The *TLE1* primers used in this study had been optimised and consistent PCR conditions were maintained in all experiments. However, melt curve analysis on RT-qPCR products obtained from puromycin-selected cells showed dissociation curves that were different relative to melt curves previously obtained from *TLE1* RT-qPCR products.

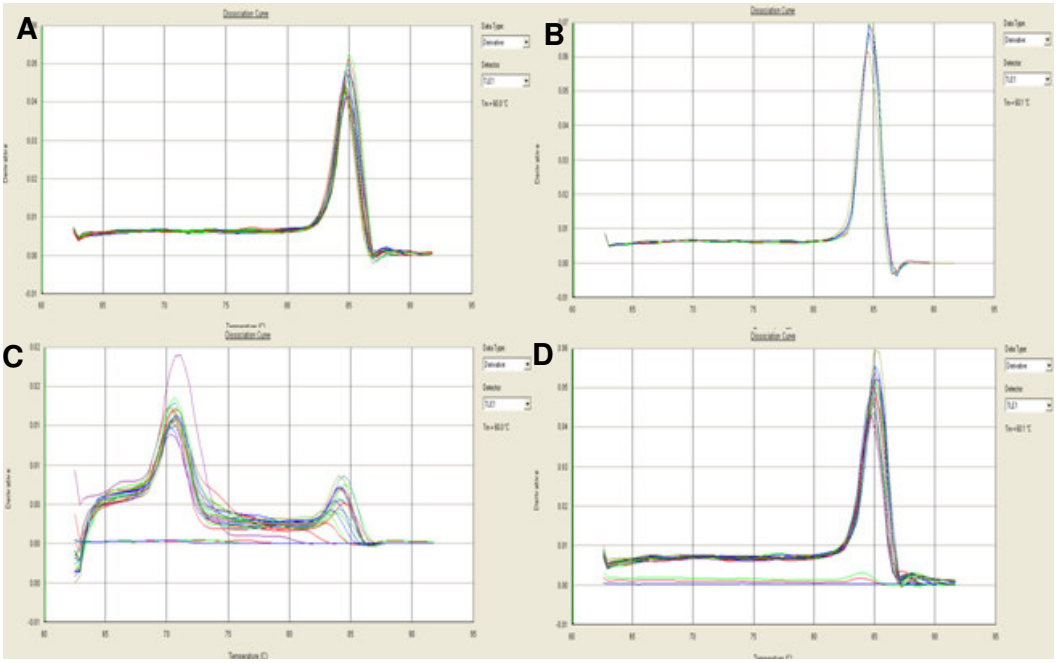


Figure 5.38: Melt curve analysis for *TLE1* PCR products.

Melt curve analysis was performed during each experiment to verify the production of a single specific product at the temperature anticipated for the *TLE1* PCR product. Melt curves obtained using **(A)** cDNA prepared from a panel of B cell lines, **(B)** IB4 cells transfected with pcDNA3-TLE1, **(C)** puromycin-selected shRNA-expressing IB4 cells, and **(D)** shRNA-expressing IB4 cells without puromycin selection. Identical conditions for RT-PCR were used in all experiments.

Sample preparation and experimental steps were scrutinised and compared to ensure that the observed unpredicted melt curve was not due to reagent interference. RT-qPCR analysis demonstrated that the expected melt curve for *TLE1* PCR product was not obtained only in the case of cells treated with puromycin. To determine the identity of the product generated from the samples with irregular melt curves, PCR products were purified following RT-qPCR and their DNA sequences determined.

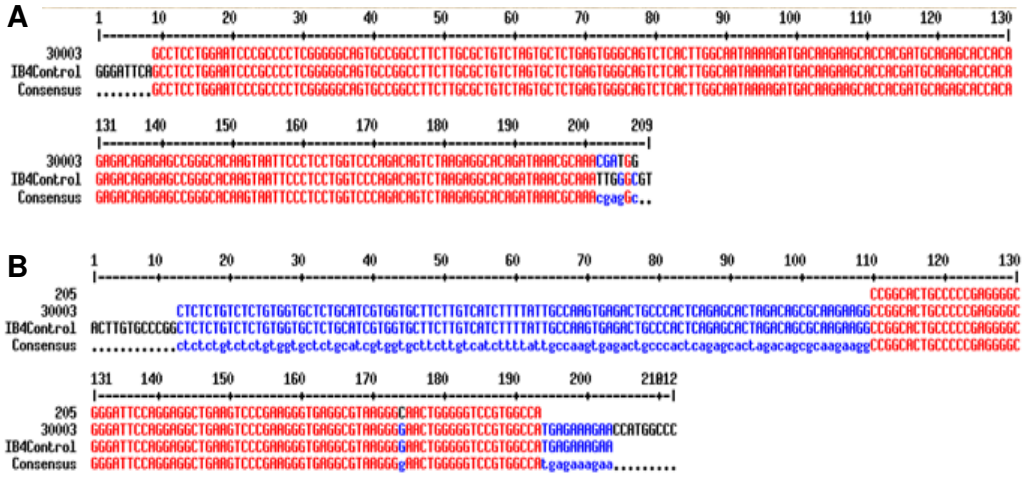


Figure 5.39: DNA sequencing analysis for *TLE1* cDNA products generated during RT-qPCR.

(A) and **(B)** DNA sequencing was performed on purified RT-qPCR products from puromycin-selected transfected IB4 cell pools (using sh205 and the scrambled sh30003 vector). The non-selected sample containing pcDNA3 TLE1 was included as a positive control for the correct *TLE1* RT-qPCR product. The alignment of the PCR products shows that the three products are the same. Red bases indicate perfect alignment between all three sequences, while blue bases indicate alignment for two of the three sequences under analysis.

DNA sequence alignments confirmed that the PCR products generated were in fact the same in every case, in spite of the fact that the dissociation curves for the puromycin-selected cells were different (Figure 5.39). BLAST analysis was used to confirm PCR product identity as that of the anticipated portion of *TLE1*. Thus, the *TLE1* PCR assay as previously validated was continuing to perform successfully.

5.3 Discussion

TLE1 is potentially an interesting negative target of EBV. A possible role for TLE1 in the regulation of two signaling pathways key to EBV latency, Notch and NF-κB, was therefore investigated in the LCL IB4. Promoter co-transfection studies demonstrated that ectopic TLE1 (i) *trans*-activated a Notch/CBF-1 responsive promoter in a dose-responsive manner, and (ii) was not concluded to affect the activity of an NF-κB-dependent promoter, in that the effect was neither consistent between experiments nor dose-dependent. The potential for TLE1-mediated regulation of Notch/CBF-1 target genes was therefore pursued.

An IB4 cell pool expressing ectopic TLE1 was then established and from this it could be seen that the rate of increase in cell numbers declined due to the transfected gene. Furthermore, ectopic TLE1 expression was seen to be lost over time (Section 5.2.4). Based on these observations it was reasoned that TLE1 had a negative impact on the LCL phenotype. The results obtained in section 5.2.14, in which *TLE1* knockdown cells appear to lead an increased growth rate relative to control cells (Figure 5.37) substantiates this conclusion. These observations are at odds with the general published data relating to the impact of TLE1 on phenotype, such as the over-expression of TLE1 as a feature of higher grade meningiomas (Cuevas, et al. 2005) and synovial sarcoma (Terry, et al. 2007). However, recent studies on haematological malignancies have indicated a contrasting role for TLE1 in oncogenesis within hematopoietic cells. Fraga and colleagues studied a panel of 49 human malignancies arising from 14 different cell types and found that *TLE1* silencing by hypermethylation was a feature of a number of haematological malignancies such as AML, non-Hodgkin's lymphoma, chronic myeloid leukaemia (CML) and B-cell diffuse large cell lymphoma (B-DLCL) (Fraga, et al. 2008). They confirmed that *TLE1* silencing was a feature of malignant cells and not healthy peripheral cells. In addition, restoration of *TLE1* activity to three leukaemia or lymphoma cell lines led to a reduction in the rate of cell growth (Fraga, et al. 2008). The tumour-suppressor activity of *TLE1* was further corroborated by the knockdown of

TLE1 in lymphoma cell lines which expressed *TLE1* which in turn led to increased cell growth. The published findings of the investigations by Dayyani and colleagues and Fraga's laboratory are in agreement with the findings in this study, which implicate *TLE1* as a negative regulator of haematological cell fate.

An analysis of the expression of selected cellular genes in the cell pool expressing ectopic TLE1 showed that TLE1 modulated the transcription of *EBNA2*, *Pax5*, *CD23*, *E2A*, *Runx3* and *Cyclin D1*. That these events impact on the levels of those individual proteins concerned, with the exception of EBNA2, awaits confirmation by western blotting. TLE1 is a transcription-associated protein however, and these findings very likely indicate that TLE1 does affect the expression of these cellular genes. TLE1 was seen to positively regulate the crucial EBV latent gene *EBNA2*; however, the regulation of EBNA2 in response to TLE1 knockdown could not be ascertained in this study due to difficulty with the RT-qPCR assay. Dissociation curve analysis indicated that a second product appeared to be generated upon the down-regulation of the characteristic EBNA2 product. In the same LCL background *EBNA1* expression remained unchanged due to ectopic TLE1 (Section 5.2.5), but demonstrated significant down-regulation in response to TLE1 knockdown (Section 5.2.8.1). Furthermore, Generally, in LCLs, both *EBNA2* and *EBNA1* are transcribed primarily from the Cp promoter as one unprocessed transcript (Reviewed in Palermo, et al. 2008). However, in IB4 only the Wp promoter is active and all EBNA transcripts (with the exception of EBNA3B which is not expressed) are transcribed from this promoter (Hurley, et al. 1991). If *EBNA2* was independently regulated relative to *EBNA1* it could be speculated that this differential regulation must occur through *EBNA2*'s second promoter, the Wp. Although, it is also possible that TLE1 affects the differential splicing of EBNA RNAs.

It was also demonstrated that *TLE1* regulated several key EBV known and potential Notch target genes.

(i) *Cyclin D1* is another positive EBV target found to be down-regulated by TLE1 in IB4 cells (Section 5.2.6.1). *Cyclin D1* has been shown to be

transcriptionally up-regulated by EBV via c-Jun up-regulation of the *Cyclin D1* promoter (Herber, et al. 1994). The results obtained in this study are in agreement with published data that suggests that knockdown of *TLE1* by shRNA in a myeloid cell line leads to the up-regulation of the cell cycle proliferation marker, *Cyclin D1* (Dayyani, et al. 2008). This EBV-mediated positive regulation of *Cyclin D1* has been found to be mediated by LMP1 (Zhao, et al. 2001, Zhao, et al. 2000). A measure of the importance of *Cyclin D1* regulation is the wide spectrum of transcription factors that can bind to and/or regulate *Cyclin D1* at the transcriptional level (Review in (Wang, et al. 2004)). In addition, due to the broad range of *Cyclin D1* regulators, *Cyclin D1* can be regulated by a number of diverse mechanisms. Dys-regulation of *Cyclin D1* is a hallmark of a number of proliferative diseases such as cancer and atherosclerosis (Reviewed in Klein and Assoian 2008). *Cyclin D1* is classified as a proto-oncogene and it functions by driving the cell cycle progression of the cell from G1 through S phase. Therefore, if TLE1 reduces the expression of *Cyclin D1* this is likely to lead to a reduced proliferation rate, as was observed in the ectopic TLE1 expressing cell pool (Section 5.2.6.1). Furthermore, in agreement with these results, *Cyclin D1* was conversely significantly up-regulated in the same LCL when *TLE1* expression was knocked down by shRNA (Section 5.2.8.2). In addition, the rate of proliferation in the shRNA knockdown cell pool increased in comparison to a cell pool carrying a scrambled shRNA-expressing vector (Section 5.2.14).

(ii) E2A, a transcription factor crucial to B cell identity, was found to be a negatively regulated by ectopic TLE1 in an LCL (Section 5.2.6.2). These results were then corroborated by the *TLE1* shRNA knockdown experiment in which *E2A* mRNA levels were induced by the knockdown of TLE1 (Section 5.2.8.3). *E2A* is expressed throughout B cell development from the earliest lymphoid stage to the mature B cell (Decker, et al. 2009). B cells arise from multi-potent progenitor cells (MPPs) generated in the post natal bone marrow. The E2A transcription factor is critical to bring about the correct formation of common lymphoid progenitors (CLPs) from MPPs (Nutt and Kee 2007). Three transcription factors essential for the commitment of CLPs to the B cell lineage are E2A, Ebf1 and Pax5 (Decker, et al. 2009). *E2A* is a

bona fide early B cell factor (*Ebf1)* regulator, and is required to initiate *Ebf1* induction (Smith, et al. 2002). Whilst this lends a critical role to E2A in the initiation of B cell development, loss of the factor is not detrimental to mature B cell maintenance. This redundancy is due to the presence of dual *Ebf1* promoters, the α (or distal) promoter and the β (or proximal) promoter (Roessler, et al. 2007). The E2A transcription factor binds the distal *Ebf1* promoter, leading to the induction of Ebf1. The *Pax5* promoter contains an Ebf1 binding site, binding to this site activates Pax5 expression. Pax5 expression is critical for both the initiation and maintenance of the B cell lineage (Nutt and Kee 2007) and has been implicated as a requirement for EBNA2 expression (Klein, et al. 2010). To further potentiate the induction loop, Pax5 binds to the proximal *Ebf1* promoter accelerating *Ebf1* transcription by triggering activity off the second promoter (Nutt and Kee 2007). Due to this positively regulated loop, E2A is indispensible only for the initiation step of the B cell lineage. After this Ebf1 and Pax5 can maintain an adequate level of activity to conserve cell line lineage. In addition to the critical role for E2A in lineage commitment, E2A has furthermore been implicated in mature B cell survival and it has been demonstrated that knocking out E2A activity results in enhanced apoptosis in non-transformed cells (Lazorchak, et al. 2006). E2A is a positive regulator for transcription factors required for BCR expression; thus, it has been speculated that reduced E2A expression could lead to reduced survival due to loss of BCR signaling. However, it must be noted that E2A can protect pre-B cells from apoptosis in a BCR-independent manner; thus, E2A may confer survival advantages to B lymphocytes involving a novel mechanism (Lazorchak, et al. 2006).

(iii) The *Pax5* transcription factor performs a dual role in the cell, as it can positively and negatively regulate transcription through direct DNA binding via its N-terminal paired-domain motif (Cobaleda, et al. 2007). The regulatory effect of *Pax5* is dictated by the presence of repressive binding partners. Pax5 alone elicits transcriptional *trans*-activation through association with the basal transcriptional machinery protein, TBP and the likely recruitment of

histone acetyltransferases (HATs) (Emelyanov, et al. 2002). However, TLE-containing repressor complexes can interact with Pax5 via a TLE-binding motif in the Pax5 protein, converting it to a transcriptional repressor (Eberhard, et al. 2000). In this study, Pax5, a down-stream effector of E2A activity, was found to be down-regulated by ectopic TLE1 in an LCL background (Section 5.2.6.3) and up-regulated following shRNA-mediated *TLE1* knockdown in the same cell line (Section 5.2.8.4). Pax5 is one of three transcription factors vital to the B cell lineage commitment. Continued Pax5 activity is required to retain B lymphocyte identity. In addition, Pax5 also positively regulates the early latent promoter, Wp. The TLE family have been found to act as co-factors along with Pax5 to elicit a repressional effect within a B cell. Pax5 activity is responsible for regulating many B cell features prominent in an EBV infected lymphocyte such as BCR signalling and *CD21* stimulation (Reviewed in Cobaleda, et al. 2007). Although *Pax5* is essential for B lineage commitment, surprisingly little is known about the regulatory mechanisms behind *Pax5* expression. One defined *trans*-activator of *Pax5* is the early B cell factor (*Ebf1)*. The E2A transcription factor stimulates the activity of *Ebf1*; this in turn induces *Pax5* expression which is essential for the initiation and maintenance of B cell lineage commitment (Nutt and Kee 2007). Earlier results in this study indicated that TLE1 expression led to the repression of *E2A* mRNA expression in an LCL (Section 5.2.6.2). As *Pax5* is an indirect target of E2A it remains to be determined in this situation whether *Pax5* down-regulation is a result of direct regulation by TLE1, or whether it is an impact of the reduced level of E2A. A recent study has indicated that a 10-fold transcriptional knockdown of *E2A* expression using shRNA results in a 2-fold reduction in Pax5 mRNA levels in the EBV-negative BL-derived DG75 cell line (Hauser, et al. 2009). Whether or not *Pax5* repression is a direct or indirect effect of TLE1, it is noteworthy that plasma cell differentiation is inhibited by *Pax5* (Fuxa and Busslinger 2007) and EBV immortalised LCLs do not readily succumb to terminal differentiation. Inhibiting the plasma cell fate provides a possible explanation for the EBV mediated down-regulation of *TLE1*, if the downstream effect of TLE1 expression is *Pax5* repression.

(iv) *Runx3*, a positively-regulated EBV cellular target gene, has been found in this study to be negatively regulated by ectopic TLE1 (Section 5.2.6.4). *Runx3* is a known target of EBNA2, although TLE1 was shown to up-regulate EBNA2 in the ectopic cell pool, the transcription of *Runx3* was down-regulated. This implies that TLE1 may directly target *Runx3*. Circulating resting B cells are in a non-proliferative state and express *Runx1* (Spender, et al. 2005). Upon EBV infection of resting B cells, *Runx3* expression is induced by EBNA2, leading to a direct decrease in *Runx1* expression (Spender, et al. 2002). Ectopic expression of Runx1 in an LCL inhibits cell proliferation (Brady and Farrell 2009). Moreover, it has been confirmed that one function of Runx3 in an EBV immortalised LCL is to repress the negative regulator of proliferation, Runx1 (Brady and Farrell 2009). The TLE recognition motif VWRPY is essential for this Runx3 led repression; as VWRPY *Runx3* deletion mutants cannot down-regulate *Runx1* activity in an LCL (Brady and Farrell 2009), implicating a TLE family member as an associated factor in Runx3 down-regulation of *Runx1*. Once infection of the LCL is established and *Runx1* expression is negatively regulated, the infected cells can then move into a proliferative growth programme (Spender, et al. 2002). Evidence points to a role for *c-myc* as a downstream negative target of Runx1. Moreover it has been verified that forced c-MYC expression in a B cell line can overcome the Runx1 elicited proliferation repression. It can be noted that BLs characteristically encompasses *c-myc* translocation that instigates over-expression of the c-MYC protein. This over-expression of c-MYC overcomes the *Runx1* activity and allows BLs to proliferate in culture in the presence of Runx1 (Reviewed in Brady and Farrell 2009). The modulation of *Runx3* by TLE1 epitomises the fine balance of gene regulation in an EBV infected cell, as it appears that EBV may down-regulate *TLE1* to potentially enhance activation of positive EBV target genes (Such as *Runx3* and *CD23*), but TLE1 is also a factor required for the function of said positive EBV targets (e.g. *Runx3*). Therefore it is logical that TLE1 expression is detected in EBV infected cells, but that the expression of the transcription factor is attenuated by EBV. However, it was also determined in this study that transient *TLE1* knockdown using shRNA did not result in significant modulation of *Runx3* activity (Section 5.2.8.5). This may have been due to

the limited time frame in which *TLE1* knockdown was elicited, or a threshold for *Runx3* expression may already have been met preventing further potentiated *Runx3* expression. In addition, no TLE1 elicited regulation of *Runx1* was observed in this study during ectopic expression of TLE1 nor during *TLE1* knockdown, this is in agreement with published studies that indicate that although AML1 (Runx1) utilises TLE1 to elicit function, it is not itself regulated by TLE1 (Imai, et al. 1998; Levanon, et al. 1998).

(v) The known positive EBV target, *CD23*, a direct target of EBNA2, was found to be down-regulated by ectopic TLE1 in an LCL in this study (Section 5.2.6.5). CD23 expression promotes the activation and proliferation of B lymphocytes (Jurisic, et al. 2008), and thus is a logical EBV target. Two isoforms of CD23 exist that demonstrate variation in the cytoplasmic domain of the protein and are expressed from two different promoters: (i) CD23a arising from the proximal promoter is expressed only in B lymphocytes and (ii) CD23b originating from the distal promoter expressed in B lymphocytes and other cells of myeloid lineage in response to IL4 stimulation (Lemieux, et al. 2007). This was concluded in part by the fact that B lymphocytes infected by the EBV *EBNA2* deletion mutant, P3HR1, failed to activate *CD23* expression (Cordier, et al. 1990). It was then established that *EBNA2* subverts the Notch signaling pathway to stimulate *CD23* expression through the CBF1 binding domain in the *CD23a* proximal promoter (Ling, et al. 1994). If TLE1 expression were to down-regulate *CD23*, this would result in reduced proliferation, coinciding with the results for the growth rate in the ectopic TLE1 expression cell pool, pcDNA3 TLE1 IB4 (Section 5.2.8.6). However, the knockdown of *TLE1* did not lead to the expected rise in *CD23* expression. No change in transcriptional activity was observed for *CD23* between the *TLE1*-shRNA knockdown cell pool and that pool expressing scrambled shRNA.

(vi) *Hes1* expression is known to be up-regulated in LCLs (Callahan, et al. 2000). In this study *Hes1* was not found to be regulated due to the ectopic expression of TLE1 in an LCL (Section 5.2.6.6), however, knockdown of TLE1 in the same LCL resulted in up-regulation of *Hes1* activity (Section

5.2.8.7). *Hes1* is a bHLH transcription factor that binds DNA and is directly *trans*-activated by Notch to elicit Notch stimulated regulation (Ross, et al. 2003). Hes1 elicits negative regulation by associating with members of the TLE1 family through the TLE recognition sequence, WRPW (Buscarlet and Stifani 2007). Recent studies have established a strong link between *Hes1* expression and quiescence. Quiescence is a reversible state in which the cell is not dividing but can be stimulated to proliferate in response to the appropriate stimuli. Quiescent cells can either be stimulated to proliferate or they can move into an irreversible terminal state (differentiation, apoptosis or senescence). Quiescence was widely accepted to be a passive state; however, accumulating evidence indicates that it may be maintained by active molecular mechanism. *Hes1* has recently been shown to be expressed in quiescent cells to prevent terminal differentiation. Furthermore, *Hes1* expression has also been found to be up-regulated in several types of tumours to protect against differentiation. Thus, it is feasible, considering that EBV infected cells are blocked from undergoing differentiation, that EBV up-regulates *Hes1* to protect the infected cell from the plasma cell fate which would otherwise lead to virus production and immune visibility (Reviewed in Sang, et al. 2010).

(vii) In this study it was observed that *Hey1* was not regulated by ectopic TLE1 in an LCL (Section 5.2.6.6); however, upon *TLE1* shRNA knockdown, the mRNA levels of *Hey1* became elevated (Section 5.2.8.8). It may be the case that in the cell pool expressing ectopic TLE1, the threshold level of endogenous TLE1 was already sufficient to modulate *Hey1*. It is acknowledged that although the proteins of the Hey family contain a conserved C-terminal motif (YRPW), strikingly similar to the TLE recognition sequence (WRPW), TLE1 cannot associate with the Hey family (Buscarlet and Stifani 2007). The association between EBV and Hey1 will be discussed in more detail later in this study (Section 6.2.1). However, from the results obtained in these experiments it appears that TLE1 may have a role in *Hey1* regulation.

