# Regulation of TGFβ and BMP signalling in human B cells by latent Epstein-Barr virus

**Lydia Eccersley**

**Thesis submitted for the degree of Doctor of Philosophy**

**Section of Virology**
**Department of Medicine**
**Imperial College London**

**February 2014**

## Abstract

Most individuals are infected with EBV, which establishes latent infection in B cells. Although usually asymptomatic, EBV persistence is associated with several types of lymphoma. TGFβ signals via TGFβ receptors (TGFβR) 1 and 2 and is generally pro-apoptotic and anti-proliferative, but EBV infection renders cells resistant to these effects. The aim of this thesis was to investigate further the mechanism by which this occurs.

Using the EBV-negative Burkitt lymphoma cell line BL31 infected with a panel of BAC-derived wild-type and recombinant EBVs, this study has confirmed by qRT-PCR that EBV down-regulates TGFβR2 transcription, leading to suppression of TGFβ signalling as detected by western blot for phosphorylated SMAD2. Use of recombinant viruses with deletions of individual latent proteins has shown that LMP1, LMP2A, EBNA3B and EBNA3C cooperate in this repression of TGFβR2 and suppression of signalling. Chromatin immunoprecipitation analysis has shown that the repression of TGFβR2 is accompanied by deposition of the repressive epigenetic mark histone H3 lysine 27 trimethylation and concomitant binding of SUZ12, a subunit of polycomb-repressive complex 2, to the TGFβR2 promoter. Infection of primary B cells with EBV also leads to repression of TGFβR2 and suppression of TGFβ signalling.

Additionally, EBV up-regulates TGFβR3, a co-receptor for signalling by both TGFβ and bone morphogenetic proteins (BMPs), a related group of ligands which signal via SMADs 1, 5 and 8. This led to an investigation into the effects of EBV on BMP signalling. Although EBV increases signalling in response to BMP2, BMP4 and BMP6 in BL31 cells, this is not via the up-regulation of TGFβR3. BMP2 and BMP4 induce G1 arrest in BL31 cells, but EBV does not alter this. EBV also up-regulates SMAD1 and down-regulates SMAD5.

The polycomb-mediated repression of TGFβR2 is a mechanism by which EBV might promote lymphomagenesis. In addition, the finding that EBV alters several components of BMP signalling suggests that BMP signalling may be important in B-cell biology.

## Declaration of Originality

All the work described in this thesis was done by me, except where clearly indicated in the text.

Lydia Eccersley

February 2014

## Acknowledgements

I wish to thank my supervisor Martin Allday for his rigorous scientific guidance throughout the course of this work. It certainly wasn't an easy process for me, having come from a completely different background and having a young family – but I hope I will have proved that it is possible to do a PhD with young children!

I should also like to thank the following: Kostas Paschos and Rob White for preliminary work on this project before I started, teaching me laboratory techniques, their patience with my initial lack of understanding of laboratory science, and for helpful suggestions on my work. Gillian Parker, for efficient management of the laboratory, practical laboratory advice and listening to my moans! Other members of the group, for advice and/or keeping me company during the many long days and weekends in the laboratory, in particular: Ian Groves, Guiyi Ho, Jens Kalchschmidt and Lenka Skalska.

I would like to thank Leukaemia and Lymphoma Research for funding me with a clinical research training fellowship to enable me to undertake this work.

I would also like to thank my parents, for always being there for me and for providing me with so many educational opportunities, and my parents-in-law for help with childcare.

Thank you in particular to my amazing husband Lionel, for putting up with me during this process, including many times when I was pretty miserable to live with – you always believed in me when I didn't believe in myself. You have been fantastic, selflessly taking on so many domestic tasks (even if you do keep ordering more and more tomatoes!!), and taking our girls out to explore all of London (and beyond) so that I could work. Also, thank you for your help with proof-reading this thesis and for your computing expertise.

Lastly, to my wonderful daughters, Annabelle and Eliana, who came into the world shortly before I started this, and halfway through, respectively. Although this often made things more challenging, seeing you on my return home each day gave me the sense of perspective I needed to carry on during the very many low points. Your parents' PhDs have dominated much of your lives; Annabelle, you even wrote several PhDs of your own… but I hope that very soon 'Little Miss Work' will become 'Little Miss Play' again!

## Table of Contents

## List of figures

## List of tables

## List of abbreviations

| | |
|---|---|
| ActR | Activin receptor |
| AID | Activation-induced cytidine deaminase |
| ALK | Activin receptor-like kinase |
| AMH | Anti-Müllerian hormone |
| APAF1 | Apoptotic protease activating factor 1 |
| APS | Ammonium persulfate |
| ATM | Ataxia telangiectasia mutated |
| BAC | Bacterial artificial chromosome |
| BART | BamHI A rightward transcript |
| BCL (2 or 6 or -$X_L$) | B cell lymphoma |
| BCR | B cell receptor |
| BFL1 | BCL2 family protein A1 |
| BH | BCL2 homology |
| BIC | B cell integration cluster |
| BIM | BCL2 Interacting Mediator of cell death |
| BL | Burkitt lymphoma |
| BLIMP1 | B lymphocyte-induced maturation protein 1 |
| BM | Bone marrow |
| BMP | Bone morphogenetic protein |
| BMPR | BMP receptor |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| CBP | CREB-binding protein |
| CBF1 | C-promoter binding factor 1 |
| CD | Cluster of differentiation |
| CDK | Cyclin-dependent kinase |
| CDKI | Cyclin-dependent kinase inhibitor |
| cDNA | Complementary DNA |
| C/EBP | CCAAT enhancer binding protein |
| c-FLIP | Cellular homologue of FLICE-inhibitory protein |
| ChIP | Chromatin immunoprecipitation |
| ChIP-seq | ChIP-sequencing |
| CLL | B-cell chronic lymphocytic leukaemia |
| CLP | Common lymphoid progenitor |
| Co-Smad | Common mediator Smad |
| Cp | C promoter |
| CpG | Cytosine-phosphate-guanine |
| CR2 | Complement receptor 2 |
| CSR | Class switch recombination |
| Ct | Threshold cycle |
| CTAR | C-terminal activation region |
| CtBP | C-terminal binding protein |
| DISC | Death-inducing signalling complex |
| DLBCL | Diffuse large B-cell lymphoma |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |

| | |
|---|---|
| DNMT | DNA methyltransferase |
| DSB | Double strand break |
| DZ | Dark zone |
| EA | Early antigen |
| EBER | EBV-encoded small RNA |
| EBNA | EBV nuclear antigen |
| EBNA-LP | EBV nuclear antigen-leader protein |
| EBNA2-ER | EBNA2-oestrogen receptor fusion protein |
| EBV | Epstein-Barr virus |
| EDTA | Ethylene diamine tetra-acetic acid |
| EED | Embryonic ectoderm development |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal-regulated kinase |
| EtOH | Ethanol |
| EZH2 | Enhancer of zeste homologue 2 |
| FC | Flow cytometry |
| FCS | Foetal calf serum |
| FDC | Follicular dendritic cell |
| FL | Follicular lymphoma |
| FLICE | FADD-like interleukin-1 beta-converting enzyme |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GC | Germinal centre |
| GDF | Growth and differentiation factor |
| GFP | Green fluorescent protein |
| GIPC | GAIP (G-alpha interacting protein)-interacting protein, c-terminus |
| GNB2L1 | Guanine nucleotide-binding protein subunit beta-2-like 1 |
| GRU | Green Raji units |
| HDAC | Histone deacetylase |
| HGF | Hepatocyte growth factor |
| HHV | Human herpesvirus |
| HIV | Human immunodeficiency virus |
| HL | Hodgkin lymphoma |
| HLA | Human leucocyte antigen |
| HRP | Horseradish peroxidase |
| HRS | Hodgkin and Reed-Sternberg |
| H3K4Me3 | Trimethylation of lysine 4 of histone H3 |
| H3K9Ac | Acetylation of lysine 9 of histone H3 |
| H3K27Me3 | Trimethylation of lysine 27 of histone H3 |
| HSC | Haematopoietic stem cell |
| HT (4HT) | 4-hydroxytamoxifen |
| HTLV1 | Human T-lymphotropic virus 1 |
| IAP | Inhibitor of apoptosis |
| ICAM-1 | Intracellular adhesion molecule -1 |
| Id or ID | Inhibitor of DNA binding, or inhibitor of differentiation |
| IE | Immediate early |
| IFN | Interferon |
| Ig | Immunoglobulin |

| | |
|---|---|
| IL | Interleukin |
| IM | Infectious mononucleosis |
| IP | Immunoprecipitation |
| IR | Internal repeat |
| I-Smad | Inhibitory Smad |
| ITAM | Immune-receptor tyrosine-based activation motif |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| kb | Kilobases |
| kbp | Kilobase pairs |
| kDa | KiloDalton |
| KO | Knockout |
| KSHV | Kaposi sarcoma herpesvirus |
| LANA | Latency-associated nuclear antigen |
| LB | Luria Bertani |
| LCL | Lymphoblastoid cell line |
| LFA | Leucocyte function-associated antigen |
| LMP | Latent membrane protein |
| LZ | Light zone |
| MALT | Mucosa-associated lymphoid tissue |
| MAPK | Mitogen activated protein kinase |
| MCL1 | Myeloid cell leukaemia sequence 1 |
| MDM2 | Murine double minute 2 |
| MHC | Major histocompatibility complex |
| MH1, MH2 | MAD homology 1, 2 |
| MIRA | Methylated CpG island recovery assay |
| miRNA or miR | MicroRNA |
| MIS | Müllerian inhibiting substance |
| mRNA | Messenger ribonucleic acid |
| NCoR | Nuclear receptor co-repressor |
| NFκB | Nuclear factor kappa B |
| NK | Natural killer |
| NPC | Nasopharyngeal carcinoma |
| OriLyt | Origin of lytic replication |
| OriP | Origin of plasmid replication |
| PARP | Poly ADP ribose polymerase |
| PB | Peripheral blood |
| PBL | Peripheral blood lymphocyte |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate-buffered saline |
| PcG | Polycomb group |
| PCR | Polymerase chain reaction |
| PEL | Primary effusion lymphoma |
| PI3-K | Phosphatidylinositol-3-kinase |
| PI | Propidium iodide |
| PMA | Phorbol myristate acetate |
| PMSF | Phenyl methyl sulfonyl fluoride |

| | |
|---|---|
| PRC | Polycomb repressive complex |
| pSMAD | Phosphorylated SMAD |
| PTLD | Post-transplant lymphoproliferative disorder |
| PUMA | p53-upregulated modulator of apoptosis |
| Qp | Q promoter |
| qPCR | Quantitative polymerase chain reaction |
| qRT-PCR | Quantitative reverse-transcriptase polymerase chain reaction |
| RAG | Recombination activating genes |
| Rb | Retinoblastoma |
| RBP-Jκ | Recombinant binding protein J kappa |
| Rev | Revertant to wild-type |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| R-Smad | Receptor-regulated Smad |
| SBE | Smad-binding element |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| SHM | Somatic hypermutation |
| shRNA | Short hairpin RNA |
| siRNA | Small interfering RNA |
| Smurf | Smad ubiquitin regulatory factor |
| STAT | Signal transducer and activator of transcription |
| sTGFβR3 | Soluble TGFβR3 |
| SUZ12 | Suppressor of zeste 12 homologue |
| TAK1 | TGFβ-associated kinase 1 |
| TBE | Tris-Borate-EDTA buffer |
| TBS | Tris-buffered saline |
| TEMED | Tetramethylethylenediamine |
| TGFβ | Transforming growth factor beta (TGFβ1 if not otherwise specified) |
| TGFβR1 | Transforming growth factor beta receptor 1 |
| TGFβR2 | Transforming growth factor beta receptor 2 |
| TGFβR3 | Transforming growth factor beta receptor 3 |
| TNF | Tumour necrosis factor |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| TR | Tandem repeat |
| TRADD | TNF-receptor-associated death domain |
| TRAF | TNF-receptor activating factor *or* TNF-receptor associated factor |
| TRAIL | TNF-related apoptosis inducing ligand |
| TSA | Trichostatin A |
| TSG | Tumour suppressor gene |
| TSS | Transcription start site |
| UTR | Untranslated region |
| VCA | Viral capsid antigen |
| Wp | W promoter |
| WT | Wild-type |

# Chapter 1 Introduction

## 1.1 Epstein-Barr virus

### 1.1.1 Overview

Epstein-Barr virus (EBV) is a gamma-herpes virus that persistently infects over 90% of the human population worldwide. In less developed countries, EBV infection is mostly acquired in childhood, whereas in more developed countries acquisition may be delayed until the teenage years (Crawford 2001). Primary infection is usually asymptomatic in children, but if acquired at an older age it can cause the syndrome of infectious mononucleosis (IM). Once infection is acquired, it persists for the life of the host within B lymphocytes (B cells), kept under control by the cellular immune response. Persistent infection is generally asymptomatic to the immunocompetent host, but in the setting of immune suppression it can be associated with development of B cell lymphoproliferative diseases thought to be due to defective immune responses. Even in apparently immunocompetent hosts, however, EBV is causally associated with several types of lymphoid malignancy, including Burkitt lymphoma (BL), Hodgkin lymphoma (HL) and certain T cell lymphomas (Crawford 2001), as well as with epithelial cell malignancies including nasopharyngeal carcinoma (NPC) and gastric carcinoma (Shibata and Weiss 1992, Young and Rickinson 2004).

### 1.1.2 The discovery of EBV as the first human tumour virus

In 1957, Denis Burkitt, a British surgeon working in Uganda, observed two cases of a tumour presenting in the jaw of children. He searched the records of Mulago Hospital for similar cases and thus described a novel tumour, composed of small round cells and subsequently known as BL, in 1958 (Burkitt 1958). Burkitt went on to show epidemiologically that this tumour was mostly confined to a particular region of Africa, subsequently known as the 'Lymphoma belt' (Smith 2012). He presented these findings at the Middlesex Hospital during a visit to England in 1961, describing the tumour's association with climatic conditions including temperature and rainfall. A medical virologist, Anthony Epstein, was in the audience, and became fascinated by this tumour, postulating that the climatic variation suggested an infectious cause for the tumours. A collaboration was established, and samples

of the tumour were transported to England to be investigated by Epstein. After multiple failed attempts to identify virus in the samples, Epstein and colleagues managed to establish the first cell line from one of the BL tumours, designated EB1, in 1964 (Epstein and Barr 1964). Examination of a fixed embedded section of EB1 cells by electron microscopy revealed virus particles, reported with his laboratory colleagues Bert Achong and Yvonne Barr (Epstein et al. 1964). Thus the Epstein-Barr virus, as it later became known, was the first human tumour virus identified (Pattle and Farrell 2006). Four years later, EBV was shown to be the causative agent of IM (Evans et al. 1968, Henle et al. 1968), although IM had been described long before this.

### 1.1.3 Infectious mononucleosis and the immune response to EBV

The life cycle of EBV has two distinct phases: the lytic cycle (for viral replication and spread to other hosts) and the latent cycle (for viral persistence). When symptomatic, acute EBV infection results in IM, typically comprising fever, malaise, pharyngitis, headaches, generalised lymphadenopathy, hepatomegaly and splenomegaly (Crawford 2001). There is an associated marked lymphocytosis, with the blood film showing atypical lymphocytes, which consist mainly of CD8 positive T cells reactive predominantly against lytic, but also against latent EBV antigens; NK cells are also increased during IM (Callan et al. 1998, Hislop et al. 2007, Balfour et al. 2013). EBV infection of B cells initially drives them to proliferate as B lymphoblasts (described in further detail below), with up to 18% of B cells being EBV-positive early after infection (Robinson et al. 1980, Hislop et al. 2007). The symptoms of IM are largely due to the massive T cell response to the infection (Crawford 2001). In IM, there is prolonged high level shedding of virus in the oropharynx for several months. EBV-specific CD8+ T cells are found in significantly reduced numbers in the tonsil compared to blood in IM, suggesting that immune control is less efficient in the tonsil than in the blood (Hislop et al. 2005).

T cell immune surveillance is thus highly important in controlling persistent EBV infection, but is unable to completely clear the virus. Normally the infected B cells are targeted promptly by cytotoxic T cells to limit their proliferation. In immunocompetent EBV carriers, an equilibrium develops between proliferation of infected B cells and the immune response.

Even in asymptomatic, immunocompetent carriers up to 5% of the total circulating pool of CD8 positive T cells are reactive against EBV antigens (Tan et al. 1999, Hislop et al. 2007), and there is continued low level shedding of virus, with low numbers of latently infected B cells in the blood (approximately 1-50 per million B cells) (Khan et al. 1996).

Defective T cell immunity predisposes to EBV-associated lymphoid proliferation. EBV-associated B cell lymphomas occur with increased frequency following iatrogenic immunosuppression, particularly following solid organ transplantation, but also less commonly after treatment of inflammatory disorders with immunosuppressive agents. An increased incidence of EBV-driven lymphoid proliferations is also seen in HIV infection as well as in several congenital immune deficiency syndromes (Swerdlow et al. 2008). Even in the absence of clinically symptomatic disease, immunosuppressed individuals have an expansion of latently infected B cells in the peripheral blood, as well as a higher EBV DNA viral load, as a result of viral replication, compared to immunocompetent carriers (Babcock et al. 1999).

There is increasing evidence that NK cells are important in the immune response to EBV [reviewed in (Chijioke et al. 2013)]. *In vitro*, NK cells can limit the transformation of B cells by EBV in the first few days after infection, due to production of interferon-gamma (IFNγ), with tonsillar NK cells producing significant amounts of IFN (Strowig et al. 2008).

### 1.1.4 Establishment of latent infection: *in vivo* and *in vitro*

As well as B cells, EBV can infect epithelial cells and rarely other cell types, although the efficiency of infection is greatest for B cells. The virus initially enters B cells by binding of its envelope protein gp350 to complement receptor 2 (CR2), also known as CD21, on the B cell surface. *In vivo*, EBV persists long-term within the population of resting memory B cells (Thorley-Lawson 2001). In this latent phase a limited set of nine viral proteins is expressed, including six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP) and three latent membrane proteins (LMP1, LMP2A and LMP2B). Expression of these nine viral genes is known as latency III expression or the growth programme.

EBV is spread between human hosts by oral transmission through saliva. Most new cases of EBV infection are acquired from an individual with an established persistent infection rather than during acute infection (Longnecker et al. 2013). Although the mechanism is not fully understood, it is thought that transmitted virus initially infects epithelial cells in the oropharynx, replicating within them and releasing virus particles locally. Although epithelial cells in the tonsil, but not in other parts of the body, express CD21 mRNA (Jiang et al. 2008), they are not thought to express CD21 protein and thus it is not clear how epithelial infection occurs (Shannon-Lowe and Rowe 2011). Local epithelial replicative infection can then infect naïve resting B cells as they circulate through the oropharynx, within tonsillar mucosa-associated lymphoid tissue (MALT).

To establish persistent infection *in vivo*, David Thorley-Lawson has proposed the 'Germinal Centre model' in which EBV utilises the normal B cell differentiation pathway in order to gain access to the population of memory B cells (Thorley-Lawson 2001, Hawkins et al. 2013, Thorley-Lawson et al. 2013) (see section 1.2 for further details of B cell differentiation pathways including the germinal centre). In this model, EBV initially infects naïve B cells and drives these to proliferate as activated B cell blasts, with differentiation within germinal centres to become centroblasts, centrocytes and finally resting memory B cells. This series of events involves silencing of EBV gene expression from latency III (in which the full set of nine latent proteins is expressed), via latency II and latency I to latency 0 in which no EBV proteins can be detected (fig. 1.1) (Thorley-Lawson and Gross 2004). The proteins expressed in each latency stage are also shown (fig. 1.1).

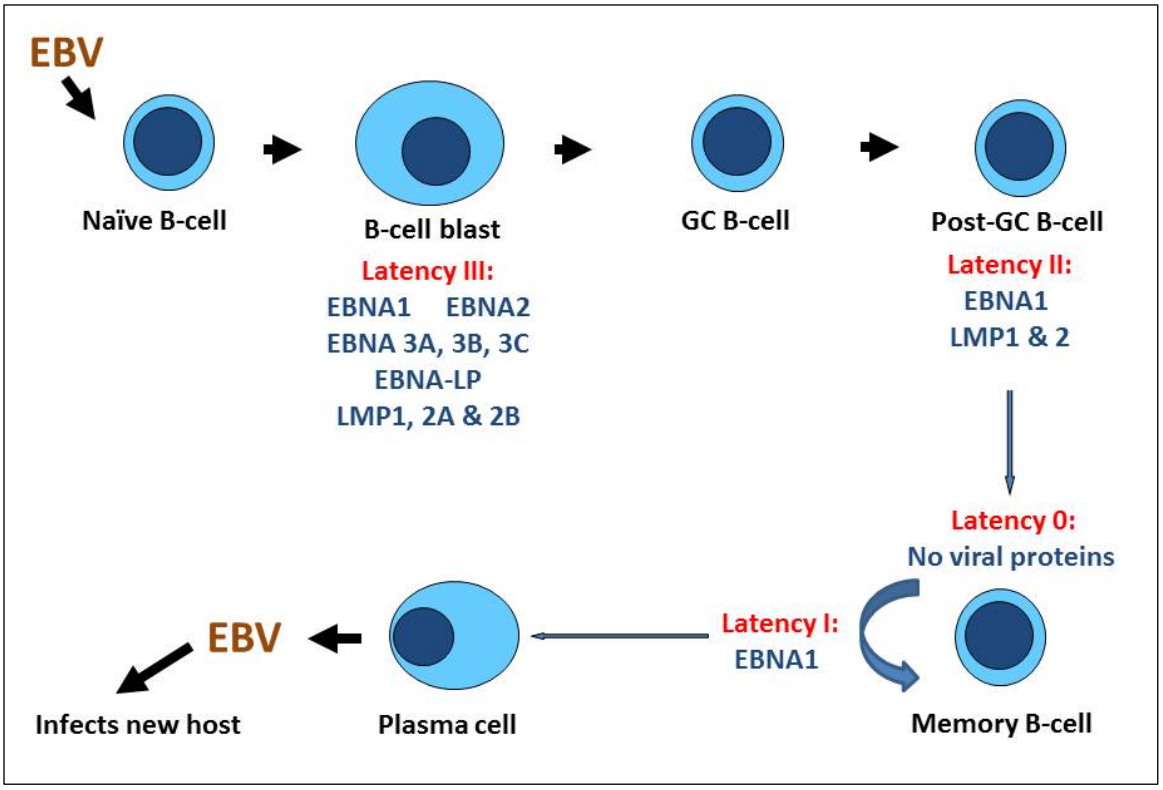



**Figure 1.1: EBV persistence**

Thorley-Lawson has proposed a model in which EBV initially infects naïve B cells and drives them to differentiate into B cell blasts, which are rapidly proliferating. At this stage the full set of nine latency proteins is expressed (latency III or the growth programme). The infected cell then goes to a germinal centre (GC) where it differentiates into a GC B cell. By the time it leaves the GC as a post-GC B cell, it has down-regulated expression of EBNA2, the EBNA3 proteins and EBNA-LP, expressing a more limited set of proteins (latency II). It then differentiates further into a memory B cell, at which stage no viral proteins are expressed, except when the cell divides, when EBNA1 is expressed to ensure viral replication. The memory B cell can subsequently differentiate into a plasma cell, becoming lytic in the process, releasing new EBV virions which can then infect further B cells or be secreted in saliva and infect a new host. Adapted from (Thorley-Lawson and Gross 2004).

Once the EBV-infected cells differentiate into memory B cells and leave the GC, some cells differentiate further to become plasma cells. This differentiation leads to activation of the BZLF1 promoter (see section 1.1.8 on lytic replication for details), resulting in lytic EBV replication (Laichalk and Thorley-Lawson 2005), hence free virus particles are released into the blood. It is also thought that infected memory B cells, which circulate through blood and lymphoid organs, can differentiate into plasma cells in the oropharynx, undergoing lytic replication there. The released virus particles can then infect oropharyngeal epithelial cells or be shed as virus directly into the saliva, thus transmitting the virus to a new host (Longnecker

et al. 2013). In asymptomatic carriers, virus is shed in saliva continuously, although the level of virus shedding varies over time within an individual. However, as the level of shedding is too great to be explained merely by tonsillar B cell infection, it has been proposed that repeated, short-lived exponential infection of tonsillar epithelial cells, initiated by lytic replication in an infected plasma cell, leads to continuous salivary viral shedding (Hadinoto et al. 2009). In addition, concurrent oropharyngeal infection, for instance with group A streptococcus, a common cause of pharyngitis, can lead to increased viral lytic replication and hence increased salivary viral shedding (Ueda et al. 2014).

*In vitro*, EBV can transform B cells to produce lymphoblastoid cell lines (LCLs), which express latency III EBV gene products. It is thought that EBV drives B cells to proliferate as lymphoblasts but they are unable to differentiate further *in vitro* and hence full latency III expression remains. More recently it has been shown that EBV infection *in vitro* leads to an initial hyper-proliferative phase in the first few days after infection, possibly equivalent to the hyper-proliferation seen within germinal centres (see section 1.2 on B cell development), with induction of cellular genes involved in proliferation, such as *MYC*. Cellular genes responsible for limiting DNA damage are also activated. This phase is then followed by a phase of slower proliferation resulting in establishment of LCLs (Nikitin et al. 2010).

Infection of epithelial cells by EBV *in vitro* is more difficult to achieve, although has been successfully demonstrated by Shannon-Lowe *et al* using direct transfer of virus from the surface of recently infected B cells (Shannon-Lowe et al. 2009). Although the epithelial cells were infected by this method, infection was not maintained, and in fact the EBV genome was gradually lost from the initially infected cells during successive cellular replications, suggesting that EBV infection does not provide a growth advantage in epithelial cells (Shannon-Lowe et al. 2009).

There is also recent evidence that one of the earliest viral products of lytic infection, BZLF1 (see section 1.1.8 on lytic replication), is also transiently expressed early after infection of naïve B cells and helps to drive the initial proliferation of B cells, without inducing lytic viral replication at this stage; in addition other lytic genes are transiently expressed early after infection (Kalla et al. 2010, Kalla and Hammerschmidt 2012).

### 1.1.5 Classification and structure of EBV

EBV is a member of the *Herpesviridae*, of which there are eight viruses infecting humans, designated Human herpesviruses (HHV) 1-8; EBV is also known as human herpesvirus 4 (HHV-4). Herpesviruses are generally found ubiquitously, with a high seroprevalence in adults. They are mostly acquired in childhood and result in persistent infection for the lifetime of the host. These infections are usually asymptomatic in immunocompetent hosts but can cause severe disease in immune-compromised hosts.

The *Herpesviridae* are subdivided into three subfamilies, the alpha, beta and gamma *herpesvirinae*, based on their biological properties. EBV is a gamma-herpesvirus; these are characterised by latency in lymphocytes and the ability to induce lympho-proliferation. There are four genera within the gamma-herpesvirinae, of which only two contain members that infect humans: the gamma-1 or *Lymphocryptovirus* genus, which includes EBV, and the gamma-2 or *Rhadinovirus* genus, which includes KSHV (HHV-8) (Pellett and Roizman 2013).

Like other herpesviruses, the EBV particle consists of a linear, double-stranded DNA core, enclosed within a nucleocapsid (containing viral capsid antigen, VCA), which is in turn surrounded by a protein tegument and then an outer envelope studded with glycoproteins (Longnecker et al. 2013). These glycoproteins are important for binding to and fusion with host cellular membranes, for example gp350 which binds to CR2 on B cells and gp42 which is needed for fusion between the virus envelope and the host cell membrane.

### 1.1.6 The EBV genome

The EBV genome is approximately 172 kilobase pairs (kbp) in size (see fig. 1.2). The genome is linear within the virus particle but becomes circularised within the host cell as an extrachromosomal episome. There is a single repeated sequence found at both terminal regions of the linear genome, the terminal repeat (TR), and also a 3 kbp tandemly repeated sequence within the genome, the internal repeat (IR1). There are usually 6-12 repeats of IR1; this is of significance as it contains the W promoter (Wp).

By 16-20 hours post-infection in B cells, the viral genome becomes circularised and then transcription of viral genes commences. The first transcripts, initiated at Wp, are EBNA2 and

EBNA-LP; these are followed by EBNA1. Transcription then switches to the C promoter (Cp) and gives rise to transcripts coding for EBNA-LP, 2, 3A, 3B, 3C and 1 (see fig. 1.2). LMP1 and LMP2 are subsequently transcribed from a promoter responding to EBNA2 (Bornkamm and Hammerschmidt 2001). The Cp and Wp promoters drive transcription of the six EBNA genes, and their function is mutually exclusive (Woisetschlaeger et al. 1989); after infection of B cells by EBV, Wp is used initially and then Cp is up-regulated.

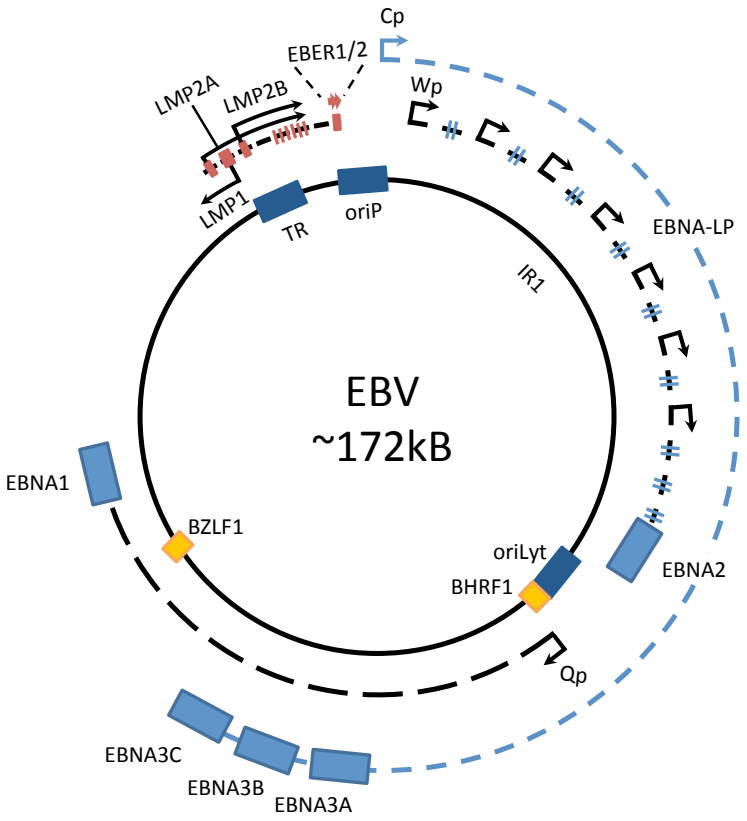



**Figure 1.2: Schematic diagram of the circularised EBV episome**

The location of the latent genes and their promoters, as well as immediate early lytic genes, are shown. OriP = origin of plasmid replication, oriLyt = origin of lytic replication. IR1 = internal repeat region. Wp, Cp and Qp are alternative promoters. The immediate early genes, BZLF1 and BHRF1, are shown in yellow. Adapted from (Kalla and Hammerschmidt 2012).

### 1.1.7 EBV latent gene products

The latency-associated viral gene products and their functions will now be described in more detail; not all are absolutely required for B cell transformation.

### 1.1.7.1 EBV nuclear antigens

**EBNA1**

EBNA1 is expressed in all forms of latency, as well as all EBV-associated tumours, and maintains the viral genome in its episomal state through sequence-specific binding to the plasmid origin of viral replication (OriP). It enhances transcription from Cp and also inhibits transcription from Qp, hence contributing to transcriptional regulation of the EBNAs (including EBNA1 itself) and LMP1 (Young and Rickinson 2004). It also tethers the EBV genome to host cell chromosomes in metaphase, ensuring partitioning of the EBV genome during cell division (Kanda et al. 2001).

**EBNA2**

EBNA2 is one of the first proteins expressed after virus entry into B cells and is absolutely required for B cell transformation to LCLs (Cohen et al. 1989, Hammerschmidt and Sugden 1989). It binds to the host DNA-binding protein recombination signal binding protein for immunoglobulin kappa J region (RBP-Jκ), also known as C-promoter binding factor 1 (CBF1), a downstream component of the Notch signalling pathway (Waltzer et al. 1994, Zimber-Strobl et al. 1994, Zimber-Strobl and Strobl 2001). It transactivates both viral and cellular genes, including the viral latency genes LMP1 and LMP2A and the cellular genes *CD21* and *CD23* (Bornkamm and Hammerschmidt 2001). It also activates *MYC* (Kaiser et al. 1999). EBNA2 also drives the switch from Wp to Cp usage that occurs early after B cell infection (Woisetschlaeger et al. 1991).

Two types of EBV have been identified, types 1 and 2 (or A and B), according to differences in the EBNA2 protein (Dambaugh et al. 1984). There are also sequence variations in EBNA3A, EBNA3B and EBNA3C and EBNA-LP between the two types (Rowe et al. 1989). Type 1 is more efficient at B cell transformation than type 2 *in vitro* (Rickinson et al. 1987), due to a stronger and faster induction of LMP1 (Cancian et al. 2011). Type 1 is thought to be more common in most populations, although the frequency of type 2 is increased in Central Africa and New Guinea such that the two types have similar frequencies in these regions (Young et al. 1987).

**The EBNA3 proteins**

EBNA3A, EBNA3B and EBNA3C are each composed of over 900 amino acids and are encoded by a short 5' exon and a long 3' exon, arranged in tandem within the EBV genome. Their transcripts are alternatively spliced from very long mRNAs initiated at Cp or Wp (Bodescot and Perricaudet 1986, Bornkamm and Hammerschmidt 2001). Full determination of the secondary structure of the EBNA3 proteins has proved elusive, but they all have a proline-rich domain (Yenamandra et al. 2009). They share approximately 40% amino acid sequence homology, suggesting they have evolved from gene duplication events (Yenamandra et al. 2009).

The EBNA3 proteins cannot bind to DNA directly but interact with RBP-Jκ, which itself binds to DNA. Hence the EBNA3 proteins act as transcriptional regulators, modulating expression of both viral and cellular genes. They compete with EBNA2 for binding to RBP-Jκ and therefore antagonise the activity of EBNA2 in transient assays (Waltzer et al. 1996). EBNA3A and EBNA3C, but not EBNA3B, were originally thought to be essential for B cell transformation/immortalisation (Tomkinson et al. 1993); however, it has recently been shown that EBNA3A can be dispensed with in immortalisation, although the outgrowth of cells is less efficient and requires the presence of feeder cells (Hertle et al. 2009). EBNA3 proteins are targets for EBV-specific CD8 T cells responses and are highly immunogenic (Murray et al. 1992), with those CD8+ T cells reactive against latent antigens being predominantly directed against the EBNA3 proteins (Hislop et al. 2007).

EBNA3C was first demonstrated to be a transcriptional regulator in the early 1990s, when it was shown to up-regulate CD21 and LMP1 expression (Wang et al. 1990a, Allday et al. 1993). It was subsequently shown that it does not bind directly to the *CD21* promoter (Radkov et al. 1997) and thus was thought not to bind directly to DNA but instead to exert its effects on transcriptional activity indirectly via interactions with DNA binding proteins such as PU1 and RBP-Jκ (Robertson et al. 1995, Zhao and Sample 2000). EBNA3C interacts with several transcriptional co-activators and co-repressors, including p300 and histone deacetylases (HDAC) (Radkov et al. 1999, Subramanian et al. 2002), and to bind the co-repressor of transcription C-terminal binding protein (CtBP) (Touitou et al. 2001, Skalska et al. 2010). EBNA3C has also been shown to have oncogenic functions by cooperating with activated Ras

to immortalise rat embryonic fibroblasts (Parker et al. 1996). Furthermore, EBNA3C deregulates cell cycle checkpoints (Parker et al. 2000, Krauer et al. 2004). EBV infection induces the DNA-damage response early after B cell infection *in vitro*, and EBNA3C is important in limiting this response and thus allowing survival of the infected cells (Nikitin et al. 2010).

EBNA3A was also subsequently shown to bind to CtBP and cooperate with Ras to transform rat embryonic fibroblasts (Hickabottom et al. 2002). EBNA3A and EBNA3C cooperate to down-regulate BIM, a pro-apoptotic protein important in the pathogenesis of Burkitt lymphoma (Anderton et al. 2008, Paschos et al. 2009), and they also interact with CtBP to repress $p16^{INK4A}$, hence allowing the proliferation of LCLs (Maruo et al. 2006, Skalska et al. 2010, Maruo et al. 2011, Skalska et al. 2013). In fact, extensive cooperation between the EBNA3 proteins in regulation of cellular genes has been shown in a global exome microarray analysis, with 390 out of 1201 differentially regulated genes requiring more than one of the EBNA3s for their regulation [www.epstein-barrvirus.org.uk (White et al. 2010)].

EBNA3B is completely dispensable for B cell transformation, yet it has not been selected against during the course of evolution, suggesting it has a role *in vivo*. It has recently been demonstrated to act as a tumour suppressor in a humanised mouse model (White et al. 2012). A case of a highly aggressive PTLD *in vivo* has been reported in which the virus had an EBNA3B deletion, further supporting the tumour suppressor effect of EBNA3B (Gottschalk et al. 2001). Thus it is thought to counteract the oncogenic properties of other EBV latent antigens, ensuring survival of the host in order to transmit infection to others.

**EBNA-LP**

EBV leader protein (EBNA-LP) is highly expressed, along with EBNA2, early after EBV-induced B cell transformation. It is transcribed from multiple copies of Wp, since Wp is contained within the internal repeat (IR1) region of the EBV genome (see fig. 1.2). Hence multiple different-sized isoforms of EBNA-LP are initially expressed during B cell transformation. Then, as immortalisation becomes established, transcription occurs increasingly from Cp rather than Wp and hence the expression of EBNA-LP decreases (Nitsche et al. 1997) (see fig.1.2). EBNA-LP is a potent co-activator of EBNA2, activating EBNA2-mediated transcriptional

activation of viral and cellular genes, and is therefore important for B cell transformation, for instance it enhances the transactivation of LMP1 by EBNA2 (Nitsche et al. 1997). EBNA-LP causes the relocation of HDAC4 and HDAC5 from EBNA2-responsive promoters to the cytoplasm (Portal et al. 2006) and displaces the transcriptional co-repressor nuclear receptor co-repressor (NCoR), which would otherwise inhibit RBP-Jκ, from EBNA2-responsive promoters, thus enhancing the effect of EBNA2 which acts to increase transcription via RBP-Jκ (Portal et al. 2011).

#### 1.1.7.2 Latent membrane proteins

**LMP1**

It was originally thought that LMP1 was absolutely required for B cell transformation (Kaye et al. 1993) and for continued growth of established LCLs (Kilger et al. 1998). In the absence of EBNA2, LMP1 was not able to maintain proliferation but did increase B cell survival (Zimber-Strobl et al. 1996). Subsequent work by Wolfgang Hammerschmidt's group, however, has shown, using LCLs with conditional expression of LMP1, that when LMP1 expression was very low, the cells were able to survive but unable to proliferate (Kilger et al. 1998). Members of this group have successfully managed to produce LCLs with LMP1 KO virus, but at markedly reduced frequency compared to wild-type, and only in the presence of irradiated fibroblast feeder cells (Dirmeier et al. 2003), confirming that LMP1 is not absolutely required for transformation but the efficiency of this is extremely reduced without LMP1 expression.

LMP1 is a membrane protein of 386 amino acids, with six transmembrane domains and a 200-amino-acid cytoplasmic domain, containing two C-terminal activation regions, CTAR1 and CTAR2, which are essential for its signalling functions. CTAR1 associates with tumour necrosis factor (TNF)-receptor activating factors (TRAFs), and CTAR2 associates with TNF-receptor-associated death domain proteins (TRADD), both activating the nuclear factor kappa-B (NFκB) pathway (Huen et al. 1995). The TRAFs interact with other kinases, resulting in activation of other signalling pathways including NFκB, phosphoinositide-3 kinase (PI3-K)/Akt, extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK)

and Janus kinase/signal transducers and activators of transcription (JAK/STAT) (Young and Rickinson 2004).

LMP1 thus functions as a constitutively active member of the TNF-receptor superfamily, providing growth and survival signals to B cells by mimicking the CD40 interaction with CD40 ligand on T cells (Kilger et al. 1998, Uchida et al. 1999). LMP1 expression in EBV-negative B cell lines results in expression of adhesion molecules (e.g. ICAM-1, LFA-1 and LFA-3) and cell clumping, as well as up-regulation of the B cell activation markers CD23, CD40 and CD44 (Wang et al. 1990a). LMP1 also down-regulates BLIMP1-α, thus disrupting differentiation to plasma cells (Vrzalikova et al. 2011).

LMP1 is oncogenic both *in vitro*, transforming rodent fibroblasts (Wang et al. 1985), and *in vivo*, where its expression results in lymphomas in transgenic mice (Kulwichit et al. 1998). Furthermore, LMP1 expression is protective against apoptosis, by up-regulation of the anti-apoptotic protein BCL2 in B cells (Henderson et al. 1991), as well as by induction of other anti-apoptotic proteins, via NFκB [reviewed in (Allday 2009)] (see section 1.5.3 for further explanation of apoptotic pathways). By mimicking CD40, leading to activation of NFκB, it induces cFLIP expression, inhibiting extrinsic apoptosis pathways [reviewed in (Spender and Inman 2011)]. LMP1 also inhibits BAX promoter activity, another means of preventing apoptosis (Grimm et al. 2005). In addition, it prevents senescence in mouse embryonic fibroblasts by inhibiting induction of the cdk inhibitor $p16^{INK4B}$ (Yang et al. 2000).

**LMP2A and LMP2B**

LMP2A and LMP2B are related integral membrane proteins, each with 12 transmembrane domains and a cytoplasmic tail, which are not required for *in vitro* immortalisation of B cells (Longnecker et al. 1993b, Speck et al. 1999). The LMP2 gene encodes 2 mRNA isoforms, with exons 2-9 common to both LMP2A and LMP2B, but a unique 5' exon for each; exon 1 of LMP2B is non-coding whereas that of LMP2A encodes the cytoplasmic N-terminal sequence, through which signalling occurs (Rechsteiner et al. 2008). The exons encoding the LMP2 proteins are found at both ends of the linear EBV genome and the promoters of both share a bidirectional EBNA2 response element (Harada and Kieff 1997) (see fig. 1.2). The LMP2A

promoter is used to transcribe exon 1 of LMP2A, whereas all the other exons are transcribed from the LMP2B promoter.

LMP2A can act as a constitutively active B cell receptor (BCR) mimic and hence can drive the proliferation and survival of B cells in the absence of BCR signalling (Mancao and Hammerschmidt 2007). Thus it is able to rescue cells lacking strong BCR stimulation due to low affinity for antigen, which would otherwise die, enabling survival of EBV-infected cells. The cytoplasmic N-terminal domain of LMP2A contains an immune-receptor tyrosine-based activation motif (ITAM). In B cells, LMP2A recruits the Src family kinase Lyn to its N-terminal region, leading to constitutive phosphorylation of multiple tyrosine residues, including in the ITAM (Fruehling and Longnecker 1997). This in turn leads to recruitment, phosphorylation and consequent activation of Syk, resulting in constitutive activation of PI3K/Akt, which promotes B cell survival (Portis and Longnecker 2004). LMP2A also activates other signalling pathways including NFκB and MAPK (Swanson-Mungerson et al. 2005, Anderson and Longnecker 2008). LMP2A protects B cells from apoptosis by several mechanisms including induction of anti-apoptotic BCL-$X_L$ and survivin (Portis et al. 2003, Portis and Longnecker 2004, Bultema et al. 2009) (see section 1.5.3 for details on apoptosis). In a murine transgenic model, LMP2A protects from apoptosis by induction of NFκB and BCL2 (Swanson-Mungerson et al. 2010). Furthermore, EBV increases proteasomal degradation of pro-apoptotic BIM via activation of the MAPK/ERK pathway, and this is likely to occur via LMP2A [(Clybouw et al. 2005), reviewed in (Allday 2009)].

The function of LMP2B in B cells is not entirely understood, although it has been shown to bind to LMP2A, blocking its phosphorylation and activation, hence regulating its activity (Rovedo and Longnecker 2007).

#### 1.1.7.3 EBV-encoded RNAs and microRNAs

As well as latent proteins, latent EBV also expresses a number of non-coding RNAs. These include the EBV-encoded small RNAs (EBERs), EBER1 and EBER2, which are of 167 and 172 nucleotides respectively, first described in 1981 [(Lerner et al. 1981) and reviewed in (Swaminathan 2008)] (see fig. 1.2). They are produced abundantly in all EBV-infected cells

and expressed in all latency types, suggesting that they have an important function. Although they are not essential for B cell transformation (Swaminathan et al. 1991), EBERs induce oncogenic properties in EBV-negative Akata cells, including up-regulation of BCL2 leading to resistance to apoptosis (Komano et al. 1999) and enhance proliferation in EBV-negative BJAB BL cells (Yamamoto et al. 2000). However, their functions are still not completely understood.

EBV also produces microRNAs (miRNAs), which are functional but non-coding RNAs of around 21-25 nucleotides in length. miRNAs bind to specific mRNA sequences, usually within their 3' untranslated regions (UTR), leading to reduced translation or enhanced destruction of the target mRNA. They are increasingly recognised as being produced by several different viruses including herpesviruses, and regulate both viral and host gene expression [reviewed in (Grundhoff and Sullivan 2011)]. EBV expresses 44 of these mature miRNAs (Grundhoff and Sullivan 2011), which are expressed from two regions of the EBV genome, the *Bam*HI A region and the *Bam*HI H region. These miRNAs are known as the BamHI A rightward transcript (BART) miRNAs and the BHRF1 miRNAs respectively. The two groups are differentially expressed in cell lines: BART miRNAs are strongly expressed in NPC cell lines, but not EBV-infected primary epithelial cell lines (Shannon-Lowe et al. 2009), and are only weakly expressed in LCLs and EBV-infected BL cell lines. The BHRF1 miRNAs are expressed in LCLs and latency III-expressing BL cell lines but undetectable in NPC cell lines (Cai et al. 2006). EBV miRNAs can regulate both viral and host cellular gene expression.

It is increasingly recognised that miRNAs expressed by oncogenic herpesviruses can contribute to oncogenesis (Grundhoff and Sullivan 2011). In the case of EBV, for example, miR-BART5 has been shown to target the pro-apoptotic protein PUMA, hence leading to resistance to apoptosis in epithelial cells (Choy et al. 2008) and the BART miRNAs can protect BL cells from apoptosis, probably by inhibiting caspase 3 (Vereide et al. 2013). In addition, the BHRF1-3 miRNAs inhibit apoptosis and promote cell cycle progression early after EBV infection of primary B cells (Seto et al. 2010). It is likely that further oncogenic functions of EBV-associated miRNAs will be identified in future.

### 1.1.8 Lytic infection

During lytic viral replication, multiple viral genes are expressed. The first event in a sequence is the expression of immediate early (IE) genes BZLF1 (also known as ZEBRA, Zta or EB1) and BRLF1 (also known as Rta) (see fig. 1.2). These in turn transactivate viral early genes, leading to expression of early antigens including early antigen diffuse component EA-D, which is regulated by the BMRF1 promoter (Holley-Guthrie et al. 1990). Then late genes are expressed, resulting in the structural components of the virions, which can then be released from the host cell, acquiring the envelope by budding through the host cell membrane, and usually killing the host cell in the process. When EBV infects epithelial cells, it generally causes lytic replication, whereas infection of B cells eventually results in persistent (latent) infection, although lytic replication is again initiated when differentiation to plasma cells occurs (Laichalk and Thorley-Lawson 2005, Longnecker et al. 2013).

In a persistently infected host, the majority of infected B cells contain latent virus, but intermittently the virus can reactivate to the lytic form in occasional cells. This is thought to occur in response to B cell stimulation by other antigens, due to stimulation of the BCR. *In vitro*, lytic replication can be induced by several mechanisms including anti-Ig, 12-O-tetradecanoylphorbol-13-acetate (TPA), transforming growth factor beta (TGFβ), histone deacetylase (HDAC) inhibitors and DNA methyl transferase (DNMT) inhibitors, although the ability of these agents to induce lytic replication varies between cell lines (zur Hausen et al. 1978, Ben-Sasson and Klein 1981, Takada 1984, di Renzo et al. 1994, Chang and Liu 2000, Fahmi et al. 2000, Inman et al. 2001, Countryman et al. 2008, Ghosh et al. 2012). These signals act on the two viral IE promoters Zp and Rp to increase their activity, resulting in IE gene expression.

## 1.2 Normal B cell development and function

### 1.2.1 Antibody production

B cells are the main effectors of the humoral immune response, being responsible for antibody production. The antigen specificity of the B cell is provided by a surface receptor, the B cell receptor (BCR), which binds to a particular epitope of an antigen. Every B cell produced has a unique antigen specificity, although all the BCRs on one cell are identical. The BCR can subsequently be secreted by a B cell as antibody. There are five main classes of antibody: IgM, IgD, IgG, IgE and IgA, which all have different effector functions. An antibody is a Y-shaped immunoglobulin molecule (fig. 1.3) consisting of two identical heavy chains and two identical light chains, bound together by disulphide bonds. The light chains have two domains, the variable domain $V_L$ at the N-terminal end and the C-terminal constant domain $C_L$. The heavy chains each have a variable domain $V_H$ at the N-terminal end and at least three (depending on the antibody class) constant domains (fig. 1.3). The variable regions of both heavy and light chains are therefore adjacent on each 'arm' of the molecule, and this is the region that binds to an epitope of a particular antigen. The stem of the molecule, known as the Fc portion, consists of the heavy chain constant regions and its structure varies according to the antibody class. This region performs the effector functions of the antibody, or is bound to the B cell surface membrane in the form of the BCR. There are five main types of heavy chain constant region, γ, μ, δ, α and ε, which determine the class (or istoype) of the antibody produced, which are IgG, IgM, IgD, IgA and IgE respectively. There are two isotypes of light chain, κ and λ.

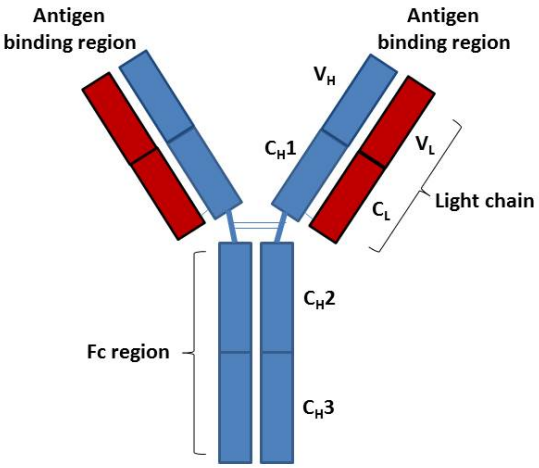



**Figure 1.3: Schematic diagram of antibody structure**

An antibody or immunoglobulin (Ig) molecule consists of a pair of heavy chains (blue) and a pair of light chains (red) joined by disulphide bonds (shown as fine blue lines). The light chain consists of a variable ($V_L$) and a constant ($C_L$) light chain domain. The heavy chain consists of a variable ($V_H$) and at least three constant ($C_H$) domains, $C_H1$, $C_H2$ and $C_H3$. $C_H2$ and $C_H3$ comprise the Fc region, which performs the effector functions of the antibody and varies according to the class (isotype). The unique antigen binding regions are made by $V_H$ combined with $V_L$ at the N-termini.

The diverse repertoire of antigen specificities is enabled by the variable regions of heavy and light chains. The human heavy chain gene locus, on chromosome 14, consists of multiple gene segments including 40 variable (V) gene segments, 25 diversity (D) segments and 6 joining (J) segments (see fig. 1.4). When a B cell makes its heavy chain, one of the D segments is chosen at random and joined to one of the J segments, in the process of DJ rearrangement. Then the DJ segment is joined to a V segment, i.e. VDJ rearrangement. The resulting VDJ is then joined to the appropriate constant region segment depending on which isotype of antibody is to be made. The light chain loci each consist of multiple V and J (but not D) segments and thus VJ rearrangements are made in a similar way to create diversity of the light chains. The recombination of the gene segments is catalysed by the V(D)J recombinase enzyme complex, which includes the proteins RAG1 and RAG2 encoded by recombination activating genes *RAG1* and *RAG2*. Each gene segment is flanked by a recombination signal sequence, and the RAG proteins introduce double strand breaks (DSBs) in the DNA between the gene segments to be joined and their recombination signal sequences (Murphy 2011). RAG then recruits DNA repair enzymes to join the segments. In

this process, a small number of nucleotides may be lost or gained between the segments, and this adds to the diversity generated on the antibody variable region.

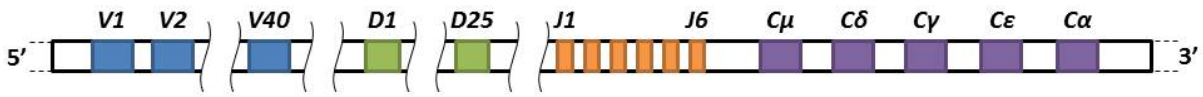
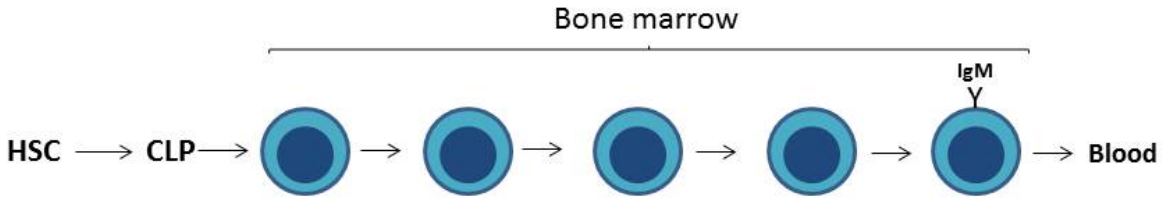



**Figure 1.4: The human heavy chain locus**

The human heavy chain gene locus on chromosome 14 consists of 40 variable (V) segments, 25 diversity (D) segments, 6 joining (J) segments and then the constant (C) segments which encode the five different classes of heavy chain, IgM, IgD, IgG, IgE and IgA respectively. Adapted from (Alberts et al. 2008).