There is endogenous TLE1 in LCLs, and this basal level of expression may have already reached the threshold for eliciting its effect on some TLE1 targets. Ectopic TLE1 might not therefore further modulate the expression of some TLE1 target genes. Gene knockdown served to lower the level of endogenous TLE1 and, therefore, results with any one target gene using the two approaches might not necessarily always appear to agree. Endogenous TLE1 in LCLs, present in lower levels than in resting B cells, may interact with transcription factors that play roles in cell subversion by EBV, given the requirement for TLE to act as a co-factor in the activities of Runx1, Runx3 and Pax5. Indeed, TLE can be recruited by positive EBV targets to elicit the repression of unwanted gene expression. One such example of this is the previously mentioned co-operation between Runx3 and a TLE family member to down-regulate *Runx1* in an LCL (Spender, et al. 2005; Brady and Farrell 2009).

The transfection efficiency of suspension cell lines of lymphoid and myeloid origin have a tendency to be quite low and thus for this project a novel method for establishing an enriched transfected cell pool was established. Using an LCL it was found that removing excess dead cells 24 hours after transfection improved the overall viability of the transfected cell pool (Section 5.2.11). A B cell-specific lectin (Con A) was also used to further 'pan-out' viable lymphocytes following transfection/drug-selection (Section 5.2.11). This novel approach greatly facilitated the isolation of cell pools enriched in drug-resistant (and hence transfected) cells. This method may have potential for improving the success of establishing stably transfected B cell lines. Con A, although unable to activate B cells, is a potent activator of T lymphocytes however, thus restricting the use of this approach to B cells. Due in large part to the success of this method, it was possible to establish cell pools transfected with individual shRNA-expressing plasmids. The cell pool exhibiting *TLE1* knockdown appeared to have a higher rate of proliferation than the LCL expressing the scrambled shRNA (Figure 5.37). These results indicated that *TLE1* had a negative impact on the growth rate of cells in an LCL background.

A previous association has been made linking TLE1 and EBV-immortalised cells (Almqvist, et al. 2005). As described in Chapter 1, EBV has four latency types (0-III) and three promoters for the activation and maintenance of these growth programmes (Cp, Qp and Wp). The activation of the Cp requires the binding of EBNA1 to a family of repeats (FR) upstream of the promoter. The FR is composed of a 30 bp sequence with a variable number of repeats and between seven and nine of these are required for EBNA1 activation of the Cp (Lindner and Sugden 2007). When bound to Cp, EBNA1 does not occupy the full FR sequence, and other proteins such as Oct1, Oct2 and Bob1 also associate with the FR. Indeed, even in the absence of EBNA1, Oct2 is a potent inducer of activity and can bind the Cp promoter unaccompanied to elicit up-regulation of the promoter. Oct1 gives a weaker response than Oct2, requiring the co-repressor Bob1 to elicit positive regulation on the promoter. TLE binds to Oct transcription factors thus modifying their function from transcriptional activators to transcriptional repressors. Studies have demonstrated the ability of TLE to regulate Oct function in relation to the *EBNA* Cp promoter. It was shown that all TLE proteins were capable of repressing Oct2-mediated enhancement of Cp. The effect of TLE proteins on Oct1-mediated induction varied and was at times contradictory to the Oct2/TLE function. The effect of Oct1/Bob1 was unchanged by the expression of *TLE2* and *TLE3*, and surprisingly expression of *TLE1* and *TLE4* actually enhanced Cp activity. The effect of the TLE proteins on *EBNA1*-driven Cp promoter activity was also looked at in that study. It was found that co-transfection of *EBNA1* with *TLE3* or *TLE4* led to up-regulated Cp activity. However, TLE1 and TLE2 had no impact on EBNA1-mediated Cp regulation (Almqvist 2005). EBV tightly regulates the expression of its latent genes, thus making the repression of a Cp deregulator, such as *TLE1,* a logical host-virus interaction.

Chapter 6: *Regulation of other candidate* EBV cellular target *genes following EBV infection* in B lymphocytes.

6.1 Introduction

The RT Profiler PCR SuperArray experiment (Chapter 4) identified a spectrum of potential cellular genes as targets of EBV. These candidate targets were reduced to a panel of fourteen based on possible relevance to EBV biology and also novel association with EBV. Here, a role for EBV in the regulation of several cellular genes from this panel, shown to be modulated in naïve B cells following infection by EBV (determined using an RT Profiler PCR SuperArray) (Table 4.1), was pursued using the conditional LCL ER/EB2.5 (described in Chapter 4). In this chapter the remaining genes were investigated, namely (i) *Hey1*, (ii) *NR4A2*, (iii) *STIL,* (iv) *Cyclin E1*, (v) *LRP5*, (vi) *CIDEB*, (vii) *c-Fos*, (viii) *c-Jun*, (ix) *TNFSF7*, (x) *MMP7*, (xi) *TRAF4* and (xii) *Fas*. Their possible relevance in EBV latency is discussed.

6.2 Results

6.2.1 Up-regulation of *Hey1* by EBV in the conditional LCL, ER/EB2.5.

A gene of particular interest in this study was Hairy/enhancer of split related YRPW motif 1 (*Hey1*). *Hey1* (also denoted *HERP2, Hesr1, HRT1 or CHF2)*, is a member of the *hairy/Enhancer of Split*-related bHLH orange family of transcription factors that mediate Notch signaling (Fischer, et al. 2002). The family comprises the three members *Hey1*, *Hey2* and *HeyL* (Steidl, et al. 2000; Leimeister, et al. 1999). *Hey1* was identified as a potential cellular EBV target gene from the Notch RT Profiler PCR SuperArray in which a 121-fold increase in the level of *Hey1* mRNA was observed 4 days post-infection of freshly isolated naïve B cells (Section 4.2.1). Primers for two *Hey* family members were included on the RT Profiler PCR SuperArray -*Hey1* and *HeyL,* of which only *Hey1* appeared to be modulated following EBV infection. Indeed, no mRNA from *HeyL* was detected in either sample.

This aim of this study was to (i) confirm that *Hey1* is an EBV target, (ii) determine which of the EBV latent proteins may be responsible for this regulation, (iii) to establish if there is a trend, with respect to *Hey1* expression, in a panel of B cell lines and (iv) attempt to assess the impact of *Hey1* expression in a B cell.

To confirm that EBV regulates *Hey1* activity, the expression of *Hey1* in the cell culture model, ER/EB2.5 was examined. ER/EB2.5 permits activation/inactivation of EBV latent gene expression with β-estradiol. Like any LCL, ER/EB2.5 cells proliferate due to the effects of EBV latent gene products, and ER/EBNA2 inactivation (in the absence of β-estradiol) leads to a cessation of the EBV growth programme. Cells were prepared for ER/EBNA2 activation as described in Section 4.2.3.

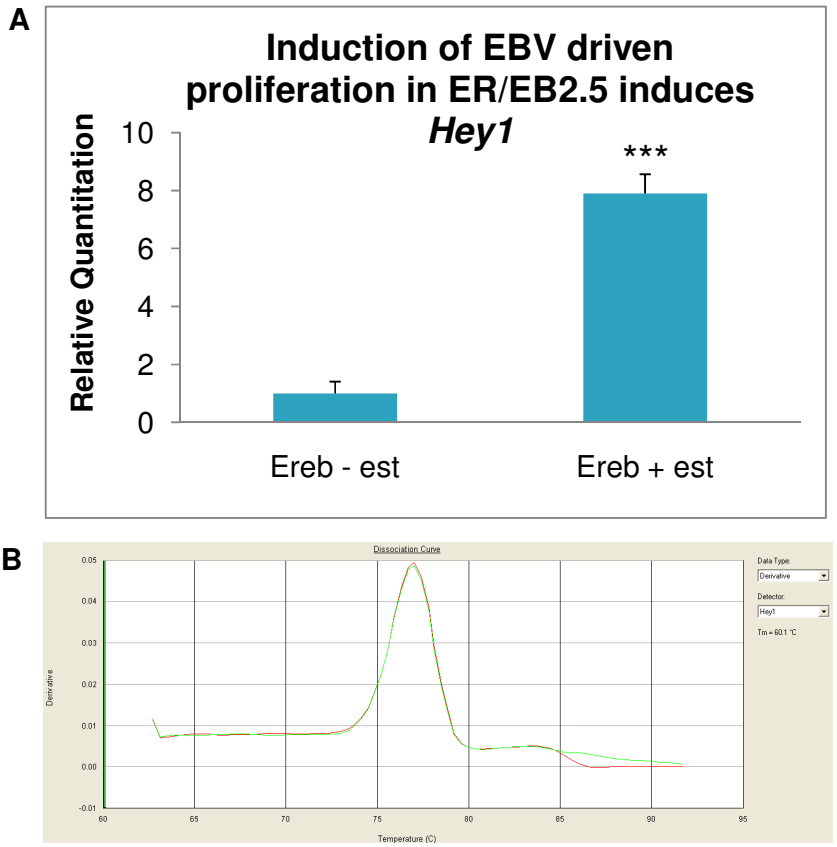


Figure 6.1: Induction of EBV-driven proliferation in ER/EB2.5 leads to an increase in the level of *Hey1* mRNA.

RT-qPCR analysis was carried out to determine the levels of *Hey1* expression in the cell culture model of resting B cells (ER/EB2.5 - est) relative to ER/EBNA2 activated cells (ER/EB2.5 + est). ER- EBNA2 was activated by the addition of 1μM β-estradiol to previously β-estradiol-starved cells. RNA samples were taken before and after the addition of β-estradiol to the growth medium and reverse transcribed using random hexamers and MMLV reverse transcriptase. Amplification of cDNA was monitored in real time by detection of fluorescence intensity using SYBR green. **(A)** Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control being EBV cycling cells (ER/EB - est): arbitrarily assigned a value of 1. (Data are Mean ±SD, *** P<0.001). **(B)** The dissociation curve for the product of the *Hey1* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The results from this experiment correlated with the data obtained from the EBV-naïve B cell infection experiment (Section 4.2.1). The level of *Hey1* expression within the ER/EB2.5 cell line increased 8-fold due to ER/EBNA2 activation (Figure 6.1). Taken together, these experiments support the positive regulation of *Hey1* by EBV at the transcriptional level.

6.2.1.1 Up-regulation of Hey1 by EBNA2

The conditional BL, BL41-K3, described in Section 4.2.4, can be induced to express ER/EBNA2, whose function is dependent on β-estradiol, but no other latent viral proteins. Total RNA samples were prepared at time points during β-estradiol activation of ER/EBNA2 in BL41-K3 and the expression of *Hey1* at the transcriptional level was investigated using RT-qPCR. BL41-K3 cells were prepared for ER/EBNA2 activation by washing twice in PBS prior to the addition of β-estradiol to a final concentration of 1 μM in supplemented RPMI medium.

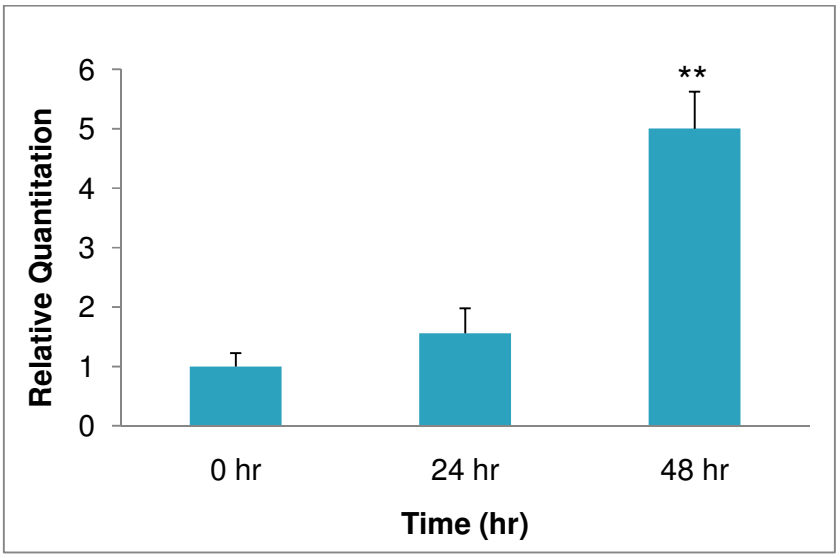


Figure 6.2: ER/EBNA2 positively regulates *Hey1* mRNA in BL41-K3.

ER/EBNA2 was activated in BL41-K3 by the addition of 1μM β-estradiol to the growth medium. RNA extracts were prepared at the times indicated following activation of ER/EBNA2 in BL41-K3. RT-qPCR analysis was performed for *Hey1* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1; results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as BL41-K3 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD, ** $P<0.01$).

Results from the RT-qPCR investigation indicated that ER/EBNA2 activation coincided with up to a 6-fold increase in the steady state levels of *Hey1* mRNA during a 48 hour period (Figure 6.2). These findings imply the ER/EBNA2 is capable of transcriptionally driving *Hey1* in an EBV-negative

BL cell line. The established conditional cell line DG75 tTA-EBNA2 (described in detail in Section 4.2.6) was next used to confirm the ability of EBNA2 to *trans*-activate *Hey1*. Here, EBNA2 can be induced as sole EBV protein in an EBV-negative BL background in response to tetracycline. DG75-tTA-EBNA2 cells were maintained in supplemented RPMI media containing 1 µg/ml of tetracycline. To induce EBNA2, the cells were washed 4 times in PBS and re-cultured in tetracycline free medium. Total RNA samples were prepared at time points following tetracycline withdrawal and the level of *Hey1* mRNA was investigated using RT-qPCR.

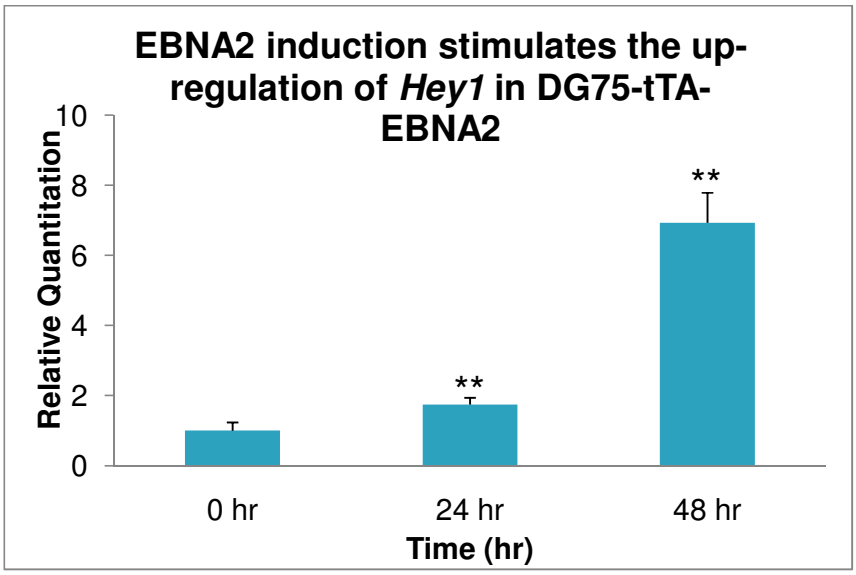


Figure 6.3: EBNA2 induction stimulates the up-regulation of *Hey1* in DG75-tTA-EBNA2.

EBNA2 expression was induced in DG75-tTA-EBNA2 by the removal of tetracycline from the supplemented RPMI medium. The figure shows RT-qPCR analysis of *Hey1* mRNA levels in DG75-tTA-EBNA2 in response to EBNA2 induction. RNA samples were prepared at the time points indicated post-induction. RT-qPCR analysis was performed as described in the legend to figure 6.1; results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as DG75-tTA-EBNA2 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD, ** $P<0.01$).

Increased *Hey1* mRNA levels were observed to coincide with the induction of EBNA2 (Figure 6.3). Forty eight hours after the removal of tetracycline from the growth medium, the *Hey1* mRNA level had increased seven fold. Together, these experiments show that *Hey1* is *trans*-activated by EBNA2.

6.2.1.2 Hey1 mRNA expression levels in a panel of BLs and LCLs

Relative *Hey1* mRNA levels were determined in a panel of B cell lines including EBV-positive and EBV-negative BL-derived cell lines and an LCL.

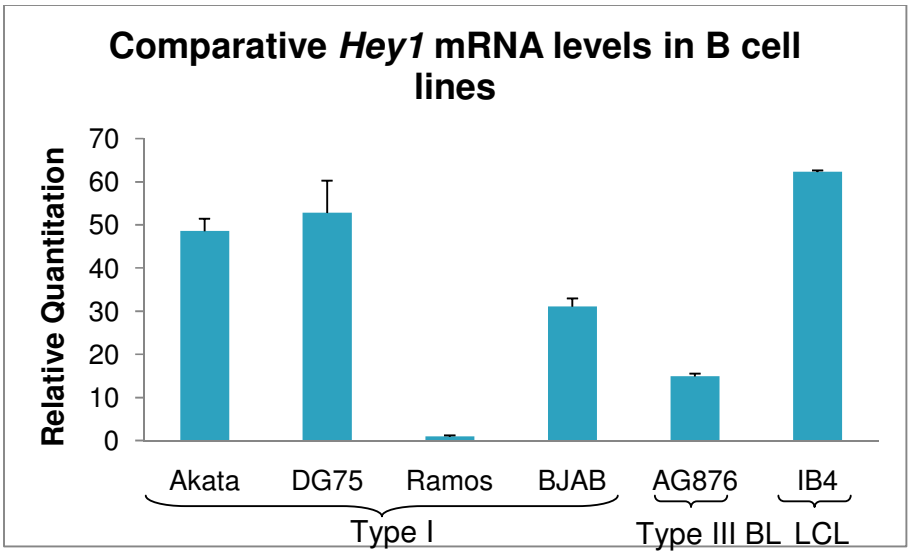


Figure 6.4: Comparative *Hey1* mRNA levels in B cell lines.

RT-qPCR was used to monitor *Hey1* mRNA levels in a range of B cell lines including the EBV-negative B cell lines DG75, BJAB, Ramos and Akata4E3, the EBV-positive AG876 (group-III BL cell line), and the EBV-immortalised LCL IB4. RNA samples were prepared from each cell line in the same time frame, and RT-qPCR analysis of *Hey1* mRNA levels was performed as described in the legend to figure 6.1. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment Ramos cells were set as control cells, arbitrarily assigned a value of 1. (Data are Mean ±SD).

From the results obtained, and taking into consideration the previous data presented in this study indicating that EBV up-regulates *Hey1* (Section 6.2.1), it was surprising to observe basal *Hey1* mRNA levels at a lower level in the EBV-positive BL, AG876, in contrast to the EBV-negative BLs DG75 Ramos and Akata4E3. Overall, no correlation between EBV/EBV gene expression appears to exist regarding *Hey1* expression in the cell lines examined.

6.2.1.3 Hey1 represses a CBF1-regulated promoter

The pGa981.6 construct is a Notch responsive reporter vector that has been described previously in section 3.2.2. To confirm the role for *Hey1* in the Notch signalling pathway in a B cell background, the EBV-negative BJAB cell line was co-transfected with the Hey1 expression vector, pSG5-hHey1, and pGa981.6.

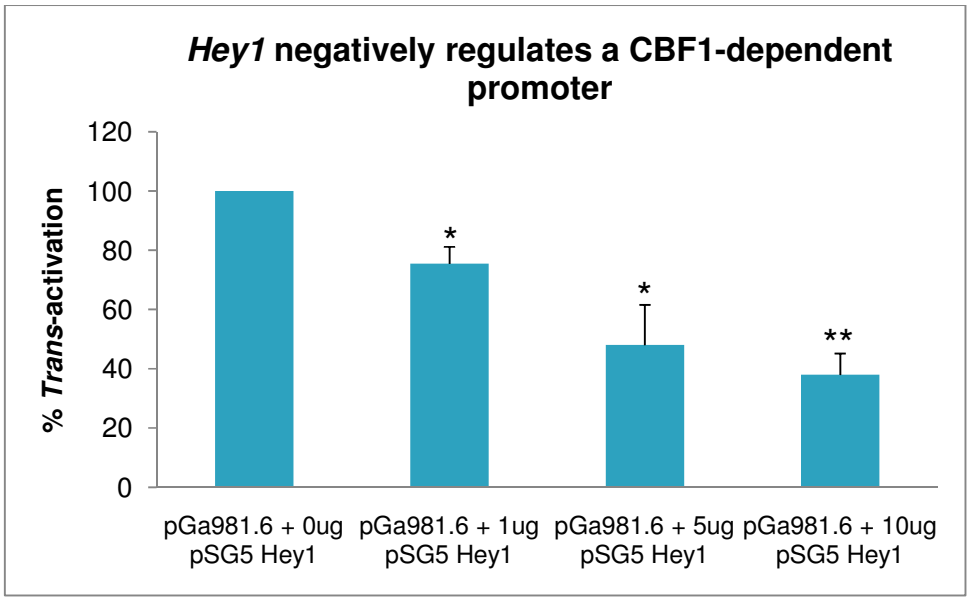


Figure 6.5: *Hey1* negatively regulates a CBF1-dependent promoter.

BJAB cells were co-transfected with increasing quantities of pSG5 hHey1 (0µg, 1µg, 5µg and 10 µg) along with 2 µg of the luciferase reporter construct pGa981.6. Cells were harvested 48 hours post-transfection and assayed for luciferase activity. Luciferase values were evaluated based on the *β*-galactosidase activity measured from a co-transfected pCMV-LacZ reporter, which was included in all transfections. Normalised values were expressed as percentage *trans*-activation relative to the corresponding data obtained from co-transfection of pSG5 and pGa981.6 vector. Data are presented as the average of three independent experiments (Data are Mean ±SD, * P<0.05, **P<0.01).

Transient transfection of the EBV-negative cell line, BJAB showed that co-transfection of the pGa981.6 luciferase reporter plasmid with increasing quantities of a Hey1 expression vector demonstrated a dose-responsive repressive effect elicited by Hey1 on promoter-driven luciferase values (Figure 6.5). These results show that Hey1 represses transcription from a

CBF1-regulated promoter. These data supports a role for Hey1 as a regulator of Notch-responsive genes in this non-BL B cell line.

6.2.1.4 An NF-κB responsive promoter is negatively regulated by Hey1 expression

EBV subversion of the NFκB signaling pathway is a widely accepted requirement for successful establishment of EBV infection. To determine if Hey1 can also influence the NFκB pathway, BJAB was co-transfected with pSG5 hHey1 and the previously described NFκB-regulated reporter construct, 3x enh κB luc (Section 5.2.2).

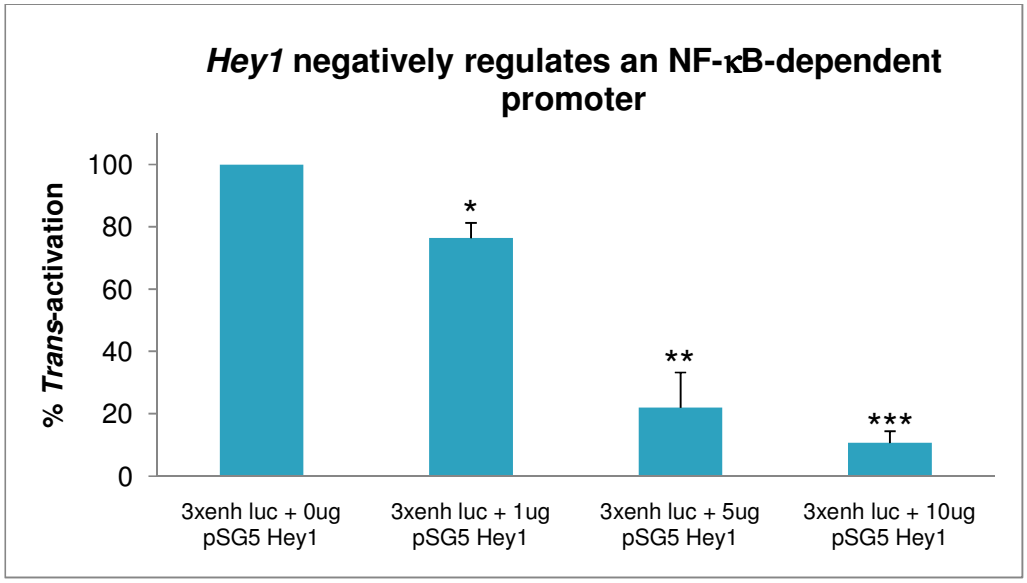


Figure 6.6: *Hey1* negatively regulates an NF-κB-dependent promoter.

BJAB cells were co-transfected with increasing quantities of pSG5 hHey1 (0 µg, 1 µg, 5µg and 10 µg) along with 2 µg of the luciferase reporter construct 3xEnh-luc. Cells were harvested 48 hours post-transfection and assayed for luciferase activity. Luciferase values were calculated as previously described in the legend to figure 6.5 and normalised values were expressed as percentage *trans*-activation relative to the corresponding data obtained from co-transfection with pSG5 and 3xEnh-Luc vector. Data are presented as the average of three independent experiments (Data are Mean ±SD, * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Co-transfection of BJAB cells with 3xEnh-Luc and increasing quantities of pSG5 hHey1 gave a consistent dose-responsive decrease in luciferase activity within the triplicate experiments performed in this study (Figure 6.6). This indicates that Hey1 plays a role in regulating NF-κB signaling in BJAB.

6.2.1.5 Determining a role for Hey1 in an LCL

The results obtained so far in this study indicate that EBV up-regulates the cellular gene *Hey1*. This part of the study involved investigating the impact of ectopic Hey1 expression on the phenotype of an LCL. To express Hey1 in an inducible manner within the cell, a *Hey1* cDNA was sub-cloned into a tetracycline (Tet) regulated expression vector, pRTS-1 (Figure 6.7). The resulting plasmid pRTS-1-hHey1 could be induced to simultaneously co-express both Hey1 and GFP.

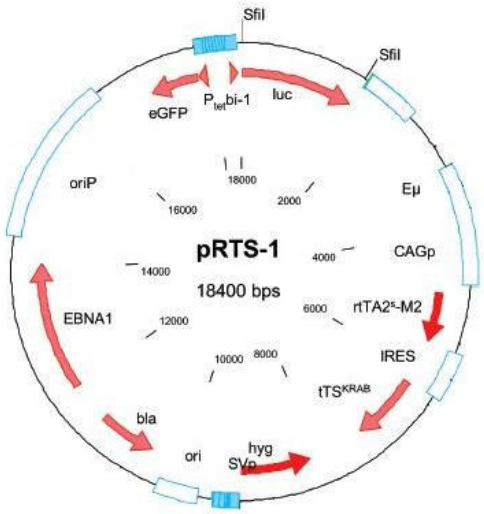


Figure 6.7: Schematic circular map illustrating pRTS-1.

pRTS-1 is an EBV-derived episomally replicating plasmid that is designed to allow tetracycline controlled regulation of expression of a gene of interest from the vector. pRTS-1 possesses a bi-directional promoter P_{tet}bi-1 from which *GFP* and *Luc* (or an inserted target gene) is expressed. The *luciferase* gene is designated as luc. The bicistronic expression cassette driving expression of rtTAs-M2 and tTSKRAB, separated by an internal ribosomal entry site (IRES), is located down-stream of the chicken β-actin intron and transcribed from a promoter/enhancer consisting of the mouse heavy chain intron enhancer (Eμ) and the chicken β-actin promoter (CAGp). OriP is the EBV episomal origin of replication which can be driven by EBNA1, the EBV gene EBNA1. SVp denotes the SV40 early promoter; ori, the bacterial origin of replication derived from pMB1; and hyg, the hygromycin phosphotransferase gene (Adapted from Bornkamm, et al. 2005).

The tetracycline-regulatable pRTS-1 vector, a novel EBV-derived episomally replicating plasmid, allows simultaneous expression of two genes (Bornkamm, et al. 2005). The pRTS-1 construct encompasses two

regulational factors, a transcriptional silencer tTS_{KRAB} and a transcriptional activator rtTA2S-M2. In the absence of tetracycline, the vector-derived silencer, tTS_{KRAB} binds to P_{tet}bi-1 preventing expression from the bi-directional promoter. Addition of tetracycline alleviates this repression, and binding of rtTA2S-M2 then activates the P_{tet}bi-1.

6.2.1.6 The construction of pRTS-1 Hey1

To generate pRTS-1-Hey1*,* a cloning strategy was employed that involved amplification of the *Hey1* target sequence from pSG5 FLAG hHey1 (a gift from Belandia, et al. 2005) by PCR (Figure 6.8A) and subsequently sub-cloneing into pRTS-1 in place of the excised *luc* gene. The pRTS-1 vector was first linearised by digestion with *Sfi* I to excise the *luc* gene (Figure 6.8B), followed by de-phosphorylation using CIP to prevent re-circularisation of the linearised DNA, and additionally to prevent re-cloning of the excised *luc* gene. The linearised, CIP-treated DNA was then purified by phenol:chloroform extraction followed by ethanol precipitation (Section 2.2.1.2). The primers used to amplify hHey1 included *Sfi* I restriction sites for cloning. Following PCR, the products were analysed by agarose gel electrophoresis (Figure 2.2.1.16) to determine that the fragment was at the expected size. The PCR products were subsequently purified using the Promega Wizard® DNA purification system (Section 2.2.1.6), digested with *Sfi* I and purified by phenol: chloroform extraction followed by ethanol precipitation. Purified PCR products were then sub-cloned between the *Sfi* I sites of pRTS-1. Ligations of vector and insert DNA were carried out overnight at 16˚C (Section 2.2.1.7). Following ligation, competent *E. coli* JM109 cells were transformed with the recombinant plasmid DNA. DNA mini-preparations were prepared from cultures inoculated with single transformed colonies. Restriction digestion analysis was performed on the DNA to monitor the presence of hHey1 insert DNA. Although both *Sfi* I sites used for cloning were different in base-pair composition it was possible for the insert DNA to ligate into the vector in either the forward or reverse orientation as the overhangs produced are palindromes. To determine which orientation the insert DNA had ligated in, restriction analysis using *Bst* EII was performed

(Figure 6.8D). The correctly orientated clones were then purified using the Qiagen® Midi Kit (Section 2.2.1.12).

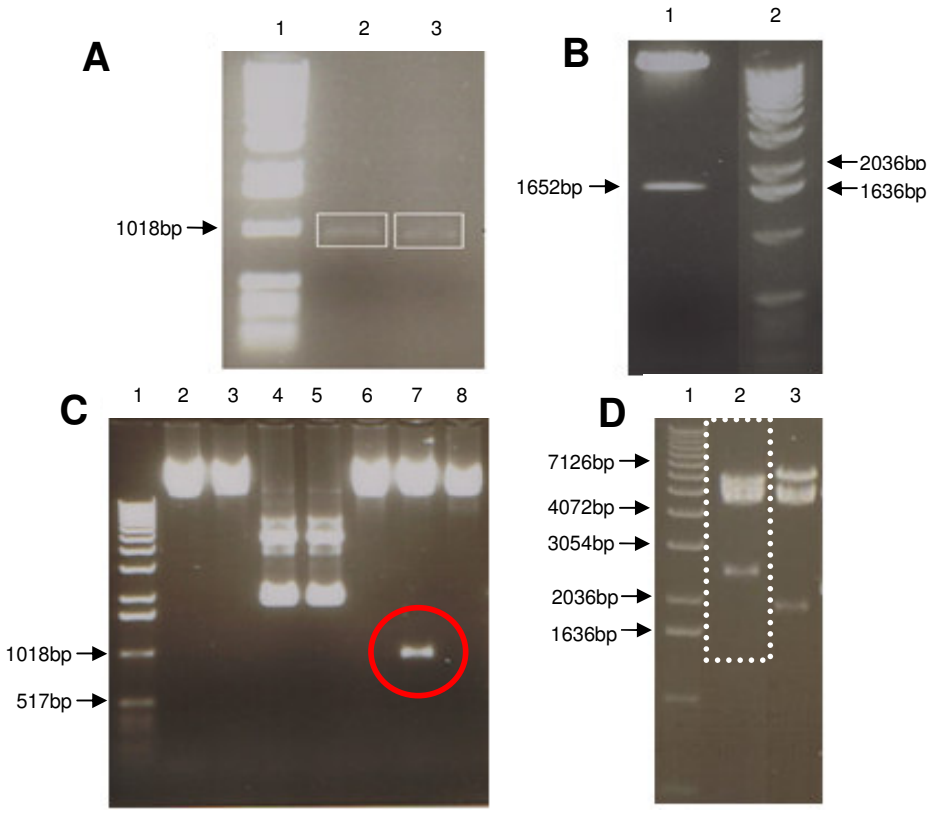


Figure 6.8: Generation of pRTS-1 hHey1.

(A) PCR product amplified from pSG5 FLAG Hey1, exhibiting a band at the expected size of approximately 955 bp. Lane 1 contains a 1 kb ladder. All DNA was analysed using 0.7-1% agarose-1X TAE gels and electrophoresis was carried out at 100V for 1 hour in 1X TAE. **(B)** Restriction digestion of pRTS-1 with the expected *Sfi* I excised *luc* gene at 1652 bp in lane 1. The remaining linearised vector was phosphatase treated by CIP, to prevent re-circularisation. **(C)** Following ligation, recombinant vectors were analysed by *Sfi* I digestion followed by agarose gel electrophoresis. Lane 1 shows a 1 kb DNA ladder, the excised hHey 1 cDNA fragment from a positive recombinant clone is highlighted in red at the expected size, 955 bp. Lanes 2, 3, 6 and 8 comprised the pRTS-1 vector alone, whilst lanes 4 and 7 contained undigested and digested pRTS-1 clones respectively, with the *luc* gene still present. **(D)** Restriction analysis of recombinant pRTS-1 hHey1 clones to determine correct insert orientation by digestion using *Bst* EII enzyme followed by agarose gel electrophoresis on a 0.7% agarose-1X TAE gel. In the correct orientation this would yield four fragments of 5597 bp, 4592 bp, 4964 bp, and 1957 bp (highlighted in lane 2). However, incorrect orientation would produce four fragments of 6145 bp, 4964 bp, 4592 bp, and 1957 bp upon digestion, as seen in lane 3. A 1 kb ladder is seen in lane 1.

To demonstrate that pRTS-1 hHey1 could be induced to express eGFP by tetracycline, DG75 cells were transfected with pRTS-1-hHey1. The

transfected cell pool was divided in two, half of the pool was cultured in tetracycline free supplemented RPMI medium, while the remaining half was cultured in the presence on tetracycline and the two cell pools were monitored by fluorescent microscopy after 48 hours.

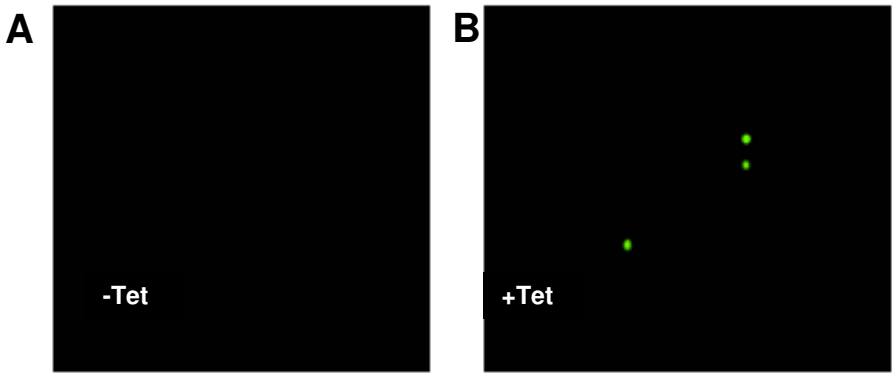


Figure 6.9: Induction of ectopic eGFP expression from pRTS-1 hHey (10x magnification).

DG75-pRTS-1 hHEy1 cell pools were induced by addition of tetracycline to express Hey1 and GFP proteins. **(A)** The un-induced control cell pool (-Tet) displaying no GFP expression. **(B)** The induction of GFP protein expression by Tet (+Tet). The cell pools were examined 48 hours post-induction using an Olympus DP-50 fluorescent microscope.

The detection of Hey1 expression by Western blot analysis in this study proved unsuccessful. RT-qPCR was employed however to confirm inducible expression of *hHey 1* mRNA from this vector. To estimate the relative levels of *Hey1* mRNA expression from a transfected cell pool in which the pRTS-1-hHey1 construct is induced, relative to an un-induced pool, the DG75 cell line was co-transfected with pRTS-1-hHey1. The transfected pool was divided and one portion was induced by the addition of 1µg/ml tetracycline to the culture medium. Subsequently, RNA samples were prepared from the un-induced pool and the induced pool 48 hours after the addition of tetracycline and RT-qPCR was performed.

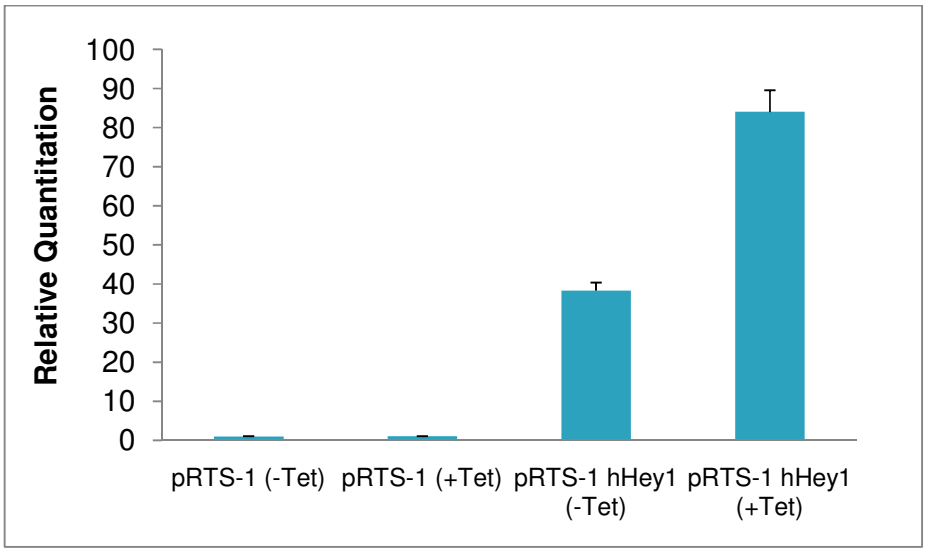


Figure 6.10: Induction of *Hey1* mRNA from pRTS-1-hHey1.

Hey1 expression was induced in DG75 cells transfected with pRTS-1 hHey1 by the addition of 1µg/ml tetracycline to the supplemented RPMI culture medium. However, increased *Hey1* mRNA expression can be observed in the un-induced cell pool relative to the empty vector controls (pRTS-1 induced and un-induced). RNA samples were taken and RT-qPCR analysis was performed as described in the legend to figure 6.1. In this experiment, the control was designated as pRTS-1 (-Tet); arbitrarily assigned a value of 1. (Data are Mean ±SD).

The RT-qPCR analysis of DG75 cells transfected with pRTS-1-hHey1 indicated that *Hey1* mRNA was detectable in the absence of tetracycline. A 38-fold increase in *Hey1* mRNA levels can be seen due to un-induced pRTS-1-hHey1, relative to the empty pRTS-1 vector. Tetracycline induction led to an 84-fold increase in the levels of *Hey1* mRNA relative to the empty pRTS-1 vector. However, relative to the un-induced construct, induction from the pRTS-1-hHey1 only increases the *Hey1* expression levels 2-fold.

To determine if the leaky expression from pRTS-1-hHey1 elicits a significant effect on a known Hey1 target, DG75 cells were co-transfected with pRTS-1-hHey1 along with a luciferase reporter construct containing a *Hey1* responsive GATA4 promoter, pGL3-GATA4. *GATA4* is a housekeeping gene that is repressed by Hey1. Once again the cell pool was divided in two, one half was induced by the addition of tetracycline to activate Hey1 expression, and the remaining half was left un-induced. DG75 was also co-transfected

with pGL3-GATA4 and pSG5 FLAG hHey1 as a positive control for *Hey1* mediated GATA4 repression.

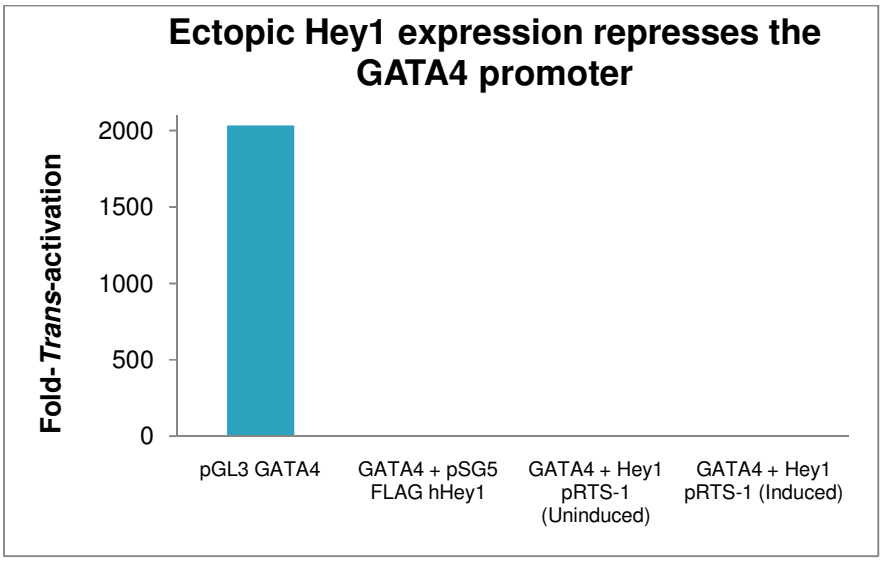


Figure 6.11: Ectopic Hey1 expression represses the GATA4 promoter.

DG75 cells were co-transfected with 5 µg of pGL3-GATA4 reporter construct, 2 µg pCMVLacZ, to allow for normalisation, and either pSG5 FLAG hHey1 or pRTS-1-hHey1. The total quantity of transfected plasmid was adjusted to 12 µg with the empty vector pSG5. Transfected cell pools were split into two and one portion was induced with tetracycline. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed as fold-activation relative to the corresponding value obtained for the pGL3 GATA4 reporter construct when co-transfected with pSG5 FLAG hHey1; arbitrarily assigned a value of 1. (Data are Mean ±SD).

It can be observed from figure 6.11, that GATA4 promoter activity is almost completely repressed by Hey1 expression, in agreement with published data (Fischer, et al. 2005). pSG5 FLAG Hey1 reduces the *trans*-activation of GATA4 over 2000-fold. In addition, induction of the pRTS-1-hHey1 construct by tetracycline down-regulated the GATA4 promoter to an equivalent degree. However, the leaky expression from the un-induced pRTS-1 hHey1 construct was sufficient to elicit a similar level of repression on the GATA4 promoter construct. It was clear at this point that regulatable expression of Hey1 from the pRTS-1 construct would not be achieved, it was decided not to proceed with this vector.

6.2.1.7 Determining the effect of EBNA2 on the Hey1 promoter in an EBV-negative BL

Results previously obtained in two independent experiments indicated that an increase in *Hey1* mRNA expression was found to coincide with increased EBNA2 protein expression (Section 6.2.1.1). Thus EBNA2 was implicated as a *trans*-activator of *Hey1* in a BL background. It was, therefore, of interest to determine if this up-regulation effect was elicited through the *Hey1* promoter. The human *Hey1* promoter-reporter construct used in this experiment was a gift from Dr. M Gessler (Zavadil, et al. 2004).

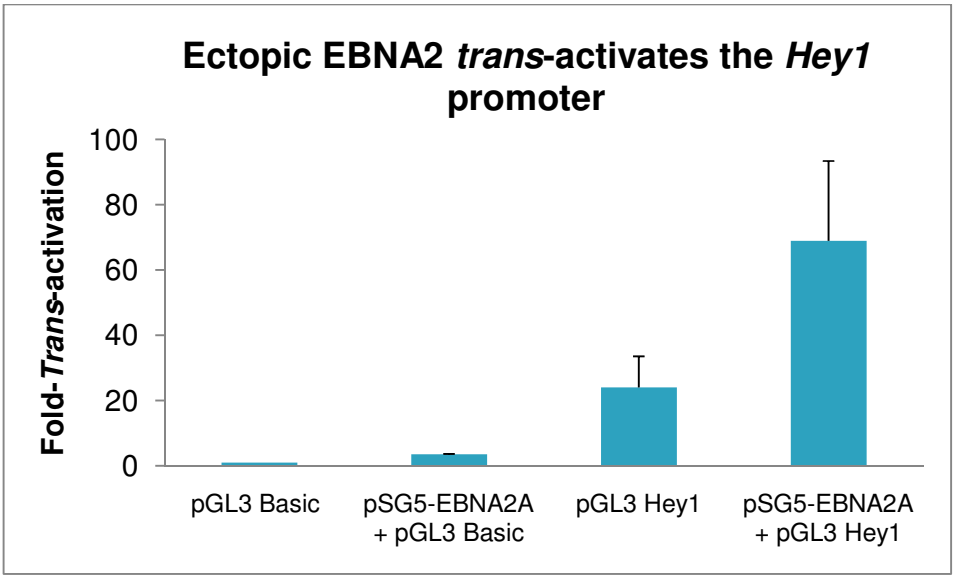


Figure 6.12: Ectopic EBNA2 *trans*-activates the *Hey1* promoter.

DG75 cells were co-transfected with 2 µg of pGL3-Hey1 luciferase reporter construct, 2 µg pCMVLacZ, to allow for normalisation, and 5 µg pSG5-EBNA2. As negative controls DG75 was also transfected with (i) 2 µg of the empty pGL3-basic vector, (ii) 2 µg of the empty pGL3-basic vector along with 5 µg pSG5-EBNA2 and (iii) 2 µg of pGL3 Hey1. The total quantity of transfected plasmid was adjusted to 9 µg with the empty vector pSG5. Transfected cells were harvested 48 hours later and luciferase and β-gal assays were performed. Normalised luciferase values were expressed as fold-activation relative to the corresponding value obtained for the empty pGL3 reporter construct; arbitrarily assigned a value of 1. (Data are Mean ±SD).

It initially appeared that EBNA2 was *trans*-activating the *hey 1* promoter in pGL3 Hey1. However, co-transfection with both pGL3-basic and pSG5-

EBNA2, resulted in a three-fold increase in luciferase activity, relative to transfection of DG75 with pGL3-basic alone. The luciferase expression levels were examined further and it was determined that luciferase production from pGL3-Hey1 increased three-fold upon co-transfection with pSG5-EBNA2. This pGL3 Hey1/ pSG5-EBNA2 *trans*-activating effect may not be due to a specific Hey1 target effect, but rather the up-regulation of luciferase production from the promoterless pGL3-basic host plasmid activity, due to EBNA2 expression. To investigate this further, DG75 cells were co-transfected with increasing quantities of pSG5-EBNA2 and a constant quantity of pGL3-basic.

In that experiment, a dose-dependent increase in pGL3-encoded luciferase activity can be observed in response to increasing quantities of pSG-EBNA2 (Figure 6.13). EBNA2 may therefore be enhancing transcription of *luc* possibly from a cryptic promoter site on pGL3-basic.