### 1.2.2 B cell development within the bone marrow

B cells develop initially in the bone marrow (BM) from CD34+ haematopoietic stem cells (HSCs), which give rise to all haematopoietic cells. During this maturation process within the BM, which is antigen-independent, the B cells gradually migrate towards the central sinus, remaining in contact with stromal cells. HSCs differentiate first into the common lymphoid progenitor cell, which can give rise to T or B cells (Pieper et al. 2013). Once committed to the B cell lineage, the cell develops through the pro-B then pre-B cell stages (see fig. 1.5). During the process of B cell maturation, immunoglobulin genes are rearranged in turn. First the IgH (Ig heavy chain) locus, with D-J rearrangement occurring in the early pro-B cell, and then V-DJ rearrangement in the late pro-B stage. In the subsequent pre-B cell stage the light-chain genes are rearranged. Once at the immature B cell stage, IgH VDJ and light chain VJ having been rearranged, IgM is now expressed on the cell surface (Murphy 2011). At this stage the cell exits the BM into peripheral blood, and also starts to express surface IgD. These mature naïve, non-proliferating, B cells now migrate via the blood to secondary lymphoid organs (the spleen, lymph nodes, or mucosa-associated lymphoid tissue, MALT) and continue to circulate through these until either they encounter the relevant antigen or die.




| | Early pro-B cell | Late pro-B cell | Early pre-B cell | Late pre-B cell | Immature B cell |
|---|---|---|---|---|---|
| **Gene rearrangement** | D - J rearranging | V-DJ rearranging | Light chain V-J rearranging | Light chain V-J rearranging | All complete |
| **Phenotype** | Proliferating lymphoblast | Proliferating lymphoblast | Proliferating lymphoblast | Non-dividing | Non-dividing small lymphocyte |
| **Expression of Ig** | | | Complete heavy chain (μ) bound to surrogate light chain, briefly expressed on surface | Cytoplasmic μ | Surface IgM with heavy and light chains |

**Figure 1.5: Stages of development of B cells within the bone marrow**

B cells are originally derived from haematopoietic stem cells (HSCs), which differentiate into a common lymphoid progenitor (CLP). The earliest stage of commitment to the B cell lineage is the pro-B cell. In this stage, RAG genes are expressed and rearrangements of the D-J segments of the heavy chain locus occur, followed by V-DJ rearrangement. Once this has successfully occurred, a complete Ig μ heavy chain is expressed, bound to a surrogate light chain as the pre-BCR which is briefly expressed on the cell surface. The cell then differentiates into a pre-B cell, which initially proliferates and production of the surrogate light chain stops. The cell then stops dividing, the RAG genes are re-expressed and light chain V-J gene rearrangements occur. Once a light chain has successfully been produced, the complete IgM molecule is expressed at the surface and the cell is released from the bone marrow as an immature B cell.

### 1.2.3 Further B cell development within secondary lymphoid tissue

B cells encounter antigens bound to follicular dendritic cells (FDCs) within secondary lymphoid tissues. If an antigen presented by FDCs is recognised by the BCR, the BCR-antigen complex is endocytosed, processed by the B cell and presented on the surface in association with major histocompatibility complex (MHC) class II molecules, allowing recognition and

help by antigen-specific CD4+ T-helper ($T_H$) cells. The binding of BCR by antigen stimulates further development of the B cell, which becomes activated, differentiates into a centroblast which proliferates and initiates a germinal centre (GC) reaction within the lymph node (or other lymphoid tissue). The GC reaction is a means of further increasing the specificity of antibody and producing immune effector cells in the form of plasma cells and memory B cells (see fig. 1.6). By light microscopy, GCs have two zones, a dark zone (DZ), consisting of rapidly proliferating B cells, and a light zone (LZ), in which B cells are interspersed with T cells (mainly CD4+) and FDCs (Klein and Dalla-Favera 2008). Within the GC, the centroblast undergoes rapid proliferation (dividing every 6-12 hours), and the enzyme activation-induced cytidine deaminase (AID) is up-regulated. This enzyme deaminates cytosine to uracil, resulting in a G:U mismatch, which is recognised by uracil DNA glycosylase or processed by one of several DNA repair pathways, leading to the formation of single nucleotide changes within the variable regions of heavy and light chain genes (Klein and Dalla-Favera 2008). This process, known as somatic hypermutation (SHM), combined with rapid proliferation, leads to multiple variants with differing affinity for antigen (Fear 2013). From these variants, any with enhanced affinity for the antigen will be selected but the remainder (the majority) will die. Interleukin-4 (IL-4) produced by $T_H2$ cells enhances proliferation/survival of B cells, plus promotes Ig isotype switching to IgG1 and IgE (Snapper et al. 1988, King and Mohrs 2009).

The proliferating centroblasts differentiate into centrocytes and at the same time migrate to the LZ of the GC. Centrocytes with the highest affinity for antigen receive survival signals, leading to down-regulation of pro-apoptotic genes. However, in the absence of such survival signals the default position of centrocytes is to die by apoptosis. GC B cells are therefore generally pro-apoptotic: BCL6, a transcriptional repressor essential for GC formation, is selectively up-regulated in GC B cells and silences anti-apoptotic BCL2 (Saito et al. 2009), and GC B cells express high levels of FAS, part of the extrinsic pathway of apoptosis (see section 1.5.3). In addition, BCL6 represses p53 (Phan and Dalla-Favera 2004), which is normally responsible for DNA damage responses, and thus allows the GC B cells to tolerate the DNA damage induced by the SHM process.

MYC is a highly important DNA-binding transcription factor which regulates multiple genes including those regulating cell cycle progression, apoptosis and senescence. Expression of MYC is increased in LZ B cells but then repressed in DZ B cells by a direct action of BCL6 on the *MYC* promoter. In addition, MYC expression is required for maintenance of GCs *in vivo* (Dominguez-Sola et al. 2012). In those cells that receive survival signals, heavy chain (isotype) class switching occurs, in order to produce IgG, IgA or IgE. This process is also dependent upon AID, and involves deletion of all coding sequences between the VDJ segment and the relevant C segment (for example, Cα if IgA is to be made; see fig. 1.4), a process known as class switch recombination (CSR).

Victora and Nussenzweig have recently proposed an updated model for the GC reaction, in which the driving force behind B cell affinity maturation and selection is competition for T cell help, with signals received through BCR being essential for survival. It has been shown that the functional distinction between the DZ and LZ is less marked than previously thought and that cells migrate cyclically through the LZ and DZ compartments, rather than passing through sequentially. Nevertheless, proliferation occurs mainly in the DZ and selection in the LZ, but these authors propose that the DZ and LZ cells represent the same cell population in different states of activation (Victora et al. 2012, Victora and Nussenzweig 2012).

The mature activated B cells which survive the GC reaction can then develop into either long-term memory B cells (which comprise approximately 40% of the B cell population) or plasma cells, which stay within lymph nodes or migrate to the BM and continue to produce antibody (Murphy 2011). The long-term memory B cells, with high affinity antibody, continue to circulate around the body for many years, and if their antigen is encountered again they are primed to differentiate rapidly into plasma cells and secrete large amounts of high affinity antibody, resulting in a secondary immune response which has a shorter lag time and a greater amount of antibody production than seen in the primary response. Such immunological memory can remain for the lifetime of the host, even if the original antigen was encountered many years previously, and is the basis for vaccination. Memory B cells re-join the pool of continuously circulating B cells.

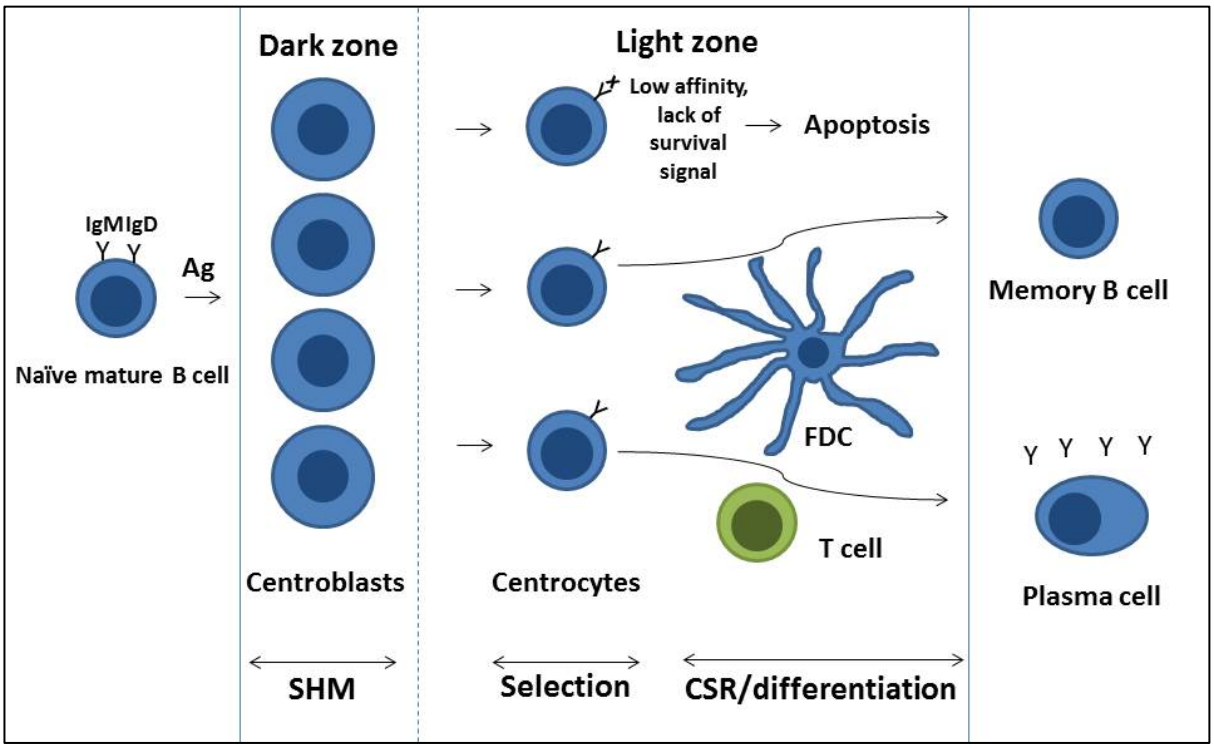



**Figure 1.6: Schematic diagram of the traditional model of the germinal centre reaction**

Resting naïve mature B cells, expressing IgM and IgD, continuously circulate through blood and lymphoid tissue. When the cell meets its antigen (Ag), it differentiates into a centroblast and starts to proliferate rapidly. During this proliferation, somatic hypermutation (SHM), occurs, catalysed by activation-induced cytidine deaminase (AID), to introduce single base-pair changes into the VDJ regions of the rearranged genes encoding the heavy and light Ig chains. This takes place mostly within the dark zone of the GC. The centroblasts then differentiate into centrocytes and migrate to the light zone, where antigen is presented by FDCs and T cell help occurs, in order to select cells with high affinity for antigen. Cells with high affinity bind to antigen and receive survival signals, whereas the default position is for cells to die by apoptosis if such signals are not received. Those cells which survive can then undergo further differentiation into memory B cells or plasma cells with class switch recombination (CSR) occurring, also catalysed by AID, to enable to cell to produce IgG, IgA or IgE antibody. The resulting memory B cells or plasma cells can then leave the lymph node and continue to circulate around the body. Adapted from (Klein and Dalla-Favera 2008).

## 1.3 EBV-associated lymphomas

EBV is associated with several different types of lymphoma, predominantly B cell lymphomas but also some T cell lymphomas; only the B cell lymphomas will be discussed here. Each type of lymphoma is thought to arise from a particular type of cell along the B cell differentiation pathway, with different tumours expressing different latent proteins (see table 1.1).

| Latency type | Viral proteins expressed | Cell/tumour type |
|---|---|---|
| 0 (latency) | None | None identified |
| I | EBNA1 | BL (group I) |
| II (default programme) | EBNA1, LMP1, LMP2 | Hodgkin lymphoma, NPC |
| III (growth programme) | EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2A, LMP2B | PTLD, HIV-associated immunoblastic lymphoma LCLs |

**Table 1.1: Viral proteins expressed in the different EBV latency types and their corresponding tumours**

In latent infection EBV expresses nine latent proteins, the EBV nuclear antigens EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-leader protein (EBNA-LP) as well as three latent membrane proteins LMP1, LMP2A and LMP2B. EBV-associated tumours have characteristic expression of these latent proteins, thought to reflect their cell of origin.

### 1.3.1 Immunodeficiency-associated lymphomas

EBV-associated lympho-proliferative disorders occur with increased frequency in immuno-compromised individuals, including as a result of solid organ or haematopoietic stem cell transplants (known as post-transplant lymphoproliferative disorders or PTLD), in HIV infection or due to other congenital or acquired immune deficiencies. Early-onset PTLD, occurring in the first year after transplant, are usually EBV-positive and express the full complement of latent proteins (latency III). This suggests that they are virus-transformed cells that are able to grow out due to the lack of effective T cell surveillance; the EBNA3s in particular are highly immunogenic such that these cells would normally be quickly destroyed in the presence of functional T cells (Murray et al. 1992, Hislop et al. 2007). As a result, such tumours respond well to adoptively transferred cytotoxic T cell therapy (Gottschalk et al. 2005). PTLD encompasses a spectrum of disease, from a polyclonal lympho-proliferation resembling IM, through polymorphic PTLD to a monomorphic lymphoma resembling diffuse large B cell lymphoma (DLBCL) or another B cell lymphoma such as BL (Swerdlow et al. 2008).

EBV-positive tumours occurring in the setting of advanced HIV infection, in contrast, are aggressive B cell lymphomas and are always monoclonal. These tumours are most commonly immunoblastic lymphomas, a morphological subtype of DLBCL, and include

primary central nervous system lymphoma (Swerdlow et al. 2008). EBV-positive DLBCL of the elderly is a recently defined EBV-positive clonal proliferation that occurs in patients aged over 50 years, without any known predisposing immunodeficiency, with increased incidence in Japan compared to Western countries (Oyama et al. 2003, Oyama et al. 2007). It is believed to develop as a result of the gradual decline in function of the cellular branch of the immune system occurring with increasing age.

### 1.3.2 Hodgkin Lymphoma

Classical Hodgkin lymphoma (HL) is characterised by the presence of occasional large mono- or multinucleated tumour cells, called Hodgkin and Reed-Sternberg (HRS) cells, within an abundant heterogeneous mixture of non-neoplastic cells. The neoplastic HRS cells of Classical HL are EBV-positive in around 40% of cases overall, with variations in EBV-positivity between different histological subtypes and depending on the clinical setting. The subtypes include lymphocyte-rich, nodular sclerosis, mixed cellularity and lymphocyte-depleted, with EBV associated mainly with the latter two subtypes. In tropical regions up to 100% of HL cases are EBV-positive (Swerdlow et al. 2008). HL also occurs with increased frequency in HIV-infected individuals, where almost all cases are EBV-positive (Swerdlow et al. 2008). In EBV-positive cases the neoplastic HRS cells express the latency II set of viral proteins, i.e. EBNA1, LMP1 and LMP2A. Isolated HRS cells have been shown to have clonal immunoglobulin gene rearrangements with a high degree of SHM, suggesting that they are derived from GC B cells which have acquired a crippling mutation rendering them unable to exit from the GC and complete differentiation to memory B cells (Kanzler et al. 1996). LMP2A is able to rescue GC B cells that would otherwise undergo apoptosis, by performing the pro-survival functions of the BCR (Mancao et al. 2005, Mancao and Hammerschmidt 2007).

### 1.3.3 Burkitt Lymphoma

BL is an extremely rapidly-growing tumour of B cells which is characterised by a chromosomal translocation involving the *MYC* proto-oncogene with one of the genes encoding the immunoglobulin heavy or light chains. This leads to aberrant constitutive

expression of MYC, resulting in uncontrolled proliferation but also a lower threshold for induction of apoptosis.

BL can be divided into three forms: endemic, which is always EBV-positive and occurs in association with malaria in children in equatorial Africa and Papua New Guinea; sporadic, which occurs throughout the world in adults with no apparent underlying predisposition and is EBV-positive in around 30% of cases; and immunodeficiency (usually HIV) - associated, in which around 40% of cases are EBV-positive. HIV-associated BL usually occurs with a higher CD4 count than other HIV-associated malignancies, thought to reflect the importance of chronic antigenic B cell stimulation rather than immunosuppression in its pathogenesis [(Kirk et al. 2001), reviewed in (Molyneux et al. 2012, Gloghini et al. 2013)].

It is thought that the BL tumour cell derives from a post-GC cell which continues to proliferate due to deregulation of *MYC.* Double strand breaks (DSB), created by AID, are a necessary intermediate in the process of CSR, with the *MYC* promoter region being essential for the recruitment of AID in order to initiate DSB [reviewed in (Fear 2013)]. Although AID can also induce mutations in many other B cell genes (Liu et al. 2008), these are repaired by different mechanisms which are less subject to error, for example the p53, ataxia telangiectasia mutated (ATM) and $p14^{ARF}$ pathways that protect against DNA damage and oncogenic stress. AID activity is therefore essential for the DSBs that allow translocation of *MYC* to IgH (Robbiani et al. 2008). Point mutations of the translocated *MYC* can also occur in BL, resulting from the action of AID. These can lead to loss of the ability of MYC to induce BIM, thus the mutants retain ability to promote proliferation, but lose ability to induce apoptosis (Hemann et al. 2005).

The exact role of EBV in pathogenesis is still unclear, but it has been proposed that EBV promotes development of BL by causing other changes in cellular genes which enable the cell to tolerate dysregulated *MYC* without undergoing apoptosis or senescence, roughly equivalent to the MYC mutants described above (Thorley-Lawson and Allday 2008, Allday 2009). EBNA3A and EBNA3C cooperate to down-regulate pro-apoptotic BIM, thus again MYC-induced proliferation can occur without induction of apoptosis (Anderton et al. 2008, Thorley-Lawson and Allday 2008, Allday 2009, Paschos et al. 2009).

Although *MYC* translocations are the hallmark of BL, they are not sufficient to cause it, as the presence of the translocation has been identified in normal individuals (Janz et al. 2003) and can occur, at lower frequency, in DLBCL or in the related category described in the 2008 WHO Classification 'B cell lymphoma, unclassifiable, with features intermediate between diffuse large B cell lymphoma and Burkitt lymphoma' (Swerdlow et al. 2008, Lin et al. 2012). The gene expression profile of BL has been identified to be distinct from that of DLBCL (Dave et al. 2006, Hummel et al. 2006) and recently several groups have demonstrated the involvement of recurrent gene mutations in BL, particularly *ID3* which acts as a tumour suppressor (Love et al. 2012, Richter et al. 2012, Schmitz et al. 2012).

### 1.4 Systems for studying the effects of EBV *in vitro* and *in vivo*

Early investigations of the effects of specific EBV latent proteins were performed using naturally occurring EBV-positive BL cell lines in which the virus lacks expression of one or more latent proteins, such as P3HR1-BL or Daudi, which both lack full EBNA2 expression. However, in order to prove that the phenotypic effects of this were due to EBNA2, it was necessary to reinsert the EBNA2 gene into these cells by transfection in order to show reversal of the phenotype [for example (Cordier et al. 1990)]. Therefore it was necessary to develop a system for rationally investigating the effects of other latent proteins for which naturally occurring mutants were not available.

Due to the large size of the EBV genome, it was not possible to adequately study the effects of mutant EBV using conventional cloning techniques. Therefore, the EBV bacterial artificial chromosome (BAC) system was developed by Delecluse *et al* in the late 1990s. They cloned the complete B95.8 EBV genome, along with a green fluorescent protein (GFP) reporter and a hygromycin resistance cassette, into an F-factor based replicon in *E. coli* (Delecluse et al. 1998). This enabled creation of recombinant EBV, which could be inserted by homologous recombination. A shuttle vector system was then used to introduce the BAC into a 293 eukaryotic producer cell line. Recombinant BAC-containing producer cells could then be selected for by addition of hygromycin, which is toxic to BAC-negative cells. The cells can then be induced to lytic replication so that recombinant infectious virions are produced (Delecluse et al. 1998, Feederle et al. 2010). The recombinant viruses obtained can then be

used to infect primary B cells or EBV-negative BL cell lines in order to investigate the phenotypic effects of deletion of individual latent genes.

Studies investigating the effects of EBV early after B cell infection have been done using purified B cells extracted from peripheral blood or occasionally from other lymphoid organs, such as extracted tonsils, or in some case peripheral blood mononuclear cells (PBMCs). LCLs are established *in vitro* by infecting resting B cells or PBMCs with EBV, following which the cells develop into a continuously proliferating immortalised cell line. In this way, the effects of EBV infection on the cellular phenotype can be investigated. LCLs express markers of B cell activation, such as CD23 (Thorley-Lawson and Mann 1985).

BL cell lines have generally been established, as in the initial case by Epstein, by setting up a suspension culture using free-floating cells from a fresh human biopsy sample (Epstein and Barr 1964). Subsequently, cell lines were established in a similar manner from samples of EBV-negative BL (including BL2 and BL31 as used in this study) and it was shown that these could be successfully infected with the prototypic wild-type EBV strain B95.8 *in vitro* to produce a continuously proliferating EBV-positive cell line (Calender et al. 1987). Phenotypically, BL generally correspond to resting B cells, being small and round, whereas LCLs have a phenotype more like activated B blasts, with increased cell size, more voluminous cytoplasm and ruffling of the cell membrane.

There are advantages and disadvantages to studying the effects of EBV using primary B cells/LCLs and *in vitro* infection of EBV-negative BL. The main problem with LCLs is that several of the EBV latent antigens are needed for establishment and continued proliferation of the LCL itself, and therefore it is not possible to directly investigate the effects of deletion/mutation of these genes. Using EBV-negative BL infected with recombinant virus, on the other hand, it is possible to investigate the effects of deletion of individual EBV latent antigens, since this should not affect the proliferation of the cell lines themselves. The disadvantage of BL, however, is that they are already highly dysregulated, having acquired multiple mutations and/or epigenetic changes in order to develop into tumour cells. They therefore match the situation in the normal EBV life cycle in humans less closely than LCLs. Furthermore, any cell line propagated *in vitro* is subject to continuous selection during

repeated passage; for instance it has been shown that changes in expression in multiple genes occur between early and late passage LCLs (Lee et al. 2010).

Since EBV is a human-only pathogen, there is no direct animal model of EBV with which to study its effects *in vivo*. However, immunodeficient mice have had components of the human immune system incorporated, in order to create a humanised mouse model of EBV infection (Yajima et al. 2008). A similar model has recently been used to study the effects of recombinant EBV *in vivo,* for example in (White et al. 2012).

### 1.4.1 Cell lines established from EBV-positive BL

EBV-positive BL can be divided into groups I-III based on their phenotype, according to their degree of phenotypic change towards that of LCLs and their expression of EBV latent proteins, with group I expressing EBNA1 only, group II expressing EBNA2 and LMP1, and group III expressing the full set of nine latent proteins. However, some BL have been shown to express EBNA2 without LMP1 (Rowe et al. 1987). When EBV-positive BL are initially cultured from a fresh biopsy, they usually express CD10, but are negative for CD23, and express type I latency EBV antigens. However, in continued culture they show a phenotypic shift to type III latency expression, along with expression of B cell activation markers including CD23, and loss of CD10 expression, accompanied by a change in morphology to more closely resemble LCLs as well as cell clumping (Rowe et al. 1987).

Group I BL use the Q promoter (Qp) for EBNA1 expression, whereas in latency III (LCLs) the promoters Wp and Cp are used (see fig. 1.2). A subset of BL has more recently been identified in which the latency III-associated promoter Wp, rather than latency-I associated Qp, is active but in which a deletion of EBNA2 results in lack of expression of LMP1, LMP2A and LMP2B and lack of activity of Cp (Kelly *et al*. 2002). These Wp-restricted BL, which express EBNA1, EBNA3A, EBNA3B, EBNA3C and a truncated EBNA-LP, but no EBNA2 or LMPs, are relatively protected from apoptosis induced by anti-IgM and ionomycin when compared to type I BL (which express only EBNA1) (Kelly et al. 2005). The naturally occurring EBV-positive BL which lack full EBNA2 expression, including Daudi and P3HR1-BL (as described above), as well as BAC-derived recombinant EBNA2 KO-infected BL, were similarly resistant

to apoptosis induced by these agents (Kelly et al. 2005). MYC, which is overexpressed in BL, generally makes them prone to apoptosis, and thus the resistance to apoptosis seen in Wp-restricted BL provides a survival advantage. This suggests that the addition of the EBNA3 proteins gives the Wp-restricted BL an advantage in terms of protection against apoptosis induced by several agents. Indeed this has been shown, as for instance EBNA3A and EBNA3C cooperate to down-regulate pro-apoptotic BIM (Anderton et al. 2008, Paschos et al. 2009). The EBNA3 proteins are normally highly immunogenic (Murray et al. 1992); however, these Wp-restricted BL were not recognised by EBV-specific CD8-positive T cells. LMP1 expression enhances antigen-processing activity and expression of human leucocyte antigen (HLA) class I molecules (Rowe et al. 1995, Brooks et al. 2009), and thus it was proposed that the lack of LMP1 expression in Wp-restricted BL allows them to avoid T cell recognition in spite of expressing EBNA3 proteins (Kelly et al. 2002).

### 1.5 Mechanisms of lymphoma development

Lymphoma is a form of cancer, i.e. a clonal proliferation of abnormal lymphoid cells. Cell division and differentiation are normally tightly controlled, but loss of control over such processes can lead to cancer development. Loss of control can generally be acquired by either abnormal over-activation of regulatory genes which control proliferation, known as oncogenes, or by loss of expression of genes which normally control processes such as cell division, differentiation and apoptosis. Such genes are known as tumour suppressor genes (TSGs).

Haematopoietic tumours including lymphoma typically develop as a result of a series of such changes, and arise originally within a single cell. As the cell proliferates abnormally, further changes can be acquired leading to a more abnormal phenotype. The dysregulation of gene expression may occur in a number of ways, including by genetic mutation (i.e. altered genetic sequence), chromosomal abnormalities such as translocations, deletions or inversions, or by changes which alter the expression of genes without altering the underlying DNA sequence, known as epigenetic changes; these will now be discussed further. TSGs include genes which regulate progression through the cell cycle, or which control apoptosis; these processes will be discussed further later in this section.

### 1.5.1 Epigenetic modifications

Epigenetic modifications affect the expression of genes without altering the underlying DNA sequence (Berger et al. 2009). They include covalent histone modifications and methylation of CpG dinucleotides of DNA. These changes can result in transcriptional silencing or activation of genes, can be heritable and are increasingly recognised as being important in the development of cancer [reviewed in (Esteller 2007)].

#### 1.5.1.1 Histone modifications

In eukaryotic cells, DNA within chromosomes is packaged by interactions with histone and non-histone proteins to form chromatin. The basic unit of chromatin packaging is the nucleosome, consisting of an octamer of histone proteins (two each of histones H2A, H2B, H3 and H4) forming a protein core around which DNA is wound (Luger et al. 1997). Histones can undergo reversible covalent modifications of their N-terminal tails, resulting in changes in the transcriptional activity of particular genes. Such modifications include, among others, methylation and acetylation of lysines. Generally, acetylation of histones, for example lysine 9 of histone H3 (H3K9Ac), is associated with transcriptional activation, as is trimethylation of lysine 4 of histone H3 (H3K4Me3), whereas trimethylation of lysine 27 of histone H3 (H3K27Me3) is associated with transcriptional repression (Kouzarides 2007).

The polycomb group (PcG) proteins are a group of proteins that were first described in *Drosophila* and are vitally important for embryonic development and cell differentiation. They have also been shown to be necessary for maintenance of identities of stem, progenitor and differentiated cells (Pietersen and van Lohuizen 2008). They function within groups called polycomb repressive complexes (PRC) and are involved in chromatin modifications leading to gene repression. PRC2 consists of the histone methyltransferase enzyme enhancer of zeste homologue 2 (EZH2), embryonic ectoderm development (EED) and suppressor of zeste 12 homologue (SUZ12) (Kirmizis et al. 2004) (see fig. 1.7). EZH2 performs H3K27 trimethylation (Cao et al. 2002). Until recently this was thought to result in recruitment of PRC1; however, there is increasing evidence that H3K27Me3 may not always recruit PRC1, since some PRC1 complexes lack the site necessary for H3K27Me3 binding; instead, PRC1 may be recruited by other means, especially in mammalian cells (reviewed in (Simon and

Kingston 2013). PRC1 catalyses the ubiquitylation of histone H2A on K119 (Cao et al. 2005) which contributes to maintenance of the created repressive chromatin state, although the mechanism of this is not currently understood (Simon and Kingston 2013). EZH2 also recruits DNA methyltransferases hence marking genes for DNA methylation (Vire et al. 2006, Schlesinger et al. 2007).

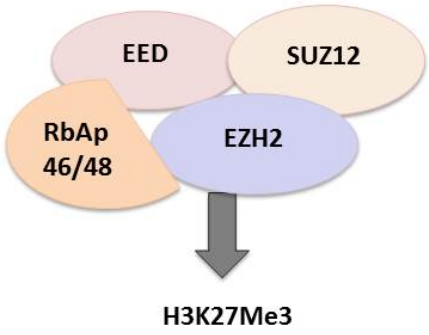



**Figure 1.7: Schematic diagram of polycomb repressive complex 2 (PRC2)**

The core components of PRC2 are enhancer of zeste homologue 2 (EZH2), embryonic ectoderm development (EED), suppressor of zeste 12 homologue (SUZ12) and the histone binding proteins, Retinoblastoma associated proteins 46 and 48 (RbAp46/48). PRC2 has histone methyltransferase activity specific for lysine 27 of histone H3, resulting in transcriptional repression by trimethylation (H3K27Me3) (Kuzmichev et al. 2002, Kirmizis et al. 2004).

It has been shown that some genes have both active (H3K4Me3) and repressive (H3K27Me3) chromatin marks simultaneously; these are known as bivalent domains (Bernstein et al. 2006). It is thought that in this way the genes are 'poised' for either activation or inactivation in preparation for differential expression during cell differentiation, suggesting that bivalency allows postponement of the decision as to whether the gene should be activated or repressed (Pietersen and van Lohuizen 2008). These genes tend to be important in developmental control and are often epigenetically silenced by promoter DNA methylation in cancer (Ohm et al. 2007).

### 1.5.1.2 DNA methylation

A further type of epigenetic modification is methylation of cytosine bases in CpG dinucleotides within genomic DNA. Within the genome there are fewer CpG dinucleotides than would be expected, assuming an equal distribution of all nucleotides. Those present are also not evenly distributed along the genome, being concentrated in particular regions known as 'CpG islands', which are often around 1kbp in length and found within the promoter regions of around 60-70% of human genes. Although CpGs outside of CpG islands are generally methylated, the DNA within CpG islands is normally unmethylated, and genes where this occurs are transcriptionally active. However, CpG methylation within CpG islands leads to transcriptional silencing of the relevant gene. When CpGs are methylated, this modification is heritable and hence epigenetic. DNA methylation is important in malignancies, where hypermethylation within gene promoters often leads to silencing of tumour suppressor genes (Esteller 2007).

### 1.5.2 The cell cycle

The cell cycle is a regulated series of events enabling the cell to divide. It consists of two active phases, the S (or DNA synthesis) phase, in which DNA replication occurs, and the mitosis (M) phase, which is followed by cytokinesis. In between these phases are two resting (Gap) phases, G1 and G2 (fig. 1.8). Cells can also exit the cell cycle completely from G1 for a longer time, entering a resting phase, G0. Senescence is a permanent exit from the cell cycle.

Progression through the cell cycle is tightly controlled by a set of cyclin-dependent kinases (CDK), whose kinase activity is in turn dependent on binding by cyclins, which are positive regulators and determine the specificity of the CDK. Cyclins are classified into four groups, the A, B, D and E cyclins. Specific cyclins are expressed at specific times during the cell cycle and thus regulate the activity of the CDKs, with the levels of cyclin expression being controlled by synthesis and degradation. Expression of specific CDKs also occurs at specific times within the cycle, with CDKs 2, 4 and 6 acting in interphase and CDK1 acting in mitosis. D cyclins are expressed early in G1 and bind to CDK4 and CDK6, allowing progression

through G1; cyclin E expression then activates CDK2 to allow progression into the S phase (fig. 1.8).

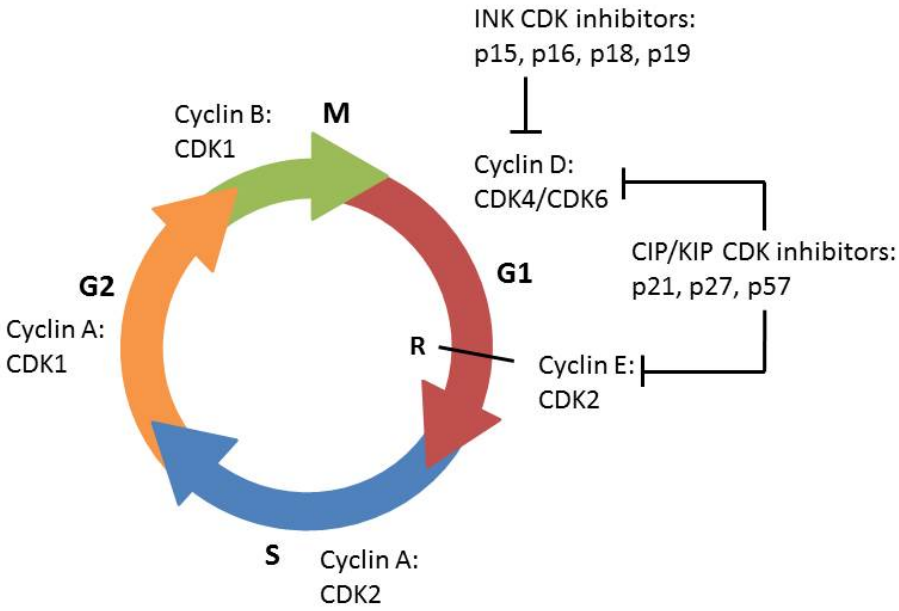



**Figure 1.8: Cell cycle regulation**

The cell cycle consists of the S phase, in which DNA synthesis occurs, the M phase for mitosis and two gap phases, G1 and G2. Progression between stages is controlled by cyclin-dependent kinases (CDKs) which are expressed at distinct stages. These are controlled by binding to specific cyclins. The CDKs are regulated by two families of CDK inhibitors, the INK and the CIP/KIP families. The restriction point R controls entry to the S phase, and the cell is committed to DNA replication once R has been passed. Adapted from (Dehay and Kennedy 2007).

There are also CDK inhibitors (CDKIs) which modulate the activity of CDKs; these fall into two groups: the CIP/KIP family ($p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$) and the INK4 family ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$). $p15^{INK4B}$ is encoded by *CDKN2B*, which is found close to the genes for two other tumour suppressors, $p14^{ARF}$ and $p16^{INK4a}$ at the INK4a/ARF/INK4b locus, which is frequently deleted in cancer. Both $p15^{INK4B}$ and $p16^{INK4A}$ bind to and inactivate CDK4/6, which promote proliferation; thus expression of $p15^{INK4B}$ or $p16^{INK4A}$ result in cell cycle arrest. $p14^{ARF}$ inhibits MDM2, leading to the stabilisation of p53 (Kim and Sharpless 2006).

The INK4 CDKIs bind specifically to CDK4/6, preventing their association with cyclin D. The CIP/KIP CDKIs interact with cyclin D-CDK4/6 and with cyclin E-CDK2 (fig. 1.8). Progression through the cell cycle is regulated at certain checkpoints: the G1 checkpoint or restriction point R (see fig. 1.8), which controls entry to the S phase, a G2/M checkpoint, and a checkpoint at the metaphase-anaphase transition. These checkpoints serve to ensure that conditions, both intracellular and in the environment, are suitable for the cell to replicate its DNA and divide. They are controlled mainly by the CDKIs. The G1 restriction point R is particularly important as, once this is passed, the cell is committed to DNA replication.

Retinoblastoma protein (Rb) is another tumour suppressor gene which controls cell cycle progression. During the cell cycle it is progressively phosphorylated and dephosphorylated. It is hypophosphorylated in non-cycling cells, and in this state it binds proteins of the E2F family, inhibiting transcription of E2F target genes. When Rb is phosphorylated, the binding to E2F is disrupted, allowing E2F to activate transcription of its target genes, which generally promote cell cycle progression, DNA replication and mitosis [reviewed in (Polager and Ginsberg 2008)].

If a cell detects unfavourable conditions, including DNA damage or mutation, then arresting in G1, for instance, allows time for the problem to be repaired, if possible. Disturbance of these checkpoints are frequently found in cancer, often due to mutations of CDKIs. This can result in replication of damaged or mutated DNA and/or uncontrolled proliferation. If these checkpoints are disrupted, and thus replication of mutated or damaged DNA occurs, this can lead to secondary mutations or genomic instability, allowing progression towards tumour development. When DNA damage or mutation is detected, or there are other particularly unfavourable conditions that cannot be rectified, the cell can also undergo apoptosis, which will now be described.

### 1.5.3 Apoptosis

Apoptosis is a form of programmed cell death, leading to destruction of the cell without causing any inflammatory response. It results in characteristic phenotypic changes, including nuclear and cytoplasmic condensation and nuclear fragmentation (Kerr et al. 1972). It is

used extensively to destroy cells which are no longer required, for example during embryonic development and in removal of auto-reactive B or T cells during their development in the immune system.

There are two distinct apoptotic signalling pathways, the extrinsic and intrinsic pathways, which nevertheless show some degree of overlap (see fig. 1.9). Both pathways involve activation of a series of caspase enzymes, found as inactive procaspases which are activated by proteolytic cleavage. The initial members activated are the 'initiator' caspases, which in turn activate downstream caspases including 'effector' or 'executioner' caspases, which carry out the specific functions leading to cellular breakdown. The extrinsic and intrinsic pathways converge at the level of effector caspases, but require different initiator caspases: the extrinsic system needs caspase 8 whereas the intrinsic pathway requires caspase 9 for initiation [reviewed in (Strasser 2005)]. The effector caspases include caspase 3, caspase 6 and caspase 7.

The extrinsic apoptotic pathway is triggered by the binding of extracellular ligands belonging to the tumour necrosis factor (TNF) family to specific cell-surface death receptors, which are in turn members of the TNF receptor family. The ligands include TNFα, FAS-ligand and TNF-related apoptosis inducing ligand (TRAIL), among others, and the receptors include FAS. The cytoplasmic portion of the transmembrane death receptor, known as the death domain, recruits intracellular proteins forming the death-inducing signalling complex (DISC), resulting in pro-apoptotic conditions within the cell. Caspase 8, the initiator caspase of the extrinsic pathway, is recruited into DISC. Once activated it then cleaves the executioner caspase 3 (Danial and Korsmeyer 2004).

The alternative, intrinsic, apoptotic pathway is initiated from within the cell in response to DNA damage, hypoxia or a lack of extracellular survival signals. The initiation of apoptosis via this pathway is largely regulated by the balance between pro-apoptotic and anti-apoptotic members of the BCL2 protein family [reviewed in (Danial and Korsmeyer 2004, Volkmann et al. 2014)]. The anti-apoptotic members contain four highly conserved BCL2-homology (BH) domains 1-4 and include BCL2 itself, BCL-$X_L$, BFL1 (also known as BCL2A1, or simply A1) and MCL-1. The pro-apoptotic members are divided into two subgroups based on

their structure: the first group share homology in the BH1-3 domains and include BAX and BAK, and the second group, the 'BH3 only' proteins (so called as they have homology with BCL2 only in the BH3 domain), includes BAD, BIK, BIM, BID, PUMA and NOXA.

BAX and/or BAK are required for intrinsic apoptosis induced by the BH3-only proteins, thus functioning downstream of the BH3-only proteins; although the process is not completely understood, BAX and BAK are thought to be redundant. BAK is localised to the outer mitochondrial membrane, whereas BAX is found within the cytosol. When activated, they create pores in the outer mitochondrial membrane, allowing release into the cytosol of proteins which execute apoptosis, leading to activation of caspase 9; once the mitochondrial membrane is permeable the process leading to cell death is irreversible (Volkmann et al. 2014). The activity of BAX/BAK is tightly regulated by the balance between the anti-apoptotic BH3-only proteins. BIM, BID and PUMA are direct activators of BAX/BAK, thought to bind directly to BAX/BAK. BID, BIM and PUMA can also form heterodimers with anti-apoptotic BCL2 family members, inhibiting their activity. The anti-apoptotic BH3 proteins such as BCL2 and BCL-$X_L$ can antagonise BAX/BAK and can also sequester the pro-apoptotic members (such as BIM and PUMA), thus preventing their action (Volkmann et al. 2014).

The intrinsic pathway involves release from mitochondria of cytochrome c, which binds to the cytosolic protein apoptotic protease activating factor 1 (APAF1), leading to a conformational change and activation of APAF1, which then interacts with caspase 9 forming a protein complex termed the apoptosome, resulting in activation of downstream effector caspases [(Li et al. 1997), reviewed in (Wurstle et al. 2012)].

One of the BH3-only proteins, BID, is activated by caspase 8, being cleaved into a pro-death C-terminal fragment, tBID, which, along with BIM and PUMA, can in turn activate BAX/BAK or inhibit the anti-apoptotic proteins such as BCL-$X_L$. BID therefore provides a link between the extrinsic and intrinsic pathways (Volkmann et al. 2014). Other proteins that can inhibit activation of executioner caspases are the Inhibitor of apoptosis (IAP) family, which inhibit caspases 3, 7 and 9 (Deveraux et al. 1997, Deveraux et al. 1998).

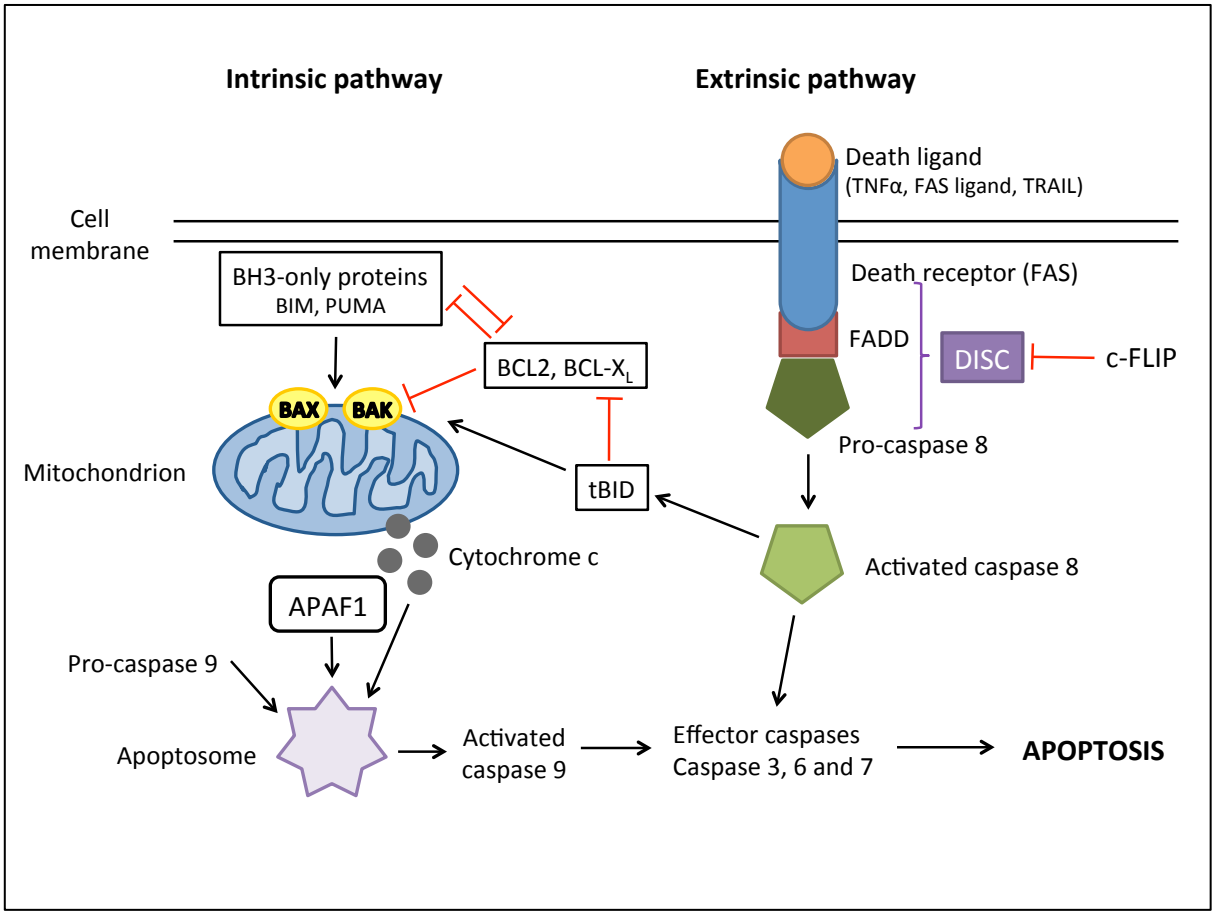



**Figure 1.9: Schematic diagram of the intrinsic and extrinsic pathways of apoptosis**

The extrinsic pathway, shown on the right, is activated by extracellular death ligands, which bind to the transmembrane death receptor, leading to activation of intracellular caspase 8 which in turn can activate effector caspases leading to the processes of apoptosis. Apoptosis can also be initiated from inside the cell, the intrinsic pathway (shown on the left), in response to hypoxia, DNA damage or lack of survival signals. Activation of this pathway is regulated by the BCL2 family of proteins, including both pro-apoptotic and anti-apoptotic members. The pro-apoptotic members such as BIM and PUMA cause activation of BAX and BAK which interact with the mitochondrion, causing release of cytochrome c. This in turn interacts with APAF1 and activated caspase 9 to form the apoptosome complex, which also activates effector caspases. The extrinsic and intrinsic pathways share cross-talk via pro-apoptotic BID. See main text for further details of these pathways.

### 1.5.3.1 Apoptosis mechanisms within the germinal centre

Within the GC, when a BCR binds to an antigen with high affinity, T cell help leads to signalling through CD40/CD40 ligand (CD40L). This leads to induction of anti-apoptotic BCL-$X_L$ and hence promotes survival of cells expressing a high affinity BCR; without the CD40/CD40L interaction, BCR stimulation leads to down-regulation of BCL-$X_L$ (Tuscano et al.

1996). CD40 signalling via NFκB induces c-FLIP expression, thus rendering cells which receive CD40 signals resistant to FAS-induced apoptosis (van Eijk et al. 2001).

The extrinsic pathway of apoptosis is also thought to be important in apoptosis of low-affinity B cells in the GC, which do not receive the survival signal CD40 ligand from T cells. FAS is required for apoptosis in GC B cells lacking T cell help (Takahashi et al. 2001). In these cells, a preformed FAS DISC is present, which does not require ligand binding for its activation. The cellular homologue of FLICE-inhibitory protein, c-FLIP, normally interferes with DISC formation, but is lost from GC B cells lacking the CD40L survival signal, thus allowing DISC to become active, resulting in apoptosis via activation of caspase 8 (Hennino et al. 2001).

In GC B cells, signalling via the BCR, in the absence of the CD40/CD40L interaction as a result of T cell help, also contributes to the default apoptotic state via induction of BIK and BIM. BIM [BCL2-interacting mediator of cell death, encoded by the gene BCL2-like-protein 11 (*BCL2L11*)] binds with high affinity to all the pro-survival BCL2 family members, inhibiting their function, and is thus an extremely potent inducer of apoptosis. BIM is expressed by most lymphoid cells and is essential for deletion of autoreactive T and B cells [reviewed in (Strasser 2005)]. BIM is an important tumour suppressor in B cells, with even loss of a single allele accelerating lymphomagenesis in an Eμ Myc mouse model (Egle et al. 2004).

In GC B cells (centroblasts), transforming growth factor β (TGFβ) also induces apoptosis by autocrine signalling, leading to SMAD3/4-dependent induction of BIK and PUMA along with down-regulation of BCL-$X_L$, thus contributing to the default apoptotic state, along with FAS (Spender et al. 2009, Spender et al. 2013). This TGFβ-induced apoptotic pathway occurs independently of both FAS and TRAIL and thus uses the intrinsic pathway (Inman and Allday 2000a). Phosphorylation of SMAD2, showing that TGFβ signalling is active, has been demonstrated by immunohistochemistry in both the LZ and DZ of GCs in normal human tonsil tissue (Spender et al. 2009). BAK/BAK are also needed for TGFβ-induced apoptosis in GCs, as BL deficient in these resist TGFβ-induced apoptosis, for example the EBV-negative BL cell line CA46 does not undergo apoptosis with TGFβ, and lacks BAX expression (Spender and Inman 2009a).

## 1.6 Transforming growth factor β and bone morphogenetic protein signalling

### 1.6.1 Overview of TGFβ and bone morphogenetic protein (BMP) signalling

TGFβ was first discovered in the early 1980s, when it was initially characterised by its ability to induce a transformed phenotype in fibroblasts (Anzano et al. 1983), hence its name. However, it has subsequently been found to be important in many processes, controlling cell proliferation, differentiation, apoptosis, developmental fate and immune regulation. In addition, TGFβ modulates human haematopoiesis by selectively regulating growth of immature haematopoietic cells, but not more differentiated cells (Sing et al. 1988) and has recently been shown to restore quiescence of HSCs following recovery of haematopoiesis after myelosuppressive chemotherapy (Brenet et al. 2013).

Subsequently, the discovery of many related ligands and their receptors has led to the definition of a TGFβ signalling superfamily which also includes the bone morphogenetic proteins (BMPs) as well as other related ligands. This superfamily is characterised by a signalling pathway consisting of extracellular ligand, type I and type II transmembrane receptors which possess serine/threonine kinases, and downstream signalling by a group of Smad proteins.

Generally in the TGFβ superfamily, a ligand homodimer binds to and brings together a dimer of type I and a dimer of type II receptors on the cell surface, resulting in phosphorylation and activation of the type I receptors by the type II receptors (see fig. 1.10). This in turn leads to recruitment and phosphorylation of a receptor-regulated Smad protein, which forms a complex with Smad4 (the common mediator Smad). The Smad complex then accumulates in the nucleus, where the Smads regulate gene expression by interacting with transcription factors and/or binding directly to DNA (reviewed in Shi and Massague 2003). TGFβ superfamily signalling influences a large number of genes and the effects are specific to both cell type and extracellular conditions. As well as type I and II receptors, there are also co-receptors (or accessory receptors; see table 1.5) which can bind TGFβ superfamily ligands and regulate ligand binding to the corresponding ligand receptors. The main components of TGFβ superfamily signalling will all be described in further detail below.

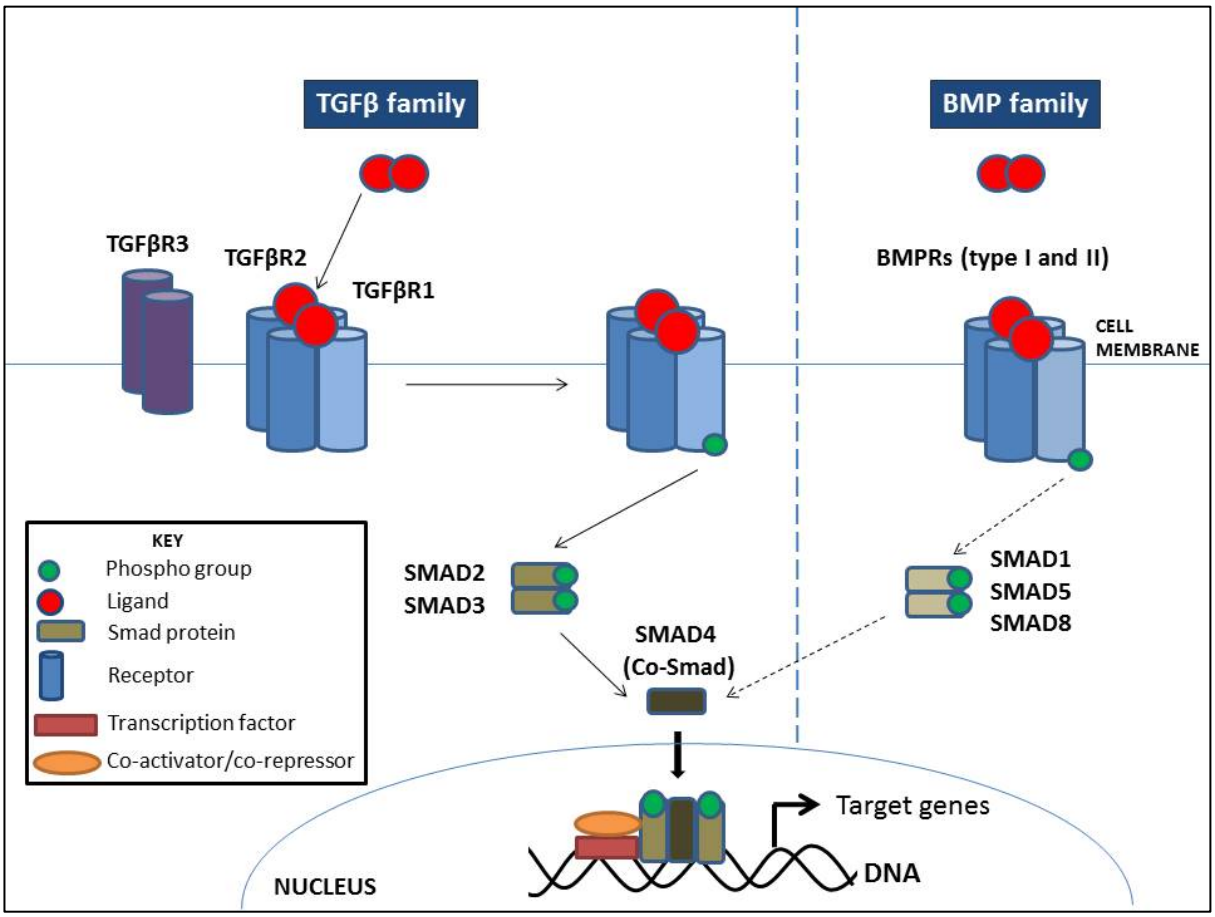



**Figure 1.10: Simple overview of the TGFβ and BMP signalling pathways**

A TGFβ1 ligand dimer binds to a homodimer of TGFβR2. Ligand binding causes association of TGFβR2 with TGFβR1 (also as a homodimer). TGFβR2 then phosphorylates and activates TGFβR1, leading to the recruitment, phosphorylation and activation of the receptor-mediated or R-SMAD, SMAD2 or SMAD3. Generally two phosphorylated R-SMADs then form a trimeric complex with the common mediator SMAD, SMAD4, and the complex accumulates in the nucleus. In the nucleus the SMAD complex binds directly to DNA or interacts with transcription factors, as well as co-activators or co-repressors, to regulate expression of multiple target genes. The BMP signalling pathway (shown on the right of the figure) is similar, but the canonical R-SMADs are SMAD1, SMAD5 and SMAD8. The co-receptor TGFβR3 facilitates ligand binding to TGFβR2.

### 1.6.2 TGFβ superfamily ligands

The TGFβ superfamily is a large group of cytokines characterised by having six conserved cysteine residues. The family is divided into two subfamilies, according to both sequence similarity and their downstream signalling pathways. These are the TGFβ/activin/nodal subfamily and the bone morphogenetic protein (BMP)/GDF (growth and differentiation factor)/MIS (Müllerian inhibiting substance, also known as anti-Müllerian hormone AMH)

subfamily (Shi and Massague 2003). Within the subfamilies there are several different ligands, some of which have alternative names, as shown in tables 1.2A and B. The two subfamilies of ligand differ in their receptor-binding characteristics: the TGFβ/activin family ligands have a high affinity for type II receptors and only bind to type I receptors in the presence of type II receptors. The BMP subfamily members, on the other hand, have a high affinity for the type I receptors and a lower affinity for type II receptors (Shi and Massague 2003).

The active form of ligand is a dimer stabilised by hydrophobic interactions (Shi and Massague 2003). TGFβ ligands are secreted in an inactive form, requiring proteolytic cleavage before they are available for binding to their receptors, whereas other ligands are secreted in active form but then bound by locally secreted antagonists (ligand binding traps), hence regulating their availability for binding to receptors (de Caestecker 2004).

### 1.6.3 BMP Signalling

BMPs have roles in formation of bone and cartilage, control of haematopoiesis and immune regulation. Members can be pro-proliferative or anti-proliferative, depending on the cell type and general context. They promote growth and differentiation in osteoblasts, and promote differentiation in early haematopoietic progenitors, whereas they are anti-proliferative in mature B cells (Kersten et al. 2005, Larsson and Karlsson 2005).

**A**

| TGFβ | | Activins | | Nodal |
|---|---|---|---|---|
| **Gene** | **Other name** | **Gene** | **Other names** | **Gene** |
| *TGFB1* | TGFβ1 | *INHA* | Inhibin α | *NODAL* |
| *TGFB2* | TGFβ2 | *INHBA* | Inhibin β A, activin A | |
| *TGFB3* | TGFβ3 | *INHBB* | Inhibin β B, activin AB | |
| | | *INHBC* | Inhibin β C | |
| | | *INHBE* | Inhibin β E, activin | |

**B**

| BMPs | | GDFs | | AMH | |
|---|---|---|---|---|---|
| **Gene** | **Other name(s)** | **Gene** | **Other name(s)** | **Gene** | **Other name** |
| *BMP2* | | *GDF1* | | *AMH* | MIS |
| *BMP3* | osteogenin | *GDF2* | BMP9 | | |
| *BMP4* | | *GDF3* | | | |
| *BMP5* | | *GDF5* | BMP14 | | |
| *BMP6* | | *GDF6* | BMP13 | | |
| *BMP7* | OP-1 | *GDF7* | BMP12 | | |
| *BMP8B* | BMP-8, OP-2 | *GDF9* | | | |
| *BMP10* | | *GDF10* | BMP3b | | |
| *BMP15* | | *GDF11* | BMP11 | | |
| | | *GDF15* | | | |
| | | *MSTN* | GDF8, myostatin | | |

**Table 1.2: Ligands of the TGFβ superfamily**

**(A):** TGFβ/activin/nodal subfamily **(B):** BMP/GDF/MIS subfamily. Alternative names are shown, where they exist.

### 1.6.4 TGFβ receptors

#### 1.6.4.1 Type I and type II receptors

There are two main types of receptor for all ligands in the TGFβ signalling superfamily: type I (which propagate signal) and type II (which activate the type I receptors). In humans there

are seven type I and five type II receptors. Type I receptors are called activin receptor-like kinases (ALK) 1-7; many have alternative names reflecting the ligand(s) they bind (see table 1.3). Both types of receptor consist of an N-terminal extracellular ligand-binding domain, a single-pass transmembrane domain and a cytoplasmic C-terminal domain which contains a serine/threonine kinase (Shi and Massague 2003). Type I receptors also have a characteristic GS domain, consisting of the specific amino acid sequence SGSGSG, immediately at the N-terminal side of the kinase domain. This GS domain is phosphorylated by the constitutively active type II receptor, hence activating the type I receptor. Phosphorylation of the type I receptor by the type II receptor leads to a conformational change: in the absence of phosphorylation by the type II receptor, the type I receptor binds the protein FK506-binding protein FKBP12, silencing the type I receptor's kinase activity, whereas once the type I receptor becomes phosphorylated this protein is released and the receptor is able to bind Smads (Huse et al. 2001).

Type I receptors also have a nine amino acid L45 loop, within the kinase domain, which determines the recruitment of the appropriate Smads and the signalling specificity, by binding to the L3 loop of the R-Smad (Feng and Derynck 1997); for instance, TGFβR1 and ALK4 have identical L45 sequences, for interactions with Smad2/3, whereas BMPRIA and BMPRIB share a different L45 sequence, as they bind to Smad1/5/8 (Chen et al. 1998). When not bound to ligand, both type I and type II receptors are found as homodimers on the cell surface. On binding of ligand, a complex forms between a ligand dimer, a homodimer of type I receptors and a homodimer of type II receptors, resulting in activation of the type I receptors which then signal to the cytoplasmic Smad proteins.

Type I receptors differ in their specificity for both ligands and other receptors. For example, TGFβR1 can only bind to TGFβ ligands (TGFβ1, 2 and 3) and only binds to TGFβR2, whereas others, such as ALK2, can bind several different ligands and/or type II receptors (Feng and Derynck 2005). Type II receptors also differ in their specificity for both type I receptors and ligands (see table 1.4). In addition, ligands vary in their binding capabilities: TGFβ1, TGFβ3 and activins can bind to their type II receptors without needing a type I receptor, whereas BMP2, BMP4 and BMP7 bind primarily to their type I receptors, BMPRIA or BMPRIB

(Derynck and Zhang 2003). TGFβ2, on the other hand, can interact only with pre-formed type II-type I-receptor complexes.

#### 1.6.4.2 TGFβR2

The *TGFβR2* gene on chromosome 3 (Mathew et al. 1994) encodes a 592-amino acid protein which, like other type II receptors, consists of an extracellular domain (including the ligand binding domain), a transmembrane domain and an intracellular domain, which contains the serine/threonine kinase. TGFβR2 binds only TGFβ ligands, but can bind to the BMP receptors ALK1 and ALK2 in addition to TGFβR1 (de Caestecker 2004). TGFβ ligands bind to TGFβR2, resulting in conformational changes within the extracellular domain (Hart et al. 2002). This results in recruitment, binding and phosphorylation of TGFβR1, which then induces phosphorylation of Smad2 and Smad3. TGFβR2 can bind ligand without requiring TGFβR1 (Lin et al. 1992), but can only signal when it forms a complex with TGFβR1 (Wrana et al. 1992). TGFβR1 and TGFβR2 bind TGFβ1 and TGFβ3 with higher affinity than TGFβ2, especially in cells lacking TGFβR3 (Cheifetz et al. 1990).

The human *TGFβR2* promoter has been fairly well characterised (fig. 1.11), and lacks a TATA box or CAAT box near its transcription start site (TSS) (Humphries et al. 1994, Bae et al. 1995). It has several Sp1 binding sites, at positions -25, -59, -102 and -143 relative to the TSS (Bae et al. 1995, Jennings et al. 2001), with Sp1 binding being necessary for basal activity of the promoter (Jennings et al. 2001). DNA methylation at Sp1 binding sites, resulting in lack of Sp1 binding, has been shown to inhibit transcription of TGFβR2 (Zhao et al. 2005, Chen et al. 2007). HDAC1 interacts with Sp1 leading to transcriptional repression (Doetzlhofer et al. 1999) and this mechanism has been shown to repress TGFβR2 transcription in human pancreatic carcinoma cells (Zhao et al. 2003).

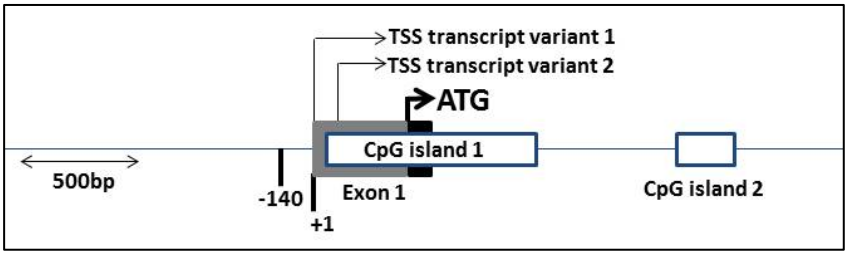



**Figure 1.11: Schematic diagram of the TGFβR2 promoter region**

The predicted transcription start site (TSS) for transcript variant 1 is shown at position +1. The 5' UTR is shown as a grey box, with the ATG start codon shown and the coding region of exon 1 shown as a black box. The locations and sizes of CpG islands are also shown.

### 1.6.4.3 TGFβR3 (betaglycan)

TGFβR3, also known as betaglycan, is a co-receptor for TGFβ signalling. It is found in higher numbers on the cell surface than TGFβR1 or TGFβR2, and is expressed on virtually every cell type except endothelial cells (Cheifetz et al. 1990) and haematopoietic progenitor cells in mice (Ohta et al. 1987). TGFβR3 is an 851 amino-acid proteoglycan which possesses a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain of 43 amino acids, which lacks a kinase (Lopez-Casillas et al. 1991, Wang et al. 1991); like other TGFβRs it exists as a homodimer. Its glycosylation by the addition of glycosaminoglycans is thought to be important for its function. The cytoplasmic domain of TGFβR3 is necessary for promotion of TGFβ signalling (Blobe et al. 2001b).

TGFβR3 directly binds multiple ligands, including TGFβ1, TGFβ2, TGFβ3 (all of which bind with high affinity), inhibin, BMP2, BMP4, BMP7, and GDF5, and facilitates their binding to the relevant type II receptors, hence enhancing TGFβ and BMP signalling (Gatza et al. 2010). TGFβR3 can also present BMP2 to the type I receptors BMPRIA or BMPRIB, increasing signalling by BMP2 (Kirkbride et al. 2008). In addition, certain ligands (inhibin and TGFβ2) require TGFβR3 for their binding to type II receptors (Gatza et al. 2010). By facilitating the binding of inhibin to both activin receptor II and BMPR2, TGFβR3 can mediate the inhibition of both activin and BMP signalling by inhibin (Lewis et al. 2000, Wiater and Vale 2003).

TGFβR3 undergoes shedding of its ectodomain by proteolytic cleavage at a site within the extracellular domain, resulting in the presence of soluble TGFβR3 (sTGFβR3) (Andres et al.

1989, Lopez-Casillas et al. 1991); the levels of this soluble form appear to correlate with that on the cell surface, suggesting that it is constitutively shed (Gatza et al. 2010). sTGFβR3 can bind to TGFβ ligands in extracellular fluid, sequestering them and preventing their binding to cell surface TGFβRs, hence negatively regulating TGFβ and BMP signalling (Lopez-Casillas et al. 1994). However, at low TGFβ concentrations, sTGFβR3 can conversely enhance binding of TGFβ ligands to cell surface receptors (Fukushima et al. 1993).

TGFβR3 can also form a complex with β-arrestin2 and BMPRIB resulting in internalisation of BMPRIB, which enhances BMP signalling (Lee et al. 2009). The cytoplasmic PDZ domain of TGFβR3 can bind to GAIP-interacting protein, C-terminus (GIPC), stabilising TGFβR3 at the cell surface and hence enhancing TGFβ signalling (Blobe et al. 2001a). Phosphorylation of a cytoplasmic domain of TGFβR3 by TGFβR2 leads to binding of TGFβR3 to β-arrestin2. The subsequent complex formation between TGFβR3:β-arrestin2 and GIPC leads to ligand-independent endocytosis of the whole TGFβ signalling receptor complex and hence down-regulation of TGFβ signalling (Chen et al. 2003, Finger et al. 2008a). TGFβR3 therefore has dual roles in modulating both TGFβ signalling and BMP signalling depending on the context and, in addition, inhibits activin signalling.

TGFβ1 binds to TGFβR3 with much lower affinity than to TGFβR1 and TGFβR2. TGFβR3 associates with TGFβR2 only in the presence of ligand, without binding to TGFβR1. It presents TGFβ1 to TGFβR2, forming a stable ligand transfer complex, and thus increases the affinity of TGFβR2 for TGFβ1. It markedly increases the affinity of TGFβ2 ligand for TGFβR2 such that in the presence of TGFβR3, TGFβ2 is nearly as potent as TGFβ1 in certain cell types (Lopez-Casillas et al. 1993). Murine hematopoietic progenitor cells which lack TGFβR3 are fairly resistant to TGFβ2 (Ohta et al. 1987), whereas cells expressing TGFβR3 are more responsive to TGFβ2. TGFβR3 is not essential for TGFβ signalling. However, it has been shown to be essential for haematopoiesis, as TGFβR3 knockout mice show embryonic lethality due to ineffective erythropoiesis (Stenvers et al. 2003).

The gene encoding TGFβR3 is on chromosome 1 and includes 16 exons transcribed from two promoters, proximal and distal (Hempel et al. 2008, Gatza et al. 2010). The gene contains three non-coding exons in its 5’ region (fig. 1.12). Two TGFβR3 mRNA transcripts have been

identified which differ in their 5' UTR regions, in keeping with the existence of two promoters (Hempel et al. 2008). The proximal promoter is approximately 25kb from the translational ATG start codon, with the distal promoter being 45kb from the start codon. The proximal promoter is located between base pairs -165 to -75 from the proximal TSS; this region contains an Sp1 site, conserved across rat, mouse and human TGFβR3, plus an upstream GC-rich region. The sequence of the proximal promoter in humans is identical to the rat and mouse TGFβR3 promoters (Wang et al. 1991, Ji et al. 1999, Lopez-Casillas et al. 2003, Hempel et al. 2008). The distal promoter has been found to be highly methylated in several cell lines, suggesting that the proximal promoter is generally more active in most tissue types (Hempel et al. 2008).

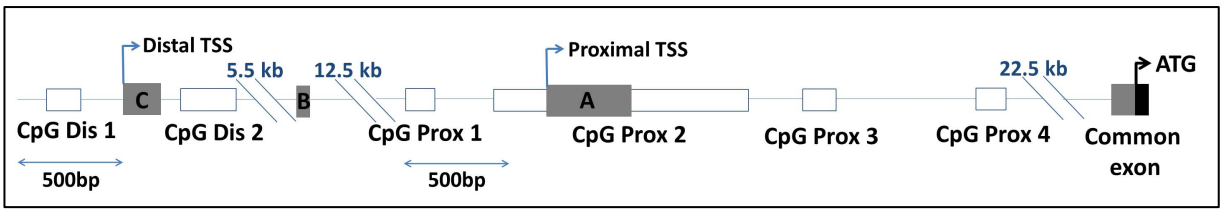



**Figure 1.12: Schematic diagram of the TGFβR3 promoters**

The TGFβR3 gene has two alternative promoters, distal and proximal, with the transcriptional start sites (TSS) shown for each. Published TGFβR3 mRNA transcripts differ in their 5' UTRs (untranslated exons shown as grey boxes), either having exons C and B, or alternatively exon A, and then share a common exon in which the ATG start codon is found (translated region shown in black). Also shown are the predicted CpG islands around the promoter regions (Dis=distal; Prox=proximal).

| Gene | Other name | Ligand(s) | Type II receptor (s) | Smads |
|---|---|---|---|---|
| *ACVRL1* | ALK1 | TGFβ, Activin A, BMP9, BMP10 | TGFβR2; ActRII, ActRIIB | 1/5/8 |
| *ACVR1* | ALK2, ACVR1A | BMP 6/7, BMP9, Activin A, AMH, TGFβ | ActRII, ActRIIB, AMHRII, TGFβR2 | 1/5/8 |
| *BMPR1A* | ALK3 | BMP2, BMP4, BMP6, BMP7 | BMPRII, ActRII, ActRIIB, AMHRII | 1/5/8 |
| *ACVR1B* | ALK4, ActRIB | Nodal, Activin A, GDF1, GDF11 | ActRII, ActRIIB | 2/3 |
| *TGFBR1* | ALK5, TGFβR1 | TGFβ1,2,3 | TGFβR2 | 2/3 |
| *BMPR1B* | ALK6 | BMP2, BMP4, BMP6, BMP7, GDF-5,6,9b, AMH | BMPRII, ActRII, ActRIIB, AMHRII | 1/5/8 |
| *ACVR1C* | ALK7 | Nodal, activins | ActRII, ActRIIB | 2/3 |

**Table 1.3: Type I receptors of the TGFβ superfamily**

The ligands and Smads which bind to the receptors are shown (de Caestecker 2004, Mueller and Nickel 2012). ALK=activin-like receptor kinase; ActR=activin receptor.

| Gene | Other name | Ligands | Type I receptors |
|---|---|---|---|
| *TGFBR2* | TGFβR2 | TGFβ1,2,3 | TGFβR1, ALK1, ALK2 |
| *ACVR2A* | ActRII | Activins/inhibins, nodal; GDF1, 5, 8, 9b, 11; BMPs 2, 6, 7, 9 | ALK1, ALK2, BMPRIA, ALK4, BMPRIB, ALK7 |
| *ACVR2B* | ActRIIB | Activins/inhibins; nodal; GDF1, 5, 8, 9b, 11; BMPs 2, 6, 7, 9 | ALK1, ALK2, BMPRIA, ALK4, BMPRIB, ALK7 |
| *BMPR2* | BMPRII | BMPs 2,4,6,7,9; GDF-5,6, 9b; inhibin A | BMPR1A, BMPR1B |
| *AMHR2* | AMHRII, MISRII | AMH/MIS | ALK2, BMPRIA, BMPRIB |

**Table 1.4: Type II receptors of the TGFβ superfamily**

The ligands and type I receptors which bind are shown (de Caestecker 2004, Mueller and Nickel 2012).

| Gene | Other name | Ligands |
|---|---|---|
| *TGFBR3* | TGFβR3, Betaglycan | TGFβ1, 2 and 3; Inhibin, BMP2, BMP4, BMP7, GDF5 |
| *ENG* | Endoglin | BMP2, BMP7, BMP9, activin; TGFβ1, TGFβ3 |
| *TDGF1* | Cripto | Nodal, GDF1,3 |

**Table 1.5: Co-receptors (accessory receptors) of the TGFβ superfamily**

Alternative names and ligands are shown (de Caestecker 2004, Mueller and Nickel 2012).

### 1.6.5 Smads

The downstream intracellular signals from TGFβ and BMP receptors are propagated by Smad proteins. There are 8 Smad proteins separated into three families: the receptor-activated or R-Smads (Smad1, Smad2, Smad3, Smad5 and Smad8, also known as Smad9), the common-mediator or co-Smad (Smad4) and two inhibitory or I-Smads (Smad6 and Smad7). Only the R-Smads can be directly phosphorylated and activated by the type I receptor kinases. Canonical signalling by the TGFβ subfamily occurs through Smad2 and Smad3, whereas the BMP superfamily ligands signal through Smad1, Smad5 and Smad8 (see fig. 1.10). The I-Smads compete with R-Smads for receptor and co-Smad binding and target the receptors for degradation.

The Smad proteins are all approximately 500 amino acids in length and consist of two conserved domains, the N-terminal MAD homology 1 (MH1) and C-terminal MAD homology 2 (MH2) domains, separated by a more divergent linker domain. The C-terminus of R-Smads has a characteristic SXS motif, the two serines of which are phosphorylated by type I receptors resulting in Smad activation. The MH2 domain contains the L3 loop region for interaction with the L45 loop on type I receptors and a region for oligomerisation with other Smads, whereas the MH1 domain enables the Smad to bind to DNA and interact with other DNA binding proteins (Attisano and Wrana 2000). Smad2 and Smad3 share an identical L3 loop, and Smad1/5/8 share an alternative identical L3 loop, thus ensuring specificity of the type I receptors for R-Smads.

In the inactive state, the R-Smads are located primarily in the cytoplasm, the co-Smad (Smad4) in the cytoplasm and nucleus, and the I-Smads predominantly in the nucleus (Shi

and Massague 2003). R-Smads undergo constant nucleo-cytoplasmic shuttling even in the absence of signalling. Phosphorylation of the R-Smad at the C-terminal SXS motif by type I receptors results in a conformational change leading to its dissociation from the receptor and allowing the R-Smad to form a complex with other Smads including Smad4 (Derynck and Zhang 2003). Activated R-Smads can form a dimer with Smad4 or a trimer with one Smad4 molecule and one other R-Smad (Inman and Hill 2002), although more recently the existence of mixed R-Smad complexes, in some cases not including Smad4, have been demonstrated (Daly et al. 2008, Gronroos et al. 2012). Formation of an activated Smad complex promotes its accumulation in the nucleus, a process facilitated by the interaction of the Smad MH2 domain with nucleoporins. R-Smad shuttling continues in the presence of signal, by continuous receptor-mediated phosphorylation in the cytoplasm and de-phosphorylation by R-Smad phosphatases in the nucleus. This enables the R-Smads to monitor receptor activity (Inman et al. 2002). Smad4 accumulates in the nucleus once bound to activated R-Smads, but also undergoes nucleo-cytoplasmic shuttling independently of signalling. It is thought that a nuclear export signal in the Smad4 linker region is hidden when Smad4 is bound to R-Smad (Watanabe et al. 2000). Nucleo-cytoplasmic shuttling is halted by blocking of the nucleoporin interaction by phosphorylation of the Smad1 linker region and binding of the E3 ubiquitin ligase Smurf1 (Sapkota et al. 2007).

Stimulation of a cell with TGFβ ligand results in simultaneous changes in expression of hundreds of genes, some being positively and others negatively regulated. These changes depend on the cell type and other conditions of the cell (Kang et al. 2003, Massague 2012). Smads cooperate both with each other and with other DNA binding proteins; the transcription factors cooperating with Smads include those from multiple families, e.g. forkhead, homeobox, Jun/Fos, and Runx [reviewed in (Attisano and Wrana 2000, Feng and Derynck 2005)]. Smad4 and R-Smads, with the exception of Smad2, bind to specific DNA sequences, called Smad-binding elements (SBEs), within the promoters of target genes. Smad3 and Smad4 bind to SBEs consisting of GTCT and its palindrome AGAC (Dennler et al. 1998, Zawel et al. 1998), found within promoters of certain TGFβ target genes such as *JUNB* and *SMAD7* (Jonk et al. 1998, Nagarajan et al. 1999). Smad1, on the other hand, binds preferentially to the GC-rich sequences GCCGNC or GRCGNC, found within the promoters of

BMP-responsive genes such as *ID1* and *SMAD6* (Ishida et al. 2000, Lopez-Rovira et al. 2002). These sequences, Smad1-binding elements, are often found near Smad3/Smad4 binding elements GTCT or AGAC, so that Smad4 bound to Smad1 within the complex can also interact with the DNA (Ross and Hill 2008).

The MH1 domain of Smad3 is able to bind to SBEs, but Smad2 is unable to bind directly to DNA and so must interact with DNA indirectly via recruitment of transcription factors. Smad2 and Smad3 share 91% amino acid homology overall, but Smad2 has an additional 30-amino-acid sequence within its MH1 domain, transcribed by exon 3, and it is thought that this sterically hinders the binding of Smad2 to DNA, since a mutated form of Smad2, lacking exon 3, is able to bind DNA (Yagi et al. 1999).

Although canonical TGFβ signalling induces phosphorylation of Smad2 and Smad3, these can have different functions and are therefore generally non-redundant, although the relative importance of each varies between cell types (Kretschmer et al. 2003, Kim et al. 2005, Brown et al. 2007). An investigation of murine embryonic fibroblasts with knockout (KO) of Smad2 or Smad3 showed that both Smads were required for TGFβ-mediated growth inhibition, whereas the induction of Smad7 and auto-induction of TGFβ1 only required Smad3 expression. Other TGFβ target genes required one or other Smad specifically, or neither (Piek et al. 2001). However, in murine mammary epithelial cells, Smad3 was not required for TGFβ-mediated growth inhibition (Yang et al. 2002). In HaCaT (human keratinocyte) cells, Smad3, but not Smad2, was needed for TGFβ-induced growth arrest, $p21^{CIP1/WAF1}$ induction and c-MYC repression (Kretschmer et al. 2003, Kim et al. 2005). A DNA array revealed several distinct sets of TGFβ target genes: (1) those that specifically required Smad2 or Smad3, (2) those requiring either Smad2 or Smad3 in a redundant fashion, or (3) a set that were entirely independent of Smad2 and Smad3 (Kretschmer et al. 2003).

The binding of Smads to DNA, even for those that do bind directly, is of low affinity, and thus they require interaction with specific transcription factors to alter transcription of target genes; this contributes to the diversity of TGFβ responses in different cellular contexts. The accumulation of Smad complexes within the nucleus as either dimers or

trimers (involving two R-Smad molecules with one Smad4) depends on which transcription factor is involved in the interaction (Inman and Hill 2002).

As well as interacting with transcription factors, Smads can recruit co-activators such as CREB-binding protein (CBP) and p300, both histone acetyl transferases, which bring sequence-specific transcription factors into close proximity with RNA polymerase II. Smads can also recruit co-repressors, such as Ski/SnoN, which repress Smad-induced transcription.

### 1.6.6 Cross-talk between TGFβ and BMP signalling pathways

In recent years the concept of distinct canonical TGFβ and BMP signalling via Smad2/3 and Smad1/5/8 respectively has been increasingly challenged. First it was demonstrated in endothelial cells that TGFβ could stimulate phosphorylation of both Smad2/3 and Smad1/5. This occurred via the formation of heteromeric receptor complexes containing TGFβR1 (which activates Smad2/3) and ALK1 (which stimulates Smad1/5/8) and required the endothelial-cell-specific co-receptor endoglin (Goumans et al. 2002). Initially this was thought to be specific to endothelial cells, as ALK1 is not expressed in other cell types. However, it was subsequently demonstrated that formation of similar mixed receptor complexes and mixed R-Smad complexes, leading to activation of both Smad2/3 and Smad1/5/8 by TGFβ, occur in other cell types including epithelial cells, fibroblasts and epithelial-derived tumour cell lines (Daly et al. 2008). In this case, the receptor complexes included a dimer of TGFβR2 complexed with a TGFβR1 monomer and a BMP receptor monomer, either ALK2 or BMPRIA (ALK3). They demonstrated the formation of mixed R-Smad complexes consisting of a phosphorylated Smad2 or 3 with a phosphorylated Smad1 or 5, without a Smad4 molecule, and proposed that these mixed R-Smad complexes could themselves accumulate in the nucleus and alter transcription of target genes (Daly et al. 2008).

It was thought that in order for TGFβ to stimulate phosphorylation of the 'BMP-type' Smads 1/5/8, one of the BMP type 1 receptors (ALK1, ALK2, ALK3 or ALK6) would have to be within the receptor complex. However, Wrighton *et al* showed that TGFβ could induce phosphorylation of Smad1 independently of BMP type I receptors in a murine mesenchymal

cell line and a human hepatoma cell line (Wrighton et al. 2009) and another group showed that TGFβ induced phosphorylation of Smad1 in a murine mammary epithelial cell line, again via the L45 loop of TGFβR1 (interacting with non-canonical Smads 1/5) and independently of BMP receptors (Liu et al. 2009). Thus the requirement for BMP receptor involvement in TGFβ-induced phosphorylation of Smad1/5 appears to vary between cell lines or cell types.

TGFβ-induced phosphorylation of Smad1/5 has now been demonstrated in endothelial, epithelial cells, fibroblasts and B cells (Goumans et al. 2002, Daly et al. 2008, Liu et al. 2009, Wrighton et al. 2009, Rai et al. 2010, Jiang and Aguiar 2014). In B cells the requirement for TGFβR2 and TGFβR1 was consistently shown, whereas BMP receptors ALK2/3 were required only in one of the DLBCL lines tested (Rai et al. 2010). Hill and colleagues have recently gone on to show, in a breast cancer-derived epithelial cell line, that TGFβ acts via TGFβR1 to induce mixed R-Smad complexes containing phosphorylated Smad1/5 and phosphorylated Smad3 (but not Smad2). These heteromeric complexes had a direct inhibitory effect on BMP-responsive elements, so that in the presence of both TGFβ and BMP ligands, the TGFβ signals antagonised BMP signals, with Smad3 being required to mediate TGFβ-induced repression of BMP-induced transcription (Gronroos et al. 2012).

### 1.6.7 Regulation of TGFβ and BMP signalling pathways

TGFβ signalling is regulated at multiple levels: by controlling the amount of ligand available, cell surface expression of receptors, availability of Smads, degradation of signalling pathway components, and by inhibitory molecules including the inhibitory Smads (I-Smads). This regulation is important for ensuring that signalling is terminated appropriately.

Internalisation of receptors is used either to enhance signalling or to degrade the receptor, depending on the mechanism of endocytosis used. Clathrin-mediated endocytosis of receptor complexes to early endosomes results in enhanced signalling, and receptors can return from there to the cell surface. Clathrin-independent receptor endocytosis to caveolin-positive vesicles, on the other hand, results in receptor degradation and thus reduced signalling (Finger et al. 2008a).

As described in section 1.6.5, Smads are continuously shuttled from the cytoplasm to the nucleus and back again, although in the presence of ligand they remain for longer in the nucleus, due to masking of nuclear export signals (Itoh and ten Dijke 2007). Smad phosphatases, found in the nucleus, dephosphorylate Smads, hence deactivating them and allowing them to be exported from the nucleus back into the cytoplasm. Smad activity can also be terminated via ubiquitin-mediated proteasomal degradation.

The I-Smads 6 and 7 inhibit signalling by several mechanisms. Smad6 is usually more specific to BMP signalling, whereas Smad7 inhibits both TGFβ and BMP signalling (Attisano and Wrana 2000). The I-Smads compete with R-Smads for binding to Smad4 (Moustakas et al. 2001). They also recruit the Smad ubiquitin regulatory factors Smurf1 and Smurf2, which ubiquitinate activated R-Smads, targeting them for proteasome-mediated degradation and hence down-regulating signalling. Smurf1 targets Smads 1 and 5 and therefore preferentially inhibits BMP signalling (Zhu et al. 1999), whereas Smurf2 targets activated Smad2 and thus down-regulates TGFβ signalling (Zhang et al. 2001). Smad7 also leads to dephosphorylation and inactivation of TGFβR1 (Ebisawa et al. 2001, Shi et al. 2004). TGFβ, activin and BMP signalling up-regulate transcription of the I-Smads, thus providing a means of ensuring the transience of signalling (Nakao et al. 1997) via a negative feedback loop.

Signalling can also be terminated by the inhibition of formation of complexes between type I and type II receptors, such as by the decoy type 1 receptor, BMP and activin membrane-bound inhibitor (BAMBI) (Itoh and ten Dijke 2007). Co-repressors such as Ski/SnoN are also important in controlling Smad function, as they inhibit Smad-induced target gene transcription. In addition, SnoN can sequester Smads in the cytoplasm, preventing their activation and nuclear accumulation. Ski and SnoN are also TGFβ target genes, thus providing another negative feedback loop mechanism to ensure signalling can be terminated (Itoh and ten Dijke 2007).

### 1.6.8 Smad-independent TGFβ signalling pathways and TGFβ receptor-independent activation of Smads

It is increasingly recognised that TGFβ can also activate other signalling pathways, via both Smad-dependent and Smad-independent mechanisms. These include MAPK, ERK, JNK and PI3K/Akt pathways [reviewed in (Massague 2012, Mu et al. 2012)]. It has recently been shown that TGFβR1 within the TGFβR complex also interacts with and activates TNF-receptor-associated factor 6 (TRAF6), causing activation of TGFβ-associated kinase 1 (TAK1), in epithelial cells [reviewed in (Landstrom 2010)]. This process is Smad-independent and leads to activation of JNK and p38 MAP kinases, resulting in promotion of apoptosis (Sorrentino et al. 2008, Yamashita et al. 2008a).

In addition, it has been shown that R-Smads can be phosphorylated, at the C-terminal, by other ligands independently of TGFβRs [reviewed in (Heldin and Moustakas 2012)], for example hepatocyte growth factor (HGF) can phosphorylate Smad2 independently of TGFβRs; the receptor kinase responsible for this is not known (de Caestecker et al. 1998). Furthermore, ERK/MAP kinases can phosphorylate Smads in their linker regions, inhibiting them by preventing their accumulation in the nucleus; p38 and JNK MAP kinases, as well as TAK1, are also able to do this [reviewed in (Heldin and Moustakas 2012)]. BMPs and TGFβ can also lead to phosphorylation of the linker region of Smad1, in addition to activation by MAP kinases, and it is thought that this may represent a means of integrating inputs from various sources, controlling the accumulation of Smad1 in the nucleus, as linker phosphorylation usually prevents its nuclear translocation (Sapkota et al. 2007). It can therefore be seen that, as more is discovered about TGFβ signalling pathways, their complexity seems to increase.

### 1.6.9 TGFβ responses

TGFβ signalling is generally anti-proliferative and/or pro-apoptotic in most cell types, although the effect varies according to the cell type and the context [reviewed in (Massague 2012)]. The mechanisms of these responses in different cell types will now be discussed in more detail.

### 1.6.9.1 TGFβ responses in epithelial cells

In epithelial cells, TGFβ can induce cell cycle arrest or apoptosis. TGFβ induces the CDKIs $p15^{INK4B}$ and $p21^{CIP1/WAF1}$ (hereafter referred to as p15 and p21 respectively), leading to cell cycle arrest in the G1 phase (Hannon and Beach 1994, Datto et al. 1995). TGFβ also leads to repressed transcription of c-Myc, via binding of Smad3 to its promoter (Frederick et al. 2004). Smad3 therefore seems to be particularly important for TGFβ-induced cell cycle arrest. The repression of c-Myc is required for induction of p15, as c-Myc normally represses p15 by binding to its promoter (Warner et al. 1999, Seoane et al. 2001); thus TGFβ and c-Myc functionally oppose each other. In epithelial cells, TGFβ causes repression of Id proteins, resulting in growth arrest (Ling et al. 2002).

The mechanism of TGFβ-induced apoptosis in epithelial cells is less well understood. Overexpression of Smad3, or to a lesser extent Smad2, can promote apoptosis in lung epithelial cells (Yanagisawa et al. 1998) and a dominant negative Smad3, or expression of Smad7, can inhibit TGFβ-mediated apoptosis in a hepatoma cell line (Yamamura et al. 2000). However, Smad7 can either promote or inhibit apoptosis in different contexts (Patil et al. 2000, Yamamura et al. 2000). Smad3 is required for TGFβ-induced apoptosis, but not TGFβ-induced arrest, in murine mammary epithelium (Yang et al. 2002), and is also necessary for TGFβ-induced apoptosis in a rat hepatoma cell line (Kim et al. 2002).

The determinant of TGFβ responses in epithelial cells, in terms of apoptosis or cell cycle arrest, may involve cross-talk between TGFβ signalling and the PI3K/Akt pathway. In hepatocytes, activated (phosphorylated) Akt can interact with unphosphorylated Smad3, sequestering the Smad3 so that it is unable to be phosphorylated and accumulate in the nucleus. Since TGFβ-induced apoptosis in epithelial cells depends on Smad3 but not Smad2, this prevents TGFβ from inducing apoptosis, but phosphorylation and nuclear accumulation of Smad2 can continue in response to TGFβ, which can thus cause TGFβ-induced arrest (Conery et al. 2004, Remy et al. 2004). Thus it has been proposed that the ratio of Smad3:Akt expression determines whether the response to TGFβ is apoptosis or arrest, i.e. a ratio above a certain threshold leads to apoptosis whereas below this threshold cell cycle arrest occurs (Conery et al. 2004).

### 1.6.9.2 TGFβ responses in normal B cells

TGFβ can also induce apoptosis or growth arrest in mature B cells. Primary B cells purified from peripheral blood or umbilical cord blood undergo spontaneous apoptosis, which is increased with TGFβ treatment (Douglas et al. 1997, Lagneaux et al. 1998). The importance of Smad3 for apoptosis is less understood in B cells than epithelial cells, although TGFβ-induced apoptosis occurs via Smad3 in murine B cells (Wildey et al. 2003), and a recent study has demonstrated a specific role for SMAD3 binding to the PUMA promoter in TGFβ-induced apoptosis in human BL cells (Spender et al. 2013). However, there is currently no evidence that the system based on a threshold of Smad3:Akt determining TGFβ response, as found in epithelial cells, occurs in B cells.

GC B cells are primed for apoptosis, with increased expression of FAS, c-Myc and BAX, and decreased expression of BCL2, compared to naïve or memory B cells (Martinez-Valdez et al. 1996). TGFβ-induced apoptosis is also important in GC B cells, leading to 'death by neglect' in cells lacking high affinity receptors for antigen (Spender et al. 2009, Spender and Inman 2011). Purified CD77+ centroblasts undergo spontaneous apoptosis, but can be partly rescued from this by the mitogen phorbol myristate acetate (PMA) (Spender et al. 2009). Blocking autocrine TGFβ signalling with the TGFβR1 inhibitor SB431542 also led to a reduction in spontaneous apoptosis, confirming that autocrine/paracrine signalling contributes to the default apoptotic state in GC B cells. This was shown to occur via induction of BIK and down-regulation of BCL-$X_L$ (Spender et al. 2009).

The majority of studies on the anti-proliferative effects of TGFβ in normal B cells have been done using B cells isolated and purified from peripheral blood or tonsil extracts. Since these are naturally quiescent, it is necessary to artificially stimulate them to proliferate before treating with TGFβ, generally by using mitogens such as PMA, anti-IgM, or cytokines such as IL-4. In these artificially stimulated B cells, TGFβ induces cell cycle arrest in G1 (Kehrl et al. 1986, Blomhoff et al. 1987, Smeland et al. 1987). TGFβ1 is secreted by resting B cells, with secretion increased upon B cell activation, thus autocrine/paracrine TGFβ1 production probably limits B cell proliferation under normal circumstances (Kehrl et al. 1986, Kremer et al. 1992).

More recently the effects of TGFβ have been investigated in human centroblasts isolated and purified (by CD77 positive selection) from human tonsil extracts (Spender et al. 2009). CD77+ centroblasts are naturally proliferating, and TGFβ inhibits their proliferation (Spender et al. 2009). Autocrine TGFβ signalling via TGFβR1 also occurs in centroblasts.

### 1.6.10 Dysregulation of TGFβ signalling in malignancies

#### 1.6.10.1 Dysregulation of TGFβ signalling in epithelial cell malignancies

In non-haematopoietic malignancies, TGFβ can have both tumour suppressive and tumour promoting effects. Often, its effects are tumour suppressive initially but then it becomes tumour promoting in association with progression of the tumour, generally in association with increased production of TGFβ1 by the tumour itself (Bernabeu et al. 2009). In this tumour promoting stage TGFβ can promote epithelial to mesenchymal transition, invasiveness and metastasis, as it reduces cell-cell adhesion and increases cell motility of epithelial cells (Elliott and Blobe 2005, Hannigan et al. 2010). Increased TGFβ1 expression and/or secretion correlate with adverse prognosis in breast, ovarian and prostate cancers (Bristow et al. 1999, Shariat et al. 2004, Desruisseau et al. 2006).

Many non-haematopoietic tumours show resistance to TGFβ signalling, often associated with mutations or reduced expression of TGFβR2 or TGFβR1, or abnormalities of other signalling components such as Smad4 mutations (Elliott and Blobe 2005). For instance, TGFβR2 has been shown to be mutated in colorectal tumours especially those associated with microsatellite instability (Markowitz et al. 1995). Loss of TGFβR2 expression, in the absence of somatic mutation, occurs in prostate cancer (Li et al. 2008), and CpG methylation of the *TGFβR2* promoter is found in lung and prostate cancers (Zhang et al. 2004, Zhao et al. 2005). Changes in histone acetylation, as well as CpG methylation, of the *TGFβR2* promoter have been shown in lung cancer cell lines, correlating with loss of growth-inhibitory response to TGFβ (Osada et al. 2001). *TGFβR2* is mutated or its expression also low in gastric carcinoma cells, correlating with loss of sensitivity to TGFβ (Park et al. 1994).

TGFβR3 also appears to have tumour suppressor functions in several non-haematopoietic malignancies. It is down-regulated in renal, endometrial, prostate, breast, lung, ovarian and

pancreatic carcinomas (Florio et al. 2005, Dong et al. 2007, Hempel et al. 2007, Turley et al. 2007, Finger et al. 2008b, Gordon et al. 2008, Margulis et al. 2008, Cooper et al. 2010). This loss of TGFβR3 expression often results in decreased responsiveness to TGFβ signalling (Cooper et al. 2010), although in some cases the effects are independent of canonical TGFβ signalling, for instance TGFβR3 signals through the MAPK pathway to cause apoptosis in renal cell carcinoma cells (Margulis et al. 2008).

#### 1.6.10.2 Dysregulation of TGFβ signalling in B cell malignancies

Unlike in carcinomas, TGFβ seems to only have tumour suppressive functions in haematopoietic cells, since although increased TGFβ levels are seen in some haematopoietic malignancies, there is no evidence that this is correlated with more aggressive disease (Dong and Blobe 2006).

**Burkitt Lymphoma**

Although several EBV-negative BL lines, including Ramos and BL41, undergo apoptosis after TGFβ treatment (Chaouchi et al. 1995), TGFβ can also induce growth arrest in BL. Some studies have shown both TGFβ-induced G1 arrest and apoptosis occurring in the same BL cell lines (MacDonald et al. 1996, Schrantz et al. 1999). However, in a study investigating the effect of TGFβ in multiple BL lines, the majority of EBV-negative BL underwent predominantly apoptosis rather than G1 arrest (Inman and Allday 2000b).

TGFβ can induce apoptosis by several different mechanisms in BL cells, including activation of caspase 3, which results in cleavage of poly ADP-ribose polymerase (PARP) (Saltzman et al. 1998, Schrantz et al. 1999, Inman and Allday 2000a). TGFβ also induces BIK, a BH3-only pro-apoptotic protein, and represses BCL-$X_L$, a pro-survival factor, in BL (Saltzman et al. 1998, Spender et al. 2009). A more recent study has shown that TGFβ can also induce early apoptosis in BL cells via up-regulation of p53-upregulated modulator of apoptosis (PUMA) (Spender et al. 2013). Expression of CD20 is required for survival of Ramos cells, and TGFβ leads to reduced expression of CD20, thus this is another mechanism by which TGFβ induces apoptosis in BL cells (Kawabata et al. 2013).

The EBV-negative BL line BL2 lacks expression of BIM but still undergoes apoptosis with TGFβ treatment, and TGFβ does not induce expression of BIM in several EBV-negative BL cell lines, suggesting that TGFβ-induced apoptosis does not occur via BIM in these cells (Spender et al. 2009). However, Bim is induced by TGFβ in the murine B cell lymphoma line WEHI (Wildey et al. 2003), and thus human and murine B lymphoma cell lines may have different mechanisms of TGFβ-induced apoptosis. It has been suggested that this differential response may be due to a difference in sequence of the BIK promoter, as the human promoter has a SMAD binding element whereas the murine and rat promoters lack this sequence (Spender et al. 2009). Thus Spender and Inman hypothesise that TGFβ-induced apoptosis occurs via up-regulation of Bim in the mouse but via up-regulation of BIK in human BL (Spender et al. 2009). Bik antagonises MCL-1, which in turn inhibits BAX/BAK (Shimazu et al. 2007), and therefore TGFβ-mediated induction of BIK relieves the inhibition of BAX/BAK, resulting in apoptosis via the intrinsic pathway.

In BL cells, in which c-Myc is deregulated, the mechanism of TGFβ-induced growth arrest appears to be different from that in epithelial cells, although the exact mechanism varies for specific cell lines. In contrast to epithelial cells, where TGFβ down-regulates Id expression, in B cells TGFβ induces Id2 and Id3 (Kee et al. 2001, Sugai et al. 2003) and in BL cells TGFβ causes induction of Id1 and Id2 (Spender and Inman 2009b).

The EBV-negative BL cell line CA46 responds to TGFβ by cell cycle arrest but not apoptosis, despite induction of BIK (Inman and Allday 2000b, Spender et al. 2009). However, these cells also fail to undergo apoptosis in response to anti-IgM (Kaptein et al. 1996); this may be explained by their very low expression of Bim (Clybouw et al. 2008) and in particular lack of BAX expression (Spender et al. 2009). The mechanism of TGFβ-induced growth arrest in CA46 BL cells occurs independently of c-Myc repression, Id repression and p15 and p21 induction; instead in these cells TGFβ represses E2F1 leading to growth arrest (Spender and Inman 2009b). However, TGFβ induces p21 in Ramos, an EBV-negative BL line (Di Bartolo et al. 2008) and thus the mechanism of TGFβ-induced arrest may differ between BL cell lines.
The down-regulation of E2F1 by TGFβ1 was confirmed in a human B lymphoma RL and the CA46 BL cell lines (Chen et al. 2012), leading to reduced levels of $p14^{ARF}$, in turn preventing

mutant p53 from inducing p21 – thus this is another mechanism by which TGFβ can induce growth arrest in B lymphoma cells (Chen et al. 2012).

**Chronic lymphocytic leukaemia**

B cell chronic lymphocytic leukaemia (CLL) is an accumulation of clonal mature B cells that are largely non-cycling *in vivo*. B cells extracted from peripheral blood (PB) of healthy individuals, or from umbilical cord blood, show increased apoptosis with TGFβ treatment, whereas B cells extracted from peripheral blood of CLL patients are resistant to TGFβ-induced apoptosis (Douglas et al. 1997, Lagneaux et al. 1998). B cells extracted from PB of healthy individuals are not able to survive for long in culture and do not proliferate; however, they will proliferate for a short time (a few days) if stimulated with certain agents such as PMA, IL2 or anti-Ig; freshly extracted B cells from CLL patients can also be stimulated to proliferate by these agents, and in some cases TGFβ inhibits their proliferation, whereas cells from other patients are completely resistant to the anti-proliferative effects of TGFβ (Lotz et al. 1994, DeCoteau et al. 1997, Douglas et al. 1997, Lagneaux et al. 1997, Schiemann et al. 2004). This TGFβ-sensitivity or resistance does not appear to obviously correlate with disease stage, although the numbers of patients were small in all these studies. No change in expression of TGFβR2 has been detected in CLL cells, whereas specific decrease-of-function mutations of TGFβR1, without alteration in mRNA expression, have been identified in TGFβ-resistant CLL cases (Schiemann et al. 2004). Lagneaux *et al* show a significant reduction in TGFβR expression in TGFβ-resistant compared to TGFβ-sensitive CLL cells, but it is not clear which TGFβR were being measured (Lagneaux et al. 1997). In some cases CLL cells thus develop resistance to the tumour suppressive effects of TGFβ *in vitro*, presumably allowing them to escape immune regulation *in vivo*; nevertheless, this does not seem to be required for CLL progression. It could be hypothesised that in those which remain sensitive to TGFβ, other apoptotic tumour suppressor pathways are de-regulated, for instance deregulation of the p53 pathway as a result of deletions on the short arm of chromosome 17 are associated with a particularly poor prognosis in CLL (Swerdlow et al. 2008). None of the quoted studies on TGFβ sensitivity in CLL have investigated this.

B cells from CLL patients, as well as B cells extracted from blood of healthy volunteers, express TGFβ1 mRNA and secrete TGFβ1, with TGFβ1 generally detectable within plasma.

There are conflicting results for whether or not the level of TGFβ1 is increased in plasma of CLL patients compared to normal controls (Kremer et al. 1992, Lotz et al. 1994, Douglas et al. 1997). However, decreased plasma TGFβ1 levels have been shown to correlate with disease progression (Friedenberg et al. 1999).

Two studies have reported increased TGFβR3 expression in B cells from CLL patients compared with normal or memory B cells on gene expression profiling (Klein et al. 2001, Jelinek et al. 2003), although the mechanism for this does not thus far appear to have been further explored.

**Diffuse Large B cell Lymphoma**

A study comparing two diffuse large B cell lymphoma (DLBCL) cell lines showed that one was resistant to TGFβ signalling and lacked expression of TGFβR2 on the cell surface compared to the TGFβ-responsive line (Chen et al. 2007). In the TGFβ-sensitive line, which expressed TGFβR2, TGFβ induced phosphorylation of Smad2 and Smad3, and ectopically expressed TGFβR2 also led to TGFβ-mediated induction of phosphorylated Smad2/3 (Chen et al. 2007). Therefore, lack of TGFβR2 could be a mechanism contributing to tumour development. In DLBCL biopsy samples, TGFβR2 was relatively over-expressed in activated B cell-like (thought to be derived from a post-GC B cell, and which have a worse prognosis) compared to GC-like subtype (Alizadeh et al. 2000). It is possible that in those with TGFβR2 expression, other apoptotic pathways had been deregulated, which if required for chemotherapy-induced apoptosis could lead to relative chemotherapy resistance and hence a worse prognosis.

**Multiple Myeloma**

In multiple myeloma, both the myeloma cells and surrounding bone marrow stromal cells secrete more TGFβ than normal B cells (Urashima et al. 1996). More recently, TGFβR3 was shown to be down-regulated in multiple myeloma, with its restoration leading to inhibition of cell growth and proliferation. However, the mechanism of this was via up-regulation of p21 and p27 when TGFβR3 was expressed rather than by the enhancement of ligand-presentation by TGFβR3 (Lambert et al. 2011).

### 1.6.11 BMP signalling in normal B cells and malignancies

Relatively little is known about the effects of BMPs in B cells. BMP2, BMP4 and BMP7 control differentiation of haematopoietic progenitors (Bhatia et al. 1999, Detmer and Walker 2002, Chadwick et al. 2003) and BMP2, BMP4 and BMP6 inhibit differentiation of mature B cells into plasma cells (Huse et al. 2011). BMP6 inhibits proliferation of B lymphoid progenitors (Kersten et al. 2006) and of resting B cells stimulated to proliferate by anti-IgM (Kersten et al. 2005). Resting B cells, including both naïve and memory B cells, express the BMP receptors ALK2 and BMPRIB (ALK6). In these cells, BMP6 induced phosphorylation of Smad1/5/8 and induction of Id1 (Kersten et al. 2005). Another study showed expression of ALK2 and BMPRIA (ALK3) in memory B cells (Seckinger et al. 2009). Treatment of naïve and memory B cells with CD40 ligand and IL-21 led to induction of ALK2 expression, which would enhance signalling by BMP6 and BMP7 (Huse et al. 2011).

Although BMPs are of major importance in the regulation of bone and cartilage formation, there is also limited evidence to suggest they may be important in development of non-haematopoietic malignancies, including promotion of invasiveness and metastasis. However, the data are generally conflicting, with several studies also showing tumour suppressive properties *in vitro* and *in vivo* [reviewed in (Thawani et al. 2010)]. Few studies have investigated the effects of BMP signalling in B cell malignancies; these have been restricted to myeloma, presumably because of the propensity for myeloma to alter the processes of bone formation and resorption, leading clinically to lytic bone lesions. BMP2, BMP4, BMP6 and BMP7 have been shown to be anti-proliferative and pro-apoptotic in myeloma cells (Kawamura et al. 2000, Hjertner et al. 2001, Ro et al. 2004, Fukuda et al. 2006b, Seckinger et al. 2009, Holien et al. 2012). In myeloma cell lines which were resistant to the growth-inhibitory effects of BMP2 and BMP4, due to a lack of type I BMP receptors ALK3 and ALK6, it was demonstrated that BMP5, BMP6 and BMP7 were anti-proliferative/pro-apoptotic (Ro et al. 2004).

## 1.7 Effects of EBV infection on TGFβ signalling

### 1.7.1 Effects of EBV on TGFβ-induced apoptosis and inhibition of proliferation

Many studies have investigated the effects of EBV on TGFβ signalling in B cells; however, the majority have assessed responses to TGFβ rather than TGFβ signalling itself. Several studies have measured the inhibition of proliferation of cells by TGFβ, but without determining whether this occurs via increased apoptosis or by growth arrest. Apoptosis and growth arrest are both regulated by multiple pathways (see sections 1.5.2 and 1.5.3), and latent EBV affects many components of these. Furthermore, these studies have been done in many different cell lines, which are mostly tumour-derived, and therefore characteristics of the particular tumours may account for some of the discrepancies between studies.

Many investigators have shown that the presence of latency III EBV in B cells renders them resistant to the effects of TGFβ (Kehrl et al. 1986, Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Arvanitakis et al. 1995, MacDonald et al. 1996, Inman and Allday 2000b). In some cases, TGFβ even appeared to increase the proliferation of EBV-infected cells (Blomhoff et al. 1987). EBV-negative B cells, and B cells expressing latency I (type I BL) generally remain sensitive to the growth-inhibitory effects of TGFβ (Blomhoff et al. 1987, Smeland et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1993, Arvanitakis et al. 1995, Chaouchi et al. 1995, MacDonald et al. 1996, Inman and Allday 2000b). However, there have been some reported exceptions, for example Akata, a group I EBV-positive BL cell line, was resistant to the effects of TGFβ (Fukuda et al. 2006a, Chen et al. 2007). There are also some discrepancies between studies, for instance Raji cells (type III BL) were sensitive to the effects of TGFβ in (Fukuda et al. 2006a), but resistant in (Wang et al. 2008) and in the EBV-negative BL line Ramos TGFβ can induce apoptosis (Chaouchi et al. 1995, Inman and Allday 2000b, Di Bartolo et al. 2008, Kawabata et al. 2013), cell cycle arrest (Blomhoff et al. 1987, Altiok et al. 1993) or be resistant to TGFβ even at high doses (Wang et al. 2008).

Previous studies investigating the effect of individual latent proteins on TGFβ responses have largely focussed on LMP1, since this had been shown to be transforming in rodent cells and nude mice (Wang et al. 1985). These studies were mostly done by stable transfection of

EBV-negative BL cell lines with an LMP1 expression vector, and have shown discrepant results for whether or not LMP1 expression was sufficient for resistance to the growth-inhibitory effects of TGFβ (Wang et al. 1988, Altiok et al. 1991, Arvanitakis et al. 1995, Inman and Allday 2000b). Expression of LMP1 alone in other cell types, including fibroblasts, epithelial cells and hepatoma cells, however, was sufficient for resistance to TGFβ-induced growth inhibition (Takanashi et al. 1999, Lo et al. 2010). Similarly, LMP2A expression in a gastric carcinoma cell line was sufficient to completely inhibit TGFβ-induced apoptosis, whereas in the EBV-negative BL line Ramos LMP2A expression only partially blocked TGFβ-induced apoptosis (Fukuda and Longnecker 2004). Thus although LMP1 or LMP2A may be sufficient to block the effects of TGFβ in epithelial cells, the situation in B cells is less clear.

There has been no investigation until now of the effect of expression of the EBNA3 proteins on sensitivity to TGFβ or TGFβR expression, even though EBNA3 proteins are expressed in latency III, which had been shown to confer resistance to TGFβ.

### 1.7.2 Effects of EBV on expression of TGFβR1 and TGFβR2

Relatively few studies have investigated possible mechanisms of the resistance to TGFβ induced by full latent EBV expression in B cells. Where TGFβR expression has been investigated, there have again been discordant results: although some suggested down-regulation of TGFβR2, correlating with TGFβ resistance, in group III BL lines and LCLs (Kehrl et al. 1989, Inman and Allday 2000b, Fukuda et al. 2006a), one study showed reductions in expression of both TGFβR1 and TGFβR2 in TGFβ-resistant lines (Kumar et al. 1991) and others investigated only for TGFβR1 but not TGFβR2 expression (Altiok et al. 1991, Altiok et al. 1993). Yet others have shown no difference in TGFβR expression between TGFβ-sensitive and -resistant cell lines (Fukuda and Longnecker 2004) or have claimed to show no difference, although the data were not shown (Arvanitakis et al. 1995, Horndasch et al. 2002). Furthermore, the mechanism of decreased TGFβR2 expression by EBV in B cells, if it does occur, is not fully established, including how its down-regulation occurs and which EBV latent proteins are responsible.