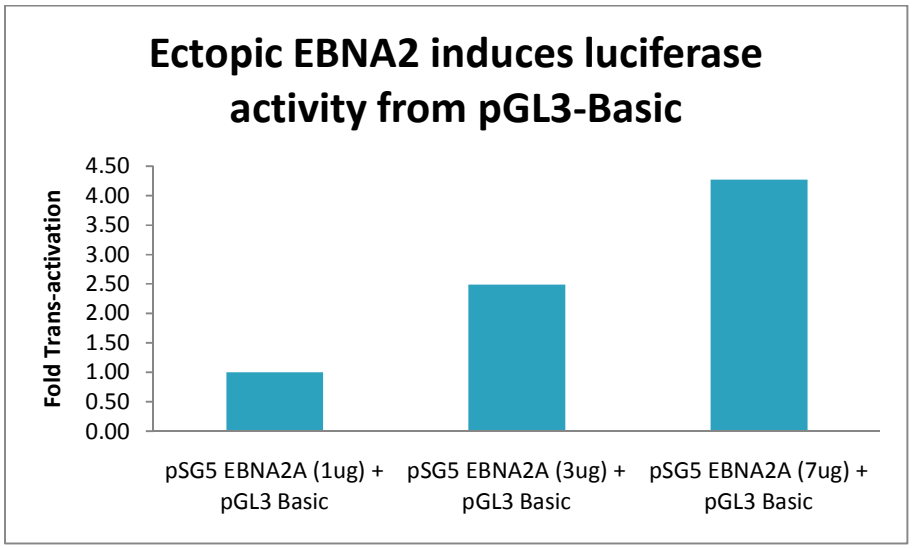


Figure 6.13: Ectopic EBNA2 induces luciferase activity from pGL3-Basic.

DG75 cells were co-transfected with 2 µg of pGL3-Basic luciferase reporter vector along with increasing quantities of pSG5-EBNA2 (1 µg, 3 µg and 7 µg). The total quantity of transfected plasmid was adjusted to 7 µg with the empty vector pSG5. The transfections were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed as fold-activation relative to the corresponding value obtained for the EBNA2A (1µg) + pGL3-basic; arbitrarily assigned a value of 1. (Data are Mean ±SD).

6.2.2 Regulation of *NR4A2* (*Nurr1*) by EBV

NR4A2 is a transcription factor that forms part of the orphan receptor family (Park, et al. 2008). This family is a branch of the steroid-thyroid hormone receptor super family of the subfamily 4 group A, composed of NR4A1, also called Nur77, NR4A2, or Nurr1 and NR4A3 alternatively known as NOR1 (Aherne, et al. 2009; Okabe, et al. 1995). These pleiotropic proteins have been implicated in steroidgenesis, neuronal development, atherogenesis and cell cycle regulation (Maxwell and Muscat 2006). The Nurr family member, Nur77, has been previously identified as a direct EBV target. EBNA2 has been shown to bind this pro-apoptotic Nur77 protein, sequestering it in the nucleus. This prevents Nur77 from eliciting its pro-apoptotic function which occurs via interactions with the mitochondria resulting in cytochrome c release. This indicates that there is precedent for modulation of this family by the activity of EBV.

NR4A2, more commonly called *Nurr1*, was identified as a potential EBV cellular target gene from the RT Profiler PCR SuperArray presented in section 4.2.1. It was found that *Nurr1* mRNA expression was down-regulated approximately 14-fold in naïve B cells after infection by EBV. To determine the effect of the activation of the EBV growth programme on the expression levels of *Nurr1* in an LCL cell culture model, ER/EB2.5 cells were cultured and RNA was prepared as described in section 4.2.3

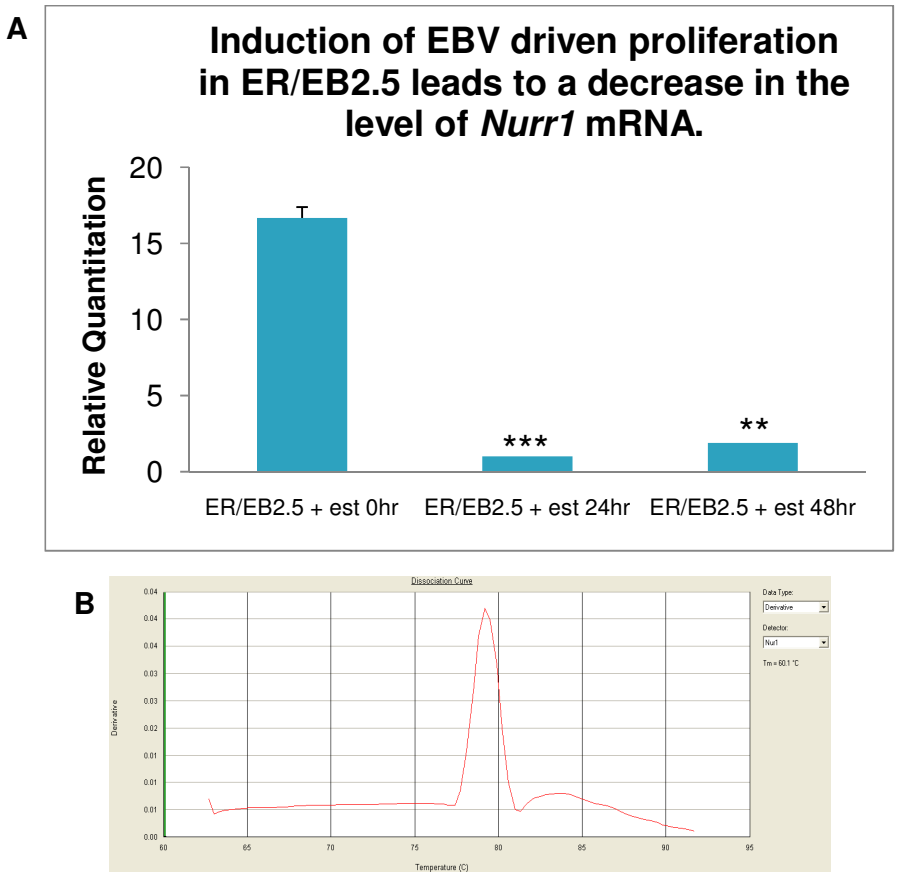


Figure 6.14: Induction of EBV driven proliferation in ER/EB2.5 leads to a decrease in the level of *Nurr1* mRNA.

RT-qPCR analysis was carried out to determine the levels of *Nurr1* expression in resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). RNA samples were prepared at the time points indicated. **(A)** RT-qPCR analysis of *Nurr1* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control for RT-Real time PCR was set as EBV cycling cells (EREB + est 24 hr): arbitrarily assigned a value of 1. (Data are Mean ±SD, *** P<0.001, **P<0.01). **(B)** The dissociation curve for the product of the *Nurr1* assay was analysed to ensure a single specific peak, confirming accuracy of the experiment, as SYBR green does not give a gene-specific response.

The results from this experiment showed that the steady state levels of *Nurr1* are down-regulated 16-fold in response to the EBV growth programme (Figure 6.14). This confirms the data from the RT Profiler PCR SuperArray, in which the expression levels of *Nurr1* were shown to be reduced following B cell infection with EBV (Section 4.2.1). To confirm this, an estrogen withdrawal experiment was again performed using ER/EB2.5 (Figure 6.15).

Cells previously cycling in the presence of estrogen were washed as described in section 4.2.3. These cells were then seeded into T75 flasks at a density of 5 X 10^5 cells/ml (ER/EB2.5 – est 0 hr) and passaged every 48 hours. Total mRNA samples were prepared at the time points indicated for the duration of time that the cells were cultured in β-estradiol free medium.

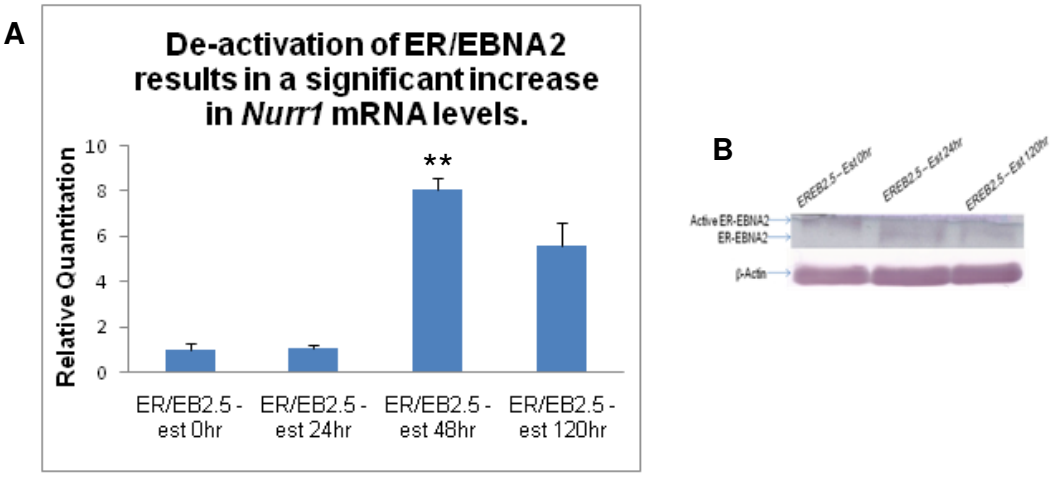


Figure 6.15: De-activation of ER/EBNA2 results in a significant increase in *Nurr1* mRNA levels.

(A) RT-qPCR was performed to determine the effect on *Nurr1* mRNA levels in response to ER/EBNA2 de-activation (β-estradiol withdrawal). RNA samples were prepared at the post-withdrawal time points indicated on the graph. Assay and normalisation of results (relative to *TBP*) was performed as described in the legend to figure 6.1. In this experiment, the control was designated as ER/EB – est 0 hr; arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01). **(B)** Western Blot showing de-activation of ER/EBNA2 in response to β-estradiol withdrawal. Protein harvested from cells cultured from β-estradiol medium (ER/EB2.5 –est 0 hr) exhibit the active 120 kDa ER/EBNA2 protein. After 24 hours, this band is detected at a lower molecular weight, indicative of inactive ER/EBNA2, due to the loss of β-estradiol. All extracts were normalised to the loading control β-Actin.

It can be seen from these results that as ER/EBNA2 is de-activated in ER/EB2.5, the *Nurr1* mRNA levels increase significantly (Figure 6.15A). The de-activation of the ER/EBNA2 was verified by Western blotting (Figure 6.15B). In this experiment a decrease in the level of chimeric EBNA2, over the duration of the time-course, was confirmed.

6.2.2.1 Nurr1 is repressed due to c-myc driven growth in the conditional LCL, P493-6

P493-6, described previously in section 4.2.7, is a conditional LCL that has been engineered from the parental cell line ER/EB2.5. When starved of estrogen, P493.6 represents a resting B-cell that can be stimulated to proliferate either in response to (i) Myc in the combined absence of tetracycline and estrogen or (ii) EBNA2 in the presence of estrogen and tetracycline (Pajic, et al. 2000; Schuhmacher, et al. 2001). Total mRNA samples were prepared from a time course that was performed in section 4.2.7 in which the P493.6 cell line was cultured on the EBV growth programme (+ β-estradiol, + Tet) or C-Myc (- β-estradiol, - Tet). RT-qPCR analysis was employed to determine if *Nurr1* was differentially regulated in LCLs cycling due to EBV, compared to those cycling due to C-Myc.

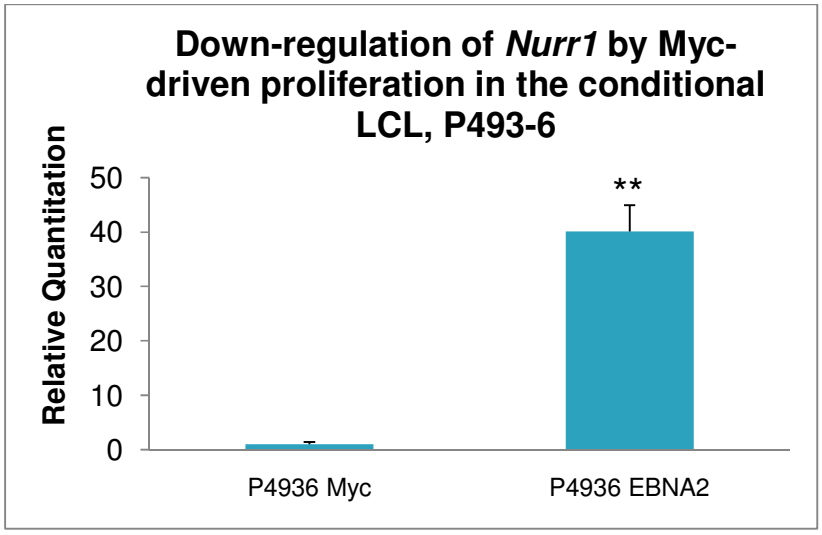


Figure 6.16: Down-regulation of *Nurr1* by Myc driven proliferation in the conditional LCL, P493-6.

The steady state levels of *Nurr1* mRNA was significantly higher in P493-6 cells proliferating due to EBNA2 compared to the same cell background cycling due to Myc. Cells from the ER/EB2.5 sub-clone, P493-6, were divided and separately cultured to permit cycling on the EBV growth programme (+ β-estradiol, + Tet) or Myc programme (- β-estradiol, - Tet). Total RNA samples were taken during each growth programme. At the cycling programme indicated, RT-qPCR was performed as previously described in figure legend 4.11. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment the control set at P493-6 Myc cells; arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01).

It was determined from the RT-qPCR analysis that the steady state level of *Nurr1* mRNA was significantly lower in cells proliferating due to C-Myc (- β-estradiol, - Tet) in contrast to the cells proliferating due to EBV (+ β-estradiol, + Tet) (see Figure 6.16). P493-6 cells proliferating due to ER/EBNA2 activation are reminiscent of the parental cell line ER/EB2.5. Therefore previous results indicate that the activation of ER/EBNA2 leads to the down-regulation of *Nurr1* in this cell background (Section 6.2.2). However, from this examination of the P493-6 cell line it can be observed that activation of the c-Myc programme in the same cell background leads to even more potent down-regulation of *Nurr1* at the transcriptional level. EBNA2 positively *trans*-activates endogenous *c-myc*, and therefore C-Myc is expressed in both cell lines but to somewhat varying degrees. From the results obtained in this study, it could be speculated that the down-regulation of *Nurr1* activity during infection of a resting B cell may be due to EBV induction of *c-myc,* consequently *Nurr1* may be a negative target of *c-myc*, and as such, an indirect target of EBV infection.

6.2.2.2 Relative Nurr1 expression is down-regulated in EBV-negative B cell lines.

As described previously, phenotypically diverse B cell lines were employed in this study. Relative *Nurr1* mRNA levels were next examined in a B cell panel comprising of several different B lymphocyte backgrounds: EBV-negative BLs, an EBV negative lymphoma cell line, an EBV-positive BL, and an LCL.

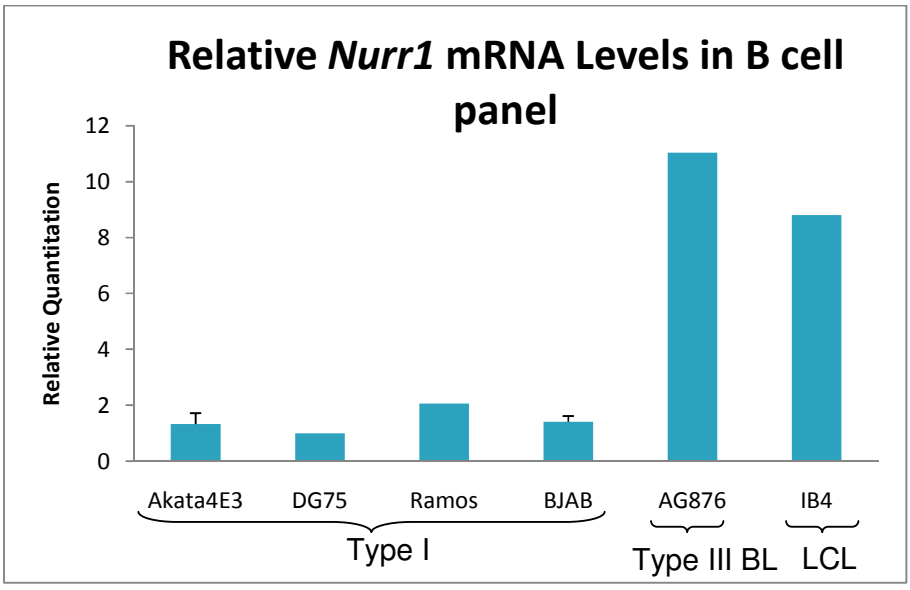


Figure 6.17: Comparative *Nurr1* mRNA levels in B cell lines.

RT-qPCR was used to monitor *Nurr1* mRNA levels in a range of B cell lines including the EBV-negative B cell lines DG75, BJAB and Akata4E3, the EBV-positive AG876 (group-III BL cell line), and the EBV-immortalised LCL IB4. RNA samples were prepared from each cell line in the same time frame, and RT-qPCR analysis of *Nurr1* mRNA levels was performed as described in the legend to figure 6.1. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment DG75 cells were set as control cells, arbitrarily assigned a value of 1. (Data are Mean ±SD).

The results obtained showed that the steady state levels of *Nurr1* mRNA were consistently lower in BL-derived EBV-negative cell lines (Figure 6.17). Taking into consideration the previous data presented in this study, indicating that *Nurr1* is down-regulated in response to c-Myc expression, it was not surprising to observe basal *Nurr1* expression levels at a lower level in these cell lines which have high c-Myc expression. However, although AG876 is a BL, and as such contains the characteristic BL translocation that results in the over-expression of c-Myc, it does not exhibit reduced *Nurr1* mRNA transcription. The increased level of Nurr1 mRNA in AG876 is consistent with it being a C-myc-responsive gene. EBNA2 in fact *represses* C-myc expression from the translocated IgMu enhancer in BLs, and so EBV-positive (Type III) express BLs less C-myc than their EBV-negative counterparts (Jochner, et al. 1996).

6.2.3 Regulation of *STIL* by EBV

S*TIL* was identified as a candidate for EBV regulation from the results obtained from the RT Profiler PCR SuperArray, in section 4.2.1. As a gene included in the Notch signaling pathway array, *STIL* was analysed and found to be up-regulated 129-fold following infection of naïve B cells with EBV. *STIL* (SCL/TAL1 interrupting Locus), also known as SCL-interrupting protein (*SIL*), is a 143kDa cytoplasmic protein that is highly conserved in vertebrates. *STIL* is composed of 18 exons and has been mapped to the chromosomal location 1q32. *STIL* has been found to be ubiquitously expressed in cells during early embryonic development and in proliferating cells. During metaphase, STIL localizes to the mitotic spindle poles to co-ordinate spindle organization (Reviewed in Kumar, et al. 2009).

As described in chapter 4, the conditional LCL, ER/EB2.5 was used to corroborate results from the RT Profiler PCR SuperArray. The ER/EB2.5 cell line was prepared for an ER/EBNA2 activation time course as described in section 4.2.3. RNA samples were harvested from ER/EB2.5 cells (i) without the presence of β-estradiol and (ii) cycling on the EBV programme due to β-estradiol activation of ER/EBNA2. *STIL* mRNA levels were examined using RT-qPCR.

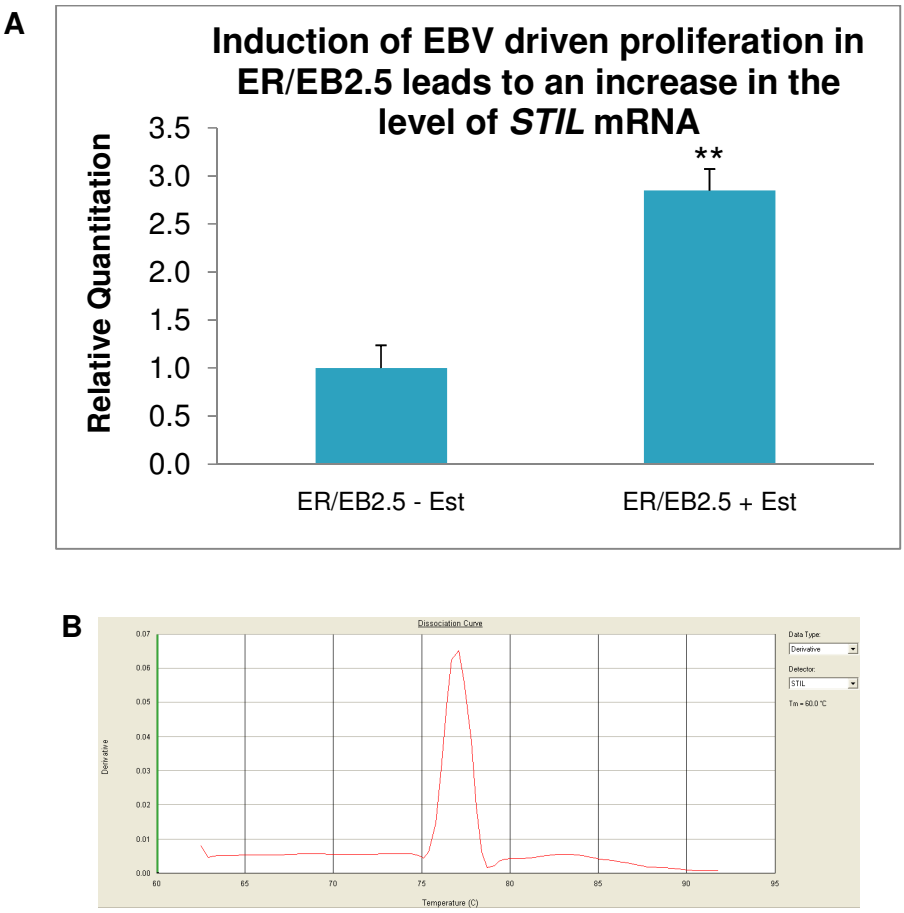


Figure 6.18: Induction of EBV driven proliferation in ER/EB2.5 leads to an increase in the level of *STIL* mRNA.

RT-qPCR analysis was carried out to determine the levels of *STIL* expression in the cell culture model of resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). RNA samples were prepared at the time points indicated. **(A)** RT-qPCR analysis of *STIL* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control for RT-Real time PCR was set as EBV cycling cells (ER/EB + est 24 hr): arbitrarily assigned a value of 1. (Data are Mean ±SD, ** P<0.01). **(B)** The dissociation curve for the product of the *STIL* assay was analysed to ensure a single specific peak, confirming accuracy of the experiment, as SYBR green does not give a gene specific response.

It can be seen that *STIL* mRNA levels increased 3-fold after the activation of the EBV growth programme. In a second experiment, *STIL* mRNA levels were again monitored at various time points in ER/EB2.5 cells (i) as estrogen was withdrawn from ER/EB2.5 cells previously cycling on activated ER/EBNA2 and (ii) while the cell line was once again induced to proliferate due to EBV through β-estradiol induction (Figure 6.18).