The effect of EBV on expression of TGFβRs has been investigated in other cell types. In LMP1-expressing murine fibroblasts, TGFβR2 mRNA expression was reported to be similar in a TGFβ-resistant clone and a TGFβ-sensitive clone, although the data were not shown (Takanashi et al. 1999). In human gastric carcinoma and normal gastric epithelial cells, expression of TGFβR1 and TGFβR2 were similar in TGFβ-responsive and resistant cell lines by northern and western blot; however, no loading controls were shown for their western blot, and expression of the housekeeping gene GAPDH was highly variable between cell lines on the northern blot (Fukuda et al. 2001). In a further study by the same group, it is stated that the LMP1-expressing HSC39 human gastric carcinoma cells, which were resistant to the effects of TGFβ, expressed TGFβR1 and TGFβR2, although no details are given of the method by which this was analysed (Fukuda et al. 2002).

### 1.7.3 Effects of EBV on TGFβ signalling

Few studies have investigated the effects of EBV on TGFβ signalling, as opposed to its growth inhibitory effects; these have been done mostly in non-lymphoid cells. LMP1 expression impaired TGFβ signalling, as detected by a TGFβ-responsive reporter plasmid, in epithelial cells and fibroblasts (Prokova et al. 2002, Mori et al. 2003). LMP1 expression was also sufficient to block induction of TGFβ target genes p21 or p15 in epithelial cells (Fukuda et al. 2002, Mori et al. 2003, Lo et al. 2010).

Some studies have investigated the effect of EBV or its latent proteins in B cells on downstream effects of TGFβ including ERK phosphorylation, p38 phosphorylation (Fukuda et al. 2006a) and junB expression (Arvanitakis et al. 1995). However, it was not shown that these effects occurred via Smad pathways. Indeed, the TGFβ-mediated activation of the MAPK/ERK pathway and of p38 are thought to be predominantly Smad-independent processes (Derynck and Zhang 2003). As far as can be determined, therefore, no published studies have investigated the effects of EBV directly on TGFβ signalling, as detected by Smad2 or Smad3 phosphorylation or a TGFβ-responsive reporter, in B cells.

### 1.7.4 Effects of EBV on TGFβ-induced immunoglobulin production

Another important function of TGFβ in the immune system is that it promotes switching to IgA but blocks class switching to all other isotypes (van Vlasselaer et al. 1992). TGFβ treatment inhibited production of IgG and IgM in tonsillar B cells treated with IL2 (Kehrl et al. 1986) and in B cells that had been induced to secrete IgG and IgM by EBV infection, at up to 30 days post-infection (Machold et al. 1993). However, established LCLs are resistant to this TGFβ-mediated inhibition of Ig production (Kehrl et al. 1986, Altiok et al. 1994).

### 1.7.5 Induction of lytic EBV replication by TGFβ and BMP signalling

In EBV-infected BL cell lines including Raji, Mutu I, Daudi and P3HR1-BL, TGFβ promotes EBV lytic cycle activation, as detected by expression of IE genes such as BZLF1. This was reported to occur via the MAPK/ERK pathway (Fahmi et al. 2000). TGFβ also induced a slight increase in EA expression in Akata cells (di Renzo et al. 1994), whereas another study demonstrated resistance to TGFβ-induced EBV reactivation in Akata (Fukuda et al. 2006a). This resistance correlated with lack of TGFβR2 expression and was reversed by TGFβR2 overexpression, suggesting TGFβ-induced lytic reactivation is mediated by TGFβR2 (Fukuda et al. 2006a).

TGFβ induces both apoptosis and lytic cycle induction in type I EBV-infected BL cell lines. However, individual cells with TGFβ-induced BZLF1 expression were protected against TGFβ-induced apoptosis. A caspase inhibitor blocked apoptosis but had no effect on lytic gene induction, suggesting that lytic induction and apoptosis occur via separate pathways (Inman et al. 2001).

BMP2, BMP6 and BMP7 can also reactivate EBV, in Mutu I (latency I) but not Mutu III (latency III) BL cells. The mechanism for resistance to BMP-induced EBV reactivation may be via miR-155, which has increased expression in latency III, since overexpression of miR-155 in Mutu I cells inhibited BMP-induced EBV lytic reactivation (Yin et al. 2010).

## 1.8 Specific background to this project

Shortly before this project started, members of the laboratory had performed a microarray analysis to investigate the effect of the EBNA3 proteins on expression of cellular genes in B cells, including in two EBV-negative BL cell lines, BL2 and BL31, as well as in EBNA3B knockout versus wild-type LCLs [www.epstein-barrvirus.org.uk and (White et al. 2010)]. This showed that in BL cells, EBV strongly down-regulated TGFβR2 and also up-regulated TGFβR3. Using cell lines with deletions of individual EBNA3 proteins, it was shown in the microarray that EBNA3B and EBNA3C both appeared to be necessary for the effects on TGFβR2 and TGFβR3.

Relatively little was known about the effects of TGFβ and, in particular, BMP signalling in B cells. Although many previous publications had shown that BL cells and LCLs expressing the full set of latent proteins were resistant to the anti-proliferative and/or pro-apoptotic effects of TGFβ, few had addressed the mechanisms of this. There was some controversy about whether or not this was due to the down-regulation of TGFβR2 by EBV (Kehrl et al. 1986, Kehrl et al. 1989, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1993, Arvanitakis et al. 1995, Inman and Allday 2000b, Horndasch et al. 2002, Fukuda and Longnecker 2004, Fukuda et al. 2006a). In addition, even in the cases where EBV had been shown to down-regulate TGFβR2, it was not known which particular latent genes were responsible for these effects or the mechanism for this down-regulation. Furthermore, recent work in the laboratory had shown that the repression by latent EBV of two other tumour suppressor genes, BIM and $p16^{INK4A}$, occurred via polycomb-mediated transcriptional repression. Therefore it was hypothesised that the repression of TGFβR2 by EBV may occur by a similar mechanism, albeit involving cooperation of a different pair of EBNA3 proteins.

At the start of this project, no studies had previously investigated the effect of EBV infection on TGFβR3 expression, and the consequences of its up-regulation were not known. Since TGFβR3 is a co-receptor for BMP signalling, it was hypothesised that EBV may alter BMP signalling as well as TGFβ signalling, and that this could also have important consequences including possible promotion of lymphomagenesis. At the time, there was very little data on BMP signalling in B cells, as discussed above.

### 1.8.1 Aims of this project

The specific aims of this project were:

1. To investigate the effects of EBV on TGFβ signalling, as distinct from TGFβ responses
2. To investigate the mechanism by which EBV down-regulates TGFβR2, including which latent proteins are responsible, and whether this occurs via polycomb-mediated repression
3. To investigate the effects of EBV on expression of TGFβR3
4. To investigate whether the up-regulation of TGFβR3 by EBV results in changes to BMP signalling

# Chapter 2 Materials and Methods

All chemical grade reagents were supplied by BDH chemicals, UK and were of AnalaR grade purity unless otherwise stated.

## 2.1 Solutions and Buffers

Unless otherwise stated, all solutions were prepared in dd$H_2O$.

**6x Agarose gel loading buffer**

20% (w/v) sucrose

0.1% (w/v) Bromophenol blue

10% (v/v) 10 x TBE

**Alkaline SDS**

1% SDS

0.2 M NaOH

**Blocking solution for western blots**

5% (w/v) skimmed milk powder (Sigma Aldrich, UK) in TBS-Tween

For certain antibodies, 5% BSA (Sigma Aldrich, UK) in TBS-Tween

**β-Estradiol**

1mM (1000x) stock was prepared by diluting 10mM β-Estradiol (gift from Claudio Elgueta Karstegl, Paul Farrell laboratory) in 95-100% ethanol, stored at -20$^{o}$C.

**4-hydroxytamoxifen (4HT)**

4mM (10 000x) stock was prepared by re-suspending 5mg of 4HT (Sigma) in 3.22 ml of 95-100% ethanol. Aliquots of the stock were stored at –20$^{o}$C in the dark.

**LB Agar**

1% (w/v) Bactotryptone

1% (w/v) Yeast extract

0.5% (w/v) NaCl

1.5% (w/v) Agar

**Lysis buffer with phosphatase inhibitors**

Tris pH 7.4 50mM

NaCl 250mM

NP40 0.1%

EDTA 5mM

NaF 50mM

PMSF 1mM

Protease inhibitor cocktail 1x

**PMSF (phenylmethylsulfonylfluoride)**

Dissolved at 100mM in isopropanol and stored in aliquots at –20°C

**Protease Inhibitor Cocktail Solution**

Protease inhibitor cocktail (Complete tablets, Roche) was made up to 25x in $ddH_2O$ and stored in aliquots at -20°C.

**10x SDS Running Buffer for SDS-PAGE**

250mM Tris

1.92M Glycine

1% (w/v) SDS

**2x SDS Sample Buffer**

100mM Tris-HCl (pH 6.8)

20% (v/v) Glycerol

1% (v/v) β-mercaptoethanol

1% (w/v) SDS

0.025% (w/v) Bromophenol blue

**STET**

8% Sucrose

0.1% Triton x 100

50mM EDTA

50mM Tris pH8

**Superbroth**

1.2% (w/v) Bactotryptone

2.4% (w/v) Yeast extract

0.4% (v/v) Glycerol

5% (v/v) 20x KPB (potassium phosphate buffer)

**10x TBE (Tris-Borate-EDTA) Buffer**

89mM Tris

89mM Boric acid

10mM EDTA

**TBS-Tween (TBS-T)**

20mM Tris

140mM NaCl

0.1% (v/v) Tween-20 (Sigma Aldrich, UK)

**TE**

10mM Tris-HCl pH 7.5

1mM EDTA pH 8

**Transfer Buffer for Western Blots**

233ml EtOH

100ml 10 x running buffer

667 ml $ddH_2O$

## 2.2 Cell culture and harvesting

### 2.2.1 BL cell lines and LCLs used in this project

The cell lines used were infected with BAC-derived mutant viruses as previously used in the laboratory (Anderton et al. 2008, White et al. 2010, White et al. 2012). For simplification the panel of BL31, BL2 and LCL cell lines are named throughout the report as shown in table 2.1.

| Cell line name | Allday lab. name | Ref. | Cell line name | Allday lab. name | Ref. |
|---|---|---|---|---|---|
| BL31 | BL31 | 1 | BL31 EBNA3C rev | BL31-3Crev-2 | 2 |
| BL31 WT (1) | BL31-wtBAC-2 | 2 | BL31 E3 KO 1 | BL31-E3KO-2.1 | 3 |
| BL31 WT 2 | BL31-wtBAC-4 | 3 | BL31 E3 KO 2 | BL31-E3KO-3.2 | 3 |
| BL31 WT 3 | BL31-wtBAC-3 | 3 | BL31 E3 rev | BL31-E3rev-2.1 | 3 |
| BL31 EBNA2 KO (G) | BL31-E2KO-GK | 4 | BL31 LMP1 KO | | 6 |
| BL31 EBNA2 KO 1 | BL31-E2KO-1.1 | 5 | BL31 LMP1 KO 2 | | 6 |
| BL31 EBNA2 KO 2 | BL31-E2KO-2.1 | 5 | BL31 LMP2A KO 1 | | 6 |
| BL31 EBNA2 rev | BL31-E2rev-1.1 | 5 | BL31 LMP2A KO 2 | | 6 |
| BL31 EBNA3A KO 1 | BL31-3AKO-4 | 3 | BL2 | BL2 | 1 |
| BL31 EBNA3A KO 2 | BL31-3AKO-1.1 | 2 | BL2 WT | BL2-wtBAC-2.1 | 2 |
| BL31 EBNA3A KO 3 | BL31-3AKO-3 | 3 | BL2 EBNA2 KO (G) | BL2-E2KO-GK | 4 |
| BL31 EBNA3A KO 4 | BL31-3AKO-1.2 | 2 | LCL-WT | LCL-wtBAC-D2.3 | 3 |
| BL31 EBNA3A rev | BL31-3Arev-2 | 3 | LCL-EBNA3B KO 1 | LCL-3BKO-D2.5 | 3 |
| BL31 EBNA3B KO 1 | BL31-3BKO-6.2 | 3 | LCL-EBNA3B KO 2 | LCL-3BKO-D2.4 | 3 |
| BL31 EBNA3B KO 2 | BL31-3BKO-1 | 2 | LCL-EBNA3B rev | LCL-3Brev-D2.3 | 3 |
| BL31 EBNA3B rev | BL31-3Brev-3 | 3 | LCL-3CHT | | 7 |
| BL31 EBNA3C KO 1 | BL31-3CKO-5 | 3 | LCL-3CHT (p16-null) | | 7 |
| BL31 EBNA3C KO 2 | BL31-3CKO-3 | 2 | | | |

**Table 2.1: Cell lines used in this project**

KO=knockout, rev=revertant. References: [1](Calender et al. 1987), [2](Anderton et al. 2008), [3](White et al. 2010), [4](Kelly et al. 2005), [5](Ian Groves, unpublished), [6](this project; HEK293 producer lines a kind gift of Wolfgang Hammerschmidt, Münich), [7](Skalska et al. 2013).

All cells were grown in RPMI-1640 medium (Gibco), all supplemented with penicillin-streptomycin 100u/ml, with additional supplementation as shown in table 2.2. Cells were

passaged two to three times weekly by diluting appropriately in fresh warmed medium. All cells were cultured at 37°C in a humidified incubator with 10% $CO_2$.

| **Cell line(s)** | **Medium** |
|---|---|
| Established LCLs (except LCL-3CHT and EREB2.5), Ramos, Akata6, Akata31 | RPMI + 10% FCS |
| BL2, BL31 | RPMI + 10% FCS + sodium pyruvate 1mM (Sigma-Aldrich) + alpha-thio-glycerol 50µM (Sigma-Aldrich) |
| BL2 and BL31 infected with recombinant BACs | RPMI + 10% FCS + sodium pyruvate 1mM (Sigma-Aldrich) + alpha-thio-glycerol 50µM (Sigma-Aldrich) + hygromycin B 100ug/ml (Roche) |
| LCL-3CHT | RPMI + 10% FCS + 400nM 4-hydroxytamoxifen (4HT, Sigma) |
| EREB2.5 | RPMI + 10% FCS + 1µM β-estradiol (gift from Claudio Elgueta Karstegl) |
| Newly infected primary B cells (for first month after infection) | RPMI + 15% selected FCS + L-glutamine 2mM |

**Table 2.2: Media used to culture B cell lines**

### 2.2.2 Freezing and thawing cells

For long-term storage of cell stocks, 5 x $10^6$ cells were pelleted and re-suspended in 1 ml freezing medium (20% FCS and 10% DMSO in RPMI). The cells were placed in cryovials in a freezing container, then transferred to liquid nitrogen following storage at -80$^o$C overnight. To recover cells from liquid nitrogen, the cryovial was thawed at 37$^o$C then the cells promptly re-suspended and dripped slowly into 10ml of medium. Cells were then pelleted, the medium aspirated and then re-suspended in fresh medium. Cells recovered from liquid nitrogen were generally cultured for at least ten days before the start of any experiment.

### 2.2.3 Harvesting cells

For all experiments, viable cell counts were performed by diluting cells 1:1 with 0.4% trypan blue (Sigma Aldrich) and placing a small volume on a haemocytometer. Living cells, which

exclude trypan blue dye, were counted under a light microscope. Cells were seeded at a density of 3 x $10^5$/ml living cells 24 hours before harvesting or performing any experiment. For RNA and protein extraction, cells were centrifuged at 1300 x *g* for 4 minutes (min) at 4$^{o}$C, the media was aspirated and the cell pellet washed with cold PBS, before being centrifuged again at 1300 × *g* for 4 min at 4$^{o}$C. This wash was repeated, the supernatant was removed again, and the pellet was re-suspended in 1ml of cold PBS and transferred into an Eppendorf. The cells were then centrifuged at 3000 × *g* for 4 min, the supernatant removed, and the dry cell pellet then snap frozen on dry ice and stored at -80$^{o}$C before extraction. When the pellet was to be harvested for RNA extraction, the pellet was lysed in RLT buffer (Qiagen) containing β-mercapto-ethanol and then stored at -80$^{o}$C before subsequent RNA extraction.

### 2.2.4 Adherent cells (HEK293)

Adherent cells were grown in RPMI + 10% FCS, supplemented with penicillin/streptomycin 100U/ml, additional L-glutamine 2mM (Gibco), and hygromycin B 100µg/ml (Roche). The cells were generally split twice weekly as follows: cells were washed once in warmed PBS and then incubated with an appropriate volume of 0.05% trypsin/EDTA (Invitrogen) at 37°C until cells detached from the flasks. An equal volume of fresh medium was added to halt further trypsinisation and cells were then split at between 1:4 and 1:6 into fresh flasks. The cell lines used are shown in table 2.3.

| Cell line | Details | Source |
|---|---|---|
| HEK293-delLMP1 (G204) | maxi-EBV deltaLMP1 (clone 2597.3) | Kind gift of Prof. Hammerschmidt (ref 1) |
| HEK293-delLMP2A (G212) | maxi-EBV deltaLMP2A (clone 2525.26) | Kind gift of Prof. Hammerschmidt (ref 2) |
| HEK293-HB9I6 | | Rob White, Allday laboratory |
| HEK293-delEBNA3B C14 | | Rob White, Allday laboratory |
| HEK293-r3B M8 | | Rob White, Allday laboratory |

**Table 2.3: Adherent cell lines used in this project**

1-(Dirmeier et al. 2003), 2-(Mancao and Hammerschmidt 2007)

### 2.2.5 Cytokine stimulation experiments

Recombinant Chinese hamster ovary (CHO)-derived human TGFβ1 (Peprotech) was made up according to the manufacturer's instructions, diluted in sterile BSA 2mg/ml in PBS, and stored in aliquots at -20$^{o}$C until use. Recombinant human BMP2, BMP4, BMP6, BMP7 and BMP9 (all R&D systems) were made up according to the manufacturer's instructions, diluted in sterile 0.5% (w/v) BSA in 4mM HCl and stored in aliquots at -20$^{o}$C until use.

In all experiments using cytokines, cells were seeded at 3 x $10^5$/ml 24 hours prior to use. At time zero, cells were re-suspended then divided into equal volumes which were then treated with cytokine (TGFβ1 5ng/ml except where otherwise specified; BMP concentrations as shown in figures) or vehicle (untreated; BSA 2mg/ml in PBS for TGFβ1 experiments and 0.5% BSA in 4mM HCl for BMP experiments). Cells were harvested under identical conditions for the cytokine-treated and untreated at the time points shown; cells harvested at time zero were not treated with vehicle.

### 2.2.6 Extraction and purification of primary B cells

PBL for infection of primary B cells were isolated from buffy coat residues (UK NHS Blood and Transplant) by density gradient centrifugation using Ficoll-Paque. Each buffy coat was made up to 200ml with cold PBS and the solution carefully layered onto Ficoll-Paque (GE Healthcare) at a ratio of 1:1 in 50ml Falcon tubes. These were then centrifuged at 1300 x *g* for 30 min at room temperature with the brake off. Leukocytes were then recovered from the cell-plasma interface and washed twice with 100ml wash buffer (RPMI + 1% FCS) before being re-suspended in wash buffer to perform a cell count.

$10^9$ cells were then transferred to a fresh Falcon tube, spun at 1300 x *g* for 5 min to pellet the cells, then re-suspended in MACS running buffer (PBS-2mM EDTA-0.5% BSA). CD19 microbeads (Miltenyi Biotech) were added and the mix left for 15 min at 4$^{o}$C. The cells were centrifuged to remove unbound microbeads, then re-suspended in MACS running buffer before being subjected to separation of CD19 positive cells (i.e. B cells) by positive selection using the AutoMACS sorting system (Miltenyi Biotec), according to the manufacturer's

instructions. The cells obtained were then re-suspended in culture medium at 2 x $10^6$ cells/ml before proceeding to virus infection.

A small sample of cells was removed for CD20 flow cytometry analysis to measure the purity of the B cell population obtained (see section 2.7.3 for details).

### 2.3 RNA extraction, reverse transcription and quantitative reverse-transcriptase PCR

RNA extraction from cell pellets was performed using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions, using additional on-column DNAse treatment (Qiagen). RNA was quantified using the Nanodrop ND-1000 spectrophotometer and appropriately diluted in RNAse-free water (Qiagen) before performing qRT-PCR.

For SYBR green-based assays, qRT-PCR was performed either in a one-step assay, using the QuantiFast SYBR Green RT-PCR kit (Qiagen), or in a two-step process, using the Superscript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen) for reverse transcription, followed by the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Reverse transcription was performed on a standard quantity of RNA (usually 750-1000ng; less in primary B cell infection experiments, where a limited amount was available at early time points), according to the manufacturer's instructions. For several experiments, analysis was done by both one-step and two-step qRT-PCR methods on the same samples, to confirm that the same results were obtained with both methods.

qRT-PCR analysis was performed by the standard curve method, in each experiment using six serial 5-fold dilutions of a mixture containing all template RNA or cDNA samples. Each sample was run in triplicate and the standard deviation (SD) calculated. Error bars in all graphs represent +/- 1 SD. qRT-PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR system. Product specificity was confirmed using dissociation curve analysis in each qRT-PCR run, with agarose gel electrophoresis used initially to confirm product specificity for each primer set. No-template controls were also included on each plate to exclude contamination or primer-dimer formation.

The PCR settings were as follows: for the one-step QuantiFast assay: fast (96-well plates only): 10 min at 50°C (reverse transcription), 5 min at 95°C (activation), then 10 sec at 95°C alternating with 30 sec at 60°C for 40 cycles; standard (384-well plates): 10 min at 50°C, 5 min at 95°C, then 15 sec at 95°C alternating with 60 sec at 60°C for 40 cycles. For the two-step Platinum SYBR green assay using cDNA template: 2 min at 50°C, 10 min at 95°C, then 15 sec at 95°C and 60 sec at 60°C alternating for 40 cycles.

Primer sequences for SYBR green-based assays were as shown in table 2.4. All primers were supplied as standard salt-free preparations and used at 200nM final concentration (Eurofins MWG Operon or Sigma). Primers for qRT-PCR for GAPDH were from Qiagen (QuantiTect Primer Assay).

| Primer | Forward sequence | Reverse sequence |
|---|---|---|
| GNB2L1 | GAGTGTGGCCTTCTCCTCTG | GCTTGCAGTTAGCCAGGTTC |
| TGFβR1 | CTATATCTGCCACAACCGCACTGTC | CGCCACTTTCCTCTCCAAACTTCTC |
| TGFβR2 | GGCTCAACCACCAGGGCA | CTCCCCGAGAGCCTGTCCAGA |
| TGFβR3 | TGTGTGCCTCCTGACGAAGC | AGGCTGCAAACGCAATGCCC |
| SMAD1 | CACAAACATGATGGCGCCT | TGTGGAGGAGGCATGGAACGC |
| TGFβ1 | GATGTCACCGGAGTTGTGCG | GTGAACCCGTTGATGTCCACTT |
| SMAD5 | CTGCCTCTGACTTGACCCAAT | AGTCAGTGGCTACCGAAAGAA |
| CDKN2B | ACTAGTGGAGAAGGTGCGAC | GCCCATCATCATGACCTGGA |
| BMPR1A | TAAAGGTGACAGTACACAGGAACA | TCTATGATGGCAAAGCAATGTCC |
| ID1 | TGGTCGCTGTCTGTCTGAG | GCCGTTGAGGGTGCTGAG |
| ID2 | ACGACCCGATGAGCCTGCTA | TCCTGGAGCGCTGGTTCTG |
| RPLPO | ACTCTGCATTCTCGCTTCCT | GGACTCGTTTGTACCCGTTG |
| $p21^{CIP1/WAF1}$ | CTGGAGACTCTCAGGGTCGAA | GCGGATTAGGGCTTCCTCTT |
| ALAS1 | TCCACTGCAGCAGTACACTACCA | ACGGAAGCTGTGTGCCATCT |
| CD23 | TGGGACACCACACAGAGTCTAAA | CCGTGGTGGCTTTCCAAGT |
| TUBB | CTTCGGCCAGATCTTCAGAC | AGAGAGTGGGTCAGCTGGAA |

**Table 2.4: Primer sequences for qRT-PCR using SYBR green**

Sequences were obtained from the following sources: GNB2L1 (Zhang et al. 2005), TGFβR1 (Bruno et al. 1998), TGFβR2 (Di Bartolo et al. 2008), TGFβR3 (Konrad et al. 2007). p21, ALAS1 and RPLPO – Lenka Skalska, PhD thesis, ID1 and ID2 (Locklin et al. 2001), BMPRIA (Feeley et al. 2005), TUBB (Zhang et al. 2005), CD23 – Lenka Skalska. The remaining primers (SMAD1, TGFβ1, SMAD5, CDKN2B) were designed by the author using Primer-Blast (Skaletsky 2000).

For qRT-PCR using the Taqman method, cDNA was amplified using the EfficienSee FAST qPCR MasterMix Plus dTTP (Eurogentec), with primers (Eurofins MWG Operon or Sigma) at a final concentration of 300nM and probes (Eurogentec) at 200nM. The qPCR settings were as follows: 2 min at 50$^{o}$C, 10 min at 95$^{o}$C, then 15 sec at 95$^{o}$C alternating with 60 sec at 60$^{o}$C for 40 cycles. The sequences of primers and probes used are shown in table 2.5. All probes were labelled with 6-carboxyfluorescein phosphoramidite (FAM) reporter dye at the 5' end and Black hole quencher 1 (BHQ-1) at the 3' end.

| **Transcript** | | **Sequence** |
|---|---|---|
| LMP1 | Exon 2 forward primer | AATTTGCACGGACAGGCATT |
| | Exon 3 reverse primer | AAGGCCAAAAGCTGCCAGAT |
| | Probe (exon 2/3) | TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT |
| LMP2A | Exon 1 forward primer | CGGGATGACTCATCTCAACACATA |
| | Exon 2 reverse primer | GGCGGTCACAACGGTACTAACT |
| | Probe (exon 2) | CAGTATGCCTGCCTGTAATTGTTGCGC |

**Table 2.5: Primer and probe sequences for Taqman qRT-PCR**

Sequences were obtained from (Bell et al. 2006).

## 2.4 Protein extraction, SDS-PAGE and Western blotting

Protein was extracted from thawed or freshly harvested cell pellets using fresh lysis buffer with phosphatase inhibitors. A volume approximately equivalent to 1-2 times the size of the pellet was added, mixed by pipetting and then vortexed to lyse. The samples were left on ice for 5-10 min and then spun at 21,000 x *g* for 15 min at 4$^{o}$C. The supernatant was transferred to fresh tubes and then protein quantified using the DC protein assay with bovine serum albumin (BSA) standard (Bio-Rad Laboratories), using six dilutions for the standard curve and analysed on the Helios spectrophotometer (Thermo Scientific). The protein samples were then diluted appropriately in lysis buffer and stored at -80$^{o}$C until use. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gels were made according to table 2.6 using the Bio-Rad mini-Protean II system. Generally 30µg of each protein sample was mixed with an equal volume of 2x SDS sample buffer, heated at 100$^{o}$C for 7 min to denature proteins and then loaded into SDS-polyacrylamide gels of an appropriate percentage.

Alternatively, in some experiments whole cell lysates were used, as follows: cell pellets were directly lysed in 2x SDS protein sample buffer and then sonicated to lyse the cells (Bioruptor UCD-200, Diagenode) on high setting for 15 min (intermittent sonication – 30 seconds on, 30 seconds off). In this case, following heating at 100$^{o}$C for 7 min an equal volume of each sample was loaded into the SDS-polyacrylamide gel.

Electrophoresis was performed at 150V for 70 min in running buffer, followed by transfer to nitrocellulose membrane (Whatman GmbH, Germany) at 100V constant voltage for 60 min in transfer buffer. Membranes were washed once briefly in TBS-T then blocked in blocking solution for 60 min at room temperature (see table 2.7 for which blocking solution was used). Primary antibodies were diluted as shown in table 2.7 in 5% skimmed milk or 5% BSA in TBS-T and left to incubate at 4$^{o}$C overnight with shaking. Membranes were washed as follows: two changes of TBS-T followed by two ten-minute washes, and then incubated with appropriate HRP-conjugated secondary antibodies (table 2.8) for 60 min followed by the same wash procedure. The reaction was developed using enhanced chemiluminescence (ECL) Western Blotting detection reagents (GE Healthcare, UK) and exposed on Amersham Hyperfilm MP (GE Healthcare). The size of proteins detected was compared to protein markers, depending on the size of proteins of interest: either High-Range Rainbow marker

(Amersham), combined 1:1 with 2x SDS sample buffer and boiled for 7 min before being loaded, or Spectra multicolour broad range protein marker (Thermo scientific) loaded without boiling, according to the manufacturer's instructions.

For all western blots using antibodies to SMAD and phosphorylated SMAD (pSMAD), gels were prepared in multiples and loaded with the same samples. This enabled direct comparison between the SMAD and the related pSMAD without stripping the membrane, since the two forms of the protein run at approximately the same size on SDS-PAGE. Each membrane was re-probed with anti-γ-tubulin to ensure that equal amounts of each sample had been loaded into the gel. In all figures showing multiple antibodies, a representative anti-γ-tubulin blot is shown.

| **Resolving gels** | **7.5%**<br>**2 gels** | **10%**<br>**2 gels** | **12.5%**<br>**2 gels** | | **Stacking gels** | **2 gels** |
|---|---|---|---|---|---|---|
| **Acrylamide/Bis** | 1.98 ml | 2.64 ml | 3.3 ml | | **Acrylamide/Bis** | 828 µl |
| **Tris pH 8.8** | 2.96 ml | 2.96 ml | 2.96 ml | | **Tris pH 6.8** | 620 µl |
| **10% SDS** | 79.2 µl | 79.2 µl | 79.2 µl | | **10% SDS** | 49.6 µl |
| **dd$H_2O$** | 2.96 ml | 2.3 ml | 1.64 ml | | **dd$H_2O$** | 3.47 ml |
| **APS** | 26.4 µl | 26.4 µl | 26.4 µl | | **APS** | 24.8 µl |
| **TEMED** | 5.3 µl | 5.3 µl | 5.3 µl | | **TEMED** | 5 µl |

**Table 2.6: Resolving and stacking gels for SDS-PAGE**

| Antibody | Species/type | Manufacturer | Dilution |
|---|---|---|---|
| Anti-SMAD1 | Rabbit polyclonal | Cell Signaling 9743 | 1:1000 |
| Anti-SMAD2 | Rabbit polyclonal | Invitrogen 51-1300 | 1:200 |
| Anti-SMAD2 | Mouse monoclonal | Cell Signaling 3103 | 1:1000 |
| Anti-SMAD5 | Rabbit polyclonal | Cell Signaling 9517 | 1:1000 |
| Anti-Phospho-SMAD1 (ser 206) | Rabbit polyclonal | Cell Signaling 9553 | 1:500 |
| Anti-Phospho-SMAD5 (ser 463/465) [EP728(2)AY] | Rabbit monoclonal | Abcam 76296 | 1:1000 |
| Anti-phospho-SMAD1 (ser 463/465), 5 (ser 463/465) and 8 (ser 426/428) | Rabbit polyclonal | Cell Signaling 9511 | 1:1000 |
| Anti-phospho-SMAD2 (ser 465/467) | Rabbit polyclonal | Cell Signaling 3101 | 1:1000 |
| Anti-gamma-tubulin (GTU-88) | Mouse monoclonal | Sigma-Aldrich T6557 | 1:10,000 |
| Anti-PARP | Rabbit polyclonal | Boehringer Mannheim 1 835 238 | 1:5000 |
| Anti-EBNA2 (clone PE2) | Mouse monoclonal | Dako M7004 (discontinued) | 1:500 |
| Anti-EBNA2 (clone PE2) | Mouse monoclonal | Abcam ab49498 | 1:500 |
| Anti-EBNA3A | Sheep polyclonal | Exalpha F115p | 1:1000 |
| Anti-EBNA3B 3B(2)6C9 | Rat monoclonal | Allday lab | 1:10 |
| Anti-EBNA3C (A10) | Mouse monoclonal | Gillian Parker (Allday lab)[1] | 1:10 |
| Anti-LMP1 CS1-4 | Mouse monoclonal | Dako M0897 | 1:500 |
| Anti-EBNA-LP 4D3 | Mouse monoclonal | Yasushi Kawaguchi[2] | 1:1000 |
| Anti-EBNA1 | Human serum (polyclonal) | Prof. Paul Farrell | 1:750 |
| Anti-LMP2A (14B7) | Rat monoclonal | Abcam ab59026 | 1:1000 |
| Anti-TGFβR3 (D11G10) | Rabbit monoclonal | Cell Signaling 5544 | 1:1000 (in 5%BSA) |

**Table 2.7: Primary antibodies for western blotting**

All were diluted in 5% milk/TBS-T unless otherwise stated. 1-(Maunders et al. 1994), 2-(Shaku et al. 2005).

| Antibody | Manufacturer | Dilution |
|---|---|---|
| Polyclonal goat anti-rabbit Ig/HRP | Dako P0448 | 1:2000 |
| Polyclonal rabbit anti-rat Ig/HRP | Dako P0450 | 1:2000 |
| Rabbit anti-sheep Ig/HRP | Dako P0163 | 1:2000 |
| ECL sheep anti-mouse IgG HRP | GE Healthcare NA931 | 1:2000 |
| Rabbit anti-human (anti-IgA, -IgG, -IgM, -κ/λ) | Dako P0212 | 1:2000 |

**Table 2.8: Secondary antibodies for western blotting**

All secondary antibodies were diluted in 5% milk/TBS-T.

## 2.5 Chromatin immunoprecipitation (ChIP)

For ChIP analysis, cells were seeded at 3 x $10^5$/ml 24 h before the experiment and then 3.6 x $10^6$ cells were fixed in 1% formaldehyde (Sigma-Aldrich) for 10 min before washing three times with ice-cold 1x PBS containing phosphatase inhibitors [PMSF 1μM, aprotinin 1μg/ml (Sigma-Aldrich) and Pepstatin A 1μg/ml (Sigma-Aldrich) final concentrations]. Cell pellets were snap frozen on dry ice then stored at -80°C until the assay was performed.

ChIP was performed using the ChIP assay kit (Millipore) according to the manufacturer's instructions. Sonication (Bioruptor UCD-200, Diagenode) was performed for 12.5 min (intermittent sonication with 30 sec on, 30 sec off) to obtain sheared chromatin with DNA of 200-1000bp in length (Kostas Paschos, personal communication). The adequacy of sonication was assessed by running the sonicated samples, after cross-link reversal, on a 2% agarose gel. The antibodies used are shown in table 2.9. Rabbit IgG (Millipore) was used as a control (2μl per ChIP).

| Antibody | Manufacturer | Species | Amount per ChIP |
|---|---|---|---|
| Anti-H3K27Me3 | Millipore 17-622 | Rabbit | 4μg |
| Anti-H3K9Ac | Millipore 17-658 | Rabbit | 5μg |
| Anti-H3K4Me3 | Millipore 17-614 | Rabbit | 3μg |
| Anti-SUZ12 | Abcam ab12073 | Rabbit | 4μg |

**Table 2.9: Antibodies used for chromatin immunoprecipitation**

Precipitated DNA was purified using the Qiaquick gel extraction kit (Qiagen), according to the manufacturer's instructions. DNA was eluted in $ddH_2O$ and then stored at -20°C prior to qPCR analysis.

qPCR was performed using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), using dissociation curve analysis to check the specificity of product, with primer sequences shown in table 2.10. The qPCR settings were as follows: 2 min at 50°C, 10 min at 95°C, then 15 sec at 95°C and 60 sec at 60°C alternating for 40 cycles. Once standard curves had been performed initially to ensure that the primer sets had comparable efficiencies, analysis was performed in triplicate using the delta-delta Ct method. IgG controls were also used in every case; however, the results with these were negligible in all cases and hence results are expressed as fold enrichment relative to input. All samples were run in triplicate and the standard deviation calculated (shown as error bars in the graphs). For marks of active transcription (H3K4Me3 and H3K9Ac), actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as positive controls and γ-globin or myoglobin used as negative controls, whereas for marks of repressed transcription (H3K27Me3 and SUZ12), actin or GAPDH were used as negative controls and γ-globin or myoglobin were positive controls.

| | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| TGFβR2 set 1 | AGCTTGGGTGCTAGGTGGAGCA | GGCAAGGGTTTGTGCCAGGGT |
| TGFβR2 set 2 | AAAATGTTTTTCTTTAGGTCGAAGT | CCAGGTGATCAATATGTACATTAAA |
| TGFβR2 set 3 | GAGAGAGCTAGGGGCTGG | CTCAACTTCAACTCAGCGCTGC |
| TGFβR2 set 4 | GGACCACTCACCCGACTTCT | CCGCTGCACATCGTCCTGT |
| TGFβR2 set 5 | TCCTGGAGACGGCCACGCTT | TGCCAAGGCGTCAGTGGAGC |
| TGFβR3 dis 1 | CCCCACCAGCCTCACAGGGAT | GGGGCACTGTGCTCATCTCGC |
| TGFβR3 dis 2 | GCCTGAAGCTGCGCATGGTCT | TGCTTCCTTGTGTGTGCTGGC |
| TGFβR3 prox1 | AGGACGTCGCACAGGCACAG | TACCGGCGTTACCCGGAGGT |
| TGFβR3 prox2 | GGGGAGAGGGCAAGAGGCTGT | GGGGACTCGCTCCCTCAAACG |
| TGFβR3 prox3 | GCGTGACAGCTTCGGTGGGA | GAACGCAAACCGCGTCCGTG |
| TGFβR3 prox4 | TTCCTCAGCGGAGAGCGGCA | GCTGAAAGATGGGAGAAGATGCATGG |
| SMAD1 A | GTGCGGGGGAGTTGGCATCA | TCGAGTAGGTAACCTTGTTGCTCCA |
| SMAD1 B | AAGTCCGCTCCACCGGGACTAAA | CCCCACGCCCCGTTTCTTCCT |
| SMAD1 C | CTCGTGCTCCCACACGGACG | TGGGACGCGGCATGAAGGGA |
| SMAD1 D | CGGCCCCAGCAAGCCTCTTT | GAGACGCAGCGGGTGTAGGC |
| SMAD1 E | GGGGAGGAGGAACAAATGCCTGC | GAAGGAACGCGCCTTTACTGGT |
| Actin | TGCACTGTGCGGCGAAGC | TCGAGCCATAAAAGGCAA |
| GAPDH | CGGCTACTAGCGGTTTTACG | AAGAAGATGCGGCTGACTGT |
| γ-globin | GCCTTGACCAATAGCCTTGACA | GAAATGACCCATGGCGTCTG |
| Myoglobin | GGAGAAAGAAGGGGAATCACAT | GATAAATATAGCCAACGCCACA |

**Table 2.10: Primer sequences for ChIP-qPCR**

With the exception of TGFβR2 set 3 (see below), the TGFβR2 and TGFβR3 primers were designed by the author, with the initial help of Kostas Paschos (Allday lab), using MethPrimer (Li and Dahiya 2002) and Primer-Blast (Skaletsky 2000). The SMAD1 primers were designed by the author. References for the other primer sets are as follows: TGFβR2 set 3 (Di Bartolo et al. 2008), actin (He and Margolis 2002), γ-globin (Bottardi et al. 2003), myoglobin (Noer et al. 2009) and GAPDH (Everett et al. 2007).

### 2.5.1 Agarose gel electrophoresis

Agarose gels were made with 1% or 2% agarose dissolved in 1x TBE depending on the size of the DNA fragment of interest. Staining SYBRsafe (Invitrogen, UK) was added to the mixture. Samples were mixed with the appropriate volume of 6x agarose gel loading buffer before loading and running in 1x TBE running buffer at 80V for approximately 1 hour before

visualization on a UV trans-illuminator. In all cases a 1kb or 100bp DNA ladder (New England Biolabs, UK) was used as a marker and run alongside DNA samples.

## 2.6 DNA methylprecipitation analysis

Genomic DNA was extracted from harvested cell pellets using the Qiagen Blood and Cell culture Midi kit, according to the manufacturer's protocol, using 5 x $10^6$ cells per sample re-suspended in sterile PBS and using Qiagen protease. The extracted DNA was precipitated with isopropanol, washed once with ice-cold 70% ethanol, spun at >5000 x *g* for 10 min at 4$^{o}$C, the supernatant aspirated, the remainder air-dried and then the DNA re-suspended in dd$H_2O$. The DNA concentration was checked using the Nanodrop spectrophotometer, then all samples were diluted to 10ng/µl in dd$H_2O$ for sonication. The samples were sonicated for 12.5 min on high setting, as before, followed by analysis on a 2% agarose gel to check the adequacy of sonication to fragments of <1000bp in size. Precipitation of methylated DNA was performed using the Methylcollector Ultra kit (Active Motif) according to the manufacturer's instructions, using 100ng of sonicated DNA per sample. Fully *in vitro* methylated Jurkat DNA (Active Motif) was used as a positive control. Two negative controls were used: one sonicated genomic DNA extracted from PBMCs extracted from the blood of a healthy donor, which are known to be unmethylated [Kostas Paschos, personal communication and (Paschos et al. 2009)] and a sample in which dd$H_2O$ was added instead of the His-MBD2b protein complex. The samples obtained were then purified using the Qiaquick gel extraction kit (Qiagen), according to the manufacturer's instructions. Samples were eluted in dd$H_2O$ then stored at -20$^{o}$C until qPCR could be performed. qPCR was performed as for ChIP-qPCR, with results expressed relative to input.

## 2.7 Flow cytometry

### 2.7.1 Propidium iodide (PI) staining

Approximately 2 x $10^6$ cells were harvested per condition and washed three times in ice-cold 1x PBS. Cells were then re-suspended into 80% ethanol, which had been pre-chilled to -20°C, and mixed by vortexing. Cells were then stored at -20°C for up to seven days before flow cytometric (FC) analysis. Prior to FC, cells were centrifuged at 900 x *g* for 4 min, the ethanol removed and then the cells re-suspended in 1 x PBS containing 8µg/ml RNase A (Qiagen)

and 18µg/ml propidium iodide (Sigma-Aldrich); samples were left on ice in the dark for at least one hour before proceeding to FC analysis. FC was performed using the FACSCanto II machine (BD Biosciences) and the data analysed using FlowJo software (Treestar, Ashland, OR, USA).

### 2.7.2 Combined Draq5 and live-dead fixable staining

Approximately 1 x $10^6$ cells were harvested per condition and washed three times in ice-cold 1x PBS. Cells were then re-suspended into ice-cold PBS containing Live/dead Fixable Violet Stain 1µl/ml (Invitrogen) and left on ice in the dark for 30 min. The samples were then centrifuged, washed once with 1x PBS and once with 1x PBS containing 1% BSA before being re-suspended in 80% ethanol (pre-chilled to -20$^o$C) whilst vortexing. Samples were then left on ice in the dark for 60 minutes. They were then centrifuged at 1500 x *g* for 5 min at 4$^o$C, the ethanol aspirated and the cells re-suspended in 1 x PBS-BSA 1% and left for 10-15 min at room temperature. They were then centrifuged, PBS-BSA removed and the cells re-suspended in 1x PBS containing Draq5 2µl/ml (Biostatus) and RNase A 10µg/ml (Qiagen), left in the dark at room temperature for 30-60 min before proceeding to FC analysis. FC was performed using either the FACSCanto II or the LSR II machine (both BD Biosciences) and data analysed using Flowjo software, as above.

### 2.7.3 CD20 staining for analysis of purity of primary B cell separation

$10^6$ cells were removed from each sample of purified primary B cells, centrifuged then washed once with ice-cold 1x PBS/0.5% BSA (PBS-BSA) before re-suspending in PBS-BSA containing 10µg of human IgG. The samples were left on ice for 15 min before adding anti-CD20 APC 5µl per sample (eBioscience). Samples were then left on ice in the dark for 60 min, washed twice with PBS-BSA before being re-suspended in MACS running buffer then proceeding to FC analysis. The percentage of CD20+ve cells were analysed by FC on the DAKO CyanADP cytometer.

## 2.8 Episomal rescue from 293 cells to verify BACS

HEK293 cells, containing recombinant BACS, were established and selected with hygromycin (100µg/ml) before harvesting cells for extraction of low-molecular weight DNA for episomal

rescue and pulsed-field gel analysis of BAC restriction digests, in order to verify the BAC constructs.

### 2.8.1 Low molecular weight DNA extraction

293 cell clones were grown to approximately 80% confluence in a flask, washed and trypsinised whilst re-passaging, as described previously, and then the cells remaining after passage were pelleted, washed in PBS and then the pellet snap frozen and stored at -80$^{o}$C until use. The frozen cell pellet was subsequently thawed and then re-suspended in 60 µl STET. Cells were lysed by addition, while vortexing, of 130 µl alkaline SDS. This was then neutralised by the addition, while vortexing, of 110 µl 7.5M ammonium acetate. The mixture was incubated on ice for 5 min before centrifuging at 18,000 x *g* for 30 min at 4$^{o}$C. The cleared lysate was transferred to fresh Eppendorfs in order to proceed to phenol-chloroform extraction.

The cleared cell lysate was transferred to a 1.5 ml MaXtract High Density Gel tube (Qiagen), 200 µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1; Sigma) added and the tube rapidly inverted for 30 s to mix before centrifuging at 9500 x *g* for 6 min. The upper aqueous layer was then transferred to a new MaXtract Gel tube, using a cut-off tip, and 200 µl of chloroform was added. The tube was again inverted to mix and centrifuged for 6 min at 9500 x *g*. The upper aqueous layer was transferred to a fresh Eppendorf and the DNA precipitated by addition of 670 µl of 100% ethanol, followed by centrifugation for 30 minutes at maximum speed, then washed with 200 µl of 70% ethanol. The pellet of episomal DNA was allowed to air-dry for 15 min before being re-suspended in 50 µl TE containing 5 µg/ml RNase A.

### 2.8.2 Transformation

1 µl of this low molecular weight DNA preparation was added to 20 µl of Electromax electrocompetent DH10B *E. coli* (Invitrogen) on ice. This was electroporated at 1.8 kV, 25 µFD and 200 Ω using a Gene pulser (BioRad) before 400 µl of SOC (Invitrogen) was added and the mixture placed in a shaking incubator at 37$^{o}$C for 1 hour to recover. The entire

culture volume was plated onto LB agar containing chloramphenicol and incubated overnight at 37°C. Colonies were chosen for miniprep DNA isolation.

### 2.8.3 Small scale isolation of plasmid DNA ('Minipreps')

Single bacterial colonies were picked into 1.5ml superbroth containing chloramphenicol 12.5 µg/ml and grown overnight at 37°C in a shaking incubator. Samples were centrifuged at 1500 x *g* for 4 min at RT to pellet the bacteria. The supernatant was removed by aspiration. 70µl STET was added to the pellet, which was re-suspended by vortexing. 200µl of alkaline SDS was added while vortexing. While still vortexing 150µl of 7.5M ammonium acetate was added, before transferring the sample immediately to ice. Samples were left on ice for 5 minutes, and then centrifuged at 18,000 x *g* for 20 min at 4°C in a pre-cooled rotor. The supernatant was then poured into a fresh Eppendorf. 240µl isopropanol was added and the DNA precipitated by inversion. The sample was centrifuged at room temperature for 6 minutes at 9,500 x *g*. The supernatant was discarded and the pellet washed in 200µl of 70% ethanol and then centrifuged at 9,500 x *g* for a further 3 min. The supernatant was then aspirated and the pellet air-dried for approximately 15 min before the DNA was re-suspended in 50µl TE supplemented with 5µg/ml RNase A. The samples were then stored at 4°C until restriction enzyme digest and pulsed-field gel electrophoresis were performed.

### 2.8.4 Restriction Enzyme Digests

Restriction enzyme digests were carried out using the following reagents, made up to 15µl with $ddH_2O$:

5µl DNA

1.5µl 10x restriction enzyme buffer

0.75µl 20x BSA

To this was added a small amount (<0.5µl) of appropriate restriction enzyme, BamHI or NHeI (New England Biolabs) and the digest was incubated for 3 hours at 37°C.

### 2.8.5 Pulsed-field gel electrophoresis

Pulsed-field gels were run in a Bio-Rad CHEF DR II system. Gels were made with 1% agarose dissolved in 0.5x TBE (without Ethidium Bromide) and were run in 2 L of 0.5x TBE. A 1:1

mixture of λ DNA-BstEII and λ DNA mono cut mix ladders (New England Biolabs) were used as size markers to run alongside DNA samples. Both DNA samples and ladders were mixed with an appropriate volume of 6x agarose gel loading buffer and the λ–based ladders were heated at 50$^{o}$C for 10 min prior to loading. The settings for pulsed-field gel electrophoresis of EBV-BACs were as follows:

| | |
|---|---|
| Initial Sweep Time | 1 sec |
| Final Sweep Time | 10 sec |
| Voltage | 6 V/cm |
| Running time | 14 hours |
| Running temperature | 14$^{o}$C |

When the run was complete, the gel was stained with Ethidium Bromide at a concentration of 0.5 μg/ml in 0.5x TBE for 45 min, with gentle agitation, before visualisation on a UV transilluminator.

## 2.9 Production of recombinant virus from producer lines and infection of cells

Once the integrity of the BAC constructs had been checked as described above, HEK293 producer cell lines were induced to produce virus by transient transfection of BZLF1 (Countryman and Miller 1985, Hammerschmidt and Sugden 1988, Delecluse et al. 1998) and BALF4 (Neuhierl et al. 2002). The cells were seeded in a 10cm dish. Two days later, each dish was transfected with 0.5 μg BZLF1 and 0.5 μg BALF4 (1 μg in total) by the LID transfection method.

### 2.9.1 Transfection of EBV-BACs into 293 cells (LID transfection system)

The LID transfection system consists of **L**ipofectin Reagent (Invitrogen), **I**ntegrin-Targeting Peptide 6 (Hart et al. 1998) and **D**NA. For 1 μg of plasmid DNA, 0.75 μl of Lipofectin Reagent (1 mg/ml) was combined with 40 μl Peptide 6 (0.1 mg/ml) and left to stand for 5 min. This solution was added to 1 μg of plasmid DNA [made up to 100 μl in OptiMEM (Invitrogen)] and left for 20 min. The medium was then removed from the cells in the 10cm dish and carefully replaced with the entire LID transfection mixture which had been made up to 5 ml with OptiMEM. Cells were incubated at 37$^{o}$C for 6 hours after which the transfection

mixture was replaced with 4 ml of fresh RPMI/10% FCS + L-glutamine. This was then left to incubate for four days before harvesting the supernatant.

The virus-containing supernatant was then harvested and filtered through a 0.45 µm filter. Infectivity of the supernatant was assayed by infection of Raji-BL cells (Green Raji Units, GRU) (Dirmeier et al. 2003). Ten-fold dilutions of viral supernatant (1 ml, 0.1 ml and 0.01 ml made up to 1 ml volume using RPMI/10% FCS) were used to infect $10^5$ Raji cells in 0.5 ml RPMI/10% FCS. After 2 days, 0.5 ml of RPMI/10% FCS containing 20 nM phorbol 12-tetradecanoate 13-acetate (TPA; Sigma) and 5 mM sodium n-butyrate (Sigma) was added to the cells, in order to enhance GFP expression. The next day, the viral titre was estimated by counting the number of GFP-positive Raji cells in various dilutions of viral supernatant using fluorescence microscopy.

### 2.9.2 Infection of EBV-negative BL cells

The EBV-negative BL cell line BL31 was infected with recombinant and wild-type EBV generated from EBV-BACs. 500µl of viral supernatant from the relevant 293 producer cell line was added to $10^5$ BL cells in 1ml of growth medium, in duplicate for each virus. After 48 hours, cells were selected in Hygromycin at a concentration of 250µg/ml to produce EBV-converted BL lines. Cells were monitored periodically for GFP expression by fluorescence microscopy. Once cell lines were established, the Hygromycin concentration was reduced to 100µg/ml. Expression of EBV latent proteins was validated by western blotting of protein extracts.

### 2.9.3 Infection of purified primary B cells

CD19-purified B cells were seeded at 2 x $10^6$ cells/ml in 5ml of medium in a 25$cm^2$ flask, then 1.5-2.0 x$10^6$ infectious units of virus (approx. 1x $10^5$ units for wild-type virus) were added to the cultures. When cells were harvested at early time points, the volume of culture removed was replaced with an equal volume of fresh medium.

# Chapter 3 The effects of EBV infection on TGFβ receptor expression and TGFβ signalling in BL31 cells

## 3.1 Introduction

TGFβ signalling is known to be dysregulated in many malignancies (Elliott and Blobe 2005), and EBV infection renders B cells resistant to the effects of TGFβ (Kehrl et al. 1986, Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Arvanitakis et al. 1995, MacDonald et al. 1996, Inman and Allday 2000b). It has previously been shown that TGFβR2 is down-regulated in B cell lines which are resistant to the effects of TGFβ (Fukuda et al. 2006a, Chen et al. 2007) including those expressing the latency III products of EBV (Inman and Allday 2000b). However, other studies have investigated only TGFβR1 expression (Altiok et al. 1991, Altiok et al. 1993), have shown that both TGFβR1 and TGFβR2 expression are reduced (Kumar et al. 1991) or that TGFβR expression appeared not to be altered by EBV (Arvanitakis et al. 1995).

Exon microarray analyses of the effect EBNA3 proteins have on cellular gene expression suggested that TGFβR2 is down-regulated by EBV in BL31 cells, with EBNA3B and EBNA3C apparently cooperating [www.epstein-barrvirus.org.uk and (White et al. 2010)]. In addition, other work by this group had shown that EBNA3 proteins cooperate to regulate many cellular genes, via epigenetic mechanisms (Anderton et al. 2008, Paschos et al. 2009, White et al. 2010, Paschos et al. 2012, Skalska et al. 2013). TGFβR2 has been shown to be repressed epigenetically in various other types of tumour including non-small cell lung carcinoma, prostate carcinoma and primary effusion lymphoma (PEL) (Zhang et al. 2004, Zhao et al. 2005, Di Bartolo et al. 2008).

Therefore, the detailed mechanism of down-regulation of TGFβR2 by EBV in BL31 cells was investigated and described in this chapter, including which latent proteins are responsible and whether this occurs via epigenetic repression of gene transcription. The effect of this on TGFβ signalling was also investigated, as well as the effect on the expression of the co-receptor TGFβR3.

## 3.2 The effect of EBV latent proteins on TGFβR2 transcription in the context of latent EBV infection of B cells

### 3.2.1 EBV down-regulates TGFβR2 in BL31 cells, with EBNA2, EBNA3B and EBNA3C being necessary

Microarray analysis had suggested that in BL31 cells, derived from an EBV-negative BL, latent EBV down-regulates TGFβR2, and that EBNA3B and EBNA3C were necessary for this [www.epstein-barrvirus.org.uk (White et al. 2010)]. Therefore a panel of uninfected, B95.8-BAC EBV (here called wild-type) and recombinant virus-infected BL31 cells was investigated for TGFβR2 expression by extracting RNA and performing qRT-PCR. This confirmed that EBV latent gene expression down-regulates TGFβR2 mRNA in BL31 cells (fig. 3.1A).

When EBNA3B and EBNA3C were deleted, expression was partially restored, suggesting that these latent proteins cooperate in the repression. The involvement of EBNA3A was less clear, as EBNA3A KO 1 appeared to behave like wild-type, suggesting that EBNA3A is not necessary for the repression, whereas in EBNA3A KO 2 partial de-repression was seen suggesting that in this case EBNA3A may be necessary (fig. 3.1A). This will be discussed further in section 4.6. In all the revertants, repression was again seen, similar to infection with wild-type virus.