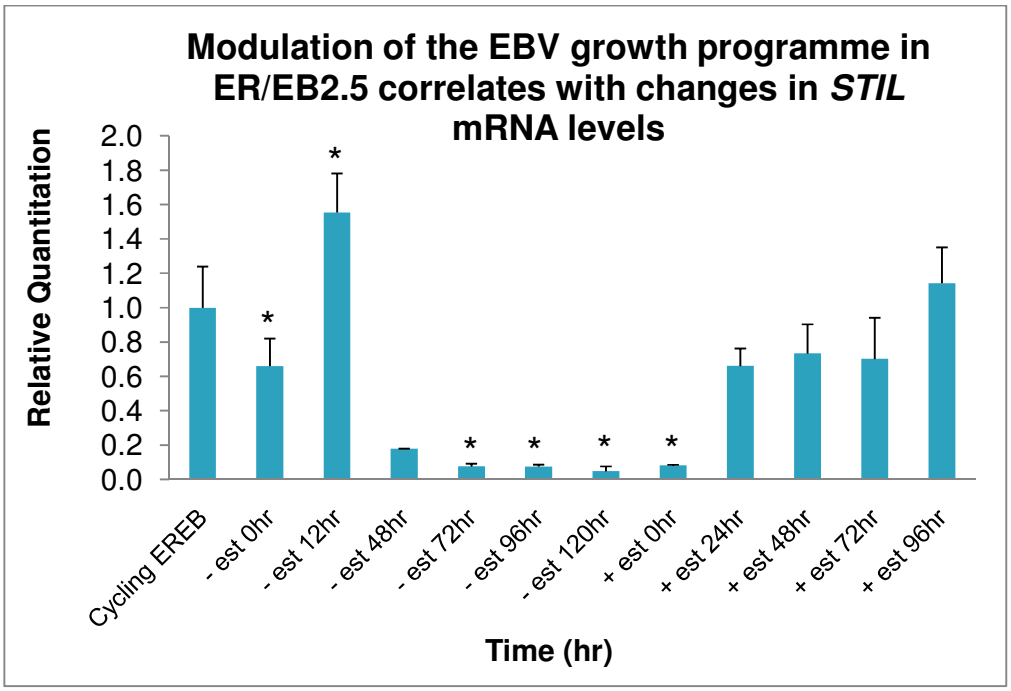


Figure 6.19: Modulation of the EBV growth programme in ER/EB2.5 correlates with changes in *STIL* mRNA levels.

To examine the modulated effect of *STIL* mRNA levels in response to activation and de-activation of EBV driven proliferation, an expanded time course of ER/EB2.5 cycling cells was performed. This experiment was described in the legend to the figure 4.6. RNA samples were monitored and normalised using *TBP* as described in the legend to figure 6.1. In this experiment, the control was designated as ER/EB cycling on estrogen; arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$).

Interestingly, the de-activation of ER/EBNA2 led to a transient increase in *STIL* mRNA levels; however, this was followed by a significant reduction by 48 hours (Figure 6.19). Re-activation of ER/EBNA2 re-induced *STIL*, restoring the expression levels observed in cells cycling due to EBV. Thus these results show that *STIL* expression positively correlates with EBV latent gene expression in ER/EB2.5.

6.2.3.1 Relative STIL mRNA levels are similar in P493-6 cells cycling due to either C-myc or EBV

STIL mRNA levels were then examined as before in P493.6 cells cycling on either the EBV growth programme (+ β-estradiol, + Tet) or C-MYC programme (- β-estradiol, - Tet).

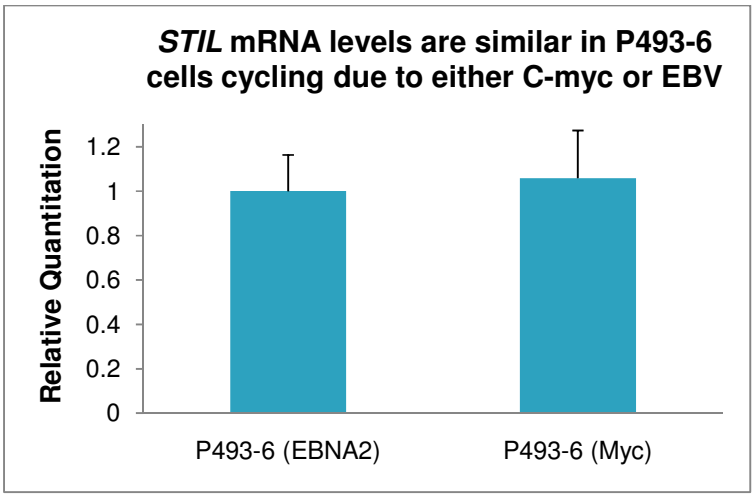


Figure 6.20: *STIL* mRNA levels are similar in P493-6 cells cycling due to either C-myc or EBV.

The steady state levels of *STIL* mRNA were similar in P493-6 cells proliferating due to EBNA2 compared to the same cell background cycling due to Myc. Cells from the ER/EB2.5 sub-clone, P493-6, were divided and separately cultured to permit cycling on the EBV growth programme (+ β-estradiol, + Tet) or Myc programme (- β-estradiol, - Tet). Total RNA samples were taken during each growth programme. At the cycling programme indicated, RT-qPCR was performed as previously described in figure legend 4.11. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment the control set at P493-6 EBNA2 cells; arbitrarily assigned a value of 1. (Data are Mean ±SD).

The results obtained in this experiment indicate that modulation of P493-6 driven growth between c-Myc driven growth and EBV-driven growth does not differentially modulate *STIL* transcriptional activity in an LCL (Figure 6.20).

6.2.3.2 Comparative STIL mRNA levels in a panel of B cell lines.

Relative *STIL* mRNA levels were examined as before in a panel of B cell lines -EBV-negative BLs including Akata4E3, DG75, and Ramos, an EBV negative lymphoma cell line, BJAB, an EBV-positive BL, AG876, and an EBV-positive LCL, IB4.

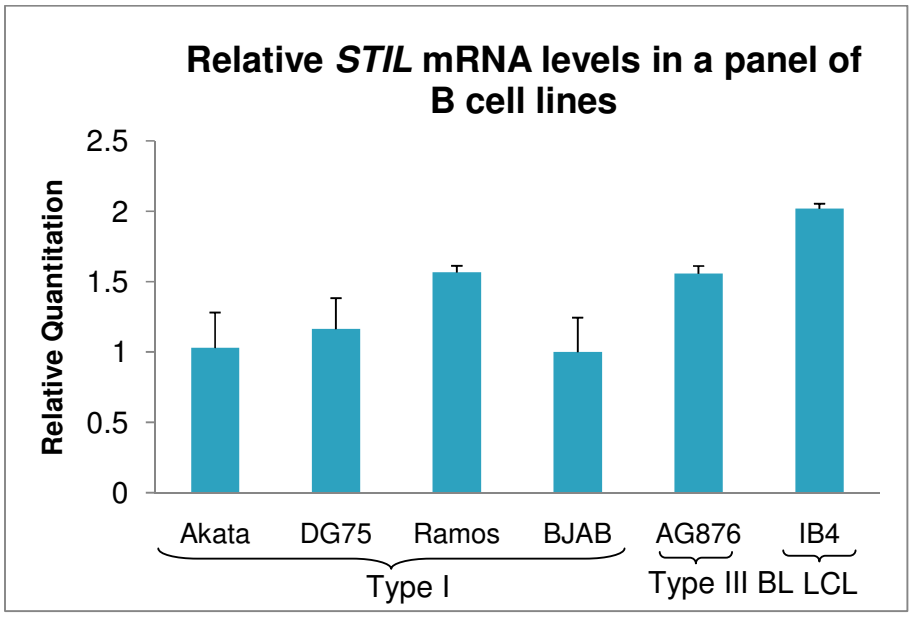


Figure 6.21: Comparative *STIL* mRNA levels in B cell lines.

RT-qPCR was used to monitor *STIL* mRNA levels in a range of B cell lines including the EBV-negative B cell lines DG75, BJAB and Akata4E3, the EBV-positive AG876 (group-III BL cell line), and the EBV-immortalised LCL IB4. RNA samples were prepared from each cell line in the same time frame, and RT-qPCR analysis of *STIL* mRNA levels was performed as described in the legend to figure 6.1. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment BJAB cells were set as control cells, arbitrarily assigned a value of 1. (Data are Mean ±SD).

The results obtained in this experiment indicate that *STIL* mRNA levels were similar across all cell lines examined (Figure 6.21). All of these results are in agreement with published data concluding that *STIL* expression is ubiquitous in proliferating cells.

6.2.3.3 No STIL regulation observed in response to EBNA2 expression in an EBV-negative BL.

STIL mRNA levels were examined in response to EBNA2 expression in the EBV-negative BL-derived cells lines DG75-tTA-EBNA2 and BL41-K3. It can be seen that EBNA2 did not modulate *STIL* mRNA levels in either case (Figures 6.22 and 6.23).

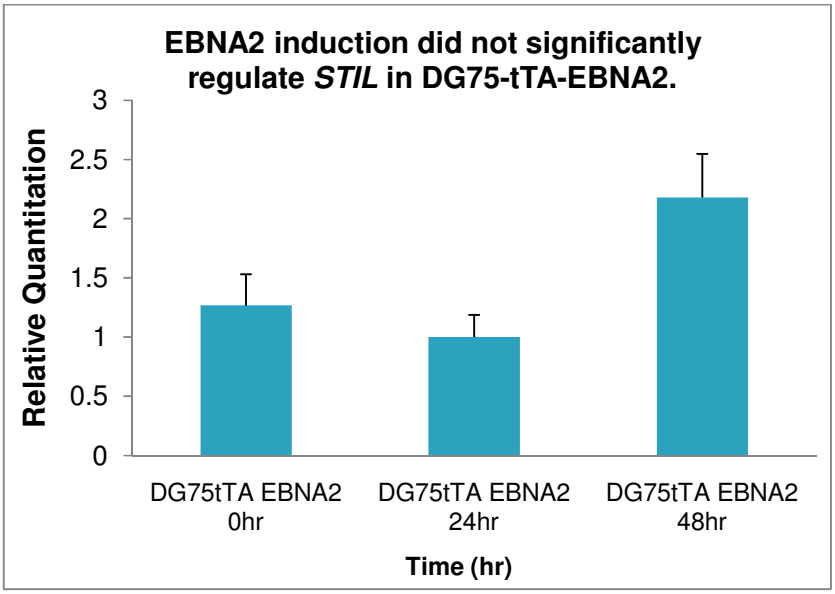


Figure 6.22: EBNA2 induction did not significantly regulate *STIL* in DG75-tTA-EBNA2.

EBNA2 expression was induced in DG75-tTA-EBNA2 by the removal of tetracycline from the supplemented RPMI medium. RT-qPCR analysis of *STIL* mRNA levels in DG75-tTA-EBNA2 in response to EBNA2 induction. RNA samples were prepared at the time points indicated post-induction. RT-qPCR analysis was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as DG75-tTA-EBNA2 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD).

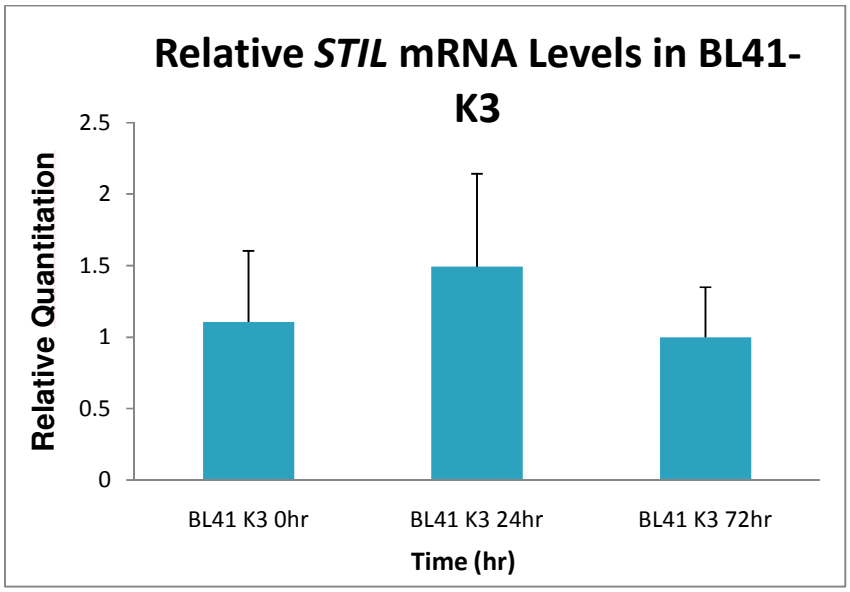


Figure 6.23: ER/EBNA2 does not regulate *STIL* mRNA in BL41-K3.

ER/EBNA2 was activated in BL41-K3 as described in the legend to figure 4.7. RT-qPCR analysis was used to determine *STIL* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated as BL41-K3 0 hr, arbitrarily assigned a value of 1. (Data are Mean ±SD).

6.2.4 Up-regulation of *CyclinE1* by EBV in ER/EB2.5 cells.

Cyclin E1 is a 45kDa nuclear protein of the Cyclin family. Cyclins are positive regulators of cell cycle progression. Cyclin E1 is not detectable in resting peripheral B cells; however, upon stimulation of cell proliferation Cyclin E1 expression is up-regulated (Hollyoake, et al. 1995, Ko, et al. 2009). Cyclin E1 expression has been found to be induced in the late G1 phase but undergoes degradation upon entry into the S phase (Datta, et al. 2000). The role of Cyclin E1 in promoting cell cycle transition involves regulating cyclin-dependent protein kinases (Cdks). In addition a role for Cyclin E1 in regulating cell cycle arrest in response to DNA damage has recently emerged (Lu, et al. 2009).

From the RT Profiler PCR SuperArray data presented previously in Chapter 4 (Section 4.2.1), it was observed that *CyclinE1* mRNA levels increased approximately 286-fold after EBV infection of a naïve B cell population. Here, this observation was corroborated using the ER/EB2.5 model, in which activation of the EBV growth programme coincided with a 4.5-fold up-regulation in *Cyclin E1* mRNA levels.

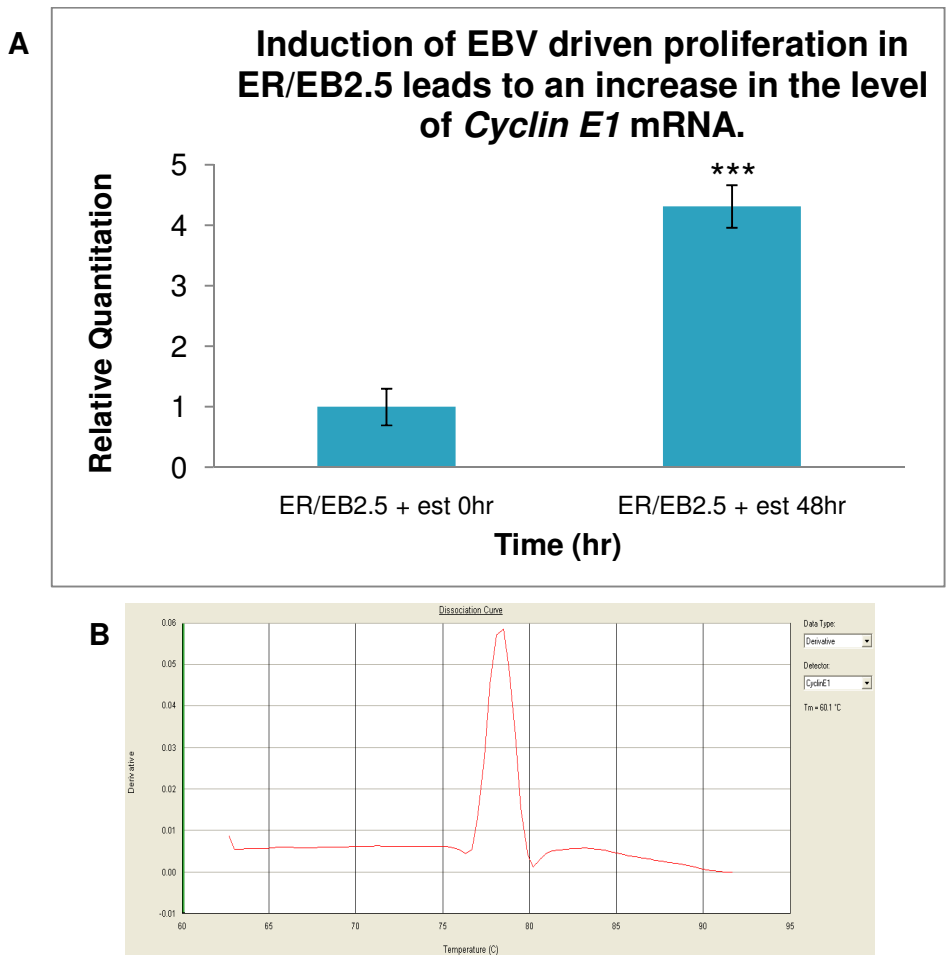


Figure 6.24: Induction of EBV driven proliferation in ER/EB2.5 leads to an increase in the level of *Cyclin E1* mRNA.

RT-qPCR analysis was carried out to determine the levels of *Cyclin E1* expression in resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). RNA samples were prepared at the time points indicated. **(A)** RT-qPCR analysis of *Cyclin E1* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control for RT-qPCR was set as EBV cycling cells (EREB + est 0 hr): arbitrarily assigned a value of 1. (Data are Mean ±SD, *** P<0.001). **(B)** The dissociation curve for the product of the *Cyclin E1* assay was analysed to ensure a single specific peak, confirming accuracy of the experiment, as SYBR green does not give a gene-specific response.

6.2.4.1 Relative CyclinE1 expression is similar in a panel of B cell lines.

An examination of a panel of B cell lines revealed that relative *Cyclin E1* mRNA levels were similar in all cases (Figure 6.25).

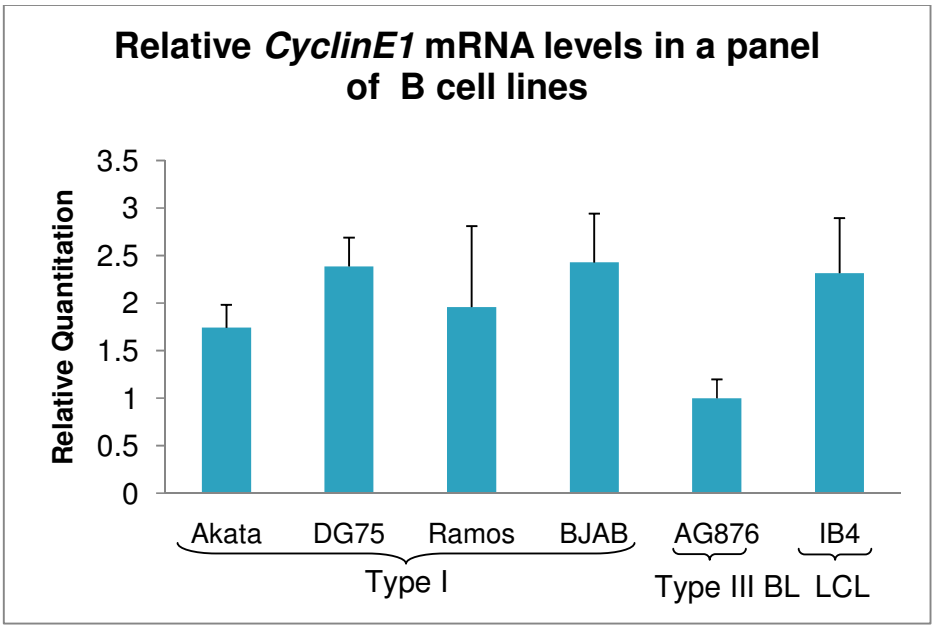


Figure 6.25: Comparative *CyclinE1* mRNA levels in B cell lines.

RT-qPCR was used to monitor *Cyclin E1* mRNA levels in a range of B cell lines including the EBV-negative B cell lines DG75, BJAB and Akata4E3, the EBV-positive AG876 (group-III BL cell line), and the EBV-immortalised LCL IB4. RNA samples were prepared from each cell line in the same time frame, and RT-qPCR analysis of *Cyclin E1* mRNA levels was performed as described in the legend to figure 6.1. Results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment AG876 cells were set as control cells, arbitrarily assigned a value of 1. (Data are Mean ±SD).

6.2.5 Activation of the EBV growth programme in ER/EB2.5 has no significant effect on the expression of LRP5

Low-density-lipoprotein (LDL) receptor-related protein 5 (LRP5) is a single-pass transmembrane protein composed of 1615 amino acid residues. LRP5 acts as a Wnt co-receptor critical for signaling in the canonical Wnt pathway (He, et al. 2004). LRP5 is classified as a member of a subfamily of the LDL family which also encompasses LRP6, another critical Wnt associated receptor (Pinson, et al. 2000; Tamai, et al. 2000).

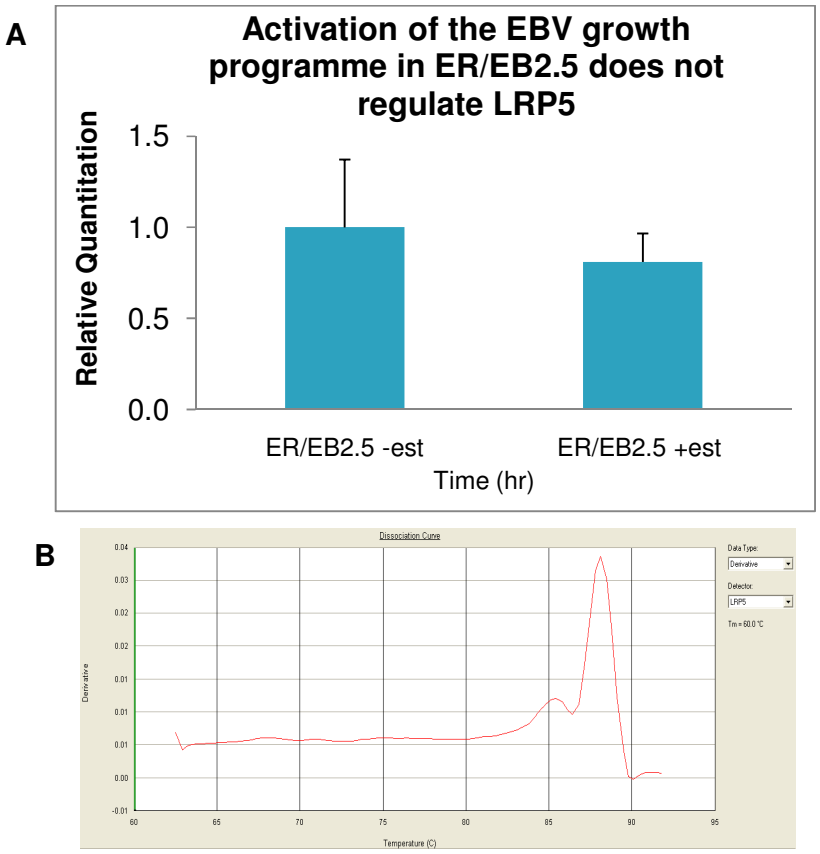


Figure 6.26: Activation of the EBV growth programme in ER/EB2.5 does not regulate *LRP5*.

An experiment was carried out using ER/EB2.5 to assess the effect of ER/EBNA2 activation on the mRNA levels of *LRP5*. RNA samples were taken during β-estradiol induction at the time points indicated. **(A)** RT-qPCR analysis was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. (Data are Mean ±SD, * P<0.05). **(B)** The dissociation curve for the product of the *LRP5* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

LRP5 primers were present in two cell signaling gene panels, *Notch* and *Wnt*, displaying a 57-fold and 18-fold up-regulation respectively for *LRP5* expression post-EBV infection (Section 4.2.1). In ER/EB2.5, there appears to be no significant effect on LRP5 gene expression due to the activation of ER-EBNA2 (Figure 6.26). Thus, the results obtained in this experiment do not corroborate the RT Profiler PCR SuperArray data obtained previously.

6.2.6 Activation of the EBV growth programme in ER/EB2.5 leads to down-regulation of *CIDEB* mRNA levels

The cell death-inducing DFFA45-like effector (CIDE) family consists of three members CIDEA, CIDEB and CIDEC/FSP27. These are conserved proteins named for the homology they share in the N terminal domain with the DNA fragmentation factor (DFF) (Inohara, et al. 1998; Da, et al. 2003). The CIDE family proteins have a number of essential responsibilities in various cellular processes including (i) metabolism regulation through lipid storage and droplet formation (Gong, et al. 2009), and (ii) as pro-apoptotic factors (Chen, et al. 2000).