Surprisingly, the deletion of EBNA2 also had a profound effect, with partial de-repression of TGFβR2, suggesting that EBNA2 also cooperates with EBNA3B and EBNA3C in the repression of TGFβR2 (fig. 3.1A). Although EBNA2 has been shown to cooperate with EBNA3 proteins in regulating cellular gene expression, in most cases these genes are activated by EBNA2 (Spender et al. 2002, Maier et al. 2006, Spender et al. 2006, Zhao et al. 2006); however, in one of these studies EBNA2 was shown to repress a limited number of genes (Maier et al. 2006). In order to confirm the effect of EBNA2 deletion, the expression of TGFβR2 was also investigated in cells derived from a different BL, BL2. Again EBV-negative, wild-type infected and those infected with EBNA2 KO virus were compared. This also showed repression of TGFβR2 by wild-type EBV, with de-repression in EBNA2 KO, confirming that EBNA2 is necessary for TGFβR2 repression (fig. 3.1B).

Despite multiple attempts using a wide variety of commercially available antibodies to TGFβR2, none were found to be satisfactory for investigation of TGFβR2 protein expression in these cell lines.

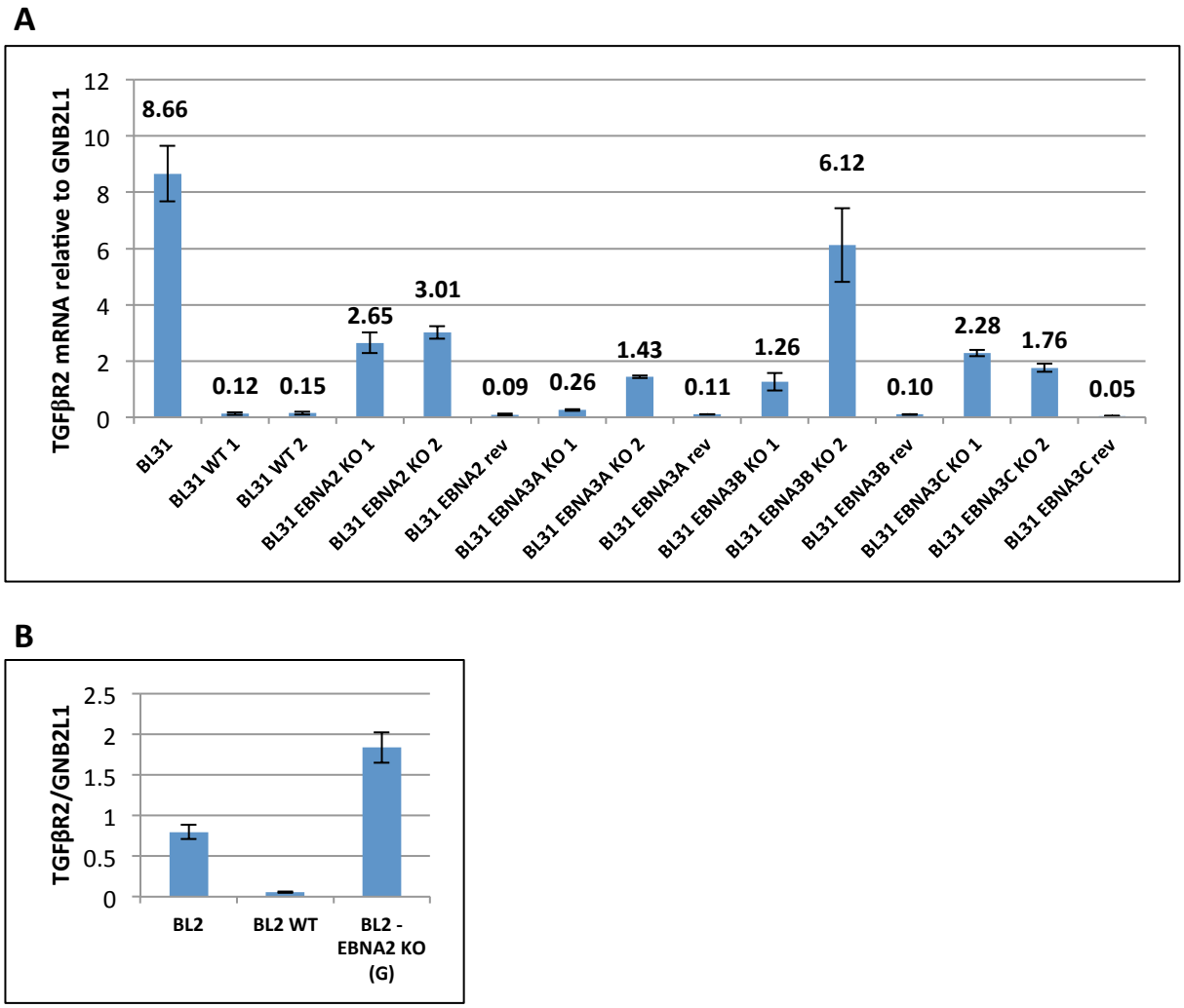



**Figure 3.1: Expression of TGFβR2 mRNA in panels of BL31 and BL2 cell lines**

**(A)** qRT-PCR on a panel of BL31 cell lines including uninfected and infected with wild-type or mutant virus as indicated. **(B)** qRT-PCR for TGFβR2 in uninfected, wild-type EBV-infected and EBNA2 KO-infected BL2 cells. The values, also shown above bars for clarification in figure 3.1A, represent the ratio of expression to the endogenous control gene *GNB2L1*. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least three separate experiments. The error bars represent standard deviations from triplicate qPCR reactions. Despite multiple attempts, no satisfactory antibody could be identified for quantifying TGFβR2 by western blot.

### 3.2.2 The down-regulation of TGFβR2 occurs via polycomb-mediated repression

It had been previously shown that EBNA3A and EBNA3C cooperate to down-regulate *BCL2L11*, the gene coding for the pro-apoptotic protein BIM, in BL31 cells, and $p16^{INK4A}$, in LCLs (Paschos et al. 2009, Skalska et al. 2010, Paschos et al. 2012). Furthermore, DNA hypermethylation of TGFβR2 has been demonstrated in response to induction of LANA, a latent nuclear protein of KSHV, in association with reduction in histone H4 acetylation of the TGFβR2 promoter (Di Bartolo et al. 2008). Therefore it was hypothesised that the repression of TGFβR2 by EBV may occur via epigenetic mechanisms.

Initially, chromatin immunoprecipitation (ChIP) was performed for H3K9Ac, a mark of active transcription, on the panel of BL31 cell lines including uninfected and cells infected with wild-type or mutant EBV. Analysis of the precipitated DNA was performed by qPCR using several sets of primers across the TGFβR2 promoter (fig. 3.2A). H3K9Ac was shown to peak at primer set 4, approximately 500 bases downstream from the TSS, in uninfected BL31 cells. EBV reduces H3K9Ac, and this was partially recovered when EBNA2, EBNA3B or EBNA3C are deleted, and reduced again in revertants (fig. 3.2B). These findings were consistent with the mRNA data (fig. 3.1A). For the EBNA3A KOs, however, the involvement is again unclear; in this case both cell lines seem to have an intermediate phenotype (fig. 3.2B).

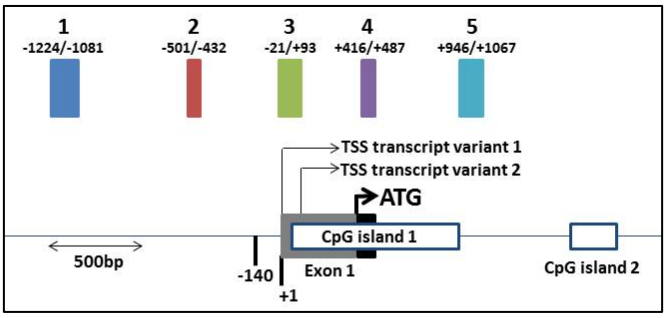
ChIP was then performed for the repressive mark H3K27Me3. This showed an increase in the deposition of H3K27Me3 induced by EBV infection, with similar values across the five primer sets suggesting a broader peak than for H3K9Ac (fig. 3.2C). When EBNA2, EBNA3B or EBNA3C were deleted, H3K27Me3 was reduced again, although in both EBNA3B KO lines and EBNA3C KO 2 the reduction was not to the same level as in uninfected BL31. Both EBNA3A KO lines showed an intermediate level of H3K27Me3. The revertant virus-infected cells showed levels of H3K27Me3 similar to those infected with WT EBV, with the exception of EBNA3C revertant, which showed reduced levels compared to wild-type in this particular experiment.

Since H2K27Me3 is deposited by polycomb repressive complex 2 (PRC2), ChIP was then performed for SUZ12, a component of the PRC2 complex (fig. 3.2D). This showed that little

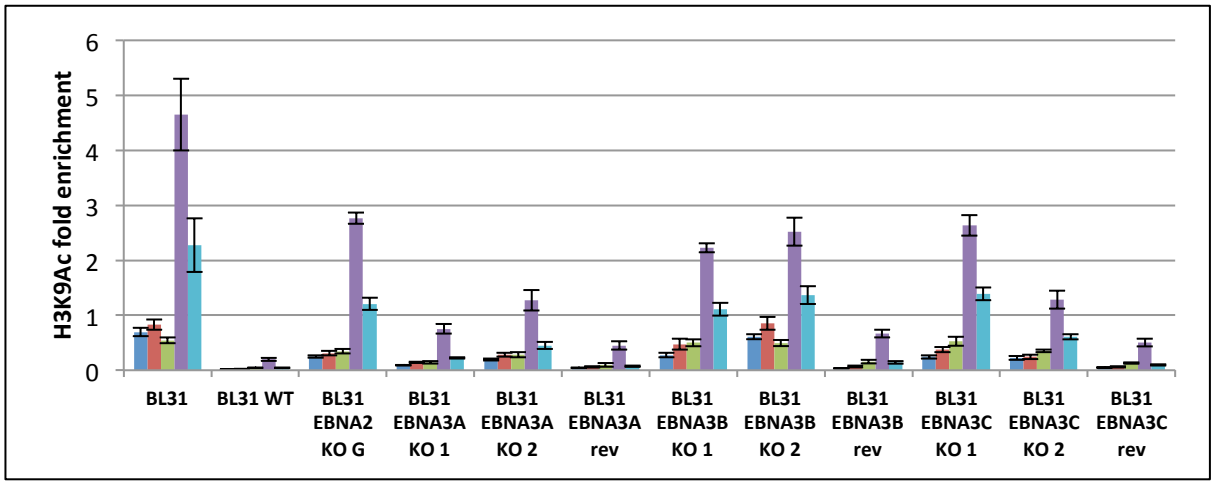
or no SUZ12 is bound in uninfected BL31 but that latent EBV increases SUZ12 binding, with a peak at primer set 4. Levels were reduced again in EBNA2 KO, EBNA 3B KO 2 and EBNA3C KO 1, and recovered in the revertants with similar values to wild-type infected cells. The other cell lines showed intermediate levels of SUZ12 binding. For both EBNA3A KO cell lines used, SUZ12 levels were increased, although for EBNA3A KO 1 the level of SUZ12 binding was intermediate (fig. 3.2D).

The binding of SUZ12 generally correlates with H3K27Me3, in turn correlating with repression of TGFβR2 mRNA transcription. Taken together, these findings suggest that EBV down-regulates TGFβR2 by PRC2-mediated repression leading to gene silencing.

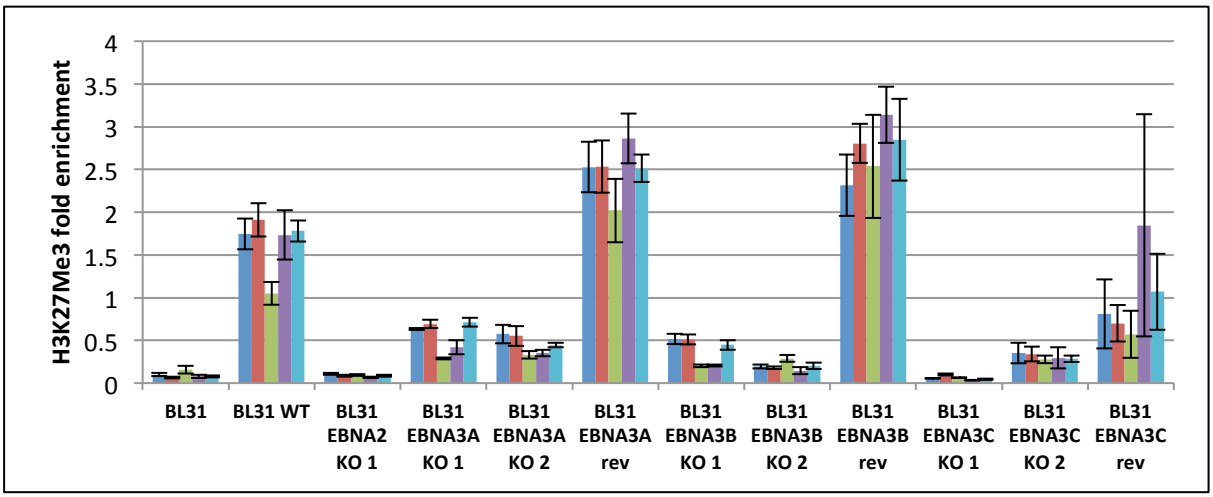
**A**


1
-1224/-1081
2
-501/-432
3
-21/+93
4
+416/+487
5
+946/+1067
TSS transcript variant 1
TSS transcript variant 2
ATG
CpG island 1
500bp
-140
+1
Exon 1
CpG island 2

**B**


H3K9Ac fold enrichment
0
1
2
3
4
5
6
BL31
BL31 WT
BL31 EBNA2 KO G
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A rev
BL31 EBNA3B KO 1
BL31 EBNA3B KO 2
BL31 EBNA3B rev
BL31 EBNA3C KO 1
BL31 EBNA3C KO 2
BL31 EBNA3C rev

**C**


H3K27Me3 fold enrichment
0
0.5
1
1.5
2
2.5
3
3.5
4
BL31
BL31 WT
BL31 EBNA2 KO 1
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A rev
BL31 EBNA3B KO 1
BL31 EBNA3B KO 2
BL31 EBNA3B rev
BL31 EBNA3C KO 1
BL31 EBNA3C KO 2
BL31 EBNA3C rev

**D**

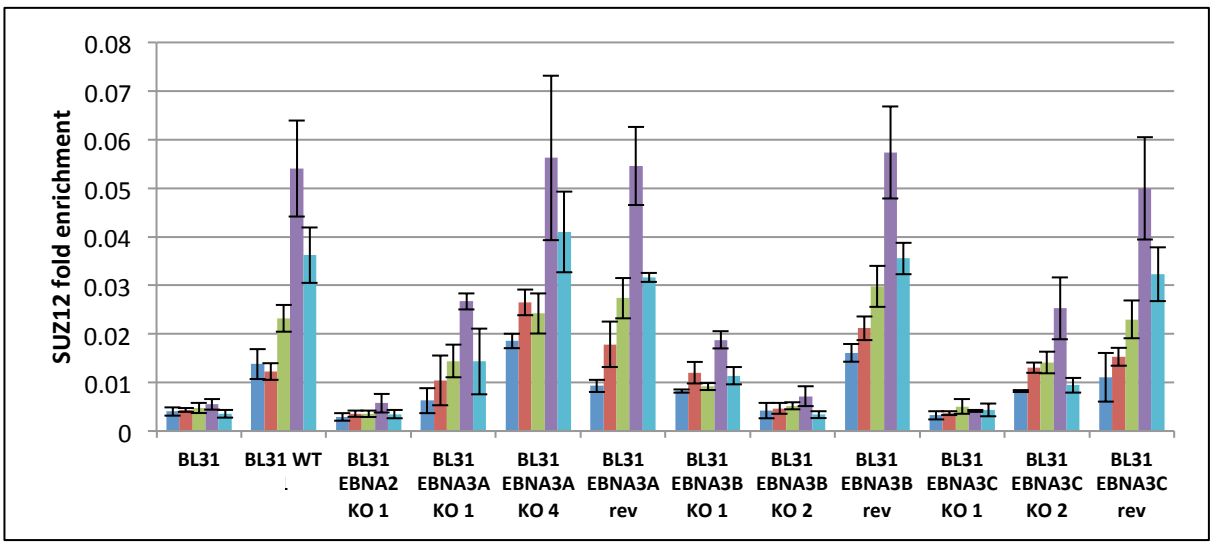

SUZ12 fold enrichment
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
BL31
BL31 WT
BL31 EBNA2 KO 1
BL31 EBNA3A KO 1
BL31 EBNA3A KO 4
BL31 EBNA3A rev
BL31 EBNA3B KO 1
BL31 EBNA3B KO 2
BL31 EBNA3B rev
BL31 EBNA3C KO 1
BL31 EBNA3C KO 2
BL31 EBNA3C rev

**Figure 3.2: ChIP for histone modifications at the TGFβR2 promoter in a panel of BL31 cell lines**

**(A)** Schematic diagram of the TGFβR2 promoter showing the location of products of primer sets used, the putative transcription start site (TSS) and CpG islands. The coloured blocks represent the primer pairs 1-5, shown as bars from left to right for each cell line in figs. B-D. **(B-D)** qPCRs showing ratio of histone modification to input DNA at the TGFβR2 promoter for **(B)** H3K9Ac **(C)** H3K27Me3 and **(D)** SUZ12. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least two separate experiments. The error bars represent standard deviations from triplicate qPCR reactions for both input and IP.

### 3.2.3 H3K4Me3 is present in all cell lines, suggesting that TGFβR2 is a bivalent promoter

ChIP was then performed for H3K4Me3, also a mark of active transcription. It has been shown that certain genes, particularly those which are important in embryonic stem cells, have large areas of H3K27Me3 with smaller areas of H3K4Me3 within. It is thought that these 'bivalent domains' are poised for activation or repression (Bernstein et al. 2006). It was hypothesised that TGFβR2, being important in embryonic development, may be such a domain. Although H3K4Me3 does appear to vary according to the presence or absence of EBV, even in the presence of EBV it remains (fig. 3.3). In those cell lines in which the level of H3K4Me3 appears to be reduced, the level of GAPDH is also lower, suggesting the reduction is due to the ChIP rather than being regulated by EBV itself.

The peak of H3K4Me3 is around primer set 4, as seen for H3K9Ac and SUZ12 (figs. 3.3, 3.2B and 3.2D). H3K27Me3, however, generally shows a broad peak with significant deposition across all primer sets used (fig. 3.2C). Therefore, in EBV-infected BL31, there appears to be a broad deposition of H3K27Me3 across the promoter, with a concomitant smaller peak of H3K4Me3, consistent with TGFβR2 being a bivalent promoter. An Encyclopaedia of DNA elements (ENCODE) search confirms increased deposition of both of these epigenetic marks around the TSS for TGFβR2 in an LCL, GM12878 (http://genome.ucsc.edu/ENCODE). This is consistent with TGFβR2 being important in embryonic development.

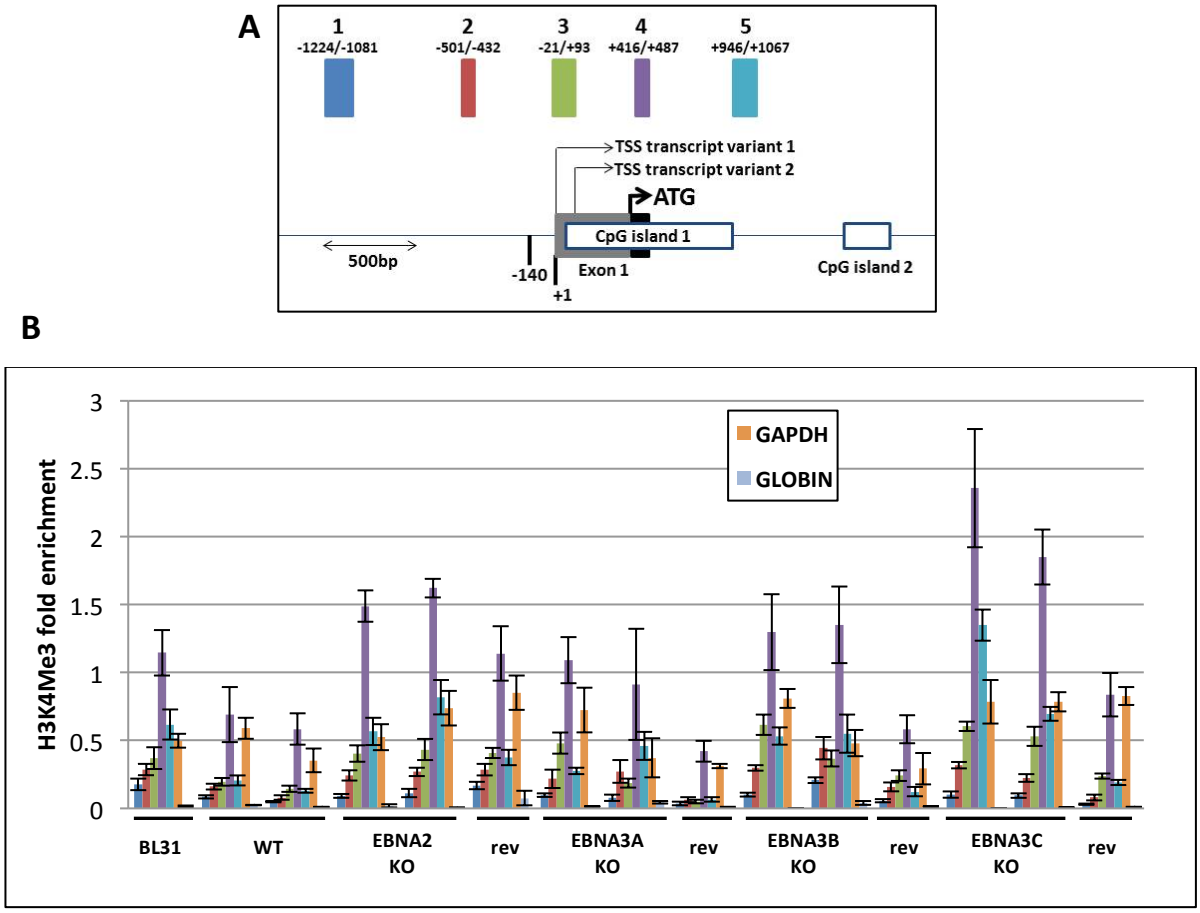



**Figure 3.3: ChIP for H3K4Me3 at the TGFβR2 promoter in a panel of BL31 cell lines**

**(A)** Schematic diagram of the TGFβR2 promoter showing the location of products of primer sets used, the putative transcription start site (TSS) and CpG islands. The coloured blocks represent the primer pairs 1-5, shown as bars from left to right for each cell line in figure B. **(B)** qPCR showing ratio of H3K4Me3 to input DNA at the TGFβR2 promoter, using the five primer sets as shown in A, and control promoter primers GAPDH (active) and globin (repressed). The error bars represent standard deviations from triplicate qPCR reactions for both input and IP.

### 3.2.4 In BL31 cell lines, DNA methylation of TGFβR2 does not occur

H3K27Me3, deposited by PRC2, leads to gene silencing. This in turn has been shown to increase the likelihood of subsequent DNA methylation, a more stable repressive epigenetic mark, by approximately 12-fold (Widschwendter et al. 2007). Therefore it was hypothesised that CpG methylation of TGFβR2 may occur in some EBV-infected cell lines in which TGFβR2 is repressed. CpG methylation was sought using a method based on the methylated CpG island recovery assay (MIRA) (Rauch and Pfeifer 2010), using a histidine-tagged recombinant methyl-binding protein complex (MBD2b/MBD3L1) that specifically binds methylated CpGs of genomic DNA fragments, followed by qPCR using primer sets 2, 3 and 4 (see fig. 3.2A). As

a positive control, fragmented DNA from Jurkat cells which had been fully methylated *in vitro* was used, with a negative control in which only the magnetic beads were added, without any His-MBD2b/MBD3L1 protein complex.

This showed that there was no DNA methylation at this locus in any of the BL31 cell lines (fig. 3.4). As expected, the positive control showed DNA methylation only in the CpG island, at primer sets 3 and 4 (see fig. 3.2A). The lack of DNA methylation in these cell lines may be because they have been infected with EBV *in vitro* relatively recently, and so DNA methylation has not yet occurred, or because PRC2 is active and H3K4Me3 is also present. Similar findings were seen for *BIM* in a similar panel of BL31 cells infected with recombinant viruses (Paschos et al. 2009).

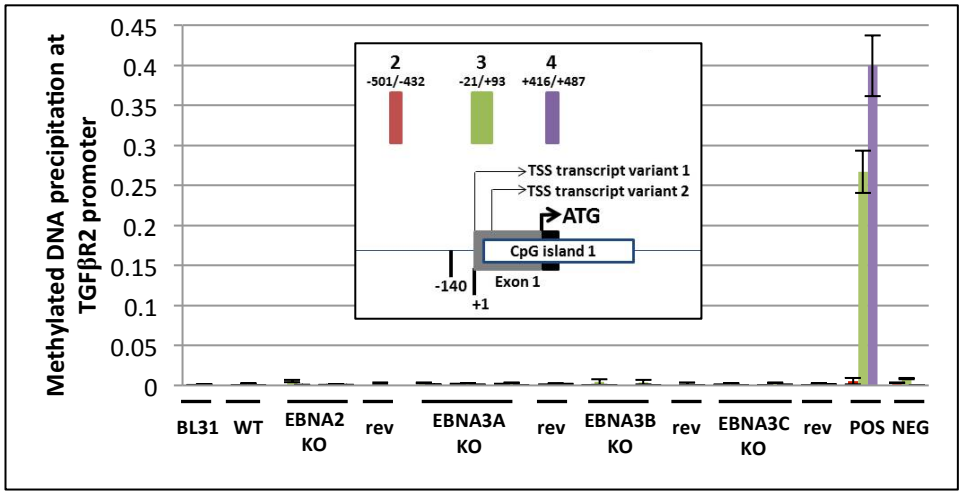



**Figure 3.4: Methylated DNA precipitation at the TGFβR2 promoter in a panel of BL31 cell lines**

Methylated DNA was precipitated followed by qPCR using primer sets 2, 3 and 4, as shown in figure 3.2A, for the cell lines shown, as a well as a positive (POS) and a negative control (NEG). The colours of the bars are the same as those of the blocks in the schematic diagram of the TGFβR2 promoter shown in figure 3.2A. The positive control was a fully *in vitro* methylated Jurkat cell line. Values are shown as ratios to input DNA and are representative of two separate experiments. The error bars represent standard deviations from triplicate qPCR reactions.

### 3.2.5 In some B cell lines showing repression of TGFβR2, DNA methylation of TGFβR2 occurs and may replace H3K27 trimethylation

Since DNA methylation of BIM had been demonstrated in other cell lines including EBV-positive BL (Paschos et al. 2009), a further set of B cell lines were investigated for TGFβR2 expression and DNA methylation of TGFβR2. Since DNA methylation of the TGFβR2 promoter region has been demonstrated in PEL cell lines (Di Bartolo et al. 2008) and a TGFβ-resistant DLBCL cell line DB as well as Akata (Chen et al. 2007), it was hypothesised that in some cell lines where TGFβR2 was repressed, DNA methylation may coexist with, or possibly replace, H3K27Me3.

TGFβR2 expression was repressed, similar to BL31-WT, in Akata6, Akata31, LCL-WT and LCL-X50.7, but was not repressed in Ramos (fig. 3.5A). For the LCLs, this was consistent with previous findings, since LCLs are known to resist the effects of TGFβ and in which TGFβR2 is repressed [see chapter 6, fig. 6.1 and (Kumar et al. 1991, Inman and Allday 2000b)]. Ramos, an EBV-negative BL cell line, has previously been shown to express TGFβR2 (Inman and Allday 2000b, Fukuda and Longnecker 2004) and to be sensitive to the effects of TGFβ (Blomhoff et al. 1987, Altiok et al. 1993, Chaouchi et al. 1995, Saltzman et al. 1998, Fukuda and Longnecker 2004, Di Bartolo et al. 2008).

The Akata cell line was originally isolated from an EBV-positive BL from a Japanese patient and expressed latency I, i.e. EBNA1 only. However, after serial passage *in vitro* selected clones from the original Akata line became EBNA1-negative, i.e. lost the EBV episome, whereas other clones remained EBNA1-positive (Shimizu et al. 1994). Akata6 is an EBV-positive (latency I) clone whereas Akata31 is an EBV-negative clone, having lost its EBV episome (Jenkins et al. 2000). The Akata cell line has previously been shown to be resistant to the effects of TGFβ and have reduced expression of TGFβR2 (Inman and Allday 2000b).

When DNA methylation was investigated, it was found that of those cell lines in which TGFβR2 was repressed, DNA methylation was seen in both Akata cell lines and in the long established LCL X50-7, but not in BL31-WT (as seen also in figure 3.4) or in the recently made LCL-WT (fig. 3.5B). In this case, DNA extracted from PBMCs was included as another negative control (in addition to beads without protein complex), as these are known to be

unmethylated [K. Paschos, personal communication and (Paschos et al. 2009)]. As expected this showed no DNA methylation (fig. 3.5B). In BL31 and Ramos, in which TGFβR2 was expressed, as expected DNA methylation did not occur at this locus.

ChIP for H3K27Me3 was performed in the same set of cell lines. This showed that, of those lines with repressed TGFβR2, H3K27Me3 was seen in BL31-WT (as before in fig. 3.2C), LCL-WT and also to some degree in LCL X50-7 (fig. 3.5C). However, no increase in H3K27Me3 was seen in either of the Akata cell lines despite repression of TGFβR2. This suggests that in the Akata cell lines, DNA methylation has replaced H3K27Me3 as a more stable repressive epigenetic modification. In BL31-WT and the recently established LCL-WT, TGFβR2 repression is associated with H3K27Me3 but no DNA methylation, whereas in LCL X50-7 there is a combination of H3K27Me3 and DNA methylation associated with the repression of TGFβR2. In BL31 and Ramos H3K27Me3 was not increased, consistent with TGFβR2 being expressed in these cell lines.

Since Akata6 and Akata31 showed DNA methylation at the TGFβR2 promoter, these cell lines were further investigated using the full set of primers in order to determine which part of the promoter region was methylated. This showed that in both cell lines, like the *in vitro* fully methylated Jurkat DNA positive control, CpG methylation occurs at both predicted CpG islands and not in the other regions analysed. The peak of CpG methylation occurred at the region covered by primer set 4, i.e. towards the distal end of the largest CpG island (fig. 3.6A-B).

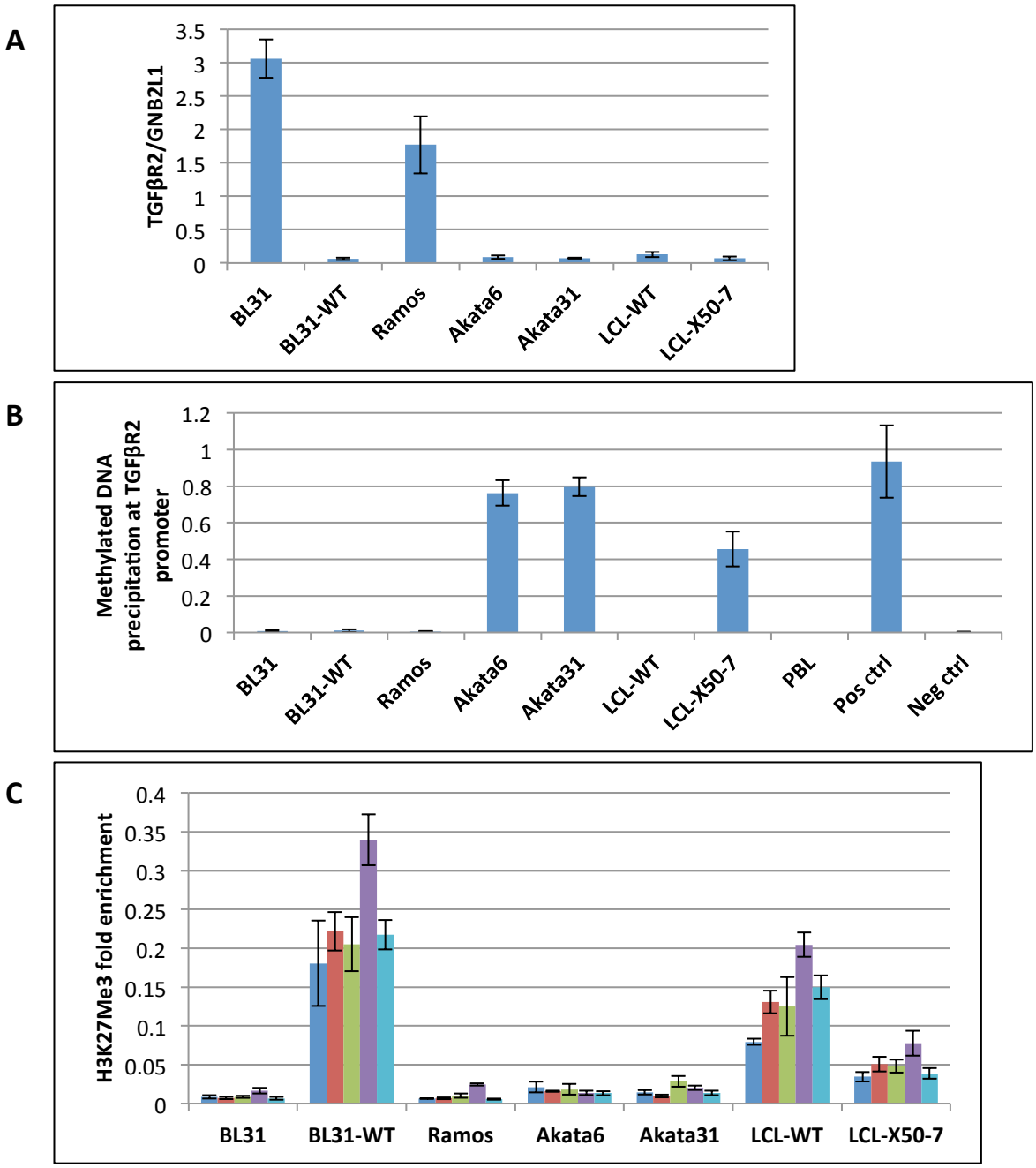



**Figure 3.5: TGFβR2 mRNA expression, H3K27Me3 and DNA methylation in a panel of B cell lines**

**(A)** qRT-PCR for TGFβR2. The values represent the ratio of expression to the endogenous control gene *GNB2L1*. Data shown are representative of at least two experiments. The error bars represent standard deviations from triplicate qPCR reactions. **(B)** Methylated DNA precipitation at the TGFβR2 promoter**:** Methylated DNA was precipitated followed by qPCR using primer set 4, as shown in figure 3.2A, for the cell lines shown, as a well as a positive and a negative control. The positive control was a fully *in vitro* methylated Jurkat cell line. Values are shown as ratios to input DNA. The error bars represent standard deviations from triplicate qPCR reactions. **(C)** ChIP for H3K27Me3 at the TGFβR2 promoter. The coloured bars represent the values at primer pairs 1-5, the locations of which were shown in figure 3.2A. qPCR showing ratio of H3K27Me3 to input DNA at the TGFβR2 promoter for the cell lines shown. Data shown are representative of at least two experiments. The error bars represent standard deviations from triplicate qPCR reactions for both input and IP.

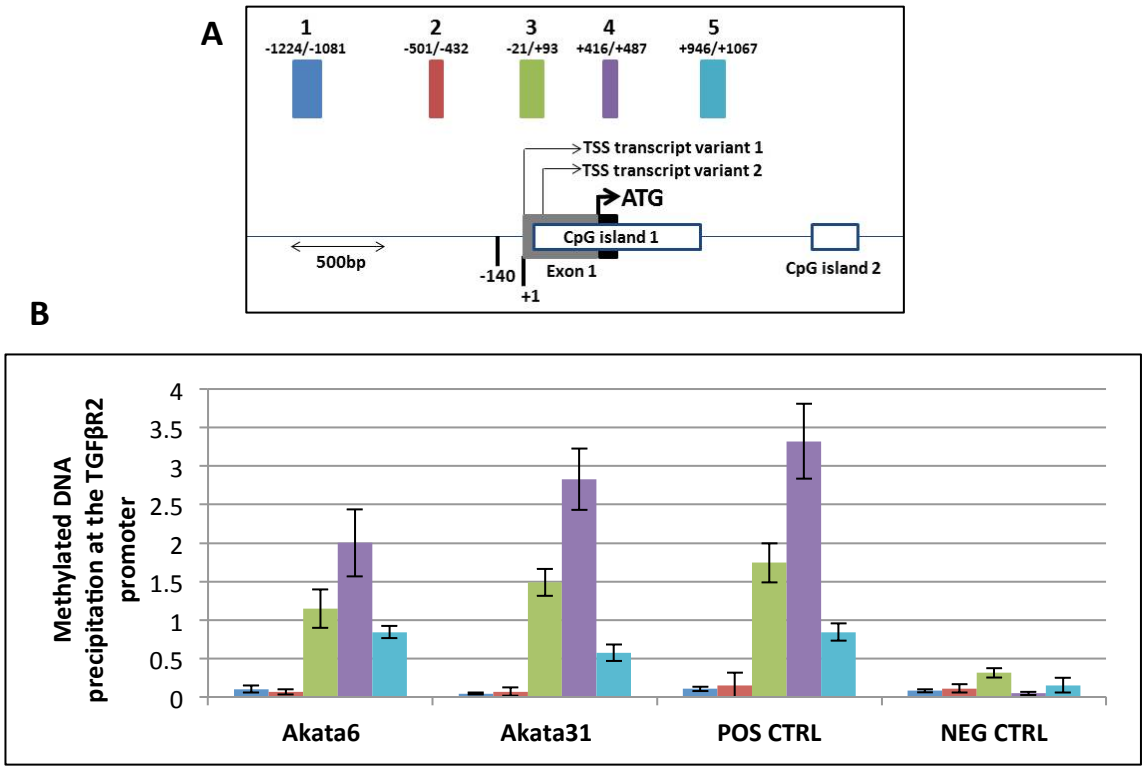



**Figure 3.6: Location of methylated DNA precipitation at the TGFβR2 promoter for Akata6 and Akata31 cell lines**

**(A)** Schematic diagram of the TGFβR2 promoter showing the location of products of primer sets used, the putative transcription start site (TSS) and CpG islands. The coloured blocks represent the primer pairs 1-5, shown as bars from left to right for each cell line in figure B. **(B)** Methylated DNA was precipitated followed by qPCR using all primer sets as shown in (A), for Akata6 and Akata31 as a well as a positive (POS) and a negative control (NEG). The positive control was a fully *in vitro* methylated Jurkat cell line. Values are shown as ratios to input DNA, with error bars representing standard deviations from triplicate qPCR reactions.

## 3.3 The effect of EBV latency on TGFβ signalling

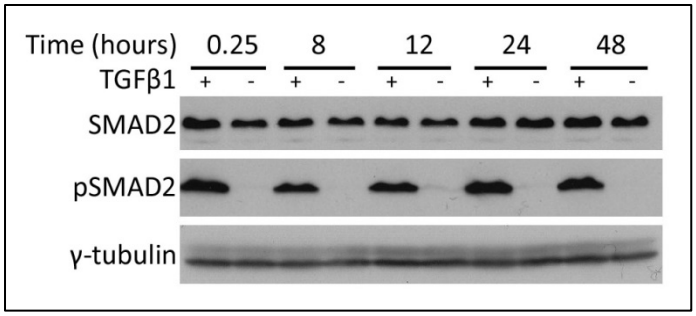
### 3.3.1 Latent EBV leads to suppression of signalling in response to TGFβ1; EBNA2, EBNA3B and EBNA3C are necessary

Canonical signalling in response to TGFβ occurs via TGFβR1 and TGFβR2 and results in phosphorylation of SMAD2 and/or SMAD3. Since EBV down-regulates TGFβR2, the effect of this on TGFβ signalling was investigated in the panel of BL31 cell lines. An initial time course was performed in which BL31 and BL31-WT were treated with TGFβ1 5ng/ml (hereafter referred to as TGFβ) and samples harvested for protein extraction at intervals up to 48 hours. SDS-PAGE followed by western blot showed phosphorylation of SMAD2 (pSMAD2) in response to TGFβ in BL31, seen at 15 minutes and maintained for 48 hours after treatment (fig 3.7A). The full panel of cell lines was therefore treated to see what effect EBV infection, and deletion of the individual latent proteins, had on TGFβ signalling as detected by pSMAD2.

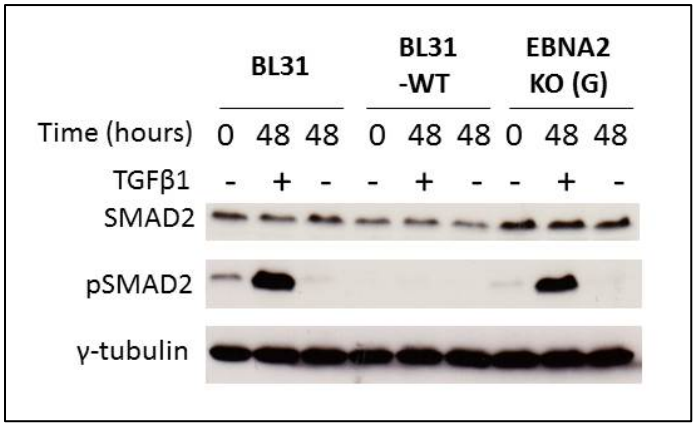
This showed that when infected with wild-type EBV, TGFβ did not induce the phosphorylation of SMAD2 (pSMAD2), i.e. signalling was suppressed. When EBNA2, EBNA3B or EBNA3C were deleted, pSMAD2 was restored (fig. 3.7B-D). These findings are consistent with the repression of TGFβR2 mRNA levels by the cooperation of these latent proteins - EBNA2, EBNA3B and EBNA3C (fig. 3.1A). Therefore, these proteins cooperate to down-regulate TGFβR2, which results in loss of TGFβ signalling via the canonical SMAD2 pathway.

Id proteins are downstream targets of both TGFβ and BMP signalling, for example TGFβ induces Id2 and Id3 in pro-B cells, inhibiting their growth and survival (Kee et al. 2001). BMPs induce Id expression in several cell types (Katagiri et al. 2002, Korchynskyi and ten Dijke 2002, Lopez-Rovira et al. 2002), however the effect of TGFβ on Id expression varies according to the cell type and TGFβ concentration (Ruzinova and Benezra 2003). In epithelial cells, TGFβ represses Id proteins (Ling et al. 2002) but in BLs it induces these (Spender and Inman 2009b). The effect of TGFβ on expression of ID1 and ID2 was investigated in BL31 cells. Both ID1 and ID2 were induced after two hours' exposure to TGFβ, but not by vehicle alone. However, consistent with the repression of TGFβR2 and suppression of TGFβ signalling by EBV in BL31 cells, ID1 and ID2 were not induced in the EBV-infected cells (fig. 3.8A-B).

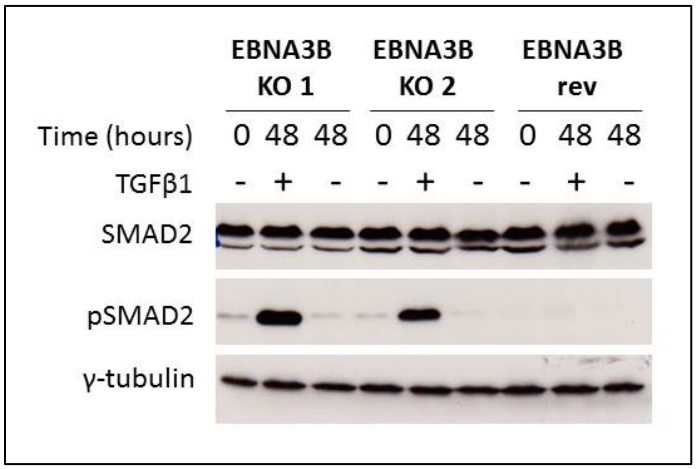
**A**


Time (hours) 0.25 8 12 24 48
TGFβ1 + - + - + - + - + -
SMAD2
pSMAD2
γ-tubulin

**B**


BL31
BL31 -WT
EBNA2 KO (G)
Time (hours) 0 48 48 0 48 48 0 48 48
TGFβ1 - + - - + - - + -
SMAD2
pSMAD2
γ-tubulin

**C**


EBNA3B KO 1
EBNA3B KO 2
EBNA3B rev
Time (hours) 0 48 48 0 48 48 0 48 48
TGFβ1 - + - - + - - + -
SMAD2
pSMAD2
γ-tubulin

**D**

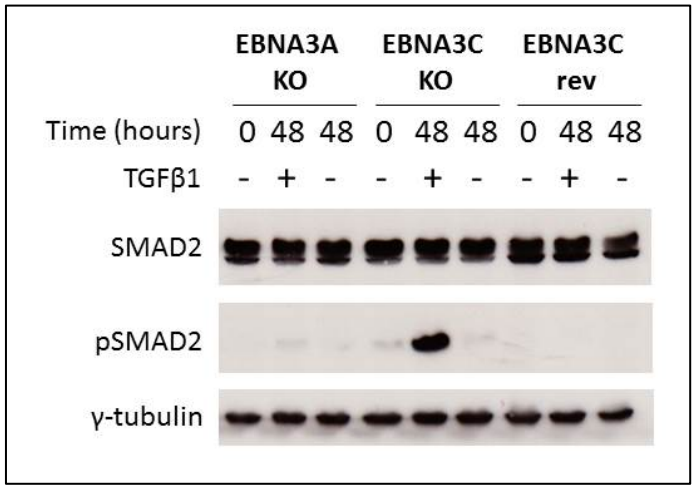

EBNA3A KO
EBNA3C KO
EBNA3C rev
Time (hours) 0 48 48 0 48 48 0 48 48
TGFβ1 - + - - + - - + -
SMAD2
pSMAD2
γ-tubulin

**Figure 3.7: The effect of exogenous TGFβ1 on SMAD2 phosphorylation in BL31 cells**

**(A)** Western blot analysis showing the effect of exogenous TGFβ1 treatment (5ng/ml) over time on levels of phosphorylated SMAD2 (pSMAD2) in uninfected BL31 cells. **(B-D)** Western blot analysis showing the effect of 48 hours' treatment with exogenous TGFβ1 (5ng/ml) on levels of phosphorylated SMAD2 (pSMAD2) in BL31 cells infected with recombinant EBV. Levels are compared to those at time 0 without any treatment, and 48 hours with vehicle (2mg/ml BSA in PBS) only. SMAD2 levels are shown for comparison and γ-tubulin was used as a loading control.

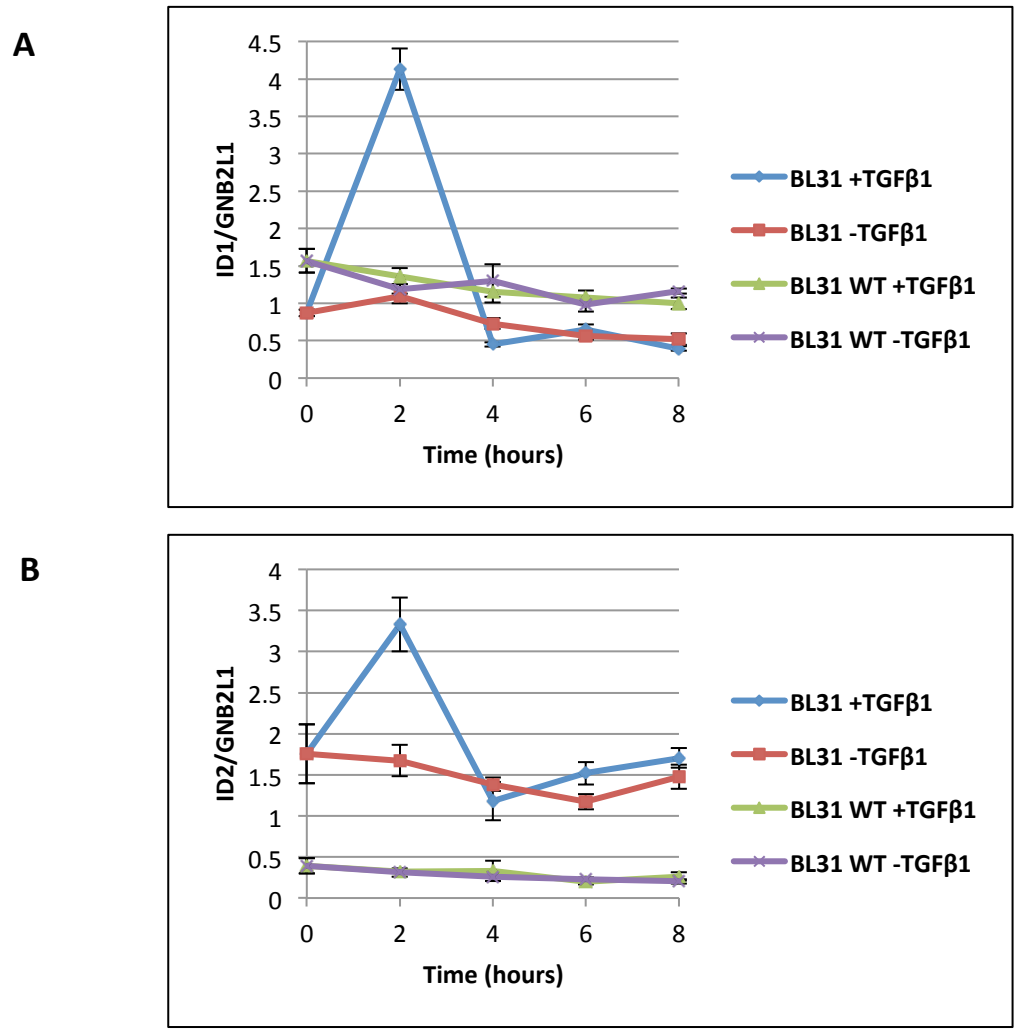



**Figure 3.8: TGFβ1 leads to induction of ID1 and ID2 two hours after treatment in uninfected, but not in EBV-infected, BL31 cells**

qRT-PCR for (A) ID1 and (B) ID2 in uninfected and EBV-infected BL31 cells with time after treatment with TGFβ1 or vehicle. Values are expressed as ratios to the endogenous control gene *GNB2L1*, with error bars representing standard deviations of triplicate qPCR reactions.

### 3.3.2 In BL31 cells, p15$^{INK4B}$ is not induced by TGFβ; however, latent EBV infection increases its expression

TGFβ is known to induce p15$^{INK4B}$ (hereafter referred to as p15) in epithelial cells, and this results in growth arrest (Hannon and Beach 1994). The effect of TGFβ treatment on p15 expression was investigated in uninfected and wild-type EBV infected BL31 cells, measuring p15 mRNA by qRT-PCR. This showed that, although wild-type EBV appears to up-regulate basal p15 expression, there is no induction of p15 in response to TGFβ (fig. 3.9). These samples were taken at the same time as protein samples taken for pSMAD2 analysis, as well as RNA for ID1 and ID2 expression (fig. 3.8A-B), showing that TGFβ was active, inducing pSMAD2, ID1 and ID2. Thus, although p15 expression is markedly repressed in BL31, it is up-regulated by latent EBV; however, consistent with other studies in BL cells (Spender and Inman 2009b), p15 is not induced by TGFβ in BL31 cells. p15 has been shown to be frequently methylated (and hence repressed) in BL (Klangby et al. 1998). The effects of EBV on p15 expression will be discussed in more detail in section 6.4.2.

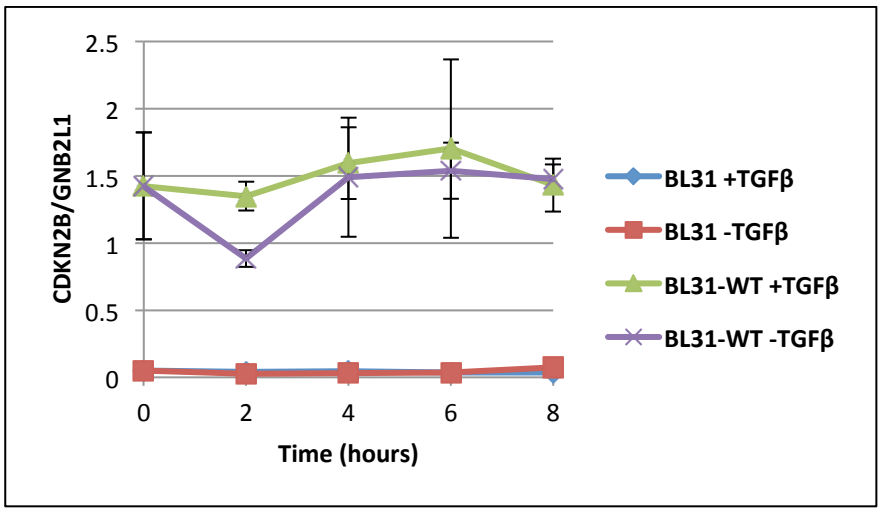



**Figure 3.9: TGFβ1 does not induce p15$^{INK4B}$ in BL31 cells, but EBV infection increases its expression**

qPCR for p15$^{INK4B}$ (CDKN2B) with time after treatment in uninfected and wild-type EBV infected BL31 cells. cDNA samples used were the same as in figure 3.8, confirming that TGFβ1 did induce ID1 and ID2 expression, but not p15, in these cells. Values are expressed as ratios to *GNB2L1*, with error bars representing standard deviation of triplicate qPCR reactions.

### 3.3.3 EBV does not significantly alter TGFβR1 expression in BL31 cells

TGFβ signalling occurs via TGFβR2 and TGFβR1. Having shown that EBV down-regulates TGFβR2 and leads to ablation of TGFβ signalling, it was also important to exclude an effect of EBV on TGFβR1 expression. Therefore qRT-PCR was performed for TGFβR1 in the full panel of BL31 cell lines. This showed very little variation in TGFβR1 expression between the cell lines (fig. 3.10). Hence EBV does not significantly alter TGFβR1 expression, and thus the effects on TGFβ signalling are likely to occur as a result of the repression of TGFβR2.

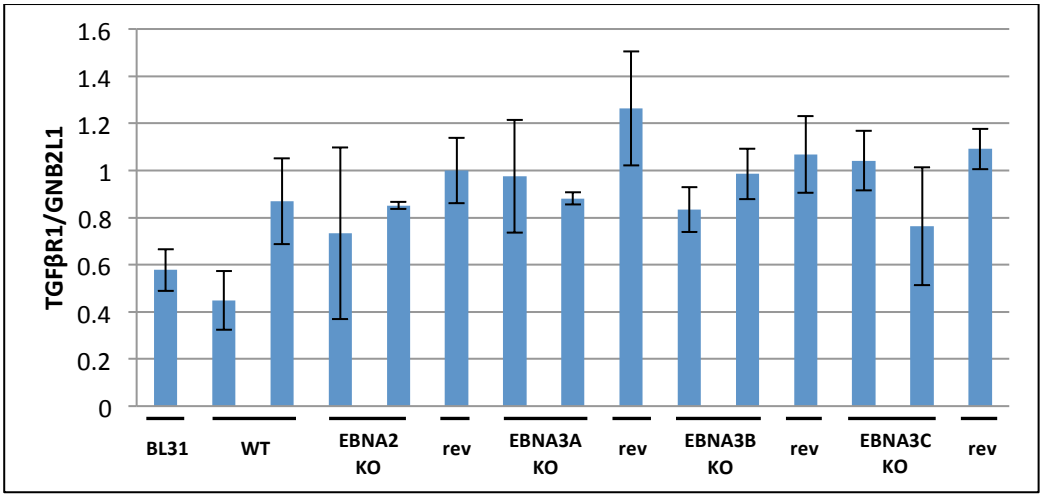



**Figure 3.10: Expression of TGFβR1 mRNA in a panel of BL31 cell lines**

qRT-PCR on a panel of BL31 cell lines including uninfected and infected with wild-type or mutant virus as indicated. The values represent the ratio of expression to the endogenous control gene *GNB2L1*. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least two separate experiments. The error bars represent standard deviations from triplicate qPCR reactions.

## 3.4 The effects of EBV and its latent proteins on TGFβR3 expression

### 3.4.1 Latent EBV up-regulates TGFβR3 expression in BL31 cells; EBNA2, EBNA3B and EBNA3C are necessary

Microarray analysis had suggested that EBV, in addition to repressing TGFβR2, up-regulates TGFβR3 in BL31 cells [www.epstein-barrvirus.org.uk and (White et al. 2010)]. Thus the expression of TGFβR3 mRNA was investigated by qRT-PCR in the panel of BL31 cell lines. This confirmed that EBV up-regulates TGFβR3 (fig. 3.11A). When EBNA2, EBNA3B and EBNA3C are deleted, the up-regulation is reduced, and in the revertants it is again seen. Western blots were also performed for TGFβR3 protein expression on extracts from the same cell lines. This confirmed the up-regulation of TGFβR3 by EBV, with EBNA2, EBNA3B and EBNA3C apparently being necessary for the up-regulation (fig. 3.11B).

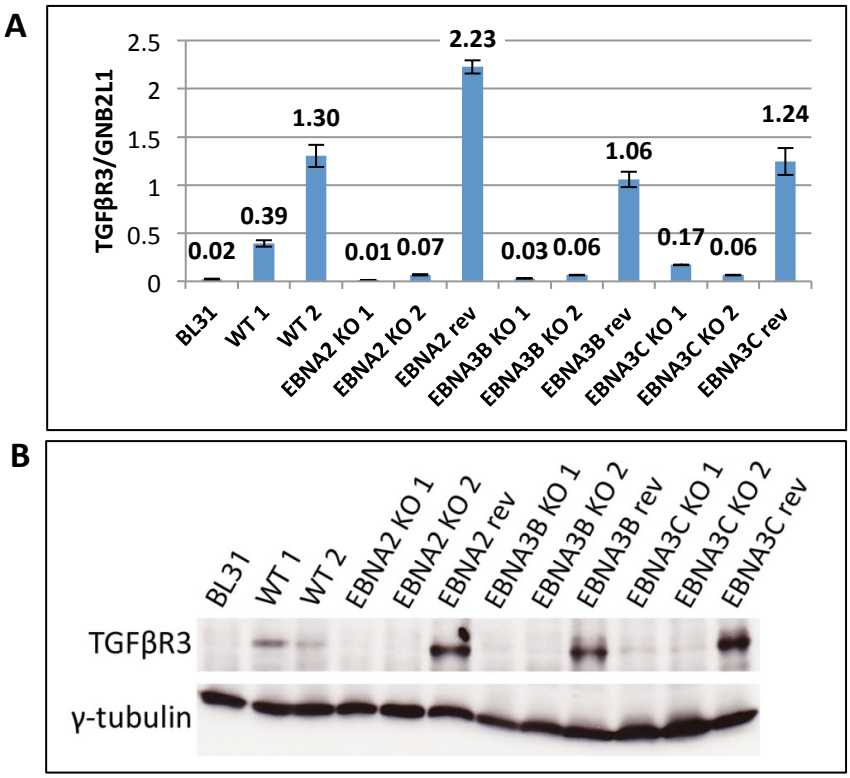



**Figure 3.11: EBNA2, EBNA3B and EBNA3C cooperate to up-regulate TGFβR3 expression in BL31 cells**

**(A)** qRT-PCR for TGFβR3 in a panel of BL31 cell lines including uninfected and infected with wild-type or mutant virus as indicated. The values, also shown above bars for clarification, represent the ratio of expression to the endogenous control gene *GNB2L1*. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least three separate experiments. The error bars represent standard deviations from triplicate qPCR reactions. **(B)** Western blot for TGFβR3 in whole cell lysates from the same cell lines, with γ-tubulin shown as a loading control.

### 3.4.2 The up-regulation of TGFβR3 by EBV in BL31 cells is also epigenetically mediated

Having shown that the repression of TGFβR2 by EBV occurs by epigenetic mechanisms involving histone modifications, the mechanism of up-regulation of TGFβR3 was investigated by performing qPCR using primers across the TGFβR3 promoters, using the same ChIP samples as had been investigated for the TGFβR2 promoter (see figs. 3.2 and 3.3).

TGFβR3 has two promoters, proximal and distal, with the proximal one being predominantly used in most human tissues (Cooper et al. 2010). There are several small CpG islands around both promoters with one larger one (of over 1000 bp) around the proximal transcription start site (fig. 3.12A). Primer sets were designed to cover both promoter regions.

ChIP for H3K9Ac, a mark of active transcription, showed increased H3K9Ac in wild-type and revertant-infected cells, and was low in EBNA2, EBNA3B and EBNA3C KO cells (fig. 3.12B), consistent with the mRNA results (fig. 3.11A). For EBNA3A KO 1, H3K9Ac was low but for EBNA3A KO 2 the level was intermediate (fig. 3.12B); however, variations in gene expression between the different EBNA3A KO cell lines have previously been demonstrated [see figs. 3.1A, 3.2B-D and (White et al. 2010)].

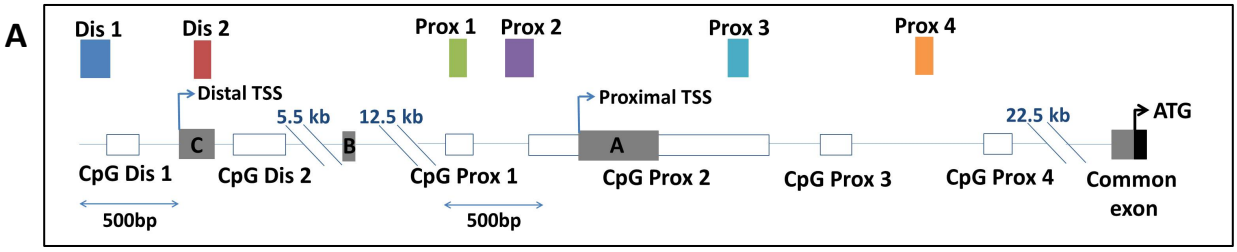
ChIP for H3K27Me3 showed relatively high levels in uninfected BL31 cells, that were reduced in wild-type and revertant infected cells, and partially restored levels in EBNA2 KO, EBNA3B KO, EBNA3C KO and both EBNA3A KO lines (fig. 3.12C). Thus, with the exception of the EBNA3A KOs, as will be discussed further in section 4.6, the results for H3K27Me3 were generally opposite to those for H3K9Ac, consistent with H3K27Me3 being a mark of transcriptional repression in the uninfected BL31 cells.

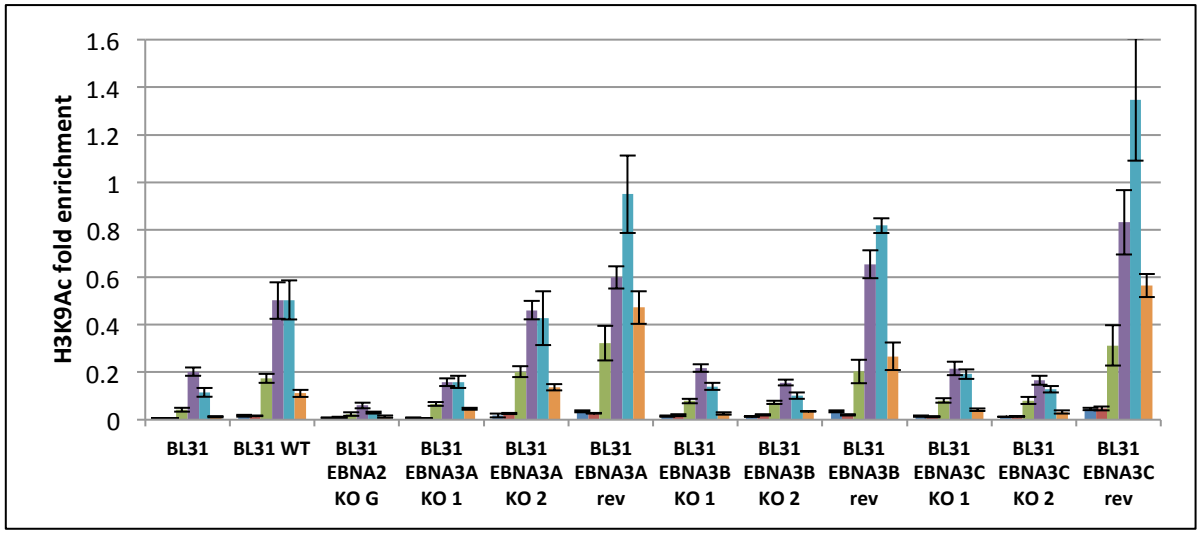
ChIP for H3K4Me3, another mark of active transcription, showed a moderate increase in wild-type and revertant cell lines, with reduced levels in EBNA2, EBNA3A, EBNA3B and EBNA3C KOs (fig. 3.12D), again consistent with the mRNA findings (fig. 3.11A) with the exception of EBNA3A KOs. However, the variation between cell lines was much less marked than for other histone modifications, suggesting that TGFβR3 might also be a bivalent promoter (see section 3.2.3).

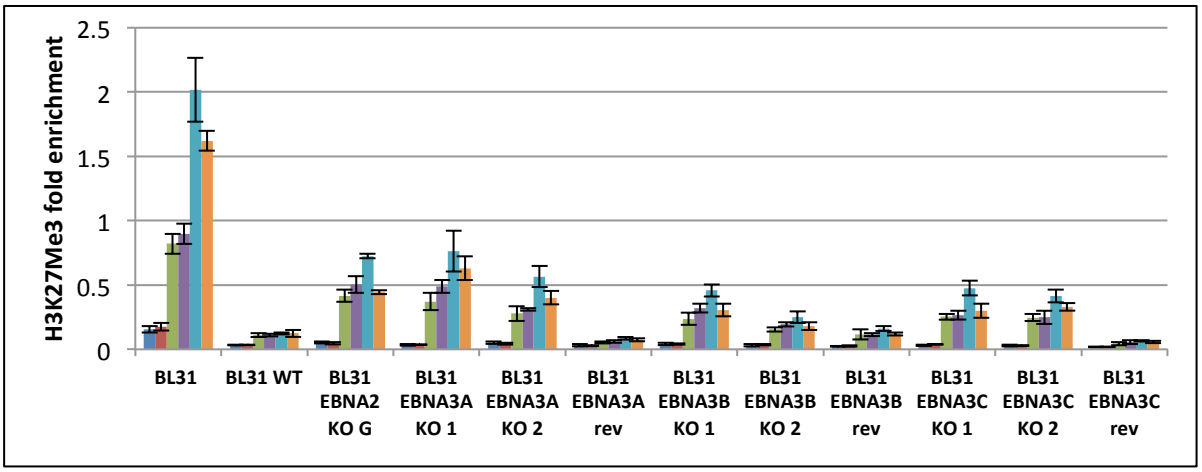
In all ChIPs performed, variation between cell lines was only seen at the proximal promoter, with levels of all histone modifications being low at the distal promoter (figures 3.12B-D). This is consistent with the regulation of TGFβR3 by EBV in BL cells acting through the proximal promoter.

For all ChIPs, the peak of binding was seen at the primer set 'proximal 3' (shown in turquoise), at the distal end of the main proximal CpG island, i.e. downstream from the proximal promoter (fig. 3.12A-D). In contrast to the findings for TGFβR2, H3K27Me3 also showed a peak around this region, rather than being equally distributed across the promoter as was seen for TGFβR2 (fig. 3.2). However, the region covered by the TGFβR2 promoter primers was smaller than that covered by the TGFβR3 proximal promoter primers (approximately 2000bp and 3000bp respectively), i.e. the primer set products were all within 500bp of each other for TGFβR2, whereas for TGFβR3 there was a distance of around 1000bp between the products of primer sets proximal 2, 3 and 4.

Taken together, these findings suggest that the regulation of TGFβR3 by EBV also occurs via epigenetic modifications at the proximal promoter.


A
Dis 1
Dis 2
Prox 1
Prox 2
Prox 3
Prox 4
Distal TSS
Proximal TSS
5.5 kb
12.5 kb
22.5 kb
ATG
C
B
A
CpG Dis 1
CpG Dis 2
CpG Prox 1
CpG Prox 2
CpG Prox 3
CpG Prox 4
Common exon
500bp
500bp


B
H3K9Ac fold enrichment
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BL31
BL31 WT
BL31 EBNA2 KO G
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A rev
BL31 EBNA3B KO 1
BL31 EBNA3B KO 2
BL31 EBNA3B rev
BL31 EBNA3C KO 1
BL31 EBNA3C KO 2
BL31 EBNA3C rev


C
H3K27Me3 fold enrichment
2.5
2
1.5
1
0.5
0
BL31
BL31 WT
BL31 EBNA2 KO G
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A rev
BL31 EBNA3B KO 1
BL31 EBNA3B KO 2
BL31 EBNA3B rev
BL31 EBNA3C KO 1
BL31 EBNA3C KO 2
BL31 EBNA3C rev

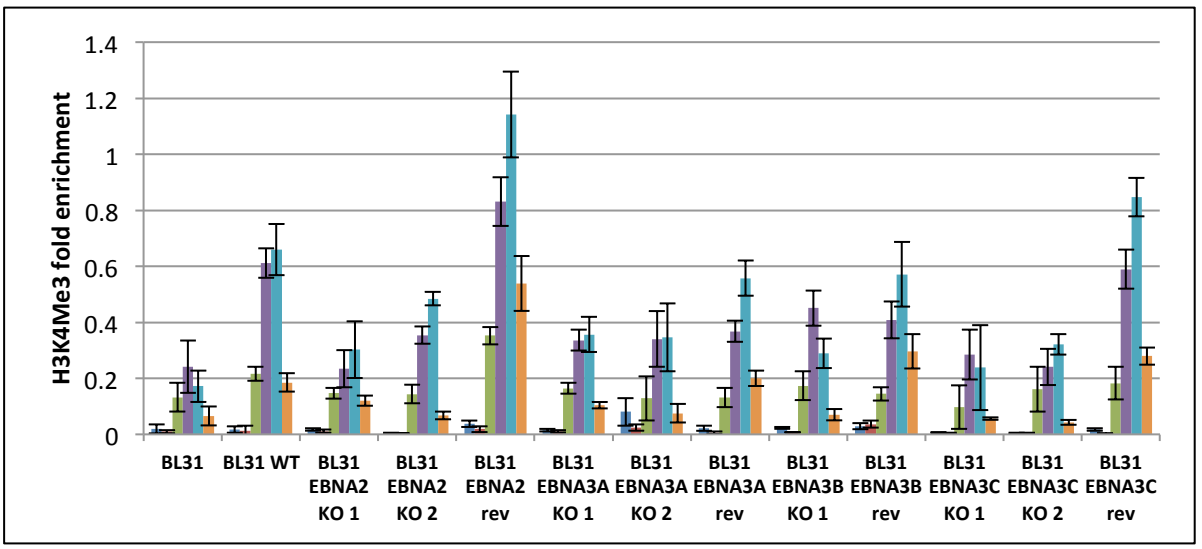

D
H3K4Me3 fold enrichment
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BL31
BL31 WT
BL31 EBNA2 KO 1
BL31 EBNA2 KO 2
BL31 EBNA2 rev
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A rev
BL31 EBNA3B KO 1
BL31 EBNA3B rev
BL31 EBNA3C KO 1
BL31 EBNA3C KO 2
BL31 EBNA3C rev

**Figure 3.12: ChIP for histone modifications at the TGFβR3 promoters in a panel of BL31 cell lines**

**(A)** Schematic diagram of distal and proximal TGFβR3 promoters showing the location of products of primer sets used, the putative transcription start sites (TSS) and CpG islands. The coloured blocks represent the primer pairs shown as bars from left to right for each cell line in figures B-D. **(B-D)** qPCRs showing ratio of histone modification to input DNA at the TGFβR3 promoters for **(B)** H3K9Ac **(C)** H3K27Me3 and **(D)** H3K4Me3. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least two separate experiments. The error bars represent standard deviations from triplicate qPCR reactions for both input and IP.

### 3.4.3 What is the relationship between TGFβR2 and TGFβR3 in BL31 cells?

In BL31 cells, EBV represses TGFβR2 and up-regulates TGFβR3. The same latent proteins (EBNA2, EBNA3B and EBNA3C) appear to cooperate in the regulation of both these genes. Thus in the majority of cell lines TGFβR2 and TGFβR3 appear to be reciprocally regulated, with the exception of some of the EBNA3A KO lines. The mechanism of the apparent reciprocality was therefore examined.

It has been shown, in breast and ovarian cancer cells, that TGFβ1 treatment leads to the down-regulation of TGFβR3 by a direct effect on the TGFβR3 proximal promoter, with this process being dependent on TGFβR1 and pSMAD2/3 (Hempel et al. 2008). It was therefore hypothesised that, if a similar process occurs in B cells, TGFβ1 present in medium produced by autocrine secretion (and/or present in the serum) could result in down-regulation of TGFβR3 in B cells where TGFβ signalling via pSMAD2 is active, i.e. in primary B cells or uninfected BL31 cells. However, when TGFβR2 is repressed by latent EBV, as in wild-type LCLs (see chapter 6) or BL31-WT, leading to suppression of TGFβ signalling via pSMAD2, this process may no longer occur and thus TGFβR3 would be de-repressed. The current study has shown that TGFβR1 expression is not altered significantly by EBV or any of the latent proteins investigated (fig. 3.10). Therefore if this effect occurs it may be via TGFβR2 rather than TGFβR1.

BL31 cells were investigated to see whether TGFβ1-induced repression of TGFβR3 may occur in a similar manner to that seen in the carcinoma cell lines described by Hempel *et al* (2008). If this is the case, blocking TGFβ signalling by any means would lead to de-repression of TGFβR3. Thus EBV-negative BL31 cells were treated with SB431542, a specific inhibitor of TGFβR1, or a DMSO control for 30 minutes, followed by treatment with TGFβ1 or vehicle for 6 hours, based upon the findings of Hempel *et al* that TGFβR3 was maximally down-regulated by 6 hours after treatment with TGFβ1. Samples were then harvested for RNA, in order to measure TGFβR3 expression, and protein, in order to confirm that SB431542 treatment had blocked TGFβ signalling.

Treatment with SB431542 was able to completely block TGFβ-induced pSMAD2 (fig. 3.13B) and caused a greater than two-fold increase in TGFβR3 expression (fig. 3.13A). Therefore, this could be consistent with TGFβ1 causing repression of TGFβR3 transcription in BL31 cells.

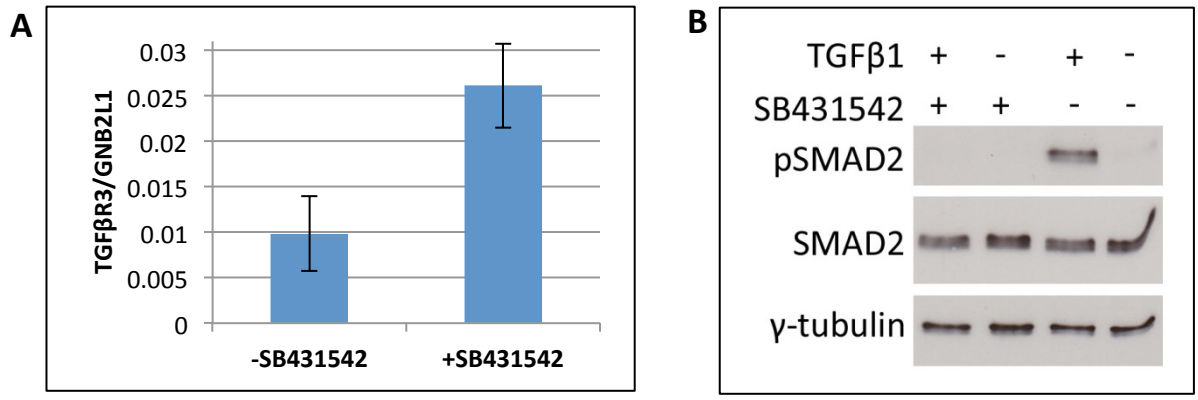



**Figure 3.13: Treatment of EBV-negative BL31 cells with the TGFβR1 inhibitor SB431542 blocks TGFβ-induced pSMAD2 and leads to an increase in TGFβR3 mRNA**

An equal number of BL31 cells were treated with 10μM SB431542 or DMSO alone for 30 minutes and then each sample split into two, of which one was treated with TGFβ1 5ng/ml and the other with vehicle alone (2mg/ml BSA in PBS). Samples were harvested after a further six hours for RNA and protein extraction. **(A)** qRT-PCR for TGFβR3 in cells treated with or without SB431542 for 30 minutes followed by vehicle alone for six hours. **(B)** Western blot for pSMAD2 after treatment with SB431542 dose or DMSO control for 30 minutes followed by TGFβ1 5ng/ml or vehicle for 6 hours. SMAD2 is shown for comparison, with γ-tubulin as a loading control.

If this TGFβ1-induced repression of TGFβR3 occurs in BL31 cells, then when TGFβ signalling is blocked by repression of TGFβR2, this repression would no longer occur and hence TGFβR3 would be de-repressed. Therefore, when TGFβR2 is not repressed and so TGFβ signalling occurs (as in EBNA3B, EBNA3C and EBNA2 KOs), then TGFβ1 can continue to repress TGFβR3 so it is not up-regulated in these cells. Generally TGFβR3 would be expressed whenever TGFβR2 is repressed. However, and particularly where expression of receptors is only partially repressed/partially up-regulated, there may be differences in the degree of repression of TGFβR3 depending on the amount of TGFβ1 available. TGFβ1 is present in serum, and in this study all experiments were conducted with cells proliferating in medium containing FCS, since it was felt that depriving them of serum may alter many processes within the cells. However, all the recombinant EBV-infected BL31 cell lines were grown in the same media and generally when an experiment was performed the same batch of medium containing FCS was used for all cell lines. Thus although there may have been differences between cell lines in the amounts of TGFβ1 present within the medium, these are likely to have been small. In addition, the doses of exogenous TGFβ1 used were

generally high and thus likely to overcome any effect of variation in TGFβ1 between cell lines.

As well as TGFβ1 being present in serum, however, there is also autocrine production by B cells (Kehrl et al. 1986, Spender et al. 2009). Therefore, in order to investigate the differences between cell lines where TGFβR2 was not fully repressed and the up-regulation of TGFβR3 did not inversely correlate with the down-regulation of TGFβR2, i.e. particularly the EBNA3A KO cell lines, it was considered whether differences in autocrine TGFβ1 production could account for the discrepancies. TGFβ1 expression was investigated in the panel of BL31 cell lines; however, because of time constraints, ELISA for secreted TGFβ1 was not performed.

Although TGFβ1 was possibly up-regulated by EBV, the difference was relatively small and there was no clear pattern of alteration of its expression by any particular latent protein (fig. 3.14). This suggests that EBV does not significantly alter autocrine TGFβ1 production (with the caveat that ELISA was not performed to confirm TGFβ1 secretion), and therefore that differences in TGFβ1 expression are unlikely to explain the lack of reciprocality between TGFβR2 and TGFβR3 in some cell lines (particularly EBNA3A KO).

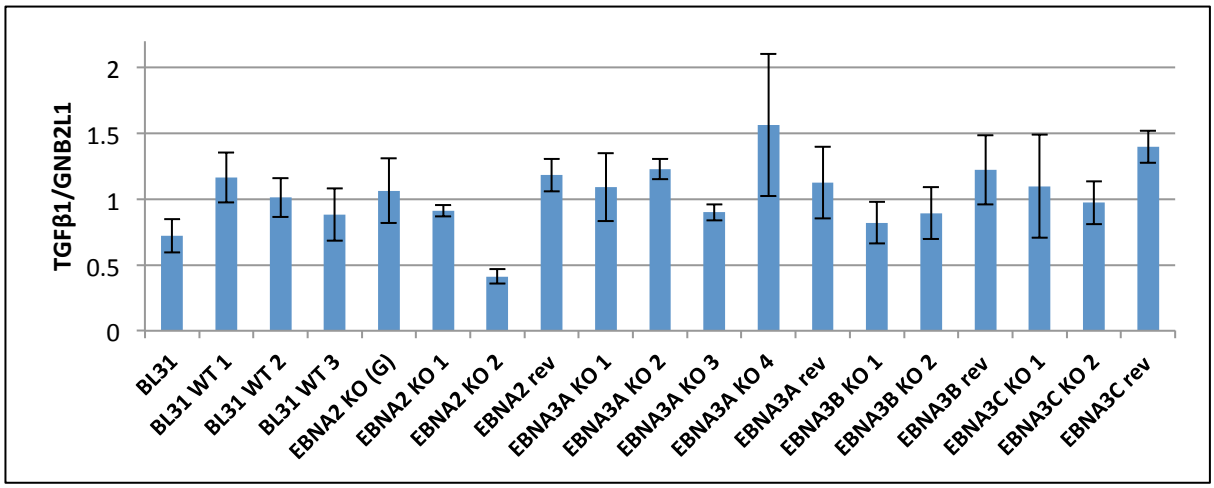



**Figure 3.14: EBV does not significantly alter TGFβ1 mRNA expression**

qRT-PCR for TGFβ1 mRNA in the panel of wild-type and recombinant EBV-infected BL31 cell lines. Values are expressed as ratios to the endogenous control gene *GNB2L1* with error bars representing standard deviation of triplicate qPCR reactions. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least three separate experiments.

## 3.5 Discussion

### EBV down-regulates TGFβR2 via PRC2-mediated transcriptional repression, leading to suppression of TGFβ signalling, in BL31 cells

This study has shown that wild-type EBV infection leads to repression of TGFβR2 and suppression of canonical TGFβ signalling, as indicated by phosphorylation of SMAD2. Many previous studies had shown that EBV infection leads to resistance to TGFβ-induced growth inhibition or apoptosis in B cells and other cell types. Although some studies in B cells had suggested that EBV down-regulates TGFβR2 (Inman and Allday 2000b, Fukuda et al. 2006a, Chen et al. 2007), others suggested that the effect of EBV on TGFβ responses was not due to changes in levels of TGFβ receptors (Arvanitakis et al. 1995). In addition, some studies had suggested that TGFβR1 and TGFβR2 were both reduced by EBV (Kumar et al. 1991) or had only investigated TGFβR1 but not TGFβR2 (Altiok et al. 1991, Altiok et al. 1993). However, no previous studies in B cells appear to have investigated the effect of EBV on TGFβ signalling, as detected by phosphorylation of SMAD proteins, use of a TGFβ–responsive reporter, or changes in transcription of known TGFβ target genes. As well as confirming the repression of TGFβR2 by EBV, the current study has demonstrated that the same latent proteins which are necessary for its transcriptional repression are also necessary for the loss of TGFβ signalling, as measured by SMAD2 phosphorylation. This suggests that the phosphorylation of SMAD2 is dependent on a threshold level of TGFβR2 expression.

Alterations in components of TGFβ signalling, including mutations of TGFβR2, have been found in many tumour types [reviewed in (Elliott and Blobe 2005, Dong and Blobe 2006)]. In addition, epigenetically mediated changes are increasingly recognised as a cause of malignancies including haematopoietic tumours. EBV had been shown to repress other tumour suppressor genes by polycomb-mediated repression (Paschos et al. 2009, Skalska et al. 2010, Paschos et al. 2012). Therefore the mechanism of repression of TGFβR2 by EBV was investigated. This showed that the transcriptional repression of TGFβR2 is accompanied by increased deposition of the repressive mark H3K27Me3, reduced deposition of the active mark H3K9Ac, and increased binding of SUZ12, one of the main components of PRC2 which mediates H3K27Me3 (fig. 3.2). These changes in histone modifications generally correlate with the changes in transcription that occur when individual latent genes are deleted.

Therefore, EBV down-regulates another tumour suppressor gene, TGFβR2, by polycomb-mediated repression.

**Significance of DNA methylation**

CpG hypermethylation of promoter regions is increasingly seen to occur in many tumours, and several treatments currently in clinical practice for haematopoietic malignancies target epigenetic lesions including DNA methylation, particularly in myelodysplastic syndrome (Khan et al. 2013). CpG methylation of the TGFβR2 promoter has been demonstrated in cancers of the lung, prostate, stomach and oesophagus as well as in some B cell lymphoma and PEL cell lines (Zhang et al. 2004, Zhao et al. 2005, Di Bartolo et al. 2008, Yamashita et al. 2008b, Dong et al. 2012, Guo et al. 2012). TGFβR2 has also been found to be hypermethylated in DNA extracted from patient samples, and associated with a worse prognosis, in multiple myeloma patients (de Carvalho et al. 2009). Deposition of H3K27Me3 is thought to precede DNA methylation and increase the likelihood of it occurring, with DNA methylation being a more stable repressive epigenetic mark than histone modifications (Schlesinger et al. 2007, Widschwendter et al. 2007).

In the panel of BL31 cell lines, although H3K27Me3 varied according to the EBV latent genes expressed, no DNA CpG methylation was seen (fig. 3.4). Investigation of some other EBV-positive and negative cell lines, including LCLs, showed that DNA methylation of the TGFβR2 promoter occurs in Akata6, Akata31 and LCL X50-7, but not in BL31-WT or a recently established LCL-WT, even though TGFβR2 was also repressed in these. Akata6 and Akata31 both showed DNA methylation of the CpG islands of the TGFβR2 promoter region, without H3K27Me3 deposition, whereas LCL X50-7 showed intermediate levels of both DNA methylation and H3K27Me3 (fig. 3.5B-C).

The original Akata cell line was established from an EBV-positive BL (Takada et al. 1991). Recently after isolation from biopsy samples, BLs usually express latency I, i.e. are of the group I phenotype (Rowe et al. 1987). Akata6 and Akata31 are distinct clones of the original Akata cell line, with Akata6 having remained EBV-positive (type I latency), whereas Akata31 had lost its episome during culture and thus become EBV-negative (Jenkins et al. 2000). The DNA methylation and consequent repression of TGFβR2 in both these cell lines suggests that

this occurred at an earlier stage in tumour development, for example as cells enter the germinal centre where the full latency III set of genes would have been transiently expressed (Thorley-Lawson and Gross 2004). In this case, the previous exposure to EBNA2/EBNA3B/EBNA3C could have resulted in polycomb-mediated repression of TGFβR2 and subsequent DNA methylation, resulting in a more stable repression of TGFβR2. Since this is heritable, TGFβR2 would have remained repressed by DNA methylation in continuous culture, even though the EBV episome was completely lost (in the case of Akata31) or the expression reverted to type I (in Akata6).

The Akata cell line has previously been shown to be resistant to the effects of TGFβ and have reduced expression of TGFβR2 (Inman and Allday 2000b, Fukuda et al. 2006a). Akata cells treated with the HDAC inhibitor trichostatin A (TSA), however, showed restoration of TGFβR2 expression and in turn restoration of sensitivity to TGFβ-induced growth inhibition (Fukuda et al. 2006a). In another study, Akata cells treated with the DNMT inhibitor 5-azacytidine became responsive to TGFβ with induction of pSMAD2 and TGFβ-induced growth inhibition (Chen et al. 2007). These observations are consistent with repression of TGFβR2 by epigenetic mechanisms including histone deacetylation and DNA methylation.

**EBNA3B and EBNA3C cooperate in the repression of TGFβR2**

The work leading up to this project included a microarray analysis investigating the effects of the EBNA3 proteins on cellular gene expression, which suggested that EBNA3B and EBNA3C cooperate to down-regulate TGFβR2 [www.epstein-barrvirus.org.uk and (White et al. 2010)]. However, preliminary investigations suggested that, as well as EBNA3B and EBNA3C, EBNA2 was necessary for the repression of TGFβR2 in BL31 cells. This was unexpected, as EBNA2 generally transactivates transcription and up-regulates both viral and cellular gene expression. Previous microarray analyses in LCLs conditionally expressing EBNA2 had indicated that, although EBNA2 does regulate a significant number of cellular genes directly or indirectly, these were all induced rather than repressed by EBNA2 (Spender et al. 2002, Spender et al. 2006, Zhao et al. 2006). A study in EBV-negative BL lines expressing a conditional EBNA2 protein, however, showed 18 genes that were repressed by EBNA2, including cell surface markers such as CD79b and CD52, but none of these were part of TGFβ signalling pathways (Maier et al. 2006). EBNA2 had been shown to cooperate with

EBNA3C in regulating multiple cellular genes in LCLs, although again these all largely involved up-regulation rather than repression by EBNA2 (Zhao et al. 2011).

The EBNA3 proteins are transcriptional regulators and have been shown both to induce or repress multiple genes. Since they are probably targeted to DNA by cellular transcription factors, it is clear that they can act to alter transcription. A global ChIP-seq analysis in Mutu III cells showed many binding sites for EBNA3C at cellular gene promoters (McClellan et al. 2012), and EBNA3C binding has been demonstrated at the promoters of the genes encoding BIM and $p16^{INK4A}$ (Paschos et al. 2012, Skalska et al. 2013). Although in the current study attempts to demonstrate binding of EBNA3C to the TGFβR2 promoter were unsuccessful, nevertheless it is likely that this could occur and be a mechanism for the repression of TGFβR2. Other investigators in the laboratory are currently investigating the binding of EBNA3B to gene promoters.

There is extensive cooperation between EBNA3 proteins, with EBNA3B/C together down-regulating 145 genes and up-regulating 144 genes (White et al. 2010). A recent microarray comparing gene expression in different BL types confirmed the down-regulation of TGFβR2 in latency III compared to latency I and in Wp-restricted compared to latency I clones of Awia-BL, i.e. consistent with down-regulation by EBNA3 proteins (Kelly et al. 2013). The microarray for genes regulated by EBNA3C in Mutu III cells, however, did not show TGFβR2 to be significantly regulated by EBNA3C (McClellan et al. 2012).

**The up-regulation of TGFβR3 by EBV and relationship between TGFβR2 and TGFβR3**

In addition to the down-regulation of TGFβR2, this study has also shown that EBV up-regulates the co-receptor TGFβR3 in BL31 cells (fig. 3.11).

The regulation, at least by EBNA3B and EBNA3C, also occurs via epigenetic regulation of the proximal promoter. Consistent with these findings for EBNA3C, a recently published microarray investigating genes regulated by EBNA3C alone in a B cell line showed up-regulation of TGFβR3 of over 4-fold by EBNA3C (McClellan et al. 2012), and up-regulation by EBNA3C has also been demonstrated in 3CHT-LCLs, as will be discussed in chapter 6 (Skalska et al. 2013).

Initial findings in this study suggested that EBNA3B, EBNA3C, and EBNA2 cooperate to both down-regulate TGFβR2 and up-regulate TGFβR3 (fig. 3.11A-B). This led to the question of whether TGFβR2 and TGFβR3 could be reciprocally regulated in some way. However, more detailed analysis showed some subtle differences between the regulation of the two genes. The involvement of EBNA3A in regulation of both genes is unclear, as will be discussed further in section 4.6.

Nevertheless, since in most other cases the expression of the two genes does appear to be reciprocal, it was hypothesised that they could somehow be co-regulated. It had been shown in carcinoma cell lines that TGFβ1 could repress transcription of TGFβR3, acting at the proximal TGFβR3 promoter, in a TGFβR1-dependent mechanism (Hempel et al. 2008). Treatment of EBV-negative BL31 cells, in which TGFβR3 expression had been found to be low, with the TGFβR1 inhibitor SB431542, led to a 2.5-fold increase in TGFβR3 mRNA expression by six hours post treatment (fig. 3.13A), with the expected concomitant blockade of TGFβ signalling as detected by pSMAD2 (fig. 3.13B). This suggests that TGFβ1-induced transcriptional repression of TGFβR3 may also occur in B cells, with the TGFβR1 inhibition and consequent disruption of signalling resulting in relief of the repression. If this is the case, then EBV infection of BL31 cells, which leads to repression of TGFβR2 and suppression of TGFβ signalling, would also cause de-repression of TGFβR3. When EBNA3B, EBNA3C, or EBNA2 are deleted, TGFβR2 is de-repressed, TGFβ signalling occurs and so TGFβ1 is again able to repress TGFβR3, thus TGFβR3 expression is low. Therefore the findings for most of the cell lines could be consistent with TGFβ1-mediated repression of TGFβR3 occurring in BL31, with the exception of some EBNA3A KO lines. In these cell lines TGFβR2 and TGFβR3 were not reciprocal, and it was confirmed that this was not due to changes in expression of TGFβR1 (fig. 3.10) or in autocrine TGFβ1 mRNA expression (fig. 3.14), although the actual secreted TGFβ1 (by ELISA) was not investigated.

The regulation of TGFβR3 also appears to be epigenetic, as variations in the histone modifications H3K27Me3, H3K9Ac and H3K4Me3 were found, generally consistent with the transcriptional TGFβR3 expression, between the different BL31 cell lines (fig 3.12). The relationship between TGFβR2 and TGFβR3 expression will be discussed further in chapter 6.

**The significance of the up-regulation of TGFβR3 by EBV**

What are the possible effects of TGFβR3 up-regulation? In many solid malignancies TGFβR3 has been shown to have tumour suppressive functions, and its expression is lost or reduced in several such malignancies, in some cases resulting in increased invasiveness (Dong et al. 2007, Hempel et al. 2007, Turley et al. 2007, Finger et al. 2008b, Gordon et al. 2008, Margulis et al. 2008). Little is known about its effects in B cells. It also has tumour suppressive functions, inhibiting cell growth and proliferation, in myeloma cells, in which its expression is decreased (Lambert et al. 2011). However, it has been shown to be up-regulated in CLL cells relative to normal B cells suggesting it may have a different, possibly tumour promoting, role in these cells (Klein et al. 2001, Jelinek et al. 2003).

TGFβR3 is specifically required for response to TGFβ2 and inhibin, since these ligands are unable to bind to their type 2 receptors without the presence of TGFβR3 (Lopez-Casillas et al. 1993, Lewis et al. 2000). Thus further work could be done to investigate the effects of EBV on signalling by TGFβ2 as well as activin/inhibins; because of time constraints, these were not investigated in this study.

In murine haematopoietic progenitor cells which responded to TGFβ1, TGFβ1 appeared to bind only to TGFβR1, suggesting that TGFβR3 may not be expressed, or possibly that TGFβ1 did not bind to it (Ohta et al. 1987). It has been suggested that on haematopoietic cells endoglin may be the main co-receptor used rather than TGFβR3, since haematopoietic cells do not respond to TGFβ2 which requires TGFβR3 to facilitate its binding to TGFβR2 (Dong and Blobe 2006). However, TGFβR3 has been shown to be expressed on B cell precursors in B cell acute lymphoblastic leukaemia, although this may represent aberrant expression due to the tumour (Buske et al. 1998).

Since it is a co-receptor for BMP signalling as well as TGFβ signalling, one possibility is that it enhances or inhibits BMP signalling; this will be addressed in chapter 7.

# Chapter 4 The effects of LMP1 and LMP2A deletion on TGFβR2 transcription and TGFβ signalling

## 4.1 Introduction

As briefly discussed in section 3.2.1, the apparent involvement of EBNA2 in TGFβR2 repression and suppression of TGFβ signalling was surprising. Since EBNA2 transactivates LMP1 and LMP2A it was hypothesised that the changes may in fact be due to one (or possibly both) of these latent proteins rather than (or as well as) EBNA2 itself. Therefore it was important to try to distinguish the effects of EBNA2, LMP1 and LMP2A on TGFβR2 expression and TGFβ signalling. In order to facilitate this, recombinant viruses containing deletions of LMP1 and LMP2A, made in the same BAC-derived system used above for the EBNA2 and EBNA3 KO lines, were obtained as a kind gift from Wolfgang Hammerschmidt, Münich, Germany. Unfortunately these viruses did not become available until relatively late in the course of the project.

## 4.2 Infection of BL31 cells to make BL31 LMP1 KO and BL31 LMP2A KO cell lines

Viruses were obtained in the form of producer lines (HEK293 cells) containing the recombinant BACs. The BAC constructs were verified by episomal rescue, restriction enzyme digestion and pulsed-field gel electrophoresis, confirming the predicted size differences of certain bands distinguishing wild-type, LMP1 KO and LMP2A KO recombinant BACS (fig. 4.1). Recombinant virus was then induced from the producer lines and used to infect EBV-negative BL31 cells. The established BL31 LMP1 KO 1 and 2 lines were established from two separate infections with the same virus, similarly for the BL31 LMP2A KO 1 and 2 lines. BL31 LMP2A KO 1 was slower to grow out than the other three cell lines, which were similar in their time to establishment.

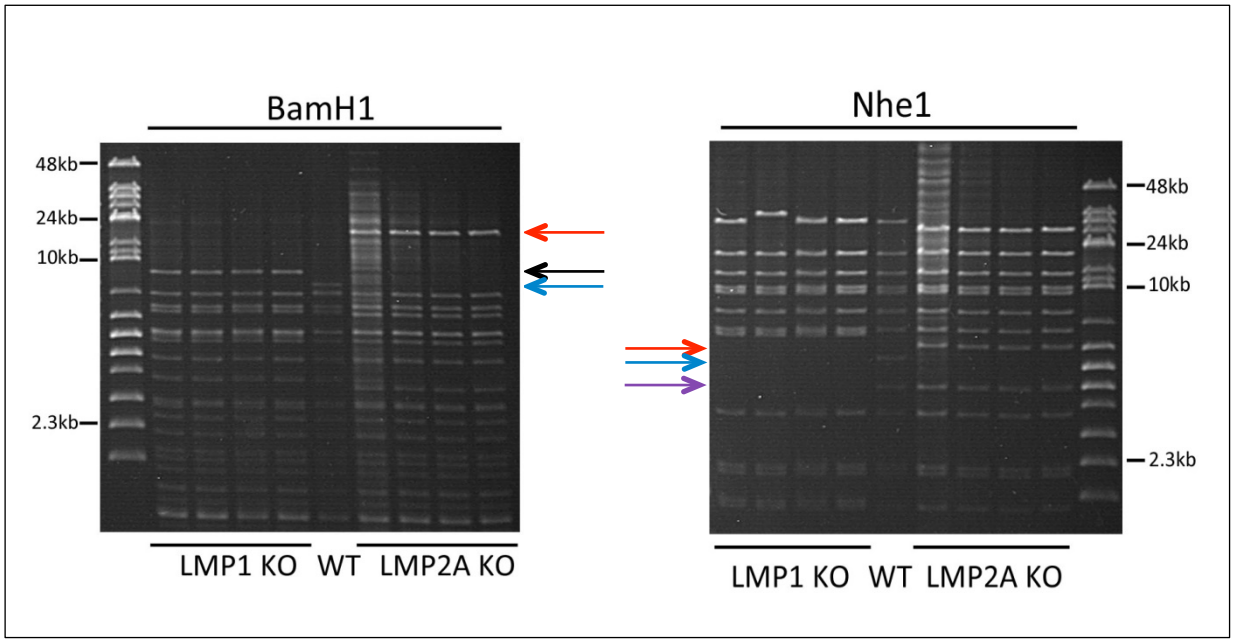



**Figure 4.1: Validation of LMP1 and LMP2A knockout EBV-BACs**

DNA from the LMP1 KO, LMP2A KO and a wild-type EBV-BAC were analysed by restriction digestion with Nhe1 and BamH1 followed by pulsed field gel electrophoresis. A mixture of λ-DNA BstEII and λ-DNA mono cut mix ladders (NEB) were used as a size marker, with the size of selected bands shown. Bands whose sizes are changed by the deletion of LMP1 and LMP2A are indicated by coloured arrows. Red = band present only in LMP2A KO, black = band present only in LMP1 KO, and blue = band present only in WT. For Nhe1, LMP1 KO is distinguished by absence of a band present in both WT and LMP2A KO, shown by purple arrow.

Once established, the expression of EBV latent proteins in the BL31 LMP1 KO and BL31 LMP2A KO cell lines were investigated, to ensure that they lacked expression of LMP1 and LMP2A as appropriate and to ensure that lack of expression of the relevant protein did not significantly alter expression of other latent proteins. This showed that the LMP1 KO and LMP2A KO lines generally expressed similar amounts of EBNA1, EBNA2, EBNA3A, EBNA3B and EBNA3C, with the exception of LMP1 KO 2 which expressed slightly higher levels of EBNA1, EBNA2, EBNA3A and EBNA3B than the other cell lines (fig. 4.2A). In addition, EBNA-LP was present in all cell lines but in varying amounts, in particular the LMP2A KOs had increased amounts of EBNA-LP. However, the level of EBNA-LP expressed in LCLs is known to be highly variable. Although the LMP1 KOs express LMP2A, expression levels varied between the two different LMP1 KO lines, being greater in LMP1 KO 2 (fig. 4.2A). This finding was also confirmed at the RNA level by qRT-PCR for LMP2A (fig. 4.2B). The western blot for LMP2A appeared to show faint bands in uninfected BL31 and in both LMP2A KO

lines (fig. 4.2A). However, this was suspected to be due to high background signal; this was confirmed by the complete absence of LMP2A mRNA expression by qRT-PCR in these cell lines (fig. 4.2B).

By western blot, neither of the LMP2A KO lines appeared to express LMP1 (fig. 4.2A), although a long exposure showed a very low expression of LMP1 in LMP2A KO 2 (fig. 4.2D). qRT-PCR confirmed that LMP2A KO 1 lacked any LMP1 expression whereas LMP2A KO 2 had a very low level of expression (fig. 4.2C).

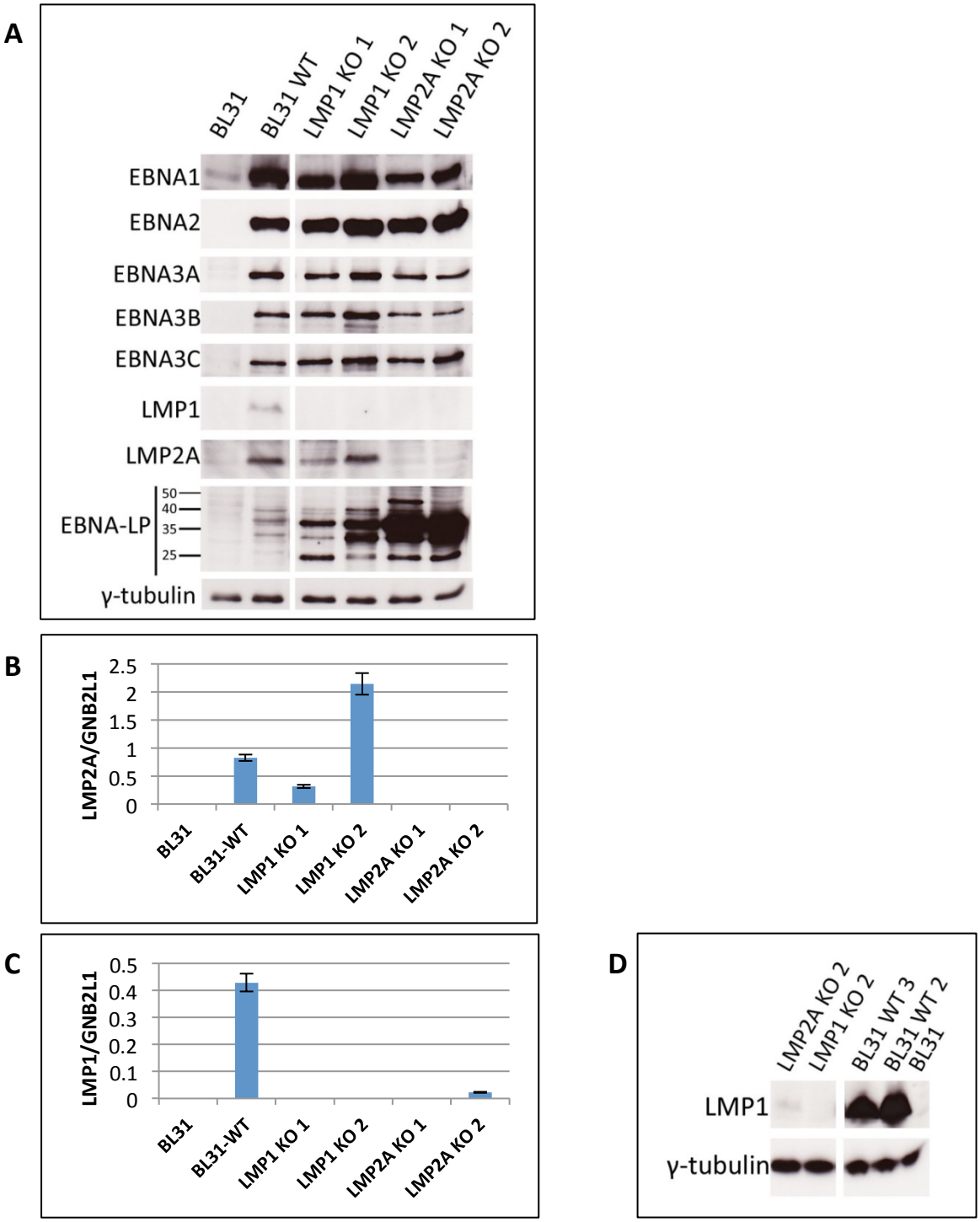



**Figure 4.2: Expression of EBV latent proteins in BL31 LMP1 KO and BL31 LMP2A KO cell lines**

**(A)** Western blot for EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2A and EBNA-LP in BL31, BL31-WT EBV and the two LMP1 KO and LMP2A KO cell lines. γ-tubulin is shown as a loading control. **(B)** qRT-PCR for LMP2A in LMP1 and LMP2A KO cell lines. **(C)** qRT-PCR for LMP1 in LMP1 and LMP2A KO lines. Values are shown as ratios to the endogenous control gene *GNB2L1* with error bars representing standard deviation of triplicate qPCR reactions. **(D)** Western blot for LMP1 in selected cell lines showing a faint band of LMP1 in LMP2A KO 2. γ-tubulin is shown as a loading control.

### 4.3 LMP1 and LMP2A are necessary for the repression of TGFβR2 and suppression of TGFβ signalling in BL31 cells

Once the BL31 LMP1 KO and BL31 LMP2A KO cell lines were established, they were used to investigate the effects of LMP1 and LMP2A deletion on TGFβR2 expression and TGFβ signalling. qRT-PCR for TGFβR2 showed expression in all LMP1 KO and LMP2A KO lines, although this was higher in LMP2A KO than LMP1 KO lines (fig. 4.3A). Treatment of LMP1 KO and LMP2A KO cell lines with TGFβ1 resulted in restoration of TGFβ signalling as detected by pSMAD2, consistent with the de-repression of TGFβR2 seen in these cell lines (fig. 4.3B).

The effects of LMP1 or LMP2A deletion, as well as deletion of the whole EBNA3 locus (EBNA3 KO), on TGFβ-induced ID1 mRNA expression was also investigated. This showed that when EBNA3, LMP1 or LMP2A were deleted, TGFβ treatment induced ID1 expression at two hours (fig. 4.3C). No induction of ID1 was seen for wild-type or EBNA3 revertant-infected cells. Expression of ID2 was also investigated in a similar manner and showed similar results; however, for clarity only ID1 data is shown here (fig. 4.3C). This supports the findings that EBNA3 proteins, LMP1 and LMP2A cooperate to suppress TGFβ signalling. These findings suggest that LMP1 and LMP2A both cooperate with EBNA3s in the repression of TGFβR2 and consequent suppression of TGFβ signalling.

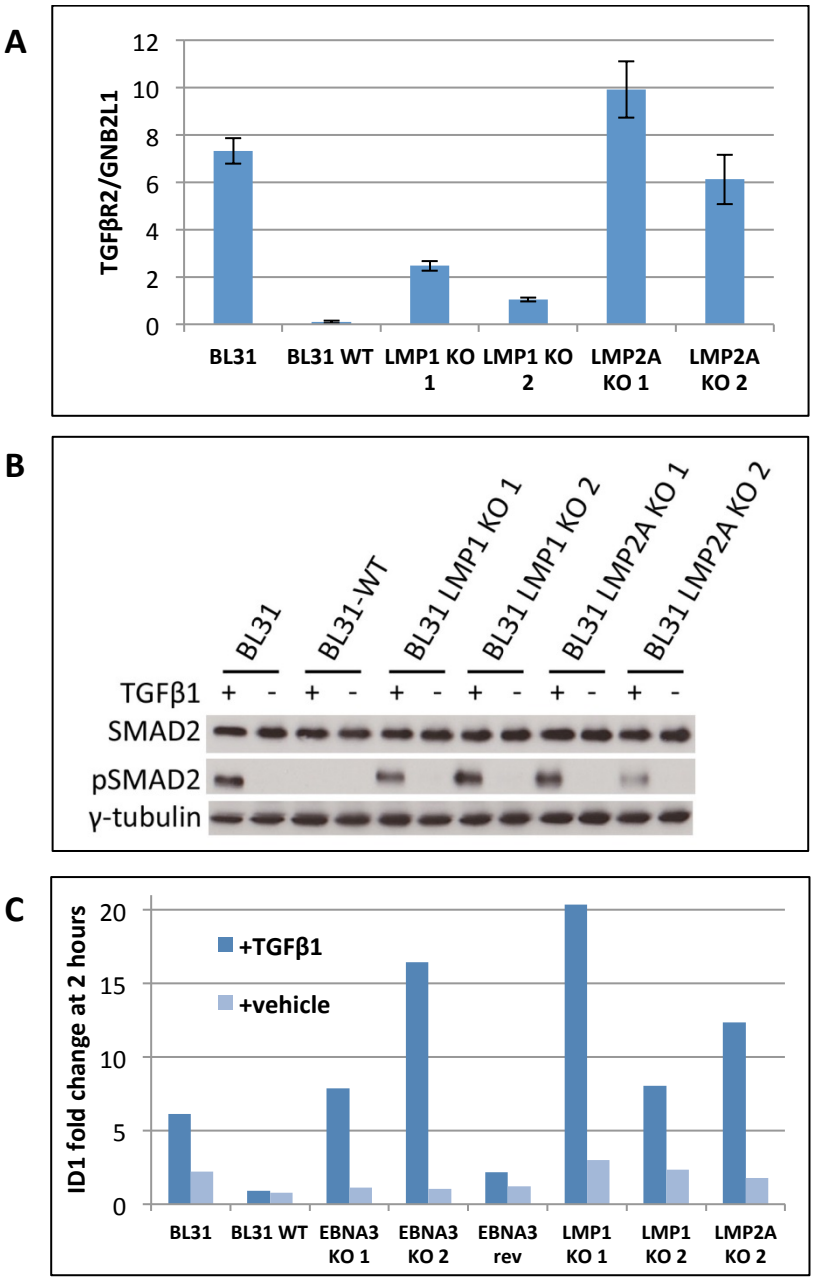



**Figure 4.3: LMP1 KO and LMP2A KO show de-repression of TGFβR2 and restoration of TGFβ signalling as detected by phosphorylation of SMAD2 and induction of ID1 expression**

**(A)** qRT-PCR for TGFβR2 mRNA in the cell lines shown. Values are shown as ratios to the endogenous control gene *GNB2L1* with error bars representing standard deviation of triplicate qPCR reactions. **(B)** Western blot for pSMAD2 after two hours of treatment with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in PBS). Total SMAD2 is shown for comparison, with γ-tubulin as a loading control. **(C)** qRT-PCR for ID1 expression in the cell lines shown after two hours' treatment with TGFβ1 5ng/ml or vehicle. Values shown are the ratio of expression at two hours to that at time zero, showing increased induction of ID1 with TGFβ treatment compared to vehicle in uninfected BL31, EBNA3 KO, LMP1 KO and LMP2A KO but no induction with TGFβ in wild-type or EBNA3 revertant-infected BL31. Values were calculated as ratios to the endogenous control gene *GNB2L1* and then the fold change was calculated as described. Similar data were obtained for ID2 expression.