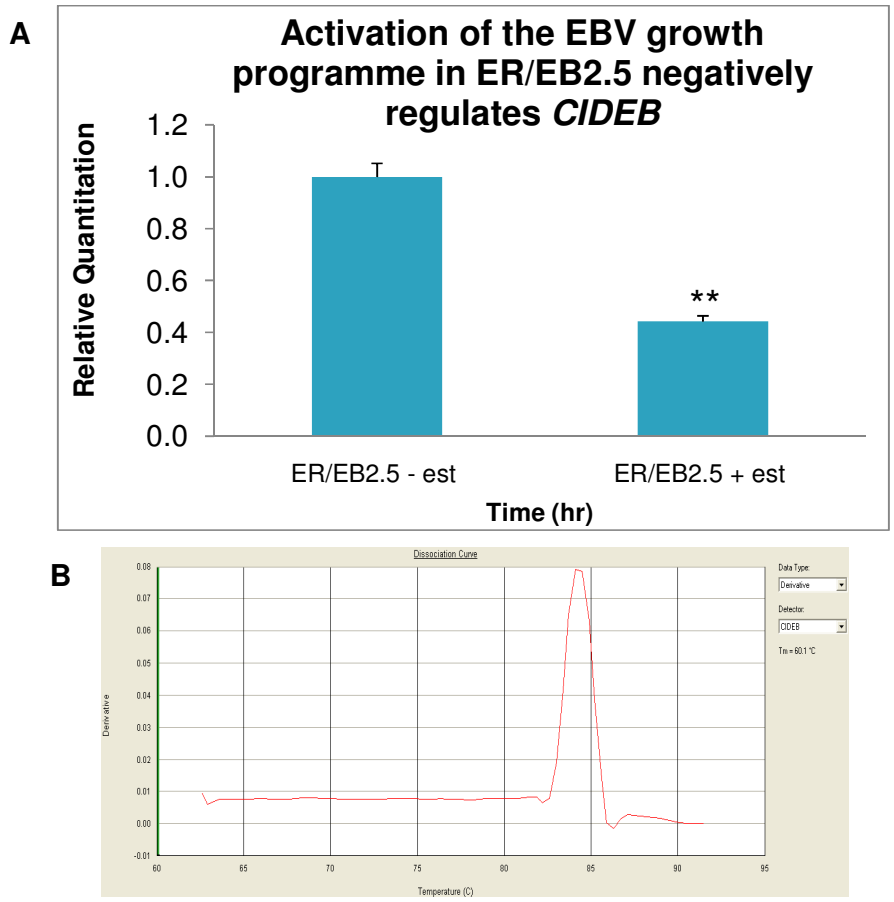


Figure 6.27: Activation of the EBV growth programme in ER/EB2.5 negatively regulates *CIDEB*.

A β-estradiol induction experiment was carried out using ER/EB2.5 to assess the effect of ER/EBNA2 activation on the mRNA levels of *CIDEB*. RNA samples were taken during β-estradiol induction at the time points indicated. **(A)** RT-qPCR analysis was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was designated ER/EB2.5 -est: arbitrarily assigned a value of 1 (Data are Mean ±SD, ** P<0.01). **(B)** The dissociation curve for the product of the *CIDEB* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The apoptosis RT Profiler PCR SuperArray indicated that *CIDEB* expression increased by 502930-fold after EBV infection of naïve B cells (Section 4.2.1). Although this was highly likely to be erroneous, *CIDEB* expression was nonetheless investigated in ER/EB2.5. Significant *CIDEB* down-regulation was observed in response to the activation of ER/EBNA2, and therefore no conclusion can be drawn with respect to the regulation of *CIDEB* by EBV.

6.2.7 Activation of the EBV growth programme in ER/EB2.5, has no significant effect on the expression of c-Fos

c-Fos is a member of the Fos family of transcription factors that forms part of the variable transcription factor complex, AP-1. The Fos family is composed of four proteins c-Fos, FosB and the splice varients Fra-1 and Fra-2 (Milde-Langosch 2005). All four contain a basic leucine-zipper region required for dimerisation; however, only the two full length members c-Fos and FosB, harbour a *trans*-activation domain at their C terminus (Wisdom and Verma 1993).

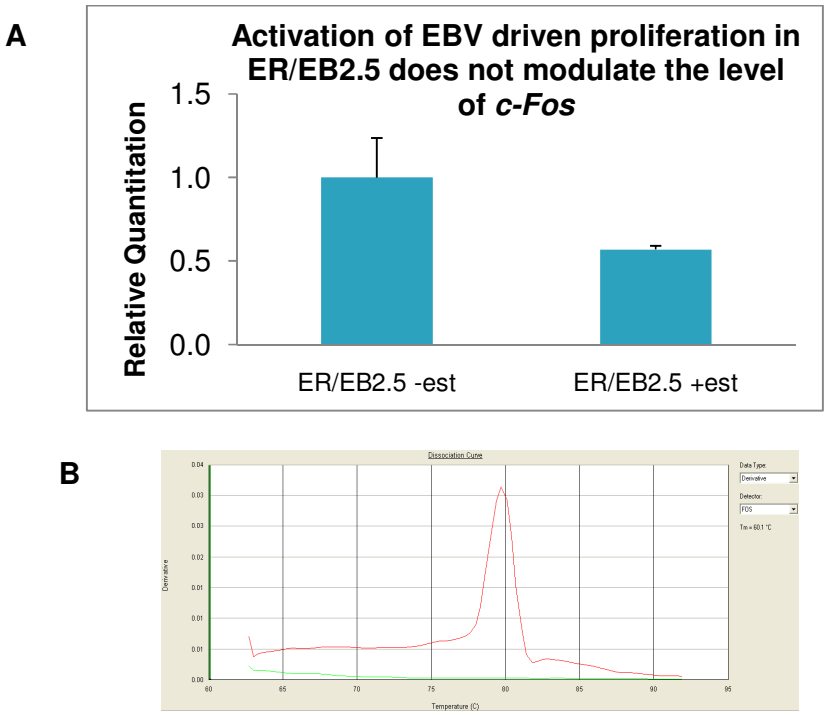


Figure 6.28: Activation of EBV driven proliferation in ER/EB2.5 does not modulate the level of *c-Fos* mRNA.

RT-qPCR analysis was carried out to determine the levels of *c-Fos* expression in resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). RNA samples were prepared at the time points indicated. **(A)** RT-qPCR analysis of *c-Fos* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. (Data are Mean ±SD). **(B)** The dissociation curve for the product of the c-*Fos* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The results from this experiment correlated with the data obtained from the EBV-naïve B cell infection experiment in which *c-Fos* levels were repressed 648-fold following EBV infection (Section 4.2.1). However, the steady state levels of *c-Fos* within the ER/EB2.5 cell line did not change due to the activation of ER/EBNA2 (Figure 6.28). Thus, the results obtained in the two experiments are not in agreement, and no conclusion can be made on the findings available. Published data indicate that c-Fos is a positively regulated target of EBV, resulting in elevated phosphorylated *c-Fos* (Vaysberg, et al. 2008). However, EBV modulation at the transcriptional level has not been demonstrated to date.

6.2.8 Activation of the EBV growth programme in the conditional LCL, ER/EB2.5, negatively regulates *c-Jun* mRNA expression

The *Jun* family consists of three members *c-Jun, JunB* and *JunD*. These proteins can homodimerise (Jochum, et al. 2001) and heterodimerise with a range of transcription factors such as those from the Fos (Angel and Karin 1991) and ATF (Hai and Hartman 2001) families and the basic leucine-zipper proteins CBP, MyoD, NFat and c-rel (Herdegen and Leah 1998). The c-Jun protein is composed of an N-terminal domain with binding and phosphorylation sites for c-Jun N-terminal kinases (JNK), followed by a *trans*-activation domain, a hinge region, a basic positively charged domain and the leucine zipper region before reaching the C terminus (Raivich 2008). Published data have revealed that c-Jun plays a pivotal role in EBV infected cells. It is a required co-factor for EBNA2-initiated LMP1 expression (Sjoblom, et al. 1998). It has been shown that *c-Jun* functions in B cells to potently inhibit differentiation (Prochownik, et al. 1990; Su, et al. 1991) and promote proliferation (Chen, et al. 2002).

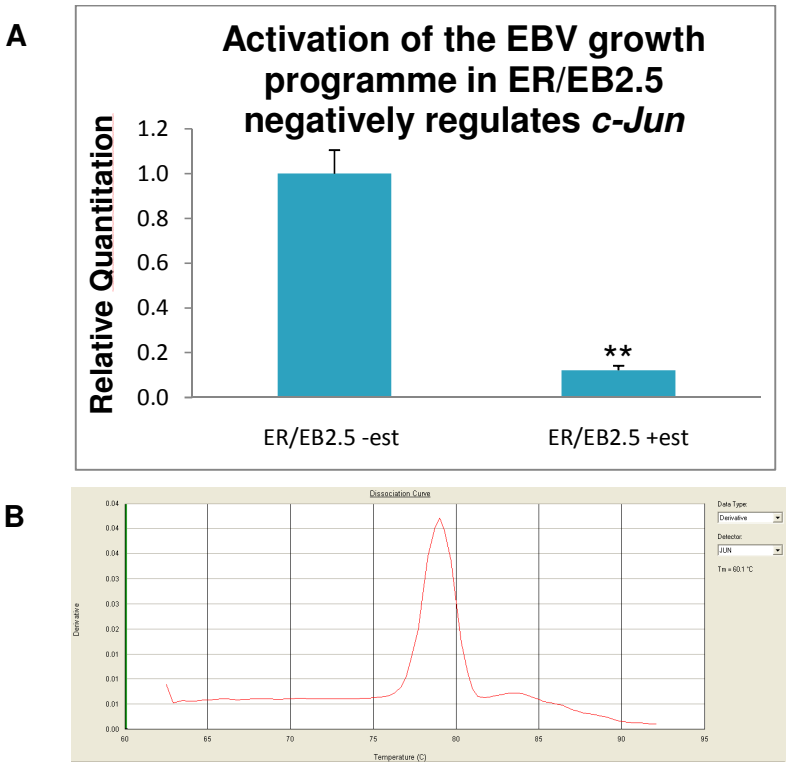


Figure 6.29: Activation of the EBV growth programme in ER/EB2.5 negatively regulates c-Jun.

An experiment was carried out using ER/EB2.5 to assess the effect of ER/EBNA2 activation on the mRNA levels of *c-Jun*. RNA samples were taken during induction at the time points indicated. **(A)** RT-qPCR analysis was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. (Data are Mean ±SD, ** $P<0.01$). **(B)** The dissociation curve for the product of the *c-Jun* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The results from the activation of ER/EBNA2 in ER/EB2.5 indicate that the steady state levels of *c-Jun* are down-regulated 8-fold in response to EBV driven growth (Figure 6.29). These data confirm the preliminary data from the Wnt RT Profiler PCR SuperArray, in which the expression levels of *c-Jun* were shown to be reduced 200-fold by EBV-mediated B cell infection (Section 4.2.1). Although the two results were in agreement, they were surprising, as studies indicate that the levels of c-Jun are elevated in EBV infected cells due to LMP2A post-translational modifications of the protein (Chen, et al. 2002).

6.2.9 Activation of the EBV growth programme in ER/EB2.5, had no significant effect on the expression of *TNFSF7*

Tumour necrosis factor superfamily member 7 (TNFSF7), also referred to as CD70 or CD27L, is part of a family of ligands required for modulation of the tumour necrosis factor (TNF) receptor superfamily. TNFSF7 can be detected in T and B cells along with monocytes at the mRNA level, and up-regulation of the protein can be detected in activated T and B lymphocytes (Cosman 1994). Although TNFSF7 is the designated ligand for CD27, it also displays independent signaling activity, activating the PI3K and MAP kinase cascades (Borst, et al. 2005; Arens, et al. 2004; Garcia, et al. 2004).

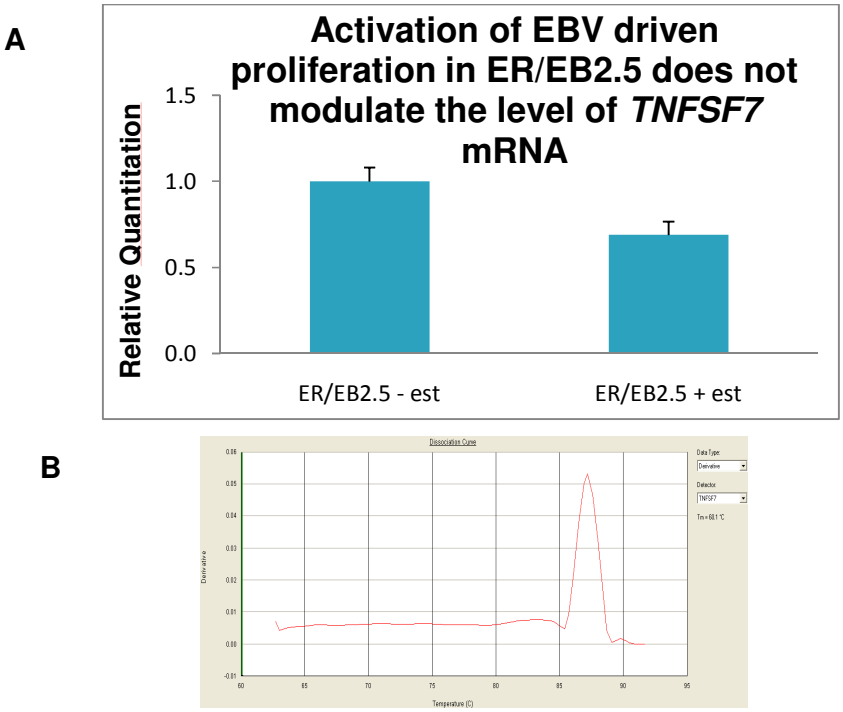


Figure 6.30: Activation of EBV driven proliferation in ER/EB2.5 does not modulate the level of *TNFSF7* mRNA

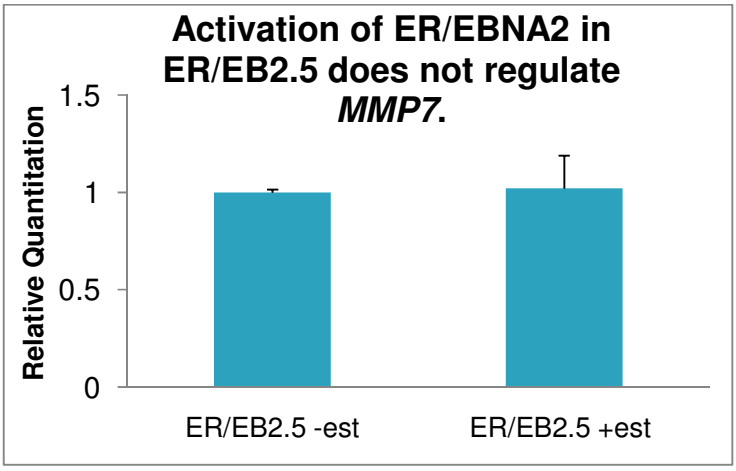
RT-qPCR analysis was carried out to determine the levels of *TNFSF7* expression in resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). RNA samples were prepared at the time points indicated. **(A)** RT-qPCR analysis of *TNFSF7* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. Data are Mean ±SD). **(B)** The dissociation curve for the product of the *TNFSF7* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

Activation of the EBV growth programme did not modulate the relative *TNFSF7* mRNA levels when compared to β-estradiol starved cells (Figure 6.30). This result is not in agreement with the data from the Apoptotic RT Profiler PCR SuperArray, which indicated that TNFSF7 was transcriptionally up-regulated 625-fold following EBV infection of naïve B cells. (Section 4.2.1).

6.2.10 Activation of the EBV growth programme in ER/EB2.5, does not regulate *MMP7* mRNA levels

Matrix metalloproteinase 7 (MMP7) is the smallest protein from a family of zinc-dependent enzymes called the matrix metalloproteinases (MMPs). MMPs display association with a wide spectrum of malignancies and chemotherapeutic resistance (Almendro, et al. 2009; Kioi, et al. 2003; Mitsiades, et al. 2001). They confer this resistance and promote metastasis through a variety of mechanisms such as modulation of angiogenesis and innate immunity, along with degradation of extracellular matrix (ECM) proteins (Kioi, et al. 2003; Chambers and Matrisian 1997).

A



B

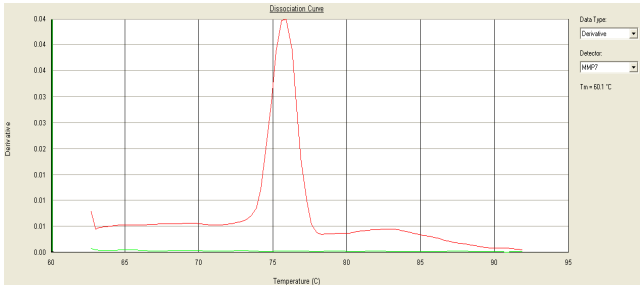


Figure 6.31: Activation of ER/EBNA2 in ER/EB2.5 does not regulate *MMP7*.

An experiment was carried out using ER/EB2.5 to assess the effect of ER/EBNA2 activation on the mRNA levels of *MMP7*. RNA samples were taken during β-estradiol induction at the time points indicated. **(A)** RT-qPCR analysis was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. (Data are Mean ±SD). **(B)** The dissociation curve for the product of the *MMP7* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The Notch RT Profiler PCR SuperArray data presented previously in Chapter 4 indicated that *MMP7* was transcriptionally up-regulated 317-fold by the EBV-infection of naïve B cells (Section 4.2.1). However, this was not corroborated by the experiments performed in the ER/EB2.5 system.

6.2.11 Activation of the EBV growth programme in ER/EB2.5, drives the expression of TRAF4

Tumour necrosis factor receptor (TNFR) associated factor (TRAF) 4 is one of seven known constituents of the TRAF family, which, with the exception of TRAF1, is ubiquitously expressed (Guo, et al. 2009). This group of adaptor proteins form stable trimer complexes to elicit a range of responses involving NF-κB, JNK and receptor-interacting kinase (RIP) (Guo, et al. 2009).

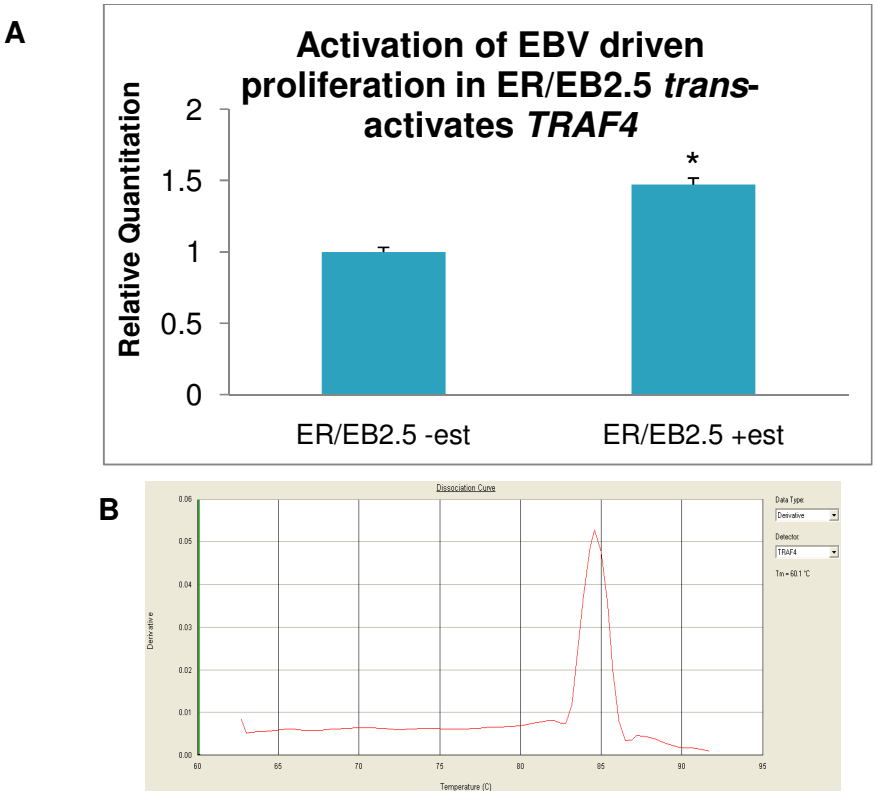


Figure 6.32: Activation of EBV driven proliferation in ER/EB2.5 *trans*-activates *TRAF4*.

RT-qPCR analysis was carried out to determine the levels of *TRAF4* expression in resting B cells (ER/EB - est) relative to ER/EBNA2 activated cells (ER/EB + est). RNA samples were prepared at the time points indicated. **(A)** RT-qPCR analysis of *TRAF4* mRNA levels in response to ER/EBNA2 activation. The post-induction time points are indicated on the graph. The RT-qPCR assay was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$). **(B)** The dissociation curve for the product of the *TRAF4* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

The apoptosis RT Profiler PCR SuperArray indicated that *TRAF4* gene expression increased 133-fold after the EBV infection of naïve B cells (Section 4.2.1). *TRAF4* transcriptional activity was then examined in ER/EB2.5 and the activation of ER/EBNA2 resulted in a marginal 1.5-fold up-regulation of *TRAF4* (Figure 6.32), corroborating the observation made in the EBV infection experiment.

6.2.12 Activation of the EBV growth programme in ER/EB2.5, induces *Fas* expression.

Fas (CD95/APO-1) is a 48kDa pro-apoptotic membrane protein of a subgroup of the TNFR family. These proteins contain an 80 aa cytoplasmic tail designated the intracellular “death domain” which is responsible for stimulation of caspase dependent apoptosis. Fas activity is crucial for healthy maintenance of the immune system through eradication of pathogen-infected, obsolete and auto-reactive cells, along with lymphocytes presenting with aberrant growth patterns (Reviewed in Strasser, et al. 2009).

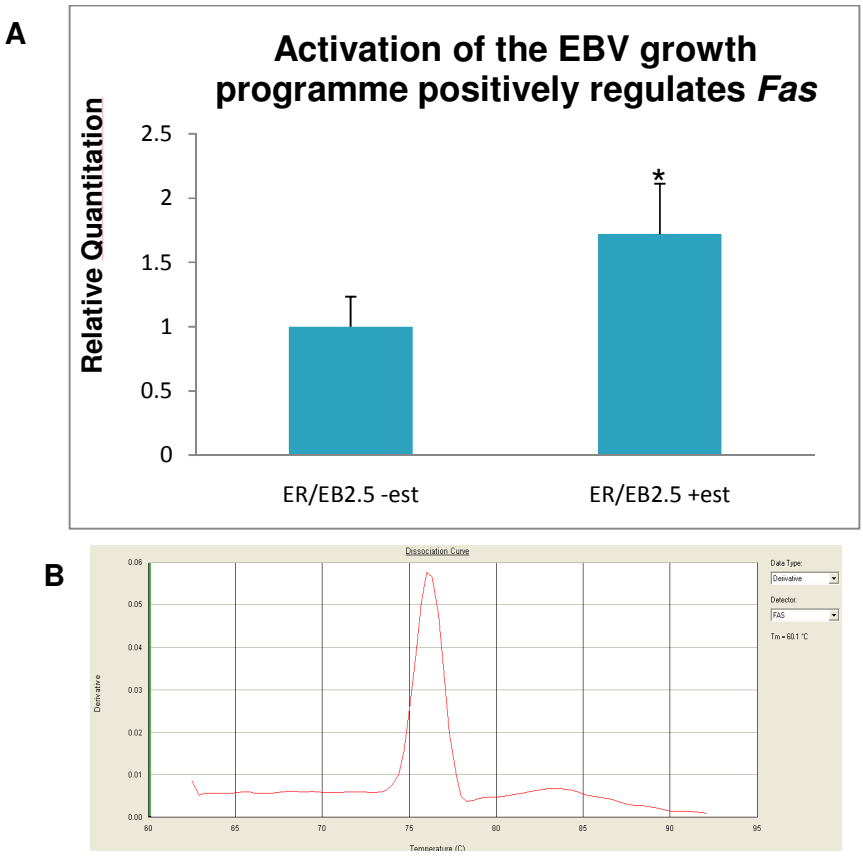


Figure 6.33: Activation of the EBV growth programme positively regulates *Fas*.

An experiment was carried out using ER/EB2.5 to assess the effect of ER/EBNA2 activation on the mRNA levels of *Fas*. RNA samples were taken during β-estradiol induction at the time points indicated. **(A)** RT-qPCR analysis was performed as described in the legend to figure 6.1, results were normalised using the internal control, *TBP*, and were expressed as relative quantitation as a function of the control. In this experiment, the control was set as ER/EB2.5 -est; arbitrarily assigned a value of 1. (Data are Mean ±SD, * $P<0.05$). **(B)** The dissociation curve for the product of the *Fas* assay was analysed to ensure a single specific peak, confirming that one specific PCR product was present.

Fas transcriptional activity was then examined in ER/EB2.5 and the activation of ER/EBNA2 also resulted in a marginal 1.8-fold up-regulation of *TRAF4* (Figure 7.8), corroborating the observation made in the EBV infection experiment.

6.3 Discussion

The findings of the RT Profiler PCR SuperArray were corroborated by the cell culture infection model ER/EB2.5 in many instances. However, not all the genes modulated in the naïve B cell pool, due to EBV infection, were found to be modulated in ER/EB2.5 in this study (Table 6.1). This may have been due to a combination of factors such as; (i) irreversible changes occurring in the naïve B cell pool after contact with the virus or; (ii) the shortcomings of an inducible cell line like ER/EB2.5, due to the presence of a potent hormone such as estrogen.

Gene	Array Name	Modulation due to EBV Infection	
		RT Profiler PCR SuperArray	Cell Culture Model (EREB2.5)
CCNE1	Notch	286	4.5
CIDEB	Apoptosis	502931	2
FAS	Apoptosis	605	1.8
Fos	Notch	648	2
Jun	Wnt	201	8
LRP5	Notch	57	-
	Wnt	19	-
MMP7	Notch	317	-
TNFSF7	Apoptosis	626	-
TRAF4	Apoptosis	133	1.5
NR4A2	Notch	14	16
STIL	Notch	129	3
Hey1	Notch	121	8
TLE1	Wnt	43	5
	Notch	44	

Table 6.1: Comparative gene expression results from the RT Profiler PCR SuperArray and the ER/EB2.5 cell pre- and post-EBV infection.
Comparison of the fold change in mRNA expression levels from; (i) samples taken from negatively purified naïve B cells against cells taken 3 days post-infection with GFP-tagged EBV from the RT Profiler PCR SuperArray and (ii) the mRNA expression levels in ER/EB2.5 after the activation of the EBV growth programme. Fold changes highlighted in blue indicate down-regulated genes, red indicate up-regulated genes while entries denoted in yellow signify genes unaffected after EBV infection. Genes that have two results are present in more than one cell signaling gene panel.

Of the thirteen novel targets analysed in this study, nine targets were found to be modulated similarly after the infection of a naïve B cell pool by GFP-tagged EBV, and due to the activation of the EBV growth programme in the ER/EB2.5 cell line. This corroborating data lends support to their potential as EBV target genes, making these nine genes interesting targets for future study. Three of the genes found to be modulated due to EBV infection of the naïve B cell pool; *LRP5*, *MMP7* and *TNFSF7* were not found to be regulated in the cell culture model after activation of the EBV growth programme. Although the results between the two experiments were not in agreement, these three genes still merit further study as they may still play a role in an EBV infected cell. The shortcomings of the cell culture model may have limited the scope of study with respect to these target genes, particularly *LRP5*, which was found to be modulated in two RT Profiler PCR SuperArray panels. Only the results of the *CIDEB* study gave conflicting results between the two infection methods, however it is worth noting that the fold up-regulation for *CIDEB* in the RT Profiler PCR SuperArray was unrealistically high.

In this study, *Hey1*, *Nurr1*, *STIL* and *CyclinE1* were investigated further using ER/EB2.5 and several additional B cell lines in an attempt to define the relationship between these genes and EBV.

Hey1

Findings from this study indicate that *Hey1* is a cellular target of EBV in a B cell. In particular, EBNA2 has been implicated here as an EBV latent protein capable of *trans*-activating *Hey1*. Activating or inducing EBNA2 expression correlated with an increase in *Hey1* expression in two independent conditional BLs although these results could not be confirmed at the protein level (Section 6.2.1.1). Whether this EBNA2-mediated *trans*-activation of *Hey1* was elicited at the promoter level by EBNA2 was not clarified in this study, as it was discovered that EBNA2 has the ability to *trans*-activate the empty luciferase reporter vector, pGL3-Basic. Whether this EBNA2-mediated *trans*-activation of *Hey1* was elicited at the promoter level by EBNA2 was not

elucidated in this study, as it was discovered that EBNA2 affected luciferase expression from the empty vector, pGL3-Basic. A future possible step would be to sub-clone the *Hey1* promoter region into a different luciferase reporter vector, such as pGL2-basic, known to be unaffected by EBNA2 activity. This could clarify the potential role of EBNA2 on the *Hey1* promoter.

Due to simultaneous characterisation of the Hey family in a variety of laboratories the Hey proteins have an array of additional names including; HRT (Hairy-related transcription factor) (Nakagawa, et al. 1999), Hesr (Hairy/Enhancer of split related protein) (Kokubo, et al. 1999), Herp (Hes-related repressor protein) (Iso, et al. 2001), and CHF (Cardiovascular helix-loop-helix factor) (Chin, et al. 2000). As these names suggest, Hey proteins present a strong likeness to the Hes family. Hey proteins are highly conserved throughout evolution and have been shown to regulate a variety of cellular decisions in different processes, such as cardiac development (Fischer and Gessler 2003), angiogenesis (Fischer, et al. 2004), neurogenesis (Sakamoto, et al. 2003) and segmentation. Dys-regulation of the bHLH family has also been implicated in an oncogenic role (Sun, Ghaffari and Taneja 2007).

The Hey family displays a dynamic expression profile throughout the body and expression has been observed in a wide range of species; however, it is notably absent from *Caenorhabditis elegans* (Fischer, et al. 2002). Hey expression is only partially overlapping, implying that each family member has unique functions and that they are differentially regulated (Maier and Gessler 2000). The Hey family members share similar domain organisation to other bHLH family members, most notably another key downstream effector of notch, the Hes family. The Hey family consists of conserved regions; a bHLH domain is followed by an Orange domain, a motif unique to bHLH factors. Proline rich regions are comparable between the members; however, the carboxy-terminal motif displays amino acid variation. Hey proteins exhibit an YRPW motif that is distinct from the highly conserved bHLH associated WRPW motif (Steidl, et al. 2000).

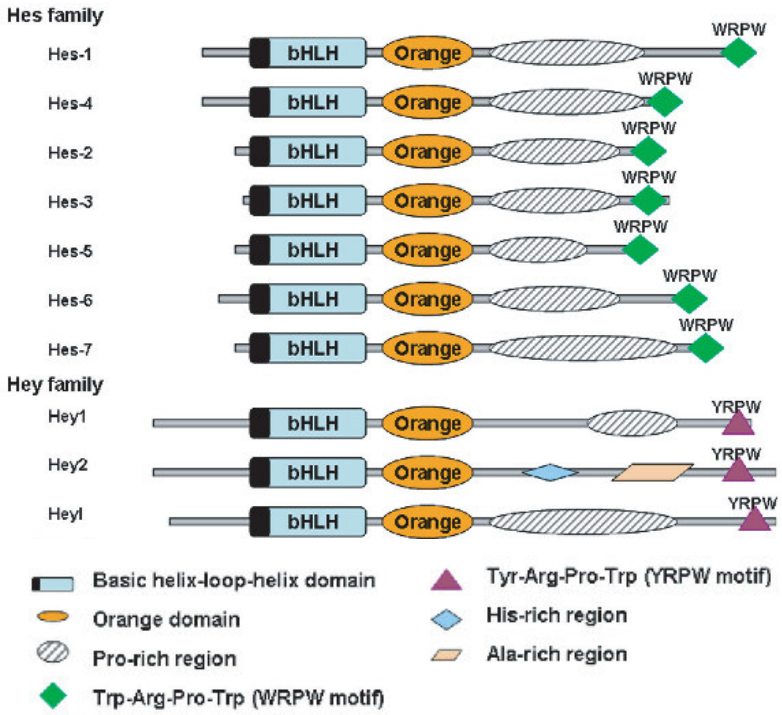


Figure 6.34: Schematic comparing the structure of Hey family members with their fellow bHLH-O family members, Hes.

The proteins have a highly similar structure as far as the bHLH and Orange domains, the proline rich regions do not differ much; however the Hes family have a WRPW motif that is involved with its interaction with the repressor protein TLE1, whilst the Hey family have a YRPW motif that to date shows no evidence of recruiting TLE1 (Sun, et al. 2007).

Generally, Hairy and Hes proteins recruit TLE to elicit the characteristic negative regulation exerted by the bHLH family. This recruitment is facilitated by the highly conserved WRPW carboxy terminal (Fisher, et al 1996; Sun, et al. 2001). Indeed, the runt (Runx) family of proteins possess a similarly conserved VWRPY motif that also interacts with TLE (Aronson, et al. 1997). Surprisingly, Hey, although displaying a conserved carboxy region, YRPW, similar to that of Hes, hairy and Runx, exhibits negligible association with TLE proteins. Mutational analysis substituting the Y residue with a W enables Hey1 to associate with TLE. This indicates that the lack of TLE binding capability by Hey1 is attributable to the deviated motif at the W residue (Fischer, et al. 2002). A number of differences in functionality and regulation have been found due to this deviated YRPW motif; however, deletion of the

YRPW motif in *Hey1* does not inhibit its transcriptional repression activity, thus suggesting different roles for the YRPW motif of Hey1 and WRPW motif of Hes1 (Sun, et al. 2001). The WRPW motif in Hes recruits TLE, and is crucial for repressor activity. However there is no evidence to indicate that the YRPW motif presented by the Hey proteins recruits the TLE co-repressor. In addition, although Hey proteins do not recruit TLE as a co-repressor, the bHLH domain and Orange domain can recruit the HDAC co-repressor complex associated with TLE function to repress transcription of target genes (Nakagawa, et al. 2000; Iso, et al. 2001).

Hey proteins are capable of homo and heterodimerisation through the HLH domain (Fischer, et al. 2002). The HLH region also facilitates association with related proteins such as the Hes family (Sun, et al. 2001). This HLH led binding effect is potentiated by the presence of the orange domain (Fischer, et al. 2002). Hey proteins elicit transcriptional repression through two known mechanisms; (i) directly binding target genes displaying a specific cognate sequence, via the basic domain. A highly specific consensus site for Hey family members has been identified. This characteristic hexamer recognition site had the core binding sequence 'CACGTG', and was found to be an E-box motif (Fischer, et al. 2002). Once bound to the target, repressor proteins are recruited via the YRPW or orange domain (Huang, et al. 2004a) or (ii) by heterodimerising with, and sequestering bHLH activator proteins. Once bound in these non-functional dimers, repression of the activator protein target gene occurs (Huang, et al. 2004a). *Hey* promoters lack TATA boxes; on the other hand, they contain other initiator elements such as GC rich regions and Sp1/Sp3 binding sites (Maier and Gessler 2000). The *Hey* family are recognised components of the Notch signaling pathway. Notch signaling stimulates the transcription factor CBF1/RBP-Jκ which in turn activates notch target genes via a distinct recognition sequence (Reviewed in Miele 2006). Several potential CBF1/RBP-Jκ, cognate sequences have been identified in the *Hey* promoters (Fischer, et al. 2002). Maier and Gessler have shown using promoter constructs for each family member, that (i) the *Hey* genes are Notch activated and (ii) that this activation requires the CBF1/RBP-Jκ

recognition motifs in the promoter regions. The Hey1 promoter contains five potential CBF1/RBP-Jκ sites; however, only two have been shown to be functional (Maier and Gessler 2000).

The Hey family are known transcriptional repressors that act as key participants in cardiac and vascular development during embryogenesis. At the initial stages of growth, all *Hey* genes are expressed in distinct compartments of the cardiac and vascular precursor cells (Fischer, et al. 2002). Evidence suggests that although *Hey1* has a central task in the vital notch signaling cascade, it appears to be a non-essential gene in this context. Studies have revealed that *Hey1* possess a redundant function, as *Hey1* knockout mice are viable and generally healthy animals inferring that other family members or cellular genes can sufficiently compensate for Hey1 function (Fischer, et al. 2002). Combined *Hey1* and *Hey2* knockout mice die in utero due to developmental failure in blood vessel development and arterial-venous differentiation (Fischer, et al. 2004). Hey family members have been shown to repress the cardiac activators GATA4 and 6, this inhibition is due to direct binding of Hey proteins to GATA4 and GATA6, preventing transcriptional up-regulation of the genes (Fischer, et al. 2005).

Besides its role in physiological developmental processes, *Hey1* has been found to up-regulate the tumour suppressor gene *p53*. *p53* is a negative regulator of cell proliferation and an inducer of apoptosis (Huang, et al. 2004a). Unexpectedly, when it is considered that *p53* exerts a repressional effect on cell proliferation, *p53* is also found to be up-regulated in EBV infected cells (Rickinson and Kieff 2001; Cannell, et al. 1998). One key regulator of p53 activity is the human double minute 2 (HDM2) protein. This regulatory factor directs ubiquitin-led degradation of p53. Evidence has revealed that *Hey1* enhances p53 activity indirectly by transcriptional repression of HDM2 (Huang, et al. 2004a). Thus, in this context, *Hey1* plays the role of a tumour suppressor. To maintain a latent infection within the host, EBV generally subverts cellular features that promote cell growth and survival whilst eliciting a minimal chance of discovery by the immune

surveillance. However, unexpectedly, evidence demonstrates that EBV stimulates the expression of the pro-apoptotic *p53* gene (Rickinson and Kieff 2001; Cannell, et al. 1998). *p53* is a crucial tumour suppressor gene that supports cell cycle arrest, senescence and apoptosis in response to stimuli such as damaged DNA, UV light and oncogene activation. In addition, as a precautionary measure, the host will attempt to eliminate any cells presenting irregularities in p53 function (Vogelstein, et al. 2000; Balint and Vousden 2001). The role of *p53* regulation in oncogenesis is well established. Indeed, it has been shown that *p53* mutation is a causative factor in approximately 50% of all human malignancies (Hainaut and Hollstein 2000). In addition, in some forms of cancer in which the *p53* gene is intact, evasion and resistance to the p53 signaling cascade is a factor in oncogenic establishment (Huang, et al. 2004a, Vogelstein, Lane and Levine 2000). Thus, ineffective responses to p53 are detrimental to healthy maintenance of cells; however, a fine balance of p53 activity must be maintained to sustain cell survival. It is most likely that EBV increases p53 expression so as not to flag the cell for destruction but only to such a level that it cannot exceed the threshold for apoptosis. Although EBV has been shown to promote p53 expression, the mechanism by which this effect is exerted has not been fully elucidated. In this study, it has been shown that EBV infection of B cells stimulates *Hey1* expression. *Hey1* is a known inducer of p53 (Huang, et al. 2004a) and thus it may be the case that EBV mediated *Hey1* expression links EBV signaling and p53 up-regulation.

A body of evidence is beginning to associate bHLH family members with oncogenesis. Expression profiling of non-small cell lung cancer (NSCLC) cells illustrated increased expression levels for *Hey1* and *HeyL* (Collins, et al. 2004). In prostate cells *Hey1* expression can be detected in both the nucleus and cytoplasm. However, it has been shown that in malignant prostate cells, *Hey1* is expelled exclusively into the cytoplasm, thus preventing *Hey1* led regulation. This implicates a potential step involving modulated *Hey1* expression and prostate cancer development (Belandia, et al. 2005). Although evidence is providing a connection between bHLH family and oncogenesis, no defined role has been elucidated for any of the *Hey* family.

Nurr1

Significant down-regulation of *Nurr1* transcription was observed just 24 hours after the activation of ER/EBNA2 in ER/EB2.5 (Section 6.2.2). De-activation of ER/EBNA2 in the same cell line restored *Nurr1* transcriptional expression. These findings indicate that *Nurr1* is negatively regulated by EBV in an LCL. However, in an isogenic LCL, P493-6, in which proliferation can be conditionally regulated to be driven due to EBV latent genes or c-Myc expression, it was found that c-Myc had a more significant negative effect on *Nurr1* in comparison to EBV driven proliferation. As EBV up-regulates *c-myc* expression upon transformation of a B cell, the down-regulation of *Nurr1* observed in the ER/EB2.5 cell line may possibly be due to up-regulation of c-Myc. This suggests that *Nurr1* may be a direct target of *c-myc* activity and not of the EBV latent proteins. The results acquired from the examination of a B cell panel corroborate this theory as *Nurr1* expression was found to be down-regulated in B cell lines with elevated c-Myc expression. Many of the cellular changes at the expression level in EBV infected cells are secondary effects of EBV-mediated c-Myc up-regulation, rather than direct modulation by EBV products. The c-Myc-mediated regulation within the EBV infected cell plays a significant role in the maintenance of EBV-led B cell immortalisation. Thus the unexplored role of *Nurr1* in B cells, and the demonstrated regulation of *Nurr1* by EBV in the same cell background, makes this an interesting target for future study.

Nurr proteins act as constitutively active ligand independent receptors that are tightly regulated (i) at the transcriptional level, (ii) by sub-cellular localisation and (iii) by post-translational modification (Reviewed in Aherne, et al. 2009). *Nurr1* has been identified as a key pan-neurological transcription factor. The Nurr1 transcription factor has been found to associate with NFκB (Wan and Lenardo 2009), and this co-regulation by the two factors in microglia and astrocytes results in inhibition of pro-inflammatory genes (Saijo, et al. 2009). This function of *Nurr1* has been implicated as a key factor in familial Parkinson's disease, as cells found in sufferers of

Parkinson's demonstrate mutations of the *Nurr1* (Le, et al. 2003). In contrast to this anti-inflammatory role, over-expression of *Nurr1* has been found to be a consistent factor for pro-inflammatory response in multiple sclerosis (Doi, et al. 2008). Little is known of the role of *Nurr1* in the immune system; however, some studies indicate that (i) that over-expression of *Nurr1* by T cells is found predominantly in MS suffers in contrast to healthy subjects and (ii) that *Nurr1* over-expression in T cells stimulates the release of the pro-inflammatory cytokines, IL-17 and IFN-γ (Reviewed in Doi, et al. 2008). Current published data indicate that *Nurr1* functions in a context-dependent manner and that any dys-regulation can play a role in debilitating diseases such as MS (Doi, et al. 2008) and Parkinson's disease (Le, et al. 2008).

Much of the work published on *Nurr1* focuses on the development and maintenance of midbrain dopaminergic neurons and the pro-inflammatory role of *Nurr1* in autoimmune diseases such as MS and rheumatoid arthritis (RA) (Davies, et al. 2005). The function of *Nurr1* appears to be very much context-dependent with *Nurr1* presenting conflicting roles in different cell types, such as its anti-inflammatory role in microglia relative to its pro-inflammatory role in T lymphocytes (Saijo, et al. 2009). This makes it difficult to determine a reason for *Nurr1* down-regulation in an EBV infected cell, as little is known of the role of *Nurr1* in the B cell setting. An observation that may be relevant to this study, however, is that there is a direct association between Nurr1 and the chemotherapeutic drug, 6-mercaptopurine (6-MP). Expression of Nurr1 has been shown to be up-regulated by 6-MP. 6-MP is an anti-proliferative and cytotoxic drug used to treat both autoimmune diseases and various types of leukaemia. The positive regulation of Nurr1 by 6-MP may imply that (in the context of a T-lymphocyte), Nurr1 is a potential mediator of the anti-proliferative and cytotoxic effect of 6-MP. Furthermore, the expression of Nurr1 correlates with the spectrum of cell types that respond to 6-MP chemotherapy.

STIL

STIL was first discovered due to its association with T-cell acute lymphoblastic leukemia (T-ALL), in which a specific deletion fusing the promoter of *STIL* to the coding region of T-Cell acute lymphocytic leukaemia 1 (*TAL1* also called SCL) occurs, inducing abnormal expression of *TAL1* (Aplan, et al. 1991). Dys-regulation of *TAL1* expression has been found in 25% of patients suffering from T-ALL (Hsu, et al. 1994). The *STIL* gene is normally expressed in T cells; however, *SCL/TAL1* is not. This translocation event results in inappropriate expression of the *SCL/TAL1* gene and contributes to the development of leukaemia (Aplan, et al. 1991). *STIL* bears no homology to other known proteins and little is known of the function of *STIL*, although its requirement for mitotic entry has been confirmed. *STIL* has been shown to be an immediate early gene that peaks during mitosis but degrades soon after (Reviewed in Erez, et al. 2007). Structural studies of the STIL protein have not identified a DNA-binding motif, a transmembrane domain or a secretion signal (Colaizzo-Anas and Aplan 2003); however, a putative nuclear localisation signal has been documented (Karkera, et al. 2002). Preliminary studies of the *STIL* promoter have revealed that the promoter contains three consensus CCAAT boxes but no evident TATA box. It also contains putative binding sites for SP1, ESF and GATA-1 (Colaizzo-Anas and Aplan 2003). Recent investigations have revealed that STIL can be regulated by the E2F transcription factor family. Studies involving siRNA knockdown of E2F family members led to a decrease in STIL expression and this coincided with a delayed entry into mitosis (Erez, et al. 2008). Studies of cancer expression profiles have revealed that an increase in STIL levels facilitated amplified mitotic activity, and that knockdown of STIL using shRNAs and siRNAs in a number of cancer cell lines (including those of colon cancer, breast adenocarcinoma, pancreatic carcinoma, prostate adenocarcinoma and non-small cell lung carcinoma), caused growth arrest of the malignant cells by preventing mitotic entry (Erez, et al. 2007).

The results obtained in this study regarding *STIL* might be explained by the growth activation properties of EBV in a B cell background. Numerous studies have indicated that cells cannot progress through mitosis without facilitation by STIL activity. Therefore, any increased proliferative activity, such as that seen due to oncogenesis, requires an increase in STIL expression. In this study, naive B cells were driven to proliferate following EBV infection (Section 4.2.1). It is possible, therefore, that activation of proliferation *per se* instigates an increase in STIL expression. Up-regulation of STIL was also observed using the ER/EB2.5 cell culture model; however, this is much the same situation in that activation of the EBV growth programme drives proliferation (Section 6.2.3). Whether EBV directly targets *STIL* or if *STIL* up-regulation is an indirect effect of transformation is unclear. Increased STIL activity is beginning to appear as a constant feature of aberrant cell growth (Erez, et al. 2007). Consistent with this, similar *STIL* mRNA levels were seen to occur here in all B cell lines examined, irrespective of the presence of EBV latent proteins (Section 6.2.3.2).

Cyclin E1

In this study it was observed that *Cyclin E1* was transcriptionally up-regulated after EBV infection of naive B cells (Section 4.2.1) and by the activation of the EBV growth programme in ER/EB2.5 (Section 6.2.4). EBV mediated up-regulation of *CyclinE1* was previously noted elsewhere and found to be due to the activation of proliferation in the EBV infected cells (Hollyoake, et al. 1995; Möröy and Geisen 2004). Cyclin D has also been shown to be an EBV-responsive gene, up-regulated upon infection of the B lymphocyte. Furthermore, *CyclinE1* activation has more recently been found to be dependent on Cyclin D expression (Möröy and Geisen 2004).