## 4.4 The effects on TGFβR2 expression and TGFβ signalling seen in EBNA2 KO lines are probably due to a combined effect of LMP1 and LMP2A

The LMP1 KO and LMP2A KO lines were established in an attempt to ascertain whether the effects seen in the EBNA2 KO cell lines were due to EBNA2 itself, or through LMP1 and/or LMP2A. The EBNA2 KO cell lines showed de-repression of TGFβR2 and consequent restoration of TGFβ signalling via pSMAD2 (see figs. 3.1 and 3.7B). The same phenotype was also seen for both LMP1 KO and LMP2A KO cell lines (figs. 4.3A-B). Therefore, in order to try to establish which of EBNA2, LMP1 and LMP2A is/are responsible for the repression, it was necessary to consider the expression levels of these latent proteins in the EBNA2 KO, LMP1 KO and LMP2A KO lines.

As previously described, EBNA2 deletion is expected to result in reduced or no expression of LMP1 and LMP2A. Three different BL31 EBNA2 KO lines were investigated, the first (designated EBNA2 KO (G)) made by Gemma Kelly (Kelly et al. 2005) and the other two by Ian Groves (Allday laboratory, unpublished data). Expression of EBNA2, the EBNA3s, LMP1 and LMP2A were first investigated in the two EBNA2 KO lines that had been more recently established in the laboratory (EBNA2 KO 1 and EBNA2 KO 2), along with the LMP1 and LMP2A KO cell lines (fig. 4.4A). As expected, both EBNA2 KO 1 and EBNA2 KO 2 lines appeared to lack expression of LMP1 by western blot (fig. 4.4A). However, in another experiment, western blot for LMP1 expression in all three different EBNA2 KO lines showed that BL31 EBNA2 KO (G) had significant amounts of LMP1, and BL31 EBNA2 KO 1 appeared to have low expression of LMP1; in contrast EBNA2 KO 2 had no apparent LMP1 expression (fig. 4.4B). The apparent discrepancy between the results for EBNA2 KO 1 in terms of LMP1 expression (between figures 4.4A and 4.4B) may be explained by having a longer exposure in figure 4.4B, although LMP1 expression is also known to vary with the state of proliferation of cells (Boos et al. 1990); this may also explain the differences in LMP1 expression seen between treated and untreated samples of each EBNA2 KO cell line (fig. 4.4B). Nevertheless, qRT-PCR for LMP1 in all three EBNA2 KO lines confirmed low LMP1 mRNA expression in both BL31 EBNA2 KO 1 and BL31 EBNA2 KO 2, but high expression of LMP1 mRNA in BL31 EBNA2 KO (G) (fig. 4.4C). All three EBNA2 KO lines were confirmed to have a very low level expression of LMP2A mRNA (fig. 4.4D). The LMP2A protein levels in EBNA2 KO 1 and EBNA2 KO 2 lines were consistent with this (fig. 4.4A).

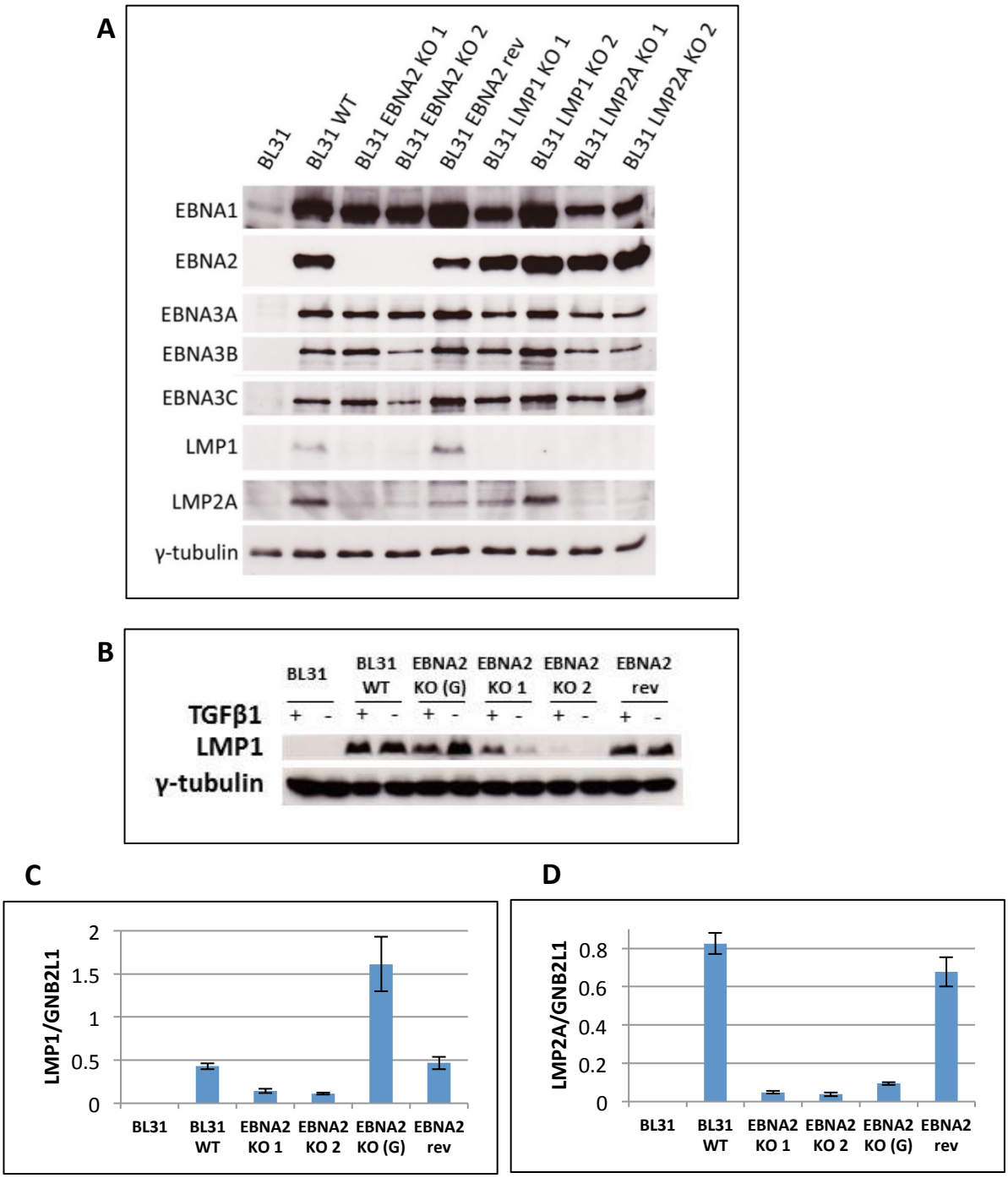



**Figure 4.4: Expression of LMP1 and LMP2A in EBNA2 KO BL31 cell lines**

**(A)** Western blot for EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1 and LMP2A in EBNA2 and LMP KO cell lines. γ-tubulin is shown as a loading control. **(B)** Western blot for LMP1 expression in BL31, BL31-WT and all three EBNA2 KO lines plus EBNA2 revertant, at 48 hours after treatment with TGFβ1 5ng/ml or vehicle. γ-tubulin is shown as a loading control. **(C)** qRT-PCR for LMP1 and **(D)** qRT-PCR for LMP2A in all three EBNA2 KO cell lines as well as BL31, BL31-WT and EBNA2 revertant. Values are expressed as ratios to the endogenous control gene *GNB2L1* with error bars representing standard deviation of triplicate qPCR reactions.

As shown previously, the LMP1 KO lines express high levels of EBNA2 (fig. 4.4A), no LMP1 and some LMP2A (fig. 4.4A-D). The LMP2A KO lines express high levels of EBNA2 but no/very low LMP1 and no LMP2A (fig. 4.4A-D).

Since all three EBNA2 KO lines, both LMP1 KO and both LMP2A KO lines all show de-repression of TGFβR2 and restoration of TGFβ signalling (figs. 3.1, 3.7A and 4.3A-C), the significance of the findings were considered for each of the three latent proteins LMP1, LMP2A and EBNA2 in turn; table 4.1 is a summary of the relevant findings for all EBNA2 KO, LMP1 KO and LMP2A KO lines.

| **BL31 cell line** | **EBNA2 expression** | **LMP1 expression** | **LMP2A expression** | **TGFβR2 expression** | **TGFβ signalling** |
|---|---|---|---|---|---|
| BL31 | - | - | - | Expressed | present |
| BL31-WT EBV | ++ | ++ | ++ | Repressed | absent |
| EBNA2 KO 1 | - | - | + | De-repressed | present |
| EBNA2 KO 2 | - | +/- | + | De-repressed | present |
| EBNA2 KO (G) | - | +++ | + | De-repressed | present |
| LMP1 KO 1 | ++ | - | + | Partially de-repressed | present |
| LMP1 KO 2 | ++ | - | ++ | Slightly de-repressed | present |
| LMP2A KO 1 | ++ | - | - | De-repressed | present |
| LMP2A KO 2 | ++ | - | - | De-repressed | present |

**Table 4.1: Summary of effects of EBNA2, LMP1 and LMP2A deletion on TGFβR2 expression and TGFβ signalling in BL31 cells**

Firstly, it was considered whether the effects seen in all these cell lines could be due to LMP1. In support of this, these effects are seen in LMP1 KOs, which express significant levels of both EBNA2 and LMP2A. Therefore, LMP1 is definitely necessary for the down-regulation of TGFβR2 and suppression of TGFβ signalling by EBV. However, the same effects are seen in EBNA2 KO (G), which expresses high levels of LMP1, and therefore the effects seen in EBNA2 KO lines cannot be entirely due to LMP1. Hence it is likely that LMP1 plus either EBNA2 or LMP2A are necessary.

Next, it was considered whether the effects could be due to LMP2A. The EBNA2 KO lines all express very low levels of LMP2A, even though they have varying expression of LMP1, yet all show de-repression of TGFβR2 and restoration of TGFβ signalling. This suggests that the phenotype could be due to LMP2A. LMP2A KOs show more marked de-repression of TGFβR2 than the LMP1 KOs. This in turn suggests that combined deletion of LMP1 and LMP2A has a greater effect than that of LMP1 KO alone, and therefore supports a model where the effects of LMP1 and LMP2A are combined. Furthermore, compared to LMP1 KO 1, LMP1 KO 2 has more LMP2A and less de-repression of TGFβR2 (figs. 4.2A-B and 4.3A); this also supports an effect of LMP2A on TGFβR2 repression. However, the effects seen in EBNA2 KO, LMP1 KO and LMP2A KO cell lines cannot be due to LMP2A alone, because the LMP1 KOs show the same phenotype despite expressing significant amounts of LMP2A.

TGFβR2 is completely de-repressed – expressed at the same level as in uninfected BL31 cells - in LMP2A KOs, suggesting that loss of LMP1 and LMP2A together are enough to fully restore TGFβR2 expression. These cell lines express normal amounts of EBNA2. Hence it seems likely that LMP1 and LMP2A cooperate (with EBNA3B and EBNA3C) to repress TGFβR2, but nevertheless an additional effect of EBNA2 cannot be completely excluded.

### 4.5 LMP2A, and to a lesser extent LMP1, are required for the up-regulation of TGFβR3 in BL31 cells

Since the same set of latent proteins, including EBNA2, initially appeared to cooperate in the up-regulation of TGFβR3 as in the repression of TGFβR2, the LMP1 and LMP2A KO BL31 cell lines were analysed for TGFβR3 expression. This showed that TGFβR3 expression remained low, similar to uninfected BL31, in LMP1 KO 1 and both LMP2A KO lines (fig. 4.5). However, partial up-regulation of TGFβR3 was seen in LMP1 KO 2, which has higher levels of LMP2A than LMP1 KO 1 (see fig. 4.2A-B). Taken together, these findings suggest that LMP2A is necessary for the up-regulation of TGFβR3. However, since TGFβR3 is only partially up-regulated in LMP1 KO 2, which has high expression of LMP2A and of EBNA2 (fig. 4.2A), this suggests that LMP1 may also contribute to the up-regulation of TGFβR3. Since all three EBNA2 KO lines have low levels of LMP2A, the finding that TGFβR3 is not up-regulated in EBNA2 KOs is consistent with LMP2A being necessary for the up-regulation. However, in

EBNA2 KO (G), which has high expression of LMP1, but very low LMP2A expression, TGFβR3 expression remains low, suggesting that LMP2A makes a greater contribution than LMP1 to the up-regulation of TGFβR3. Nevertheless, since it has not been possible to investigate a cell line that expresses LMP2A without expressing EBNA2, it is again not possible to exclude some effect from EBNA2 itself upon up-regulation of TGFβR3. Unfortunately, the TGFβR3 antibody for western blot was poor, with high background, and despite several attempts no adequate western blot could be performed in the LMP1 and LMP2A KO cell lines. Table 4.2 summarises the findings for TGFβR3 in the EBNA2 KO, LMP1 KO and LMP2A KO lines.

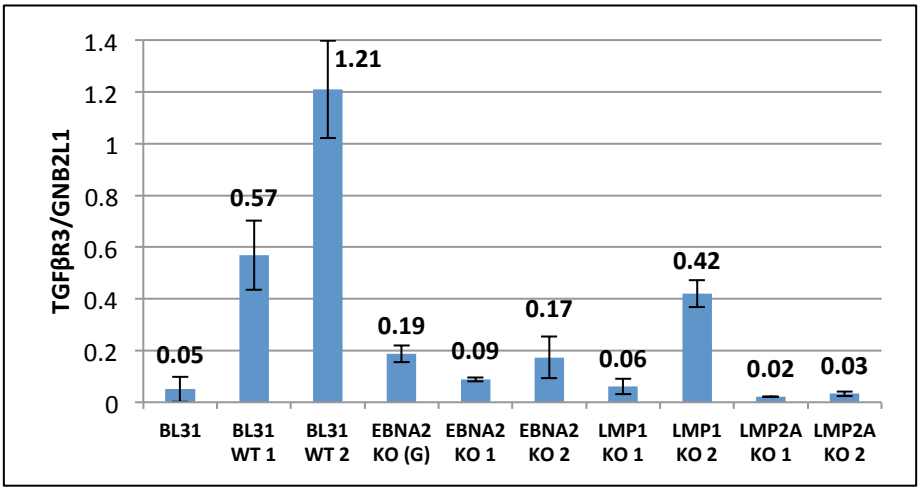



**Figure 4.5: TGFβR3 mRNA expression in EBNA2 KO, LMP1 KO and LMP2A KO BL31 cell lines**

qRT-PCR for TGFβR3, with values (shown above each bar) expressed as ratio to the endogenous control gene *GNB2L1*. Error bars represent standard deviation of triplicate qPCR reactions. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type infected cell lines is representative of three separate experiments.

| BL31 cell line | EBNA2 expression | LMP1 expression | LMP2A expression | TGFβR3 |
|---|---|---|---|---|
| **BL31** | - | - | - | Low |
| **BL31-WT EBV** | ++ | ++ | ++ | High |
| **EBNA2 KO 1** | - | - | + | Low |
| **EBNA2 KO 2** | - | +/- | + | Low |
| **EBNA2 KO (G)** | - | +++ | + | Low |
| **LMP1 KO 1** | ++ | - | + | Low |
| **LMP1 KO 2** | ++ | - | ++ | Intermediate |
| **LMP2A KO 1** | ++ | - | - | Low |
| **LMP2A KO 2** | ++ | - | - | Low |

**Table 4.2: Summary of effects of EBNA2, LMP1 and LMP2A deletion on TGFβR3 expression in BL31 cells**

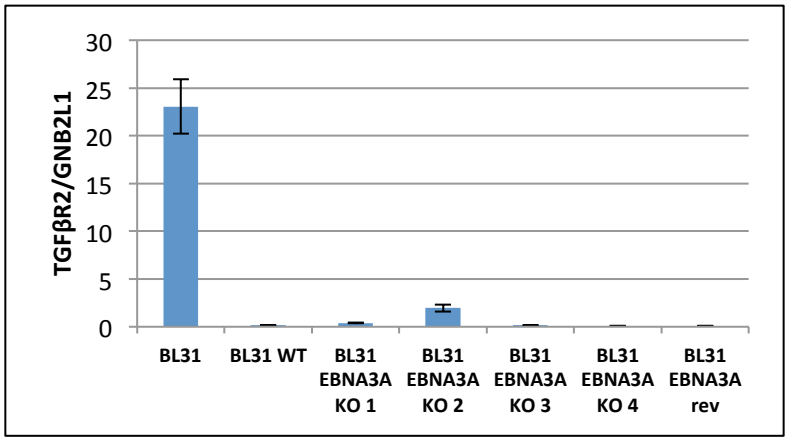
### 4.6 The variation in involvement of EBNA3A in regulation of TGFβR2 and TGFβR3

As noted in section 3.2.1, there was variation between the different EBNA3A KO cell lines in TGFβR2 expression (fig. 3.1A), also seen in the levels of binding in the ChIP assay (see figs. 3.2D and 3.12B).

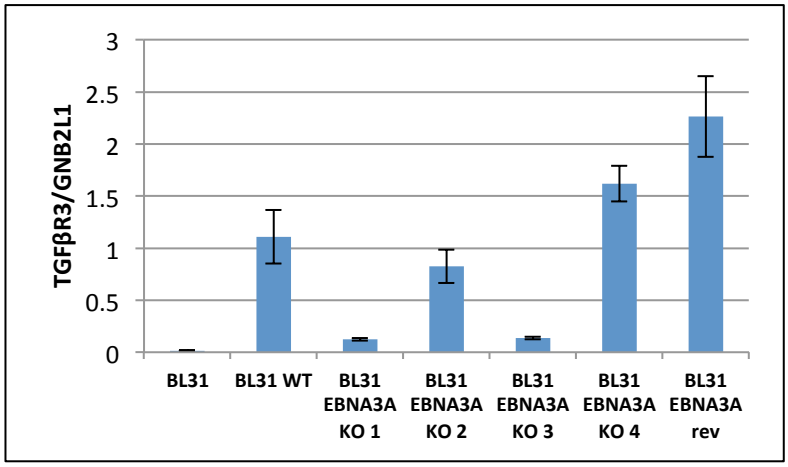
To investigate this further, TGFβR2 expression was investigated several times in the full set of four BL31 EBNA3A KO cell lines available – see figure 4.6A for representative data. Although there was some minor variation between experiments, generally EBNA3A KOs 1, 3 and 4 behaved like wild-type EBV, i.e. TGFβR2 was repressed in these, suggesting that EBNA3A is not necessary for the repression. However, in EBNA3A KO 2 TGFβR2 was partially de-repressed, but generally to a lower extent than for EBNA2, EBNA3B or EBNA3C KOs (fig. 4.6A), although in the particular experiment shown in fig. 3.1A the level of de-repression was approaching that of the EBNA2, EBNA3B and EBNA3C KOs (fig. 3.1A).

Similarly, for TGFβR3 expression and ChIP data, there was some variation between the different EBNA3A KO cell lines (figs. 3.11A-B and 3.12B-D). When all four available EBNA3A KO lines were investigated for TGFβR3 expression, they were generally seen to fall into two distinct groups: EBNA3A KO 1 and KO 3 showed low levels of TGFβR3, behaving like uninfected BL31, whereas EBNA3A KO 2 and KO 4 showed up-regulation of TGFβR3 like wild-type EBV-infected BL31 (fig. 4.6B). There was a difference in the way in which these cell lines were originally grown out: EBNA3A KOs 1 and 3 were grown out initially in medium supplemented with sodium pyruvate and alpha-thioglycerol, whereas KOs 2 and 4 were grown out without these and took a long time to grow out [Rob White, personal communication and (White et al. 2010)]. Similar clustering into these two distinct groups was noted for many genes in the original microarray [www.epstein-barrvirus.org.uk and (White et al. 2010)]. However, this was not the case for TGFβR3 in the microarray, in which TGFβR3 was up-regulated in all four EBNA3A KO lines, suggesting that EBNA3A was not necessary (fig. 4.6D). The findings for TGFβR2 were also generally different from those in the microarray, since in the microarray TGFβR2 expression did cluster into two separate groups, with TGFβR2 being fully repressed in two cell lines and partially de-repressed in the other two (fig. 4.6C).

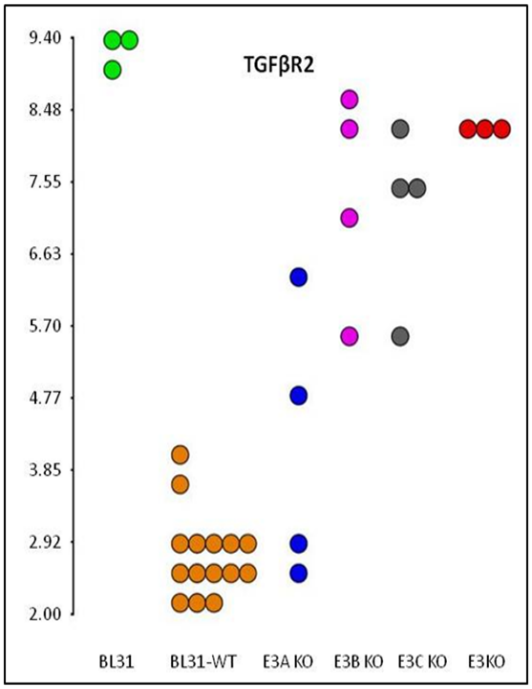
**A**


TGFβR2/GNB2L1
30
25
20
15
10
5
0
BL31
BL31 WT
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A KO 3
BL31 EBNA3A KO 4
BL31 EBNA3A rev

**B**


TGFβR3/GNB2L1
3
2.5
2
1.5
1
0.5
0
BL31
BL31 WT
BL31 EBNA3A KO 1
BL31 EBNA3A KO 2
BL31 EBNA3A KO 3
BL31 EBNA3A KO 4
BL31 EBNA3A rev

**C**


TGFβR2
9.40
8.48
7.55
6.63
5.70
4.77
3.85
2.92
2.00
BL31
BL31-WT
E3A KO
E3B KO
E3C KO
E3KO

**D**

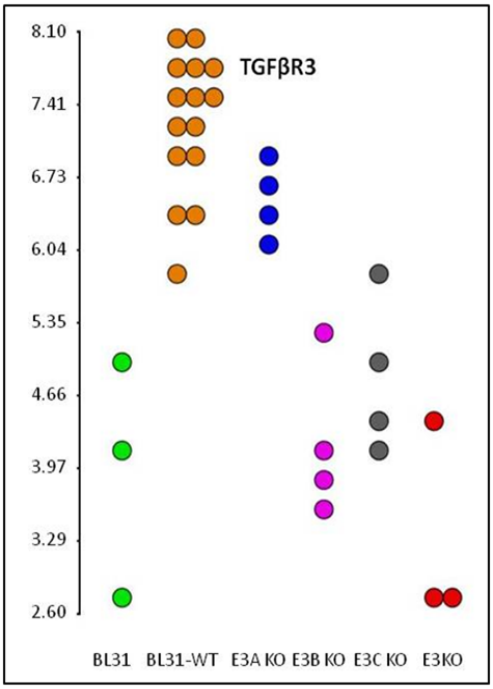

TGFβR3
8.10
7.41
6.73
6.04
5.35
4.66
3.97
3.29
2.60
BL31
BL31-WT
E3A KO
E3B KO
E3C KO
E3KO

**Figure 4.6: The expression of TGFβR2 and TGFβR3 varies markedly between different BL31 EBNA3A KO cell lines**

qRT-PCR for **(A)** TGFβR2 and **(B)** and TGFβR3 in the panel of EBNA3A KO BL31 cell lines, with BL31, BL31-WT and EBNA3A revertant. Values are expressed as ratios to the endogenous control gene *GNB2L1*, with error bars representing standard deviation of triplicate qPCR reactions. Data shown are from an experiment in which the pattern of variation of values for uninfected, knockout and wild-type/revertant infected cell lines is representative of at least three separate experiments. **(C-D)** Microarray expression of **(C)** TGFβR2 and **(D)** TGFβR3 in BL31 cells infected with wild-type (WT, including revertants) and EBNA3A KO, EBNA3B KO, EBNA3C KO and total EBNA3 KO viruses. The vertical axis represents quantity of mRNA on a $\log_2$ scale. A value of <3 generally indicates that a gene is not detectably expressed. Each point represents a single cell line. From [www.epstein-barrvirus.org.uk and (White et al. 2010)].

In order to investigate other possible reasons for the differences in expression between the EBNA3A KO cell lines, it was hypothesised that this may be due to differences in expression of LMP2A, as this had been shown to alter TGFβR2 and TGFβR3 expression. Western blot for LMP2A in the panel of EBNA3A, EBNA3B and EBNA3C KO and revertant cell lines showed marked differences in expression between the different EBNA3A KO cell lines, whereas for the EBNA3B KO and EBNA3C KO lines expression levels were similar to each other and to the revertants and wild-type viruses (fig. 4.7). In particular, EBN3A KO 3 has extremely low levels of LMP2A expression, and EBN3A KO 2 also has reduced levels compared to the other cell lines. However, these findings do not explain the differences in behaviour of the EBNA3A KO lines for either TGFβR2 or TGFβR3 expression.

It was also determined whether differences in TGFβR2 or TGFβR3 expression could be due to differences in proliferation rate at the time of experiments; however, no correlation was identified between the cell count 24 hours prior to harvest and the TGFβR2 expression in a particular experiment (data not shown); nevertheless, it was generally observed that the EBNA3A KO 2 and 4 cell lines, particularly EBNA3A KO 2, proliferated at a significantly slower rate than other cell lines, correlating with the original rate of outgrowth of the cell lines (White et al. 2010). However, it is not clear how the differences in proliferation rate would explain the differences in TGFβR3 expression.

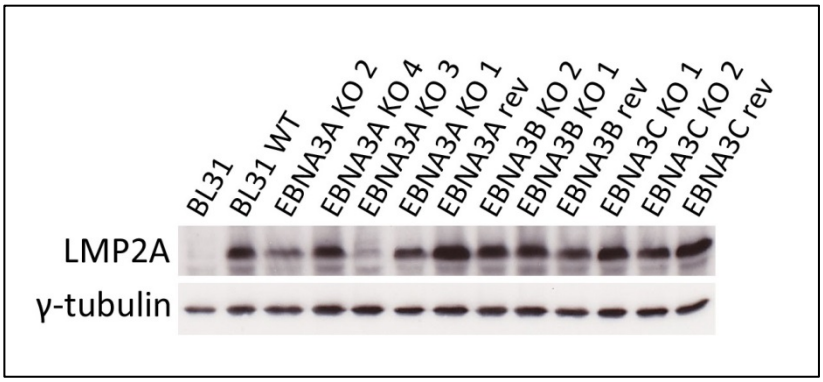



**Figure 4.7: LMP2A expression varies between EBNA3A KO cell lines**

Western blot for LMP2A in a panel of EBNA3A, EBNA3B and EBN3C KO cell lines and their revertants. γ-tubulin is shown as a loading control.

## 4.7 Discussion

### The involvement of LMP1 and LMP2A in repression of TGFβR2

Since the EBNA2 KO cell lines also showed expression of TGFβR2 and restoration of TGFβ signalling (figs. 3.1A-B and 3.7B), this led to an investigation of whether LMP1 and/or LMP2A might be responsible, since EBNA2 drives the expression of LMP1 and LMP2A. To explore their role, LMP1 KO and LMP2A KO viruses were used to infect EBV-negative BL31 cells, thus establishing new BL31 LMP1 KO and BL31 LMP2A KO cell lines. Investigation of the effects of LMP1 and LMP2A deletion suggested that both LMP1 and LMP2A are necessary for the repression of TGFβR2 and suppression of TGFβ signalling (figs. 4.3A-C), although an effect of EBNA2 itself also could not be entirely excluded.

LMP1 and LMP2A are both membrane receptors and thus do not have direct transcription factor activity. In epithelial cells LMP1 and LMP2A cooperate to regulate microRNA expression (Du et al. 2011), and synergistic cooperation between LMP1 and LMP2A has also been shown to promote carcinogenesis in a transgenic mouse model (Shair et al. 2012). In addition, LMP2A has been shown to augment signalling by LMP1 indirectly in epithelial cells, by reducing the turnover of LMP1 (Dawson et al. 2001).

However, the two proteins can also sometimes have non-synergistic effects in B cells. LMP1 requires co-expression of LMP2A for NFκB activation in B cells, suggesting that LMP1 is dependent on the presence of LMP2A for its function, rather than the two working synergistically (Guasparri et al. 2008). In a transgenic mouse model, LMP1 alone induced expression of TRAF2 (TNF-receptor associated factor 2) in B cells, but when LMP2A was co-expressed with LMP1 this induction was no longer seen. It is thought therefore that LMP2A modulates the hyperproliferation of B cells caused by LMP1, as TRAF2 mediates cell proliferation via NFκB activation (Vrazo et al. 2012).

In B cells, LMP1 and LMP2A both alter several signalling pathways and thus can alter gene transcription indirectly. A microarray analysis of splenic CD19 positive B cells in transgenic mice expressing LMP1, LMP2A or both showed that LMP2A expression regulated 3808 B cell genes, LMP1 alone regulated 1660 genes and co-expression of LMP1 and LMP2A regulated

1656 genes. Of genes regulated by co-expression of LMP1 and LMP2A, 30% were uniquely regulated by the co-expression of LMP1 and LMP2A (Shair and Raab-Traub 2012).

In the transgenic mouse model microarray analysis, components of the TGFβ signalling pathway were altered by LMP2A expression (39 genes within the pathway) and by LMP1/2A expression (18 genes within the pathway) but none by LMP1 alone. Although all the data for these genes is not available, there is a suggestion that LMP2A expression alone down-regulated TGFβR2 in this system (Shair and Raab-Traub 2012), i.e. that LMP2A alone may be sufficient to down-regulate TGFβR2, but the addition of LMP1 did not alter this. However, in the current study expression of both LMP1 and LMP2A appears to contribute to the repression of TGFβR2 - although because of the variations in expression of the other latent proteins in the knockout cell lines it is not possible to be entirely sure of the contribution of each. Ideally, to determine which of EBNA2, LMP1 or LMP2A are necessary for repression of TGFβR2, cell lines would be needed which express full amounts of every latent protein but none of the protein of interest, i.e. LMP1+/LMP2A+/EBNA2-, EBNA2+ LMP1+ LMP2A- and EBNA2+/LMP2A+/LMP1-. However, in the current study only one of these combinations was available, namely LMP1 KO 2 which had full expression of EBNA2 and LMP2A; all the other EBNA2/LMP1/LMP2A KO lines had reduced expression of an additional latent protein to that which was knocked out (see table 4.1). The reasons for lack of/reduced expression of the additional latent proteins in the knockouts will be discussed further below.

In summary, EBNA3B, EBNA3C, LMP1 and LMP2A cooperate to up-regulate TGFβR3, as well as down-regulate TGFβR2, although the contribution of LMP1 appears to be less than that of LMP2A.

**LMP1 expression in the EBNA2 KO and LMP2A KO lines**

The initial finding that EBNA2 KO cell lines also showed de-repression of TGFβR2 led to the hypothesis that the changes may have been due to LMP1 or LMP2A, since EBNA2 generally transactivates LMP1, LMP2A and LMP2B. Prior to that the findings were confirmed by investigating all three BL31 EBNA2 KO lines. In all three, TGFβR2 was expressed and TGFβ signalling restored. However, investigation of the expression of LMP1 in these cell lines surprisingly showed that EBNA2 KO (G) had full expression of LMP1 at both RNA and protein

levels (fig. 4.4B-C). Of the other two lines, EBNA2 KO 1 also had a low level of LMP1 expression, but EBNA2 KO 2 did not (figs. 4.4A-C).

When the BL31 EBNA2 KO (G) cell line was first established, it was confirmed that it did not express any EBNA2 or LMP1 protein (Kelly et al. 2005); yet in the experiments performed in the current study, LMP1 is expressed at a significant level (fig. 4.4B-C). This suggests that over time, with repeated passage *in vitro*, the BL31 EBNA2 KO (G) cell line has acquired expression of LMP1 – and this in turn suggests that LMP1 expression may provide a growth advantage. LMP1 functions in multiple ways to protect cells from apoptosis (Henderson et al. 1991, Faqing et al. 2005, Kim et al. 2012), as well as to protect cells from ligands which inhibit proliferation (Takanashi et al. 1999, Lo et al. 2010, De Leo et al. 2011). Thus the acquisition of LMP1 expression in a sub-clone would provide a growth advantage in culture.
During these experiments it was noticeable that the EBNA2 KO cell lines generally proliferated more rapidly than wild-type or other knockout cell lines. However, there was no obvious difference between the growth rates of the particular EBNA2 KO lines, although this was not formally investigated.

In type III latency transcription of LMP1 is under control of EBNA2, as EBNA2 regulates the viral LMP1 promoter (Wang et al. 1990b). However, in type II latency EBNA2 is not present, so transcription of LMP1 must occur via an alternative mechanism. In a reporter assay system, CCAAT enhancer binding protein (C/EBP) was shown to activate the LMP1 promoter independently of EBNA2 (Noda et al. 2011). In B cells, LMP1 can be induced in the absence of EBNA2 by treatment with IL-4, IL-10, IL-13 or CD40 ligand (Kis et al. 2005, Kis et al. 2006, Kis et al. 2011). In addition, it has been shown that exposure to IL-21 induces LMP1, without EBNA2 induction, in B cells (Kis et al. 2010).

In the presence of EBNA2 (i.e. in latency III), EBNA3C can enhance or inhibit the activation of the LMP1 promoter by EBNA2, depending on the cell type and growth conditions (Marshall and Sample 1995, Lin et al. 2002). EBNA3C maintains the level of LMP1 (Allday and Farrell 1994) and binds to and regulates the bidirectional LMP1/LMP2B promoter (Jimenez-Ramirez et al. 2006). Thus in EBNA2 KO lines, EBNA3C could drive expression of LMP1 in the absence of EBNA2. However, the major caveat is that all the EBNA3C experiments were based on

transient reporter assays and in KO and conditional lines, EBNA3C does not appear to alter LMP1 expression.

It is not clear exactly how BL31 EBNA2 KO (G) would have acquired an initial mutation/epigenetic lesion enabling it to express LMP1. It would be interesting to sequence the LMP1 region in the BL31 EBNA2 KO (G) line, as used in the current study, in order to ascertain whether and how the sequence differs from that of the original cell line (Kelly et al. 2005).

The two BL31 LMP2A KO cell lines, LMP2A KO 1 and LMP2A KO 2, were also unexpectedly found to express no, or extremely low LMP1, respectively at both mRNA and protein levels (fig. 4.2A,C-D). The reason for this was not clear. However, in a recent study investigating the effects of LMP2A and LMP2B on early EBV infection of primary B cells, using the same LMP2A KO virus construct as in the current study, LMP1 expression was reduced with viruses lacking LMP2A alone, or lacking both LMP2A and LMP2B, compared to wild-type virus in the first six days after infection (particularly for the double LMP2A/2B knockout virus), although this difference was not statistically significant. When primary B cells were infected with the viruses in the presence of soluble Ig as a BCR stimulating agent, the expression of LMP1 mRNA was significantly reduced in the LMP2A KO, and even more markedly in the LMP2A/B KO, compared to wild-type virus. This was despite similar LMP1 mRNA expression in wild-type, LMP2A KO and LMP2A/B KO cell lines in the BAC-containing HEK293 cells (Wasil et al. 2013). Nevertheless, the reason for this is unclear.

# Chapter 5 The effects of latent EBV on TGFβ-induced apoptosis and cell cycle arrest in BL31 cells

## 5.1 Introduction

TGFβ1 generally induces apoptosis or cell cycle arrest in B cells, and several studies have shown that latency III EBV protects against these effects. However, the exact mechanism of this is not fully understood, in particular whether TGFβ-mediated apoptosis or arrest occurs via TGFβR2 and pSMAD2. TGFβ has been shown to induce cell cycle arrest in EBV-negative BL (Blomhoff et al. 1987, Smeland et al. 1987, Wang et al. 1988, Arvanitakis et al. 1995, Chaouchi et al. 1995, MacDonald et al. 1996, Inman and Allday 2000b). This has also been demonstrated in some group I EBV-positive BLs, which express only EBNA1 (MacDonald et al. 1996, Inman and Allday 2000b). However, EBV-positive BL lines are resistant to TGFβ-induced growth arrest (Blomhoff et al. 1987, Altiok et al. 1991, Kumar et al. 1991, Arvanitakis et al. 1995, MacDonald et al. 1996, Inman and Allday 2000b).

In epithelial cells, TGFβ-induced apoptosis is dependent on or enhanced by SMAD3 (Yanagisawa et al. 1998, Kim et al. 2002, Yang et al. 2002). Although the importance of phosphorylated SMAD3 for TGFβ-induced apoptosis had also been shown in murine B cells (Wildey et al. 2003), at the start of this study the importance of SMAD3 relative to SMAD2 for apoptosis in human B cells was not known. Furthermore, many cellular pathways are involved in regulation of apoptosis and growth arrest, and thus TGFβ may induce these effects via non-canonical pathways or by cross-talk with other signalling pathways.

Latent EBV infection protects against apoptosis induced by many other agents [reviewed in (Allday 2009, Spender and Inman 2011)]. For example, EBV prevents apoptosis in response to ionomycin or staurosporine by blocking the accumulation of pro-apoptotic NOXA (Yee et al. 2011), EBNA3A and EBNA3C cooperate to repress pro-apoptotic BIM (Anderton et al. 2008, Paschos et al. 2009), and expression of LMP1 and LMP2A are protective against apoptosis, via activation of NFκB, PI3K/Akt and ERK/MAPK pathways (Henderson et al. 1991, Portis and Longnecker 2004, Mancao and Hammerschmidt 2007). Therefore, the effect of

EBV on TGFβ-induced apoptosis/arrest may not occur directly via alterations in canonical TGFβ signalling.

In this chapter the effect of latent EBV on TGFβ-induced apoptosis and/or growth arrest was investigated in BL31 cells. The effect of deletion of individual latent proteins was investigated, in order to determine whether the repression of TGFβR2 and suppression of signalling via pSMAD2 was associated with the protection against TGFβ-induced apoptosis or growth arrest.

**5.2 Latent EBV protects against TGFβ-induced apoptosis in BL31 cells**

TGFβ is generally pro-apoptotic and/or anti-proliferative in B cells, although the effect varies depending on the particular cell line, even among BL-derived cell lines (Inman and Allday 2000b). In addition, some studies have shown that both G1 arrest and apoptosis can occur in the same cell line in response to TGFβ, via distinct mechanisms, with G1 arrest followed by subsequent apoptosis (MacDonald et al. 1996, Schrantz et al. 1999). The effect of TGFβ on BL31 survival was not known.

The presence of latency III EBV expression generally confers resistance to the effects of TGFβ, and therefore it was hypothesised that BL31 cells would undergo either apoptosis or G1 arrest in response to TGFβ, and this would be abrogated by EBV infection. EBV-negative BL31 and B95.8-BAC infected BL31 cells (BL31-WT) were initially treated with TGFβ at a concentration of 5ng/ml or vehicle alone. Samples were then harvested at various times up to 48 hours. Samples were analysed by western blot for total and cleaved poly ADP ribose polymerase (PARP), a measure of apoptosis, as well as propidium iodide (PI) staining analysis by flow cytometry (FC) to show the DNA content.

Treatment of BL31 and BL31-WT with TGFβ or vehicle for 48 hours revealed that TGFβ induced PARP cleavage in uninfected BL31, but in BL31-WT, PARP remained uncleaved and thus no apoptosis occurred (fig. 5.1A). When the samples were analysed by PI staining and FC for DNA content, there was a modest increase in the sub-G1 component in TGFβ-treated compared to untreated BL31 at 48 hours (10.3% versus 4.6%, fig. 5.1B, upper figures),

consistent with apoptosis having occurred. However, this was not as marked as has generally been seen for apoptosis induced by TGFβ1 or other agents in other cell lines [see for instance (Inman and Allday 2000b)]. This may be because cells are dying in all phases of the cell cycle, i.e. PARP cleavage does not correlate entirely with sub-G1 on FC analysis, although a previous study generally found good correlation between these within each particular cell line (Inman and Allday 2000b). In BL31-WT, no difference in the cell cycle profile was seen with or without exogenous TGFβ1 (fig. 5.1B, lower figures). These results together suggest that in BL31 TGFβ induces apoptosis, and that wild-type EBV suppresses apoptosis and enhances survival.

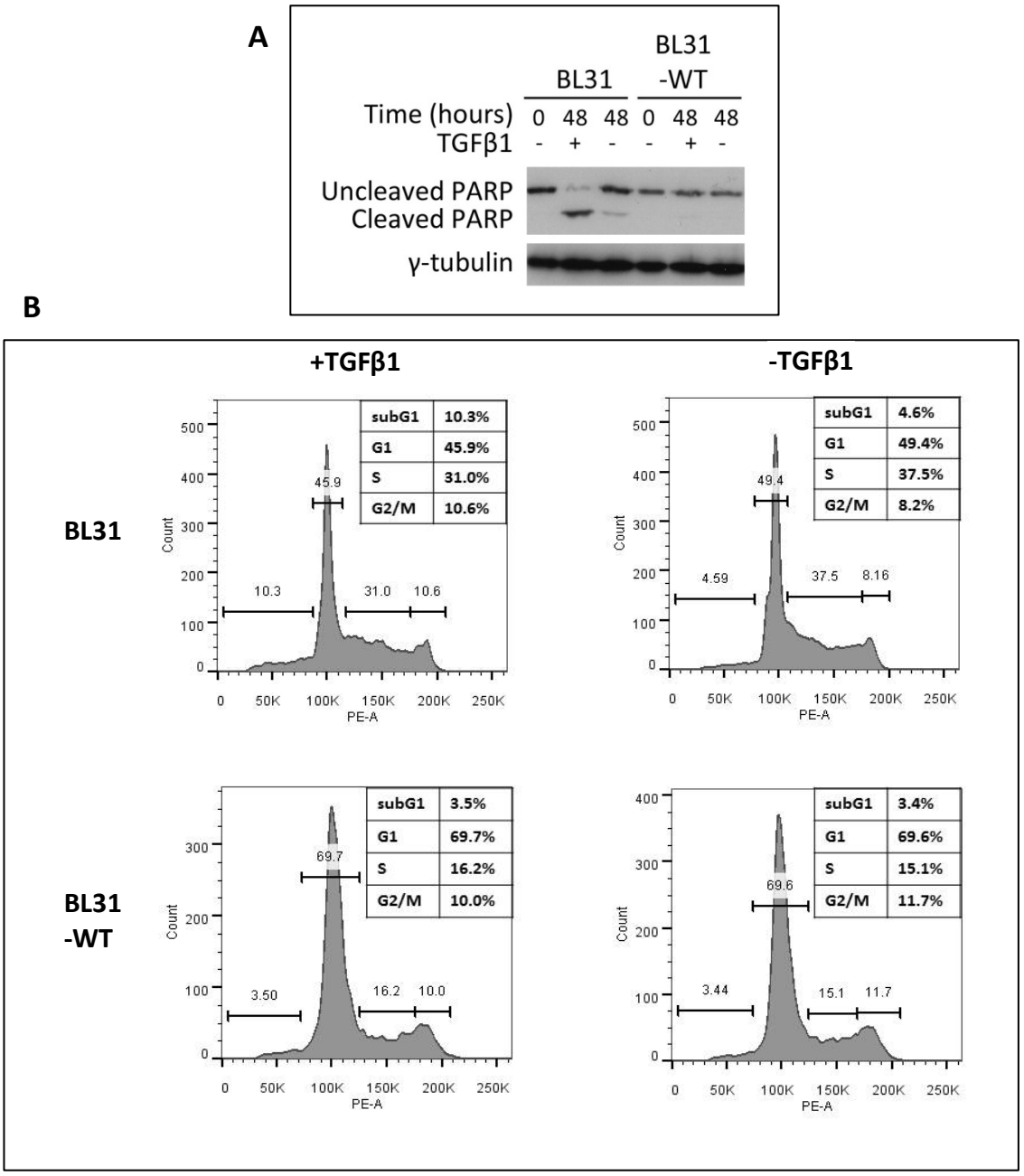



**Figure 5.1: TGFβ1 induces apoptosis in EBV-uninfected BL31 cells, but wild-type EBV protects against this**

**(A)** Western blot for PARP in uninfected and wild-type EBV-infected (BL31-WT) BL31 cells before (time 0) and 48 hours after treatment with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in PBS, -TGFβ1). γ-tubulin is shown as a loading control. **(B)** Cell cycle analysis by propidium iodide staining and FC in uninfected (upper) and wild-type infected (lower) BL31 cells treated with TGFβ1 5ng/ml (left side) or vehicle alone (right side), showing an increased sub-G1 component, consistent with increased apoptosis, with TGFβ1 treatment in BL31 but not BL31-WT.

### 5.3 EBNA3B and EBNA3C are not necessary for protection against TGFβ-induced apoptosis

It was hypothesised that TGFβ-induced apoptosis in BL31 may occur via TGFβR2 and induction of pSMAD2, and therefore that the same latent proteins which cooperate to repress TGFβR2 and suppress TGFβ signalling would protect against TGFβ-induced apoptosis. The effect of EBNA3B and EBNA3C deletion on TGFβ-induced apoptosis were investigated.

The panel of EBNA3B KO and EBNA3C KO, as well as respective revertants to wild-type, BL31 cell lines were treated with TGFβ or vehicle for 48 hours and samples harvested, as before, for western blot for PARP and for PI-stained FC analysis. This showed that there was no PARP cleavage in EBNA3B KO or EBNA3C KO, as seen in the revertants, whereas increased PARP cleavage was again seen in uninfected BL31 treated with TGFβ1 compared to vehicle, consistent with TGFβ-induced apoptosis (fig. 5.2A). Analysis of the same cell lines by PI-stained FC showed no difference in the cell cycle profile between TGFβ or vehicle treatment in EBNA3B or EBNA3C KOs (fig. 5.2B). These findings suggest that, although EBNA3B and EBNA3C are necessary for the repression of TGFβR2 and suppression of signalling via pSMAD2, they are not necessary for protection against TGFβ-induced apoptosis in BL31 cells. In addition, TGFβ does not induce G1 arrest in EBNA3B or EBNA3C KO BL31 cells.

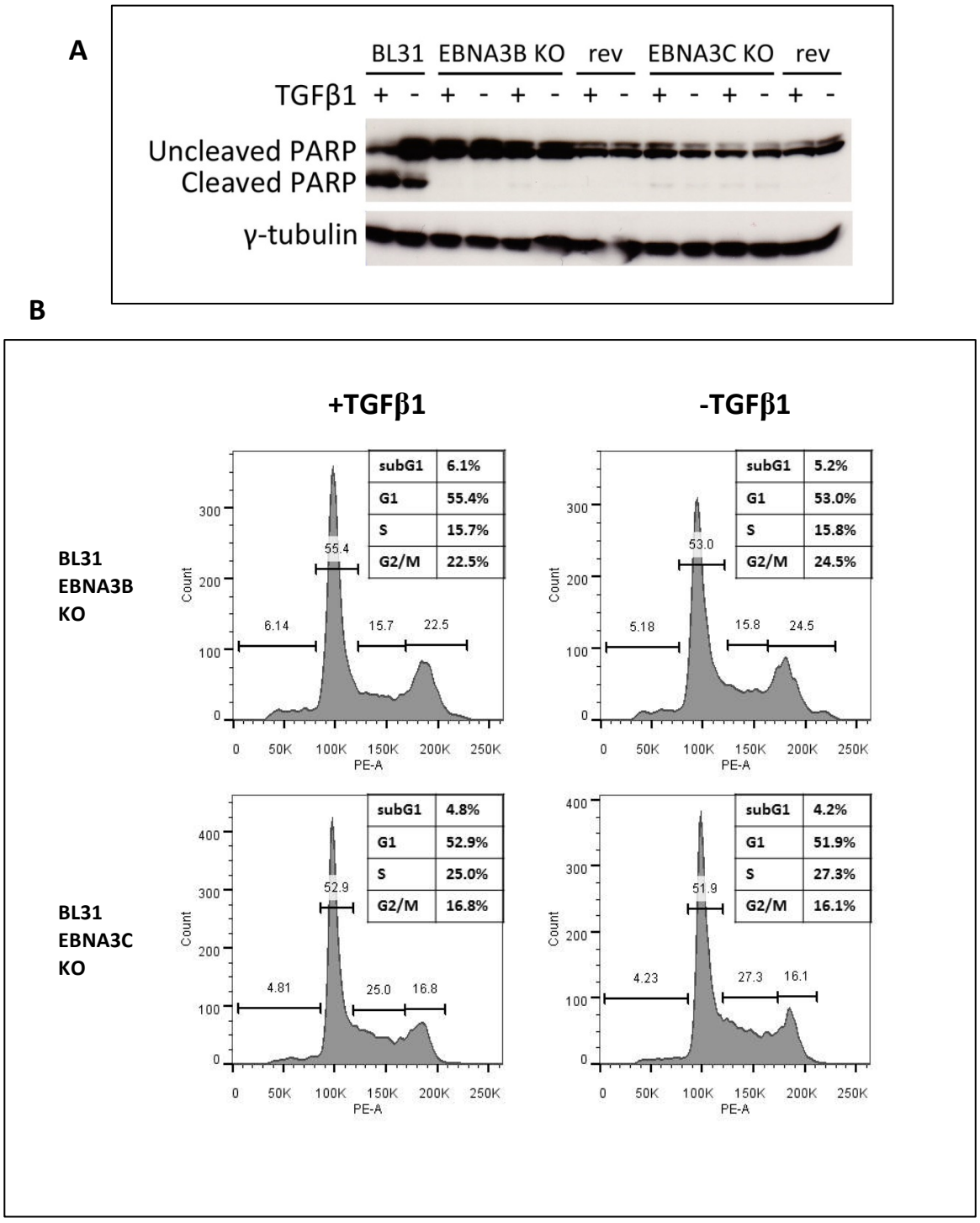



**Figure 5.2: TGFβ1 does not induce apoptosis or G1 arrest in EBNA3B KO or EBNA3C KO BL31 cells**

**(A)** Western blot for PARP in the cell lines shown after 48 hours' treatment with TGFβ1 5ng/ml or vehicle. γ-tubulin is shown as a loading control. **(B)** Cells were treated with TGFβ1 5ng/ml or vehicle for 48 hours and then samples harvested, stained with propidium iodide and analysed by FC. This experiment was repeated three times in two different EBNA3B KO and EBNA3C KO BL31 cell lines as well as revertants, with representative data shown here.

### 5.4 In EBNA2 KO BL31 cells, TGFβ induces growth arrest but not apoptosis

The effects of EBNA2 KO on TGFβ-induced apoptosis/growth arrest were assessed initially at time points up to 48 hours, using the BL31 EBNA2 KO (G) cell line. This showed that in EBNA2 KO (G), no increased PARP cleavage occurred with TGFβ1 treatment at any time point (fig. 5.3). This lack of TGFβ-induced apoptosis in EBNA2 KO cells occurred despite restoration of signalling via pSMAD2 (see fig 3.7B). This suggests that EBNA2 is not necessary for the resistance to TGFβ-induced apoptosis, even though it is necessary for TGFβ signalling via pSMAD2.

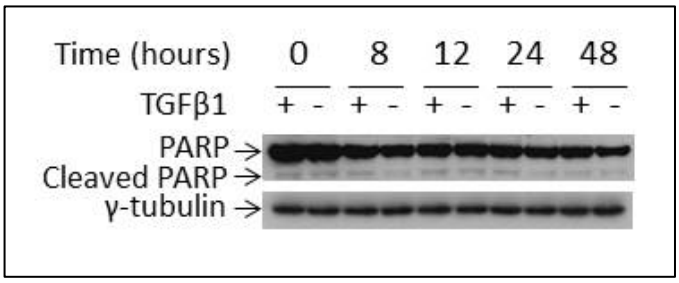
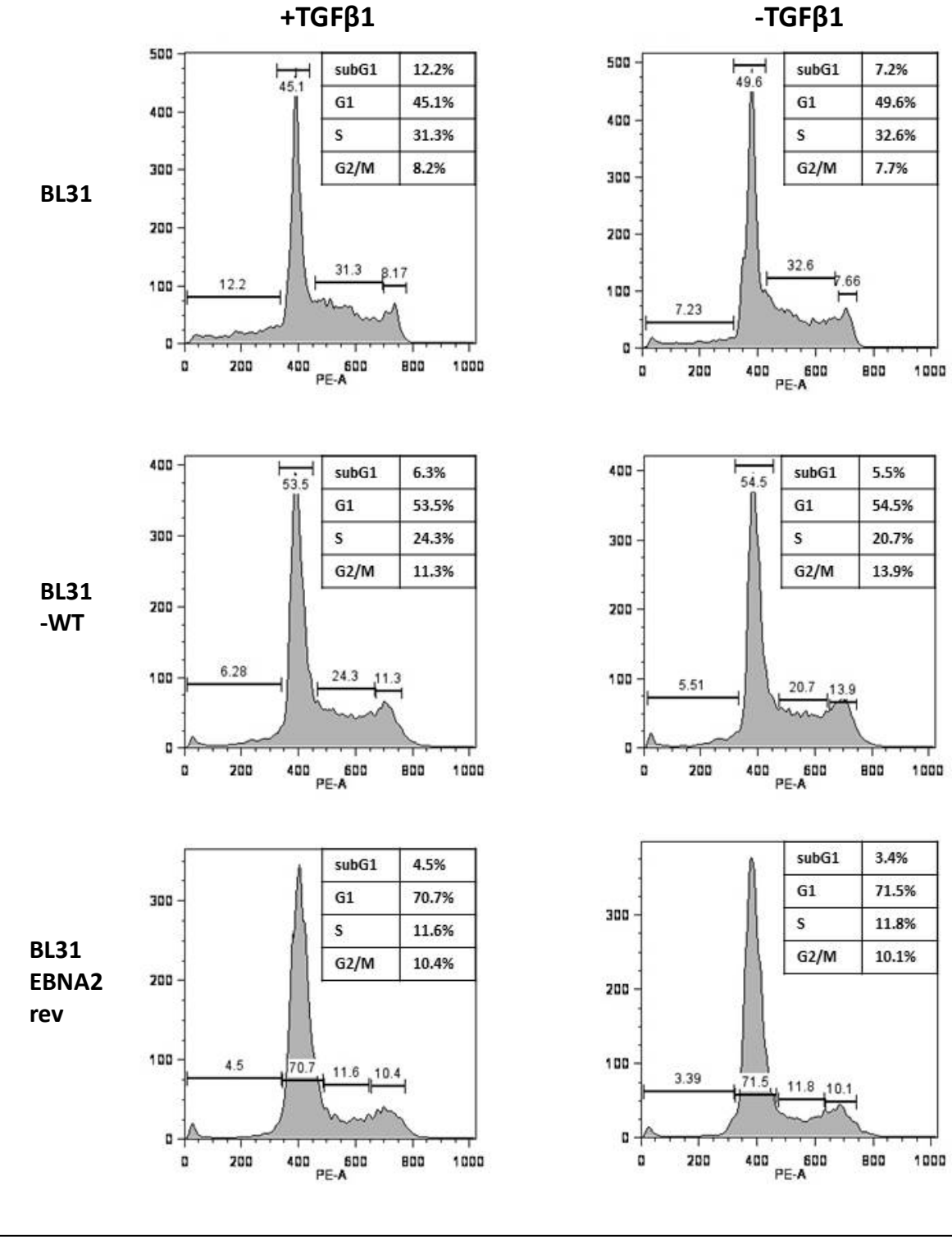



**Figure 5.3: TGFβ1 does not induce PARP cleavage in BL31 EBNA2 KO (G) cells with up to 48 hours treatment**
Western blot for PARP in BL31 EBNA2 KO (G) cells at the times shown after treatment with TGFβ1 5ng/ml or vehicle alone (2mg/ml BSA in PBS). γ-tubulin is shown as a loading control.