Elevated *Cyclin E1* expression has been associated with a diverse spectrum of malignancies, and in numerous studies, signifies a poor patient prognosis. Indeed, amplified *Cyclin E1* expression has repeatedly been shown to be a feature of advanced stage carcinoma (Ko, et al. 2009). The development of all tumours involves similar events to facilitate tumour progression. In early

stages, key factors in malignant establishment include angiogenesis and cellular adhesion, as the tumour progresses activity moves more towards driving cellular proliferation. *Cyclin E1* expression in later stages of carcinoma facilitates cellular proliferation. It has been demonstrated that increased *Cyclin E1* mRNA levels are present in advanced nasopharyngeal carcinoma (NPC) tumours (Ko, et al. 2009). Several studies have reported similar findings, confirming elevated *Cyclin E1* expression levels in later clinical stage tumours such as non-small cell lung cancer (Fukuse, et al. 2000), papillary thyroid cancer (Brzezinski, et al. 2004), testicular germ cell cancer (Datta, et al. 2000), gastric cancer (Bani-Hani, et al. 2005) and breast cancer (Peters, et al. 2004). A direct correlation has been demonstrated between high *Cyclin E1* expression and reduced survival rates in NPC (Ko, et al. 2009), breast cancer (Peters, et al. 2004) and non-small cell lung cancer (Müller-Tidow, et al. 2001). This evidence, when taken together, brings to light the value of Cyclin E1 as a potential prognostic factor for some tumours.

Remaining candidate genes

The results obtained in this chapter for the remaining genes identified as candidate EBV cellular targets, indicated that: (i) although *TNFSF7*, *LRP5* and *MMP7* were transcriptionally up-regulated following EBV/naïve B cell infection (by RT Profiler PCR SuperArray), no significant changes to their mRNA levels were seen upon activation of the EBV growth programme in ER/EB2.5; (ii) *CIDEB,* shown to be up-regulated following EBV infection, was seen to be down-regulated in the ER/EB2.5 model, (iii) the expression of *c-Fos,* found to be down-regulated following EBV infection, was not seen to change in the ER/EB2.5 system; (iv) *c-Jun* was down-regulated following both EBV/B cell infection and activation of the EBV growth programme in ER/EB2.5, (v) both *TRAF4* and *FAS* were up-regulated following EBV/B cell infection and activation of the EBV growth programme in ER/EB2.5.

Although no conclusion could be drawn for *MMP7* regulation in this study with the results available, evidence has been reported in agreement with the

RT Profiler PCR SuperArray results. Data have been published linking elevated *MMP7* expression with EBV stimulated infection. Hertle and colleagues compiled a genome wide expression panel of EBNA3A target genes using an Affymetrix gene array. Among the 167 up-regulated targets identified in the study, *MMP7* was found to be up-regulated 49-fold in comparison to EBNA3A knockout LCLs (Hertle, et al. 2009).

In vivo studies have shown that transition from a naïve B cell to an activated blast involves a significant phenotypic shift. One feature of the phenotype change involves the induction of Fas expression in the centroblast (Spender and Inman 2009). It may be argued that the Fas up-regulation is due to cellular activation rather than direct EBV modulation; however, a number of studies have demonstrated Fas modulation by the EBV latent protein LMP1 in both BLs (Henriquez, et al. 1999) and LCLs (Le Clorennec, et al. 2006; Falk, et al. 1992). Thus implicating Fas as an EBV target therefore not a novel cellular gene to pursue for this investigation.

Chapter 7: *Overall Conclusions*

In this investigation a panel of potential novel EBV target genes was compiled from the data obtained from a naïve B cell infection study. Genes that were significantly modulated in the RT Profiler PCR SuperArray experiment were assessed with respect to their novelty and potential for a role in an EBV infected cell. This entailed cross referencing the published data available for each gene and identifying the various role/s for each gene (Table 7.1). The development and maintenance of latent EBV infection in B cells is a balance of immune evasion and propagation. EBV subverts cellular pathways that control differentiation, proliferation, and apoptosis so as to ensure the long-term survival of the infected cell (Cader, et al. 2010). The genes selected for further study have a function in many of the cellular processes frequently targeted by EBV activity (Table 7.1).

Gene	Regulation (SuperArray)	Cell Signaling Array Panel	Role/s
CCNE1 (Cyclin E1)	*Up*	Notch	Cell cycle
CIDEB	*Up*	Apoptosis	Activates Apoptosis
FAS	*Up*	Apoptosis	Activates Apoptosis and NF-kB signaling
Fos	*Down*	Notch	Positive Proliferative effect, Associated with Transformation
Jun	*Down*	Wnt	Positive Proliferative effect, Associated with Transformation.
LRP5	*Up*	Notch Wnt	Component of Wnt signaling
MMP7	*Up*	Notch	Proliferation, Migration and Apoptosis
TNFSF7	*Up*	Apoptosis	Proliferation, and B cell activation
TRAF4	*Up*	Apoptosis	Activates NF-kB and JNK Signaling. Role in apoptosis.
NR4A2 (Nurr1)	*Down*	Notch	Cell cycle (implicated)
STIL	*Up*	Notch	Cell cycle and Proliferation
Hey1	*Up*	Notch	Notch responsive transcriptional repressor
TLE1	*Down*	Wnt Notch	Represses NF-kB and Wnt signaling. Potential role in cell cycle.

Table 7.1: Summary of the known role/s for selected target genes, and the signaling pathways involved.
Summary of the role of each studied gene, and the RT Profiler PCR SuperArray panel in which they were analysed. The modulation observed in response to EBV infection of the naive B cell pool can be found in second column on the left

One important mechanism contributing to survival of the EBV-infected cell is the dysregulation of certain *Bcl-2* family members, and of particular interest in this study was the modulation of anti-apoptotic member *bfl-1* by the LMP1 and EBNA2. The Bcl-2 family maintain a tight equilibrium of pro- and anti-apoptotic members in a healthy cell (Kuroda and Taniwaki 2009), and EBV can distort this balance in favour of inappropriate cell survival (Le Clorennec, et al. 2008). The EBV-mediated transcriptional regulation of *bfl-1* in B lymphocytes was examined in this study. It was determined that two

transcription factor recognition sites in the *bfl-1* promoter, from two different pathways, Notch and NF-κB, are vital for EBV-mediated up-regulation of *bfl-1* (Section 3.2.4). In addition, the presence of one of these functional binding sites can compensate for the loss of the other; thus, the inhibition of both LMP1 and EBNA2 is required to prevent *bfl-1* modulation by EBV.

Several novel EBV regulated cellular target genes were identified in this investigation including (i) *TLE1*, (ii) *Hey1*, (iii) *STIL*, (iv) *NR4A2*, and *Cyclin E1*. *TLE1* was demonstrated to be a negative transcriptional target of EBV in naïve B cells and an LCL (Section 4.2.2 and 4.2.3). *TLE1* expression levels were found to be more potently down-regulated in BLs than LCLs (4.2.8). The lower levels of TLE1 in the BL-derived cell lines was in agreement with recently published findings that implicate *TLE1* as a negative regulator of haematological cell fate (Fraga, et al. 2008). Ectopic TLE1 in an LCL led to a reduced rate of proliferation of that cell line (Section 5.2.4) and expression of the exogenous protein was lost over time. These findings indicated that elevated TLE1 conferred a negative growth advantage in this cell setting, giving a rationale for its negative regulation by EBV. Examination of expression of selected genes indicated that ectopic TLE1 down-regulated key genes such as *Cyclin D1* (Section 5.2.6.1) and *Runx3* (Section 5.2.6.4), which are required for proliferation in B lymphocytes. In addition to this, ectopic TLE1 also led to increased levels of the EBV latent protein EBNA2 (5.2.5). The level of EBNA2 expression is tightly maintained within an EBV infected cell and excessive expression of EBNA2 is known to have a negative impact on cell survival (Floettmann, et al. 1996).

To further understand the role of TLE1 in an LCL, shRNA expressing vectors were used to knockdown *TLE1*. Due to the low transfection efficiency obtained in these cells, a new protocol was established to permit the isolation of an enriched pool of viable transfected cells (Section 5.2.7). The use of a combined purification method involving Ficoll and positive selection with the lectin Con A, facilitated the analysis of transfected cell pools with reduced background from dead or dying cells. Phenotypic analysis demonstrated an

increased proliferative capacity in the TLE1 shRNA knockdown cell pools, relative to a cell pool encompassing a scrambled shRNA vector (Section 5.2.11). This corroborated previous results indicating that ectopic TLE1 reduced the proliferative capacity of an LCL (Section 5.2.4). The growth advantage conferred by the knockdown of *TLE1* may be due, at least in part, to the modulation of *Cyclin D1*, which showed significant up-regulation in response to *TLE1* knockdown (Section 5.2.8.2)

Although designated primarily a virus of the B cell compartment, other cell types such as epithelial cells and a number of T and NK cell lines can also be infected by EBV. Growth transformation does not occur in these cells however, and infection is often cytotoxic. A restricted EBV gene expression pattern can be found that relates to those found in corresponding EBV-associated tumours of epithelial, T or NK cell origin (Reviewed in Tierney, et al. 2007). The latency III growth programme of EBV is unique to cells of the B cell lineage. As previously described, EBV latency can arise from three viral promoters: the Qp, the Wp or the Cp. Cp stimulation leads to the transcription of a single alternatively spliced and alternatively polyadenylated transcripts that give rise to EBNA-LP, EBNA2, EBNA1, EBNA3A, -B, or –C. This transcription of the full panel of EBV latent genes is initially directed by the Wp, with a subsequent switch to Cp, and this form of latency occurs only in B lymphocytes (Rickinson and Kieff 2007). Tierney and colleagues determined that the B lymphotrophism of EBV is due to the recruitment of the B cell specific Pax5 (Tierney, et al. 2007). Upon initial B cell infection by EBV, EBNA2 and EBNA-LP are the first viral latent proteins expressed originating from the Wp (Kieff and Rickinson 2007). Tierney's research laboratory identified two *Pax5* binding sites in the Wp and mutational/deletion analyses demonstrated that in the absence Pax5 binding, viral infection of B lymphocytes did not succeed (Tierney, et al. 2007). Moreover, the infection of epithelial cells with these recombinant viruses led to latency I and II states as efficiently as was the case with wild-type EBV (Tierney, et al. 2007). It was concluded that the EBV latency III growth programme requires the B cell specific Pax5. In the present study, *Pax5* was identified as a negative target of TLE1 in an LCL, making EBV-mediated repression of *TLE1* a logical

interaction from the standpoint of this B cell immortalising virus. *Pax5* is expressed endogenously in B cells and is consequently present when EBV infection occurs, facilitating Wp activation. The down-regulation of *TLE1* upon EBV infection of B lymphocytes may have the advantage of relieving *TLE1*-mediated *Pax5* repression, thus potentiating Pax5 driven Wp activity, permitting EBV to express its *EBNAs*.

The transfected IB4 cell pool preferentially lost ectopic TLE1 over time, thus the cell pool did not appear to be stable in the long term. RT-qPCR analysis of potential TLE1-regulated genes showed that *E2A* and *Pax5* were both down-regulated at the mRNA level at earlier times in the cell pool expressing ectopic TLE1 (Section 5.2.6.2 and section 5.2.6.3). *Pax5* is a downstream E2A-regulated gene, and as such, it cannot be discerned whether *Pax5* repression was due to E2A down-regulation or directly due to TLE1. Down-regulation of E2A would have an impact on the stability of a B cell population; however, as E2A *trans*-activates the key B lymphocyte transcription factor, Ebf1 (Smith, et al. 2002). Ebf1 is the intermediary activator to Pax5 driven expression (Roessler, et al. 2007). *Ebf1* also has a second promoter; however, that is driven by Pax5, thus enabling continued *Pax5* and *Ebf1* activation with a positive feedback loop between the two promoters. Nevertheless it might be the case that the loss of ectopic TLE1-expressing cells over time could be in part due to the down-regulation of these key B cell regulators *E2A* and *Pax5*.

In this study it was not possible to establish whether *TLE1* down-regulation was due to EBNA2. In BL41-K3, EBNA2 appeared to positively regulate *TLE1,* however this was not confirmed in a second BL cell line in which EBNA2 was also expressed as sole EBV protein ((DG75 tTA-EBNA2); Section 4.2.6). In the pcDNA3-TLE1-transfected IB4 cell pool, significant down-regulation of *CD23* can be observed (Figure 5.2.6.5). EBNA2 has already been shown to *trans*-activate *CD23*. *TLE1*-down-regulation might potentiate this pro-survival interaction; however, EBNA2 targets CBF1-binding sites in the *CD23a* promoter. In addition, the presence of two putative Pax5-binding sites was also confirmed in the CD23 promoter

(Hubmann, et al. 2002). Results presented in this thesis indicate that *Pax5* is a negative target of TLE1. The CBF-1 binding sites allow EBNA2 to induce *CD23a* expression, likewise the presence of *Pax5* motifs are evidence of a possible indirect route for TLE1 to down-regulate this gene. The down-regulation of both *Pax5* and *CD23* would be a disadvantage to the EBV-infected cell, thus giving a clear rationale for EBV-mediated *TLE1* repression.

Considering the *Pax5* down-regulation observed due to ectopic TLE1, and taking account of a positive role for Pax5 in the induction of Wp activity, the expression of *EBNA1* and *EBNA2* was therefore next examined. Surprisingly, *EBNA2* mRNA levels were up-regulated in comparison to the control cell pool (Figure 5.7, Section 5.2.5). However, in the same experiment *EBNA1* mRNA levels remained unchanged between cell pools (Figure 5.8 Section 5.2.5). Bearing this in mind, and the fact that the IB4 cell line does not possess a functioning Cp, regulation of EBV latent gene expression by TLE1 in this cell line is likely to occur through the Wp. Transcription from the Wp is regulated by several transcription factors including the YY1, RFX, Pax5 and CREB (Tierney, et al. 2000; Kirby, Rickinson and Bell 2000). Furthermore, it has been demonstrated that methylation of the Pax5- and CREB-binding sites in the Wp can prevent binding by their cognate transcription factors (Tierney, et al. 2000). Of the three *EBNA* promoters on the EBV episome only Wp has been shown to display a methylated state (Park, et al. 2007). Park and colleagues quantified the methylation status of Wp regulatory region and discovered that in every case the CREB-binding site was subject to a greater degree of hypermethylation that the Pax5-binding site (Park, et al. 2007). This group then demonstrated a correlation between LCL passage number and increased Wp methylation. Moreover this enhancement of Wp hypermethylation coincided with a decrease in *EBNA2* expression in three different established LCLs (Park, et al. 2007). TLE1 may regulate the Wp via Pax5 down-regulation. Considering this, however, one would have expected to observe EBNA2 down-regulation due to ectopic TLE1, which was not the case. Considering that TLE proteins have been shown elsewhere to regulate

the Cp (Almqvist 2005), a central role for TLE proteins in setting the level of EBV latent gene expression is emerging and warrants further investigation.

In addition to down-regulating TLE1, another negative target of EBV was identified in this study, *Nurr1* (Section 6.2.2). However, due to the fact that *Nurr1* expression was found to be significantly more potently down-regulated in an LCL driven to proliferate due to C-Myc, in comparison to the same cell line cycling due to the EBV-growth programme, it was speculated that *Nurr1* may be a negative target of C-Myc (Section 6.2.2.1). This then implied that the EBV-mediated increase in C-MYC expression in an LCL resulted in the down-regulation of *Nurr1*, thus making *Nurr1* an indirect target of EBV activity. This was confirmed somewhat by the analysis of a panel of B cell lines, in which cell lines encompassing C-MYC over-expression demonstrated more potent down-regulation of Nurr1 in contrast to two EBV positive cell lines (Section 6.2.2.2).

The Notch associated transcription factor, Hey1, was identified as a positive EBV target following EBV infection of naïve B cells, and after the activation of the EBV-driven growth programme in a conditional LCL (Section 4.2.1 and 6.2.1). Hey1 was shown to repress the activity of both a Notch and NF-κB responsive promoters in an EBV-negative BL (Sections 6.2.1.3 and 6.2.1.4). Further evidence indicated that EBNA2 was responsible for the induction of *Hey1* expression (Section 6.2.1.1). It has been shown elsewhere that Hey1 mediates the activation of *p53*, an occurrence that is coincident with EBV infection (Huang, et al. 2004a).

Central to B-cell immortalisation by EBV is the modulation of host cellular gene expression by viral gene products. In this study, potential EBV-host interactions were identified that may positively contribute to the survival of the virus-infected cell. Further characterisation of these may shed light on the mechanisms that drive cell transformation, the establishment of life-long latency and potentially the development of EBV-associated B cell malignancies. A better understanding of the EBV B cell immortalisation

process may present therapeutic targets of relevance to EBV associated and non-associated B cell diseases.

Chapter 8: *Bibliography*

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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
6 g	NaOH pellets

The pH was adjusted to 8.0 by addition of 5 M NaOH 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

5g	Tryptone
2.5g	Yeast extract
5g	NaCl

The volume was adjusted to 500ml, followed by autoclaving for 20 minutes 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.

NZY+ Broth

10g	NZ amine (casein hydrolysate)
5g	Yeast extract
5g	NaCl

The Volume was adjusted to 1lt with dH_2O, the pH was adjusted to pH7.5 using NaOH followed by autoclaving. Add the following filter sterilised supplements prior to use;

12.5ml	MgCL2 (1M)
12.5ml	MgSO4 (1M)
20ml	Glucose (20% w/v) or 10ml Glucose (2M)

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_20, 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 100µg/ml (Ampicillin), after cooling the LB agar to ~50˚C. Plates were stored at 4˚C.

SOB medium

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

The pH was adjusted to 7.0 with 5M NaOH.
The volume was adjusted to 500ml with dH_20 and the medium autoclaved.
2.5ml of 2M $MgCl_2$ was added after cooling the broth to 5˚C and the medium was stored at 4˚C.

SOC medium

98ml	SOB medium
2ml	1M glucose (filter sterilised)

Stored at 4˚C.

Solutions for Preparation of Competent Cells

1M $MgSO_4$

24.65g	$MgSO_4.7H_20$
100ml	dH_20

Sterilised by filtering and stored at room temperature.

TFB1

30mM	Potassium acetate
10mM	$CaCl_2$
50mM	$MnCl_2$
100mM	RbCl
15%	Glycerol

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

TFB2

100mM	MOPS (pH 6.5)
75mM	$CaCl_2$
10mM	RbCl
15%	Glycerol

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

Solutions for DNA preparations

Buffer P1 (Re-suspension buffer)

50mM	Tris-Cl (pH 8.0)
10mM	EDTA (pH8.0)
100µg/ml	RNase A

Stored at 4˚C following addition of RNase A.

DNase-free RNase

RNase A (1mg/ml) in upH_2O.
Heated to 100˚C for 30 minutes.
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)**)**

29.6g	Potassium acetate
50ml	dH_2O
11.5ml	Glacial acetic acid

Adjust volume to 100ml with dH_2O.
The resulting solution is 3 M with respect to potassium and 5M with respect to acetate.
Stored at room temperature.

Buffer QBT (Equilibration buffer)

750mM	NaCl
50mM	MOPS (pH 7.0)
15% (v/v)	Isopropanol
0.15% (v/v)	Triton-X 100

Stored at room temperature.

Buffer QC (Wash buffer)

1M	NaCl
50mM	MOPS (pH 7.0)
15% (v/v)	Isopropanol

Stored at room temperature.

Buffer QF (Elution buffer)

1.25M	NaCl
50mM	Tris-Cl (pH 8.5)
15%	Isopropanol

Stored at room temperature.

50 % (v/v) Glycerol

25ml	glycerol
25ml	dH_2O

The solution was autoclaved and stored at room temperature.

Annealing buffer

100mM	Potassium acetate
30mM	Hepes-KOH, pH 7.4
2mM	Magnesium acetate

Stored at 4 – 8 ˚C.

Solutions for Agarose Gel Electrophoresis

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

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

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

1X TAE (Working solution)

20ml	50X TAE
980ml	dH_2O

Stored at room temperature.

Agarose gel loading dye

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

Stored at room temperature.

Ethidium bromide

10mg	Ethidium bromide
1ml	dH_2O

The solution was stored in the dark at 4ºC. 100 µl of stock solution was added to 1L of dH_2O for TAE gel staining.

Solutions for Cell Culture

Media and Supplements

Supplemented RPMI 1640 (200 ml)

176ml	RPMI 1640
20ml	Foetal bovine serum (de-complemented at 50˚C for 30 mins)
2ml	200mM L-glutamine
2ml	Penicillin/streptomycin (10 mg/ml)

Estrogen (β-Estradiol)

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

Tetracycline

A 5 mg/ml stock was prepared in 100% ethanol and stored at -20˚C.

Geneticin (G418)

A 50 mg/ml stock was prepared in cell culture medium and stored at -20˚C

Puromycin

A 25 mg/ml stock was prepared in distilled water 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 DEAE-Dextran Tranfections Protocol

Tris buffered saline (TBS)

25mM	Tris-Cl (pH 7.4)
137mM	NaCl
5mM	KCL
0.7mM	$CaCl_2$
0.5mM	$MgCl_2$
0.6mM	Na_2HPO_4

To make 200ml TBS:

5ml	1M Tris-Cl (pH 7.4)
5.48ml	5M NaCl
0.5ml	2M KCl
1.4ml	100mM $CaCl_2$
0.1ml	1M $MgCl_2$
0.2ml	0.6M Na_2HPO_4
187.32ml	upH_2O

TBS was prepared from autoclaved stocks, aliquoted and filter sterilised before use.

DEAE dextran

DEAE dextran was prepared at a concentration of 1mg/ml in TBS on the day of use and filter sterilised.

β-Galactosidase Assay

100X Mg solution

0.1M	$MgCl_2$
4.5M	2- β-mercaptoethanol

Stored at -20˚C.

0.1M sodium phosphate buffer

41ml	0.2M $Na_2HPO_4.2H_2O$
9ml	0,2M $NaH_2PO_4.2H_2O$
50ml	dH_2O

1X ONPG substrate

ONPG was prepared at a concentration of 4 mg/ml in 0.1 M sodium phosphate buffer (pH 7.5) and stored at -20˚C.

1M sodium carbonate

10.59g	
100ml	dH_2O

Stored at room temperature.

EDAC

EDAC was prepared at a concentration of 75mg/ml in PBS on the day of use.

Con A

Con A was prepared at a concentration of 1mg/ml in ultra pure water and stored at -20˚C.

Working solutions were prepared on the day of use at a concentration of 100 µg/ml in PBS.

Solutions for RNA Analysis

DEPC-treated H_2O

1ml	DEPC
1000ml	dH_2O

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

RNA loading buffer

50%	Glycerol
1mM	EDTA (pH 8.0)
0.25%	Bromophenol blue
0.25%	Xylene cyanol FF
1µg/ul	Ethidium bromide

Solutions for Protein Analysis

Solutions for Protein Isolation

Suspension buffer

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

100mM	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
1.5M Tris (pH 8.8)	2.5	0
1M Tris (pH 6.8)	0	0.312
dH_2O	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

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

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

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1X Tris-glycine running buffer

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

Transfer buffer

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

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

1X Tris buffered saline (TBS)

6.1g	Tris base
8.8g	NaCl
800ml	dH_2O

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

TBS-T

1L	1X TBS
1ml	Tween 20

Stored at room temperature.

Blocking Buffer

5g	non fat dry milk powder
100ml	TBS-T

Stored at 4˚C.

The goal of science and engineering is to build better mousetraps. The goal of nature is to build better mice.