The same samples were analysed by PI FC, along with wild-type infected and uninfected BL31 cells. Surprisingly, this showed that TGFβ1 induced a partial G1 arrest, rather than apoptosis, compared to those treated with vehicle alone, in EBNA2 KO cells. After these experiments were first done, a colleague in the laboratory had established two new BL31 EBNA2 KO cell lines, here named EBNA2 KO 1 and EBNA2 KO 2, as well as an EBNA2 revertant (Ian Groves, unpublished). In order to confirm the findings seen in BL31 EBNA2 KO (G), the newly established cell lines were investigated alongside it by treatment with TGFβ1 for 48 hours with similar analysis. This showed that TGFβ induced G1 arrest in all three BL31 EBNA2 KO lines (fig. 5.4B). No difference in cell cycle profile was seen with TGFβ1 compared to vehicle alone in the EBNA2 revertant, as in BL31-WT, and in uninfected BL31 an increase in sub-G1, consistent with apoptosis, was again seen (fig. 5.4A). No significant increase in the sub-G1 component occurred with TGFβ1 in any of the EBNA2 KO lines (fig. 5.4B), suggesting that TGFβ1 did not induce apoptosis in EBNA2 KO, as shown for EBNA2 KO (G) by PARP cleavage (fig. 5.3). Therefore, the effect of TGFβ in EBNA2 KO cells was different to

either wild-type EBV-infected or uninfected BL31 cells, with TGFβ inducing a partial G1 arrest but not apoptosis. This suggests that EBNA2 may be necessary for the cells to proceed through a G1 checkpoint in this cell context.

**A**


+TGFβ1
-TGFβ1
BL31
BL31 -WT
BL31 EBNA2 rev
subG1
G1
S
G2/M
12.2%
45.1%
31.3%
8.2%
7.2%
49.6%
32.6%
7.7%
6.3%
53.5%
24.3%
11.3%
5.5%
54.5%
20.7%
13.9%
4.5%
70.7%
11.6%
10.4%
3.4%
71.5%
11.8%
10.1%
PE-A

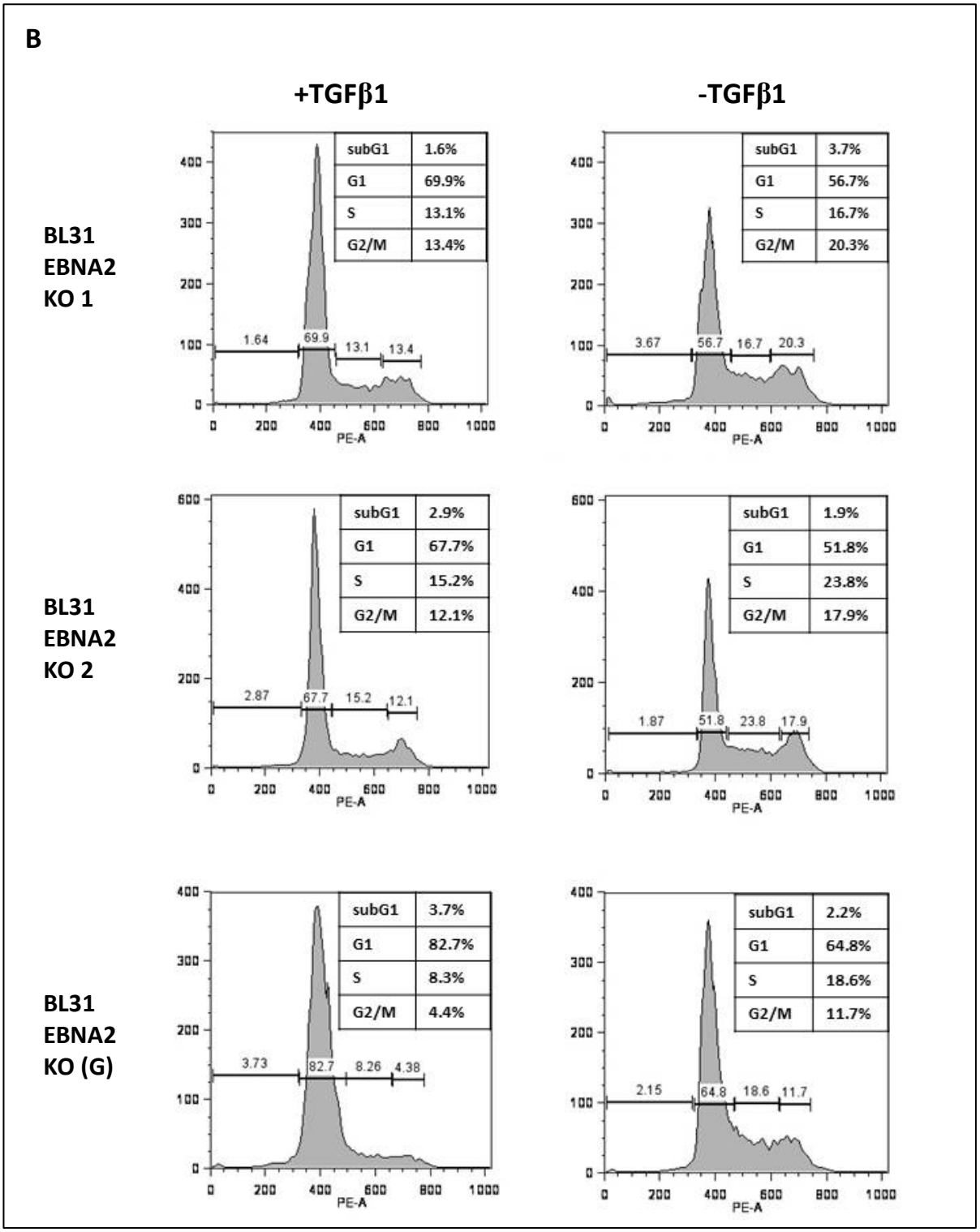



**Figure 5.4: TGFβ1 induces G1 arrest in EBNA2 KO BL31 cell lines**

FC analysis for DNA content in **(A)** BL31, BL31-WT, EBNA2 revertant and **(B)** three EBNA2 KO cell lines. Cell numbers are shown on the y axis and PI staining for DNA content on the x axis. Cells were treated with TGFβ1 5ng/ml (left) or vehicle (right) and harvested after 48 hours for PI staining and FC analysis. The percentages of cells in each phase of the cell cycle (sub-G1, G1, S and G2/M from left to right in each histogram) are indicated. This is representative data from experiments performed three times.

**5.5 Resistance to PARP cleavage varies with LMP1 expression in EBNA2 KO cell lines**

As shown in fig. 5.4, the effect of TGFβ treatment was investigated in three different BL31 EBNA2 KO lines. All three EBNA2 KO cell lines showed some growth arrest induced by TGFβ1 after PI staining and FC analysis (fig. 5.4B). However, the initial time course for PARP cleavage had only been done in the EBNA2 KO (G) cell line, showing no PARP cleavage at any time point (fig. 5.3). In order to confirm this lack of PARP cleavage in the other EBNA2 KO lines, western blots were performed for PARP after 48 hours' treatment with TGFβ1 or vehicle using extracts from all three EBNA2 KO lines. This showed some differences between the cell lines, with moderately increased PARP cleavage with TGFβ1 compared to vehicle in the two recently established BL31 EBNA2 KO cell lines, although the degree of PARP cleavage remained markedly less than for uninfected BL31 cells (fig. 5.5). Again no PARP cleavage was seen in the longer established EBNA2 KO line, EBNA2 KO (G). In this experiment significant PARP cleavage was seen in BL31 cells even after treatment with vehicle alone, suggesting a high baseline level of apoptosis in these cells. Although a small amount of PARP cleavage was also seen in BL31-WT, this was similar with and without TGFβ1 treatment, again indicating some baseline apoptosis occurring in these cells, but nevertheless suggesting that EBV protects against apoptosis specifically induced by TGFβ1.

EBNA2 can transactivate LMP1 and LMP2A, and thus EBNA2 KO cell lines would be expected to have a marked reduction in expression of these latent proteins. Several previous studies have investigated the effect of LMP1 on TGFβ-induced growth inhibition and apoptosis in B cells, with some studies suggesting that LMP1 is necessary for resistance to TGFβ-induced growth inhibition (Arvanitakis et al. 1995) and apoptosis (Arvanitakis et al. 1995, Kenney et al. 2001) but others suggesting it is neither necessary nor sufficient (Inman and Allday 2000b).

Therefore, it was hypothesised that variable LMP1 expression in the EBNA2 KO lines might result in differential amounts of TGFβ-induced apoptosis. Hence, the LMP1 expression in these cell lines was investigated by performing western blot (fig. 5.5). This showed that LMP1 expression varied between the three EBNA2 KO lines, in particular that EBNA2 KO (G) surprisingly expressed significant amounts of LMP1. Moreover, there appeared to be an inverse correlation between the level of expression of LMP1 and the degree of TGFβ1-

induced PARP cleavage in these three cell lines, with EBNA2 KO (G) having full LMP1 expression and no TGFβ-induced PARP cleavage, EBNA2 KO 1 having an intermediate level of both, and EBNA2 KO 2 having no detectable LMP1 expression and also the most TGFβ-induced PARP cleavage of the three cell lines (fig. 5.5). However, it was observed that even in EBNA2 KO 2, the degree of PARP cleavage was much less than for uninfected BL31. These findings led to the hypothesis that LMP1 may protect against TGFβ-induced apoptosis. LMP1 is known to promote survival by several mechanisms, including up-regulation of BCL2, induction of c-FLIP and inhibition of BAX (Henderson et al. 1991, Grimm et al. 2005, Allday 2009, Spender and Inman 2011). Taken together with the previous findings in section 5.4, it was hypothesised that LMP1 protects against TGFβ-induced apoptosis whereas EBNA2, or possibly LMP2A since this is also transactivated by EBNA2, protects against TGFβ-induced growth arrest in BL31 cells.

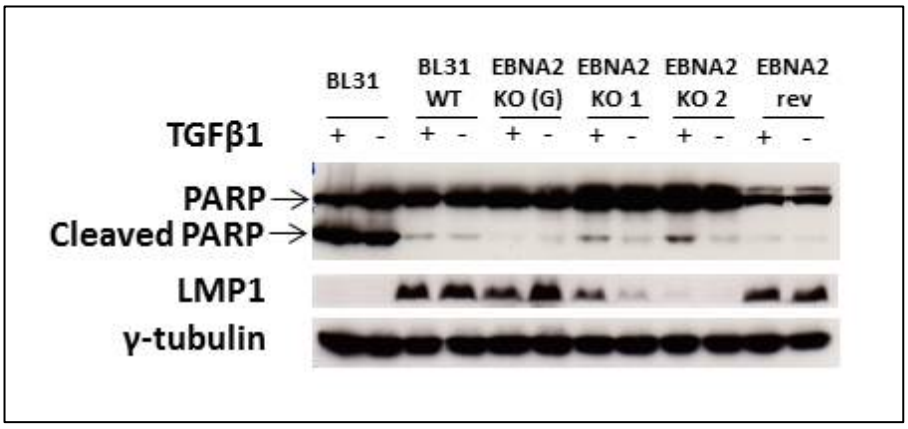



**Figure 5.5: TGFβ-induced PARP cleavage varies with LMP1 expression in EBNA2 KO BL31 cell lines**

Western blot for PARP and LMP1 expression after 48 hours' treatment with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in PBS) in BL31, wild-type EBV-infected, three different EBNA2 KO and the EBNA2 revertant BL31 cell lines. γ-tubulin was used as a loading control.

### 5.6 LMP2A is necessary for protection against TGFβ-induced growth arrest

As described earlier in section 5.4, the EBNA2 KO lines all showed TGFβ-induced G1 arrest (fig. 5.4B), but varied in their response in terms of PARP cleavage, with the cell line with more LMP1 apparently being protected from TGFβ-induced PARP cleavage (fig. 5.5), leading to the hypothesis that LMP1 may protect against TGFβ-induced apoptosis. In addition, since all EBNA2 KO lines showed G1 arrest with TGFβ, regardless of their LMP1 expression, this

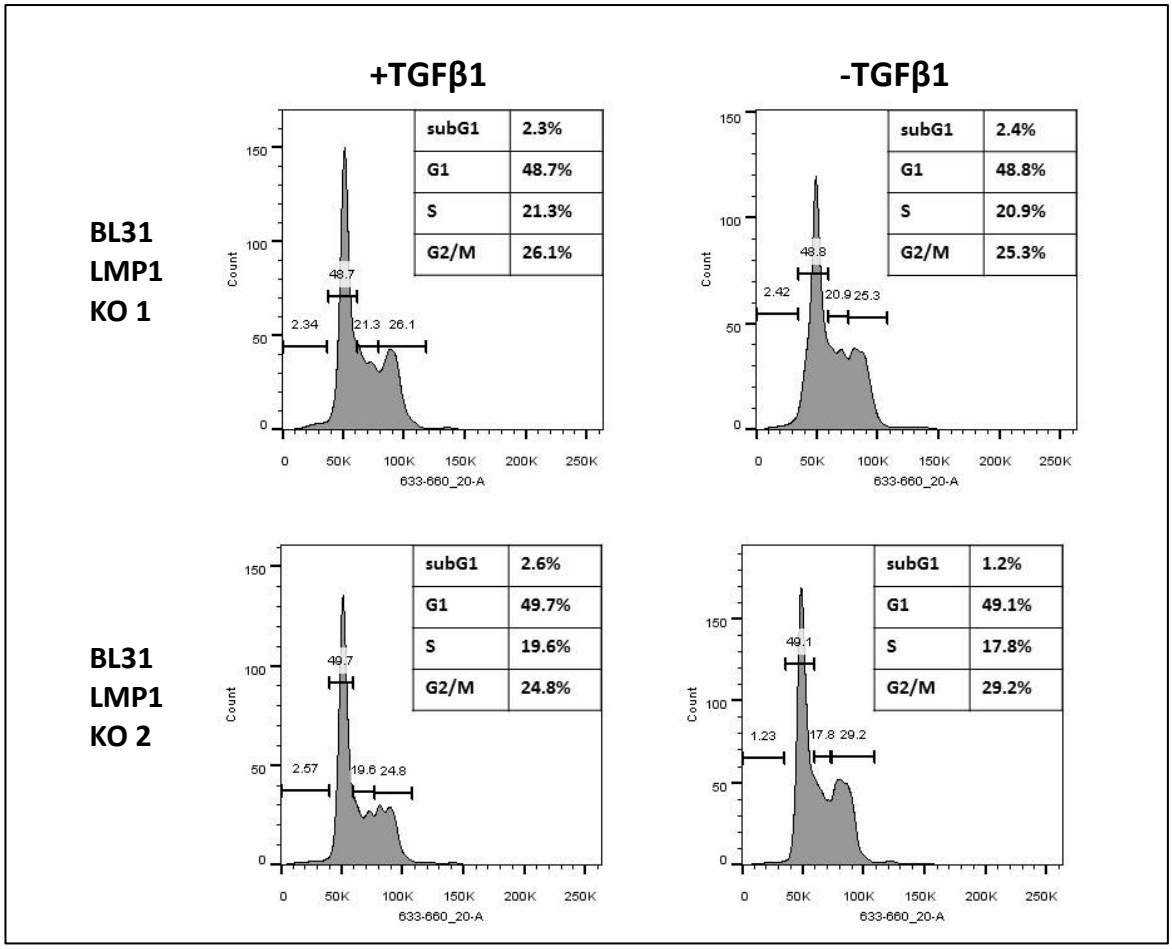
suggested that EBNA2, or possibly LMP2A, may be necessary for the cells to proceed through a G1 checkpoint.

Therefore the LMP1 KO and LMP2A KO BL31 cell lines were treated for 48 hours with TGFβ or vehicle, with samples then harvested for protein extraction, SDS-PAGE and western blot for PARP, as well as for cell cycle analysis by FC, in this case using Draq5 as the DNA stain. FC analysis showed that there was no difference in cell cycle profile between TGFβ treated and untreated for both LMP1 KO lines (fig. 5.6A). Western blot also showed no PARP cleavage with TGFβ in either of the LMP1 KO lines (fig. 5.6B). These findings suggest that LMP1 is not necessary for protection against TGFβ-induced apoptosis.

Both LMP2A KO lines, however, showed G1 arrest with TGFβ (fig. 5.7A), similar to that seen in EBNA2 KO lines (fig. 5.4B). There was no increase in the sub-G1 component with TGFβ and again no increase in PARP cleavage with TGFβ in the LMP2A KO lines (figs. 5.7A-B). These findings suggest that LMP2A is necessary for cells to proceed through the G1 checkpoint, but is also not necessary for protection against TGFβ-induced apoptosis.

The findings for PARP cleavage in the three different EBNA2 KO lines (fig. 5.5) had suggested that LMP1 might protect against TGFβ-induced apoptosis. If that were the case then it would be expected that increased PARP cleavage would be seen with TGFβ in the LMP1 KO lines. However, no increased PARP cleavage was seen in either LMP1 KO line, suggesting that LMP1 alone is not necessary for protection against TGFβ-induced apoptosis. Nevertheless, it remains possible that the presence of either LMP1 or EBNA2 are required for protection against TGFβ-induced apoptosis, since increased PARP cleavage was seen in cells lacking both EBNA2 and LMP1 (BL31 EBNA2 KO 2, fig. 5.5) but not in the LMP1 KO lines which are deficient only in LMP1 but not EBNA2 (fig. 5.6A-B).

**A**




**B**

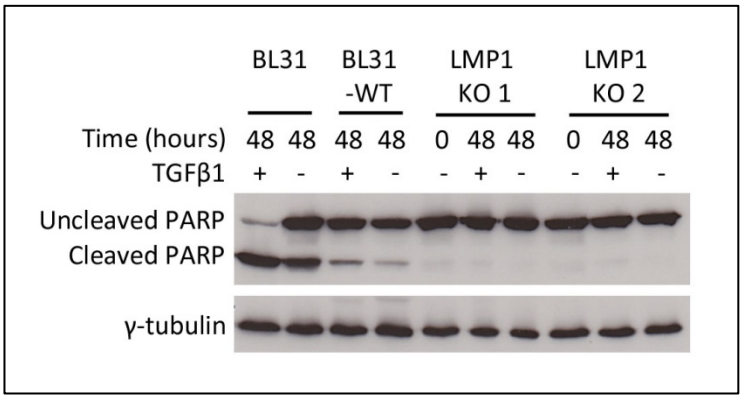
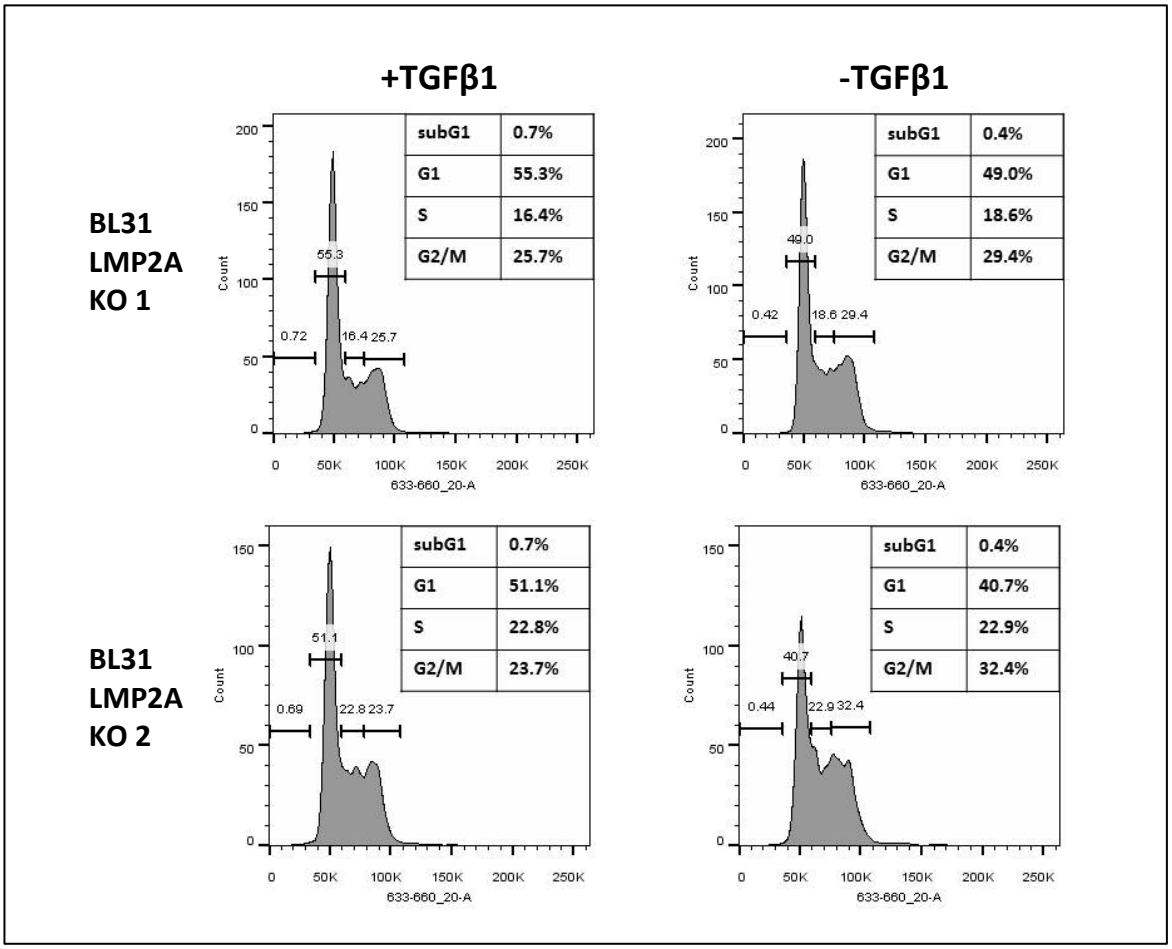



**Figure 5.6: TGFβ1 does not induce apoptosis or G1 arrest in LMP1 KO BL31 cells**

The two LMP1 KO cell lines, along with BL31 and BL31-WT, were treated with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in PBS) for 48 hours followed by harvesting for protein extraction and FC analysis. **(A)** FC for cell cycle DNA content after staining with Draq5 in both BL31 LMP1 KO cell lines treated with TGFβ1 (left side) or vehicle (right side). Percentages of cells in each phase of the cell cycle are shown in tables. **(B)** Western blot for PARP in both LMP1 KO lines with TGFβ or vehicle, with time 0 shown for comparison, as well as BL31 and BL31-WT. γ-tubulin was used as a loading control.

**A**




**B**

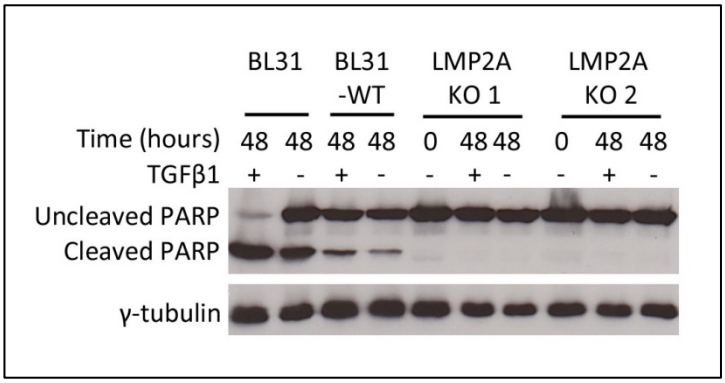



**Figure 5.7: TGFβ1 induces G1 arrest but not apoptosis in LMP2A KO BL31 cells**

The two LMP2A KO cell lines, along with BL31 and BL31-WT, were treated with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in PBS) for 48 hours followed by harvesting for protein extraction and FC analysis. **(A)** FC for cell cycle DNA content after staining with Draq5 in both BL31 LMP2A KO cell lines treated with TGFβ1 (left side) or vehicle (right side). Percentages of cells in each phase of the cell cycle are shown in tables. **(B)** Western blot for PARP in both LMP2A KO lines with TGFβ or vehicle, with time 0 shown for comparison, as well as BL31 and BL31-WT. γ-tubulin was used as a loading control.

In summary, TGFβ appears to induce apoptosis, but not G1 arrest, in EBV-negative BL31 cells, as detected by PARP cleavage (fig. 5.1A). However, as noted previously, the effect of TGFβ1 on the sub-G1 component by FC analysis seemed to be less marked than has been observed for other cell lines by previous investigators [for example (Inman and Allday 2000b)] (fig. 5.1B). The presence of wild-type EBV is protective from TGFβ-induced apoptosis (figs. 5.1A-B).

When the effects of individual latent proteins on this were investigated, firstly this showed that EBNA3B and EBNA3C deletion do not lead to TGFβ-induced apoptosis or growth arrest, hence EBNA3B and EBNA3C are not needed for protection against TGFβ-induced apoptosis or arrest (fig. 5.2A-B). However, they are both necessary for the repression of TGFβR2 and suppression of TGFβ signalling via pSMAD2 (figs. 3.1A and 3.7C-D).

When the effect of EBNA2 deletion was investigated, this showed that TGFβ induced G1 arrest, without any increase in sub-G1, in all three EBNA2 KO cell lines (fig. 5.4B). However, the three cell lines showed differing amounts of PARP cleavage induced by TGFβ, with some increased PARP cleavage in EBNA2 KO 1 and particularly EBNA2 KO 2, but no PARP cleavage in BL31 EBNA2 KO (G) (fig. 5.5). These findings suggest that increased PARP cleavage does not always correlate with an increase in cell cycle sub-G1 component in these cells – as was also suggested by the marked PARP cleavage but less dramatic increase in sub-G1 in TGFβ-treated BL31 cells (figs. 5.1A-B).

In view of these differences between PARP cleavage and cell cycle analysis in BL31, several attempts were made to repeat the FC analysis in BL31 cells. In more recent attempts, it was noted that TGFβ treatment also did not induce PARP cleavage as significantly as previously (fig. 5.8A compared with fig. 5.1A). It was hypothesised that the apparent reduction in TGFβ sensitivity in PARP cleavage may be due to the BL31 cells having developed resistance to TGFβ during continued culture. Therefore a batch of BL31 cells that had been cryopreserved at a similar time to that used for the initial experiments (as seen in fig. 5.1) was re-established from frozen and then investigated in a similar manner alongside the batch that had been more recently tested. However, the original batch of BL31 cells also yielded similar results, i.e. a much less marked increase in PARP cleavage than previously. Additionally in

the more recent experiments, the increase in sub-G1 after TGFβ treatment was even less significant than originally.

In these experiments, even though the batch of TGFβ was different from that used in the initial experiments (but from the same manufacturer), TGFβ still caused strong induction of pSMAD2 in BL31 cells. A fresh batch of TGFβ1 also yielded the same results, suggesting the findings were not due to lack of potency of the particular batch of TGFβ1. In addition, experiments were attempted with a higher dose of TGFβ1 (10ng/ml rather than 5ng/ml) and a 72 hour time point was used. Still, there was generally little difference between treated and untreated BL31 in terms of PARP cleavage or sub-G1 component.

In a further attempt to investigate the effects of TGFβ, BL31 cells were also stained with a live-dead stain prior to FC analysis, since it was hypothesised that TGFβ could be inducing cell death without the characteristic pattern of DNA fragmentation seen in apoptosis. Live-dead staining confirmed an increased proportion of dead cells after treatment of BL31 cells with TGFβ compared to vehicle (fig. 5.8B, upper figures). However, when the same cells (including both live and dead components) were analysed by cell cycle profile, there was little difference in the profiles, in particular no increase in the sub-G1 component (fig. 5.8B, lower figures).

Review of laboratory records showed that the batch of foetal calf serum (FCS) used to supplement media for growth of all cell lines in the laboratory had changed shortly after the initial set of experiments on TGFβ-induced apoptosis were done, fairly early in the course of this project. The second batch of serum had been used for all subsequent experiments. Therefore it seems likely that some factor in the serum rendered cells relatively resistant to the apoptotic effects of TGFβ, whilst maintaining the effects of TGFβ on pSMAD2. TGFβ was still able to cause increased death in the cells (fig. 5.8B); however, since this had not been investigated in the original experiments it is not known whether the newer batch of serum had also altered the amount of TGFβ-induced cell death. These findings therefore suggest that TGFβ-induced death, PARP cleavage and sub-G1 component do not always correlate, at least in BL31 cells.

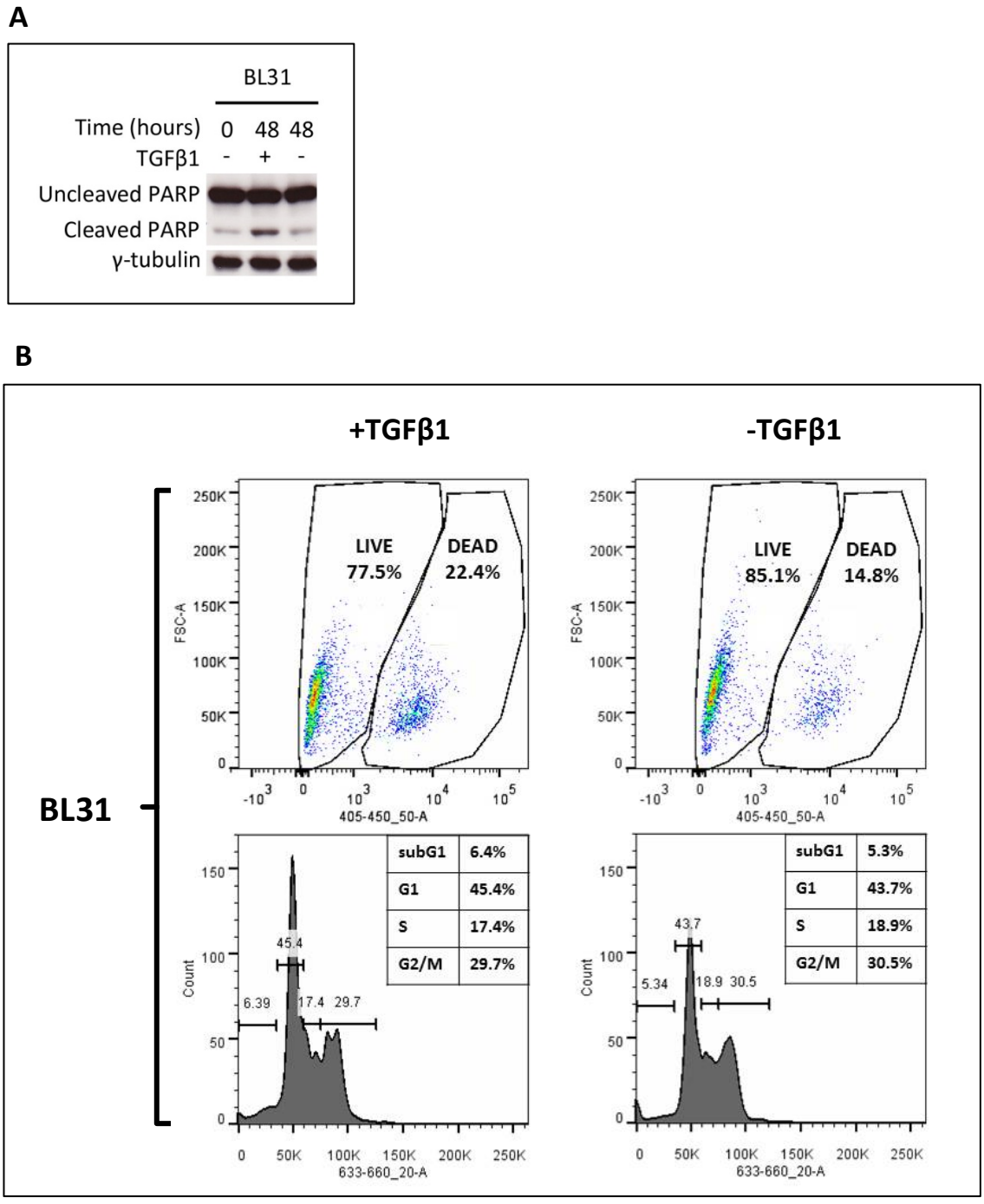



**Figure 5.8: TGFβ-induced PARP cleavage, sub-G1 component and cell death do not correlate in BL31 cells**

**(A)** Western blot for PARP cleavage at time zero, untreated and after 48 hours' treatment with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in PBS) in EBV-negative BL31 cells. γ-tubulin is shown as a loading control. **(B)** BL31 cells were harvested 48 hours after treatment with TGFβ1 (left side) or vehicle (-TGFβ1, right side), stained dually with violet live-dead fixable stain and Draq5, then subjected to flow cytometric analysis, showing proportions of live and dead cells (upper figures) with cell cycle DNA content (lower figures).

## 5.7 Discussion

TGFβ is anti-proliferative and/or promotes apoptosis in most cell types, with the effect varying according to cell type and context. TGFβ can induce both G1 arrest and apoptosis in the same cells (MacDonald et al. 1996, Schrantz et al. 1999). Having shown that EBV down-regulates TGFβR2 leading to suppression of signalling, the effects of EBV latent protein expression on TGFβ responses were investigated in BL31 cells.

### The effects of EBV infection on TGFβ-induced apoptosis

Initial experiments showed that TGFβ induced a very modest level of apoptosis, as shown by PARP cleavage and an increased sub-G1 component revealed by FC analysis, in uninfected BL31 cells (fig. 5.1). BL31 cells infected with wild-type EBV were resistant to TGFβ-induced apoptosis, confirming previous reports that full EBV expression is protective against TGFβ-induced apoptosis in BLs (fig. 5.1) (Inman and Allday 2000b, Inman et al. 2001).

The effects of the individual latent proteins were then investigated, since it was hypothesised that the down-regulation of TGFβR2 may lead to resistance to TGFβ-induced apoptosis. The responses to TGFβ in the different cell lines are summarised in table 5.1.

| Cell line | EBNA2 | LMP1 | LMP2A | EBNA3B | EBNA3C | TGFβ response |
|---|---|---|---|---|---|---|
| BL31 | - | - | - | - | - | Apoptosis |
| BL31-WT | + | + | + | + | + | No effect |
| EBNA3B KO | + | + | + | - | + | No effect |
| EBNA3C KO | + | + | + | + | - | No effect |
| EBNA2 KO (G) | - | + | - | + | + | G1 arrest |
| EBNA2 KO 1/2 | - | - | - | + | + | Arrest + apoptosis |
| LMP1 KO | + | - | + | + | + | No effect |
| LMP2A KO | + | - | - | + | + | G1 arrest |

**Table 5.1: Summary of TGFβ responses in selected BL31 cell lines, indicating expression levels of the relevant EBV latent proteins**

+ = protein expressed, - = expression low or absent.

In EBNA3B KO and EBNA3C KO cells, no increased apoptosis or G1 arrest was seen (fig. 5.2), suggesting that these latent proteins, although necessary for repression of TGFβR2 and suppression of signalling (figs. 3.1A and 3.7C-D), are not necessary for the resistance to TGFβ-induced apoptosis or growth arrest.

TGFβ induced G1 arrest in all three EBNA2 KO lines by PI FC analysis (fig. 5.4C). Western blot for PARP showed some TGFβ-induced PARP cleavage in those cells lacking or having low expression of LMP1, whereas EBNA2 KO (G), which had full expression of LMP1, did not show any PARP cleavage (fig. 5.5). This suggested that LMP1 was required to protect against TGFβ-induced apoptosis. However, when the BL31 LMP1 KO lines were treated with TGFβ, no apoptosis was seen by PARP cleavage or FC (figs. 5.6), despite the fact that TGFβR2 was de-repressed, with restoration of signalling via phosphorylation of SMAD2, in these cell lines (figs. 4.3A-B). Thus the results for the effect of LMP1 on apoptosis initially appeared to be discrepant. However, looking at the summary in table 5.1, it is possible that expression of either LMP1 or EBNA2 is needed for protection against TGFβ-induced apoptosis, since apoptosis occurs when neither are expressed, whereas when either one is expressed TGFβ does not induce apoptosis.

LMP1 promotes protection against apoptosis by multiple mechanisms [reviewed in (Allday 2009, Spender and Inman 2011)], including up-regulation of the anti-apoptotic proteins BCL2, BFL1 and MCL-1 (Henderson et al. 1991, D'Souza et al. 2004, Kim et al. 2012), via activation of NFκB, which is generally pro-survival. LMP1 also inhibits BAX/BAK and induces expression of c-FLIP, which inhibits extrinsic apoptotic pathways. Furthermore, LMP1 up-regulates expression of miR-155, which inhibits pro-apoptotic PUMA. EBNA2, on the other hand, has more limited anti-apoptotic effects, but does induce anti-apoptotic BFL1 (Pegman et al. 2006).

Although the mechanisms of TGFβ-induced apoptosis in B cells are not yet fully understood, those that have been elucidated include several mechanisms which are directly antagonistic to the effects of LMP1. For example, TGFβ up-regulates PUMA, via SMAD3 (Spender et al. 2013), up-regulates BIK, which in turn inhibits the anti-apoptotic protein MCL-1, and represses BCL-$X_L$ (Saltzman et al. 1998, Spender et al. 2009). TGFβ also inhibits NFκB, leading

to apoptosis in B cells (Arsura et al. 1996). TGFβ and LMP1 therefore have opposing effects on NFκB activation. TGFβ also activates BAX/BAK, whereas LMP1 inhibits it. Therefore it would be predicted that LMP1 expression could inhibit TGFβ-induced apoptosis.

LMP2A also has several means of protection against apoptosis, including activation of NFκB, again leading to induction of anti-apoptotic proteins BCL2 and BFL1. LMP2A also inhibits apoptosis via induction of BCL-$X_L$ (Portis and Longnecker 2004, Bultema et al. 2009). In addition, LMP2A inhibits BIM, via MAPK/ERK. However, in the current study, LMP2A expression was not necessary to protect against TGFβ-induced apoptosis (see table 5.1 and figs. 5.7A-B).

In the current study, deletion of individual latent proteins including EBNA3B, EBNA3C, EBNA2, LMP1 and LMP2A did not result in TGFβ-induced apoptosis in any case (see table 5.1). TGFβ-induced apoptosis has been previously shown to occur via SMAD3 in epithelial cells (Yanagisawa et al. 1998, Kim et al. 2002, Yang et al. 2002), a murine B cell line (Wildey et al. 2003) and more recently human BL cell lines (Spender et al. 2013). However, in those cell lines in which TGFβR2 is repressed, it would be predicted that TGFβ would not induce phosphorylation of SMAD3, although this was not investigated in this study due to time constraints; the correlation between TGFβ-induced phosphorylation of pSMAD3 and TGFβ-induced apoptosis in BL31 cells could be investigated in future. Nevertheless, the current findings suggest that TGFβ-induced apoptosis may occur via a non-canonical TGFβ pathway not involving TGFβR2, for example by cross-talk with other intracellular pathways such as PI3K, MAPK, or other Smad-independent TGFβ responses.

Furthermore, EBV blocks apoptosis by multiple mechanisms [reviewed in (Allday 2009, Spender and Inman 2011)], including those involving LMP1, LMP2A and EBNA2 as already described. In addition, EBNA3A and EBNA3C cooperate to repress pro-apoptotic BIM, and EBV inhibits pro-apoptotic NOXA. miR-BART5 inhibits PUMA, and BHRF1 also inhibits BAX. EBNA1 is anti-apoptotic, by lowering p53 levels and inhibiting p53-induced apoptosis (Kennedy et al. 2003, Saridakis et al. 2005) and EBV miRNAs can protect BL cells from apoptosis (Vereide et al. 2013). Therefore, these other mechanisms may also alter the apoptotic response to TGFβ, possibly explaining why very little apoptosis was seen in cell

lines other than EBV-negative BL31. Hence, even when particular latent proteins are deleted, the remaining latent proteins can protect against TGFβ-induced apoptosis.

LMP2A expression in a gastric carcinoma cell line was sufficient to completely inhibit TGFβ-induced apoptosis, whereas in the EBV-negative BL line Ramos, LMP2A expression only partially blocked TGFβ-induced apoptosis (Fukuda and Longnecker 2004). Thus although LMP1 or LMP2A may be sufficient to block TGFβ-induced apoptosis in epithelial cells, they appear to have only a partial effect in B cells, suggesting that other latent proteins contribute in B cells.

**The effects of EBV infection on TGFβ-mediated inhibition of proliferation**

Although many studies have investigated the effects of EBV on responses to TGFβ in B cells, these have mostly investigated the anti-proliferative rather than pro-apoptotic effects of TGFβ. In EBV-negative BLs, different responses are seen for individual cell lines, and even within the same cell line in different studies, for example in the Ramos cell line (Blomhoff et al. 1987, Altiok et al. 1993, Chaouchi et al. 1995, Inman and Allday 2000b, Di Bartolo et al. 2008, Wang et al. 2008, Kawabata et al. 2013). Several studies have shown that full latency III EBV expression leads to resistance to the anti-proliferative effects of TGFβ (Kehrl et al. 1986, Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Arvanitakis et al. 1995, MacDonald et al. 1996, Inman and Allday 2000b).

In the current study, TGFβ1 induced G1 arrest in all three EBNA2 KO and both LMP2A KO BL31 cell lines (see figs. 5.4B and 5.7A). These were all characterised by low or no expression of LMP2A (see table 5.1). The remaining cell lines, including EBNA3B KO, EBNA3C KO and LMP1 KOs, did not undergo arrest (or apoptosis) with TGFβ1 (figs. 5.2 and 5.6). These findings suggest that LMP2A expression is protective against TGFβ-induced arrest, i.e. that LMP2A expression drives progression through the G1 checkpoint.

In epithelial cells, TGFβ-induced cell cycle arrest occurs via repression of Id proteins (Ling et al. 2002). TGFβ-also down-regulates c-Myc, which normally represses p15 and p21, leading to induction of p15/p21 and hence cell cycle arrest (Alexandrow et al. 1995, Warner et al.

1999). However, in B cells TGFβ generally induces rather than represses Id expression: in murine B lymphoid progenitors TGFβ induces Id3 expression, resulting in G1 arrest (Kee et al. 2001) and in human EBV-negative BL cell lines, TGFβ induces Id1 and Id2 (Spender and Inman 2009b). The mechanism of TGFβ-induced growth arrest in CA46 BL, however, is by repression of E2F1, rather than via c-Myc repression, Id repression or induction of p15/p21; (Spender and Inman 2009b). However, TGFβ induces p21 in Ramos, another EBV-negative BL line (Di Bartolo et al. 2008), which has also been shown to undergo G1 arrest with TGFβ (Blomhoff et al. 1987, Altiok et al. 1993). Thus the mechanism of TGFβ-induced arrest differs between BL cell lines, reflecting the fact that they are tumour cell lines in which normal cell cycle control mechanisms have already been deregulated.

LMP1 expression in other cell types including fibroblasts, epithelial cells and hepatoma cells led to Id1 induction and resistance to the anti-proliferative effect of TGFβ (Takanashi et al. 1999, Lo et al. 2010). LMP1 expression was sufficient to inhibit TGFβ-mediated p21 induction and cell cycle arrest in epithelial cells (Fukuda et al. 2002, Lo et al. 2010).

The effect of LMP1 expression alone in EBV-negative BL cell lines is less clear, with conflicting results seen even in the same BL41 background. LMP1 expression was sufficient (Arvanitakis et al. 1995), or not sufficient (Altiok et al. 1991) to confer resistance to the anti-proliferative effects of TGFβ; the discrepant results may be explained by the 3-4-fold lower dose of TGFβ used in the first study (Arvanitakis et al. 1995), suggesting that LMP1 expression may lead to partial resistance to TGFβ. However, expression of LMP1 in a different EBV-negative BL cell line, Louckes, surprisingly led to an enhanced effect of TGFβ on cell cycle arrest (Wang et al. 1988).

Expression of EBNA2 in BL41 cells, investigated in a single study, was also not sufficient to cause resistance to the anti-proliferative effect of TGFβ (Altiok et al. 1991).

Previous studies have investigated the anti-proliferative effects of TGFβ in BL cell lines which are infected with EBV lacking full EBNA2, and therefore LMP, expression, including P3HR1, Daudi and Jijoye. In most cases these cell lines have been sensitive to TGFβ-induced inhibition of proliferation (Altiok et al. 1991, Altiok et al. 1993, Arvanitakis et al. 1995,

Fukuda et al. 2006a), although in two studies it was noted that higher doses of TGFβ were required to inhibit proliferation of P3HR1 than EBV-negative BL cell lines, (Altiok et al. 1993, Arvanitakis et al. 1995). Another study in P3HR1 showing resistance to the anti-proliferative effects of TGFβ1 had used up to 10-fold lower doses (Kumar et al. 1991). Taken together these studies suggest that P3HR1 has partial resistance to the anti-proliferative effects of TGFβ1. However, Ramos-AW, another BL with EBV lacking full expression of EBNA2, was entirely resistant to the effects of TGFβ1 (arrest and apoptosis) even at high doses (Blomhoff et al. 1987, Inman and Allday 2000b); this is likely to be due to the nature of that particular cell line.

The current study in the BL31 cell line has confirmed TGFβ-mediated induction of ID1 and ID2 mRNA expression, but no induction of p15 (figs. 3.8 and 3.9). In cells infected with wild-type EBV, no induction of ID1 or ID2 occurred, suggesting that EBV blocks this effect (fig. 3.8). However, deletion of the whole EBNA3 locus, LMP1 or LMP2A, led to TGFβ-mediated induction of ID1 and ID2, suggesting that these latent proteins are necessary to inhibit TGFβ-mediated induction of ID1 and ID2 (fig. 4.3C). These same latent proteins are necessary for the repression of TGFβR2 and loss of TGFβ signalling suggesting that TGFβ-mediated induction of ID1 and ID2 occurs via TGFβR2 and phosphorylation of SMAD2. However, LMP1 deletion allowed TGFβ-mediated induction of ID1/2, as well as phosphorylation of SMAD2, yet did not result in cell cycle arrest or apoptosis with TGFβ1 (figs. 4.3B-C and 5.6). Therefore, although in other cell types LMP1 alone appears to be sufficient to protect cells from TGFβ-induced growth inhibition, in B cells LMP1 has not consistently been found to be sufficient for protection. In the current study, LMP1 has been shown not to be necessary for protection against TGFβ-induced growth arrest. Several papers published during the course of, or since completion of, this project have suggested that the anti-proliferative effects of TGFβ in normal B cells and B cell lymphomas may in fact be mediated via a non-canonical signalling pathway in which TGFβ induces phosphorylation of SMAD1/5 (Bakkebo et al. 2010, Rai et al. 2010, Liu et al. 2012, Jiang and Aguiar 2014), which had also been suggested previously for SMAD1 (Munoz et al. 2004). One of these studies also showed a lack of correlation between TGFβ-induced phosphorylation of SMAD2 and its anti-proliferative effects in B cells, and suggested that TGFβ-mediated anti-proliferative effects via pSMAD1/5 may also involve activation of the p38 kinase pathway (Bakkebo et al. 2010).

Overexpression of TGFβR2 in the Akata cell line, which lacked endogenous TGFβR2 expression, resulted in two distinct clones with differing levels of TGFβR2 expression. Both clones were sensitive to the anti-proliferative effects of TGFβ, whereas only the clone expressing higher levels of TGFβR2 also underwent apoptosis, suggesting that TGFβ-induced apoptosis occurs via TGFβR2 but that expression of TGFβR2 must exceed a particular threshold for the apoptotic response (Fukuda et al. 2006a). Furthermore, the cell line CA46 does not undergo apoptosis with TGFβ despite induction of BIK; instead, it undergoes G1 arrest. It lacks expression of BAX hence is unable to undergo apoptosis (Spender et al. 2009). Taken together, these studies suggest that TGFβ induces G1 arrest by default, but that if certain other conditions are present, including TGFβR2 expression above a threshold, then the predominant response is of apoptosis. This may be equivalent to the situation in hepatocytes, where Smad3 overexpression leads to apoptosis, but in the presence of Akt, which sequesters unphosphorylated Smad3 rendering it unable to accumulate in the nucleus, TGFβ instead induces arrest (Conery et al. 2004, Remy et al. 2004).

**Discordance between PARP cleavage and sub-G1 component on FC analysis**

In the current study, initial experiments showed almost complete cleavage of PARP in BL31 after 48 hours of TGFβ; however, the increase in sub-G1 component by FC was less marked (fig. 5.1A-B). Furthermore, although EBNA2 KOs 1 and 2 showed a small degree of TGFβ-induced PARP cleavage (fig. 5.5), TGFβ did not induce any increase in sub-G1 on FC analysis in these cells (fig. 5.4). In later experiments, although there was still some increased PARP cleavage with TGFβ in BL31, and an increase in the proportion of dead cells, there appeared to be no change in the cell cycle profile with TGFβ (fig. 5.8). Therefore, in these cells PARP cleavage appeared to be more sensitive than FC analysis for detection of apoptosis. An explanation may be that some cells have undergone apoptosis from the G2/M phase, since this would not reduce the DNA content sufficiently for the cells to appear in the sub-G1 component. In the EBV-negative BL cell line BL41, treatment with TGFβ induced apoptosis mainly from the G1 but also from the G2/M phases of the cell cycle (Inman and Allday 2000a).

In later experiments in the course of this project, the amount of both TGFβ-induced PARP cleavage and sub-G1 on FC became less marked than originally (figs. 5.1 and 5.8). This was

thought to be due to a change in the serum used to supplement the media in which the cells were grown, but did not alter the occurrence of TGFβ-induced G1 arrest in the relevant cell lines, confirming that arrest and apoptosis occur via distinct pathways as previously demonstrated in B cells (Chaouchi et al. 1995, Schrantz et al. 1999, Inman and Allday 2000a). The apparent resistance to the effects of TGFβ after the serum change did not alter the induction of SMAD2 phosphorylation, suggesting that some factor in the second serum, but not the first, was interfering with the apoptotic pathways in BL31 cells.

# Chapter 6 The effects of EBV on TGFβR expression and TGFβ signalling in newly infected primary B cells and LCLs

## 6.1 Introduction

Chapters 3 and 4 investigated TGFβ signalling and expression of TGFβR2/TGFβR3 in BL31 cells plus or minus infection with latent EBV. However, EBV-infected BL31 cells are derived from highly selected tumour cells, whereas LCLs are derived from normal primary B cells. Therefore it was important to see whether similar findings are seen early after infection of primary B cells and in established LCLs, although it is harder to investigate the effects of individual latent proteins since several of them are required for the transformation to and maintenance of LCLs. In addition, several previous studies on the effects of EBV on TGFβ responses have been done in established LCLs, which may also have undergone clonal selection in culture and thus may be heterogeneous (Heath et al. 2012). Therefore, in this chapter the effects of EBV infection early after infection of normal primary B cells was investigated, as well as the effects in established LCLs.

LCLs are known to resist the effects of TGFβ (Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Inman and Allday 2000b, Kenney et al. 2001, Horndasch et al. 2002) and it had been shown that expression of TGFβR2 is repressed in LCLs (Inman and Allday 2000b). Having shown that four latent proteins cooperate to repress TGFβR2, the question was therefore whether the same latent proteins repress TGFβR2 early after infection of primary B cells and in established LCLs.

In addition, since EBV also up-regulates TGFβR3 in BL31 cells and the findings in BL31 suggested that TGFβR2 and TGFβR3 may be reciprocally regulated, this was investigated further in infected primary B cells and LCLs.

## 6.2 Regulation of TGFβR2, and TGFβ signalling, in newly infected B cells and LCLs

### 6.2.1 TGFβR2 is epigenetically repressed by EBV in LCLs, leading to suppression of TGFβ signalling

In BL31 cells, EBV down-regulates TGFβR2, and the same latent proteins are necessary for suppression of signalling via pSMAD2 as for down-regulation of TGFβR2, suggesting that the down-regulation of TGFβR2 leads to the suppression of signalling (see chapter 3, figs. 3.1A and 3.7).

In order to confirm that TGFβR2 is also repressed in LCLs, as has been shown previously (Inman and Allday 2000b), qRT-PCR was performed for TGFβR2 in several different LCLs carrying wild-type EBV, established at different times using primary B cells from different donors, and compared to expression in wild-type EBV-infected and uninfected BL31 cells. This confirmed the down-regulation of TGFβR2 in LCLs, to a similar level as in wild-type-infected BL31 cells (representative data is shown in fig. 6.1A).

The effect on TGFβ signalling was also investigated by western blot for pSMAD2 in cells treated with TGFβ1 or vehicle alone. This showed that in wild-type LCLs, TGFβ does not induce pSMAD2, as seen in wild-type infected BL31 cells (fig. 6.1B), whereas TGFβ1 does induce pSMAD2 in uninfected BL31 as seen previously (see fig. 3.7). This suppression of TGFβ signalling is consistent with the repression of TGFβR2 by EBV in LCLs.

Chromatin immunoprecipitation was performed for H3K27Me3 at the TGFβR2 promoter, in order to determine whether the repression is also epigenetically mediated in LCLs. This showed H3K27Me3 on the TGFβR2 promoter, in a similar pattern to but to a slightly lesser amplitude than in BL31-WT (fig. 6.1C-D). This suggests that the down-regulation of TGFβR2 in LCLs is also associated with polycomb-mediated repression, as in BL31 cells.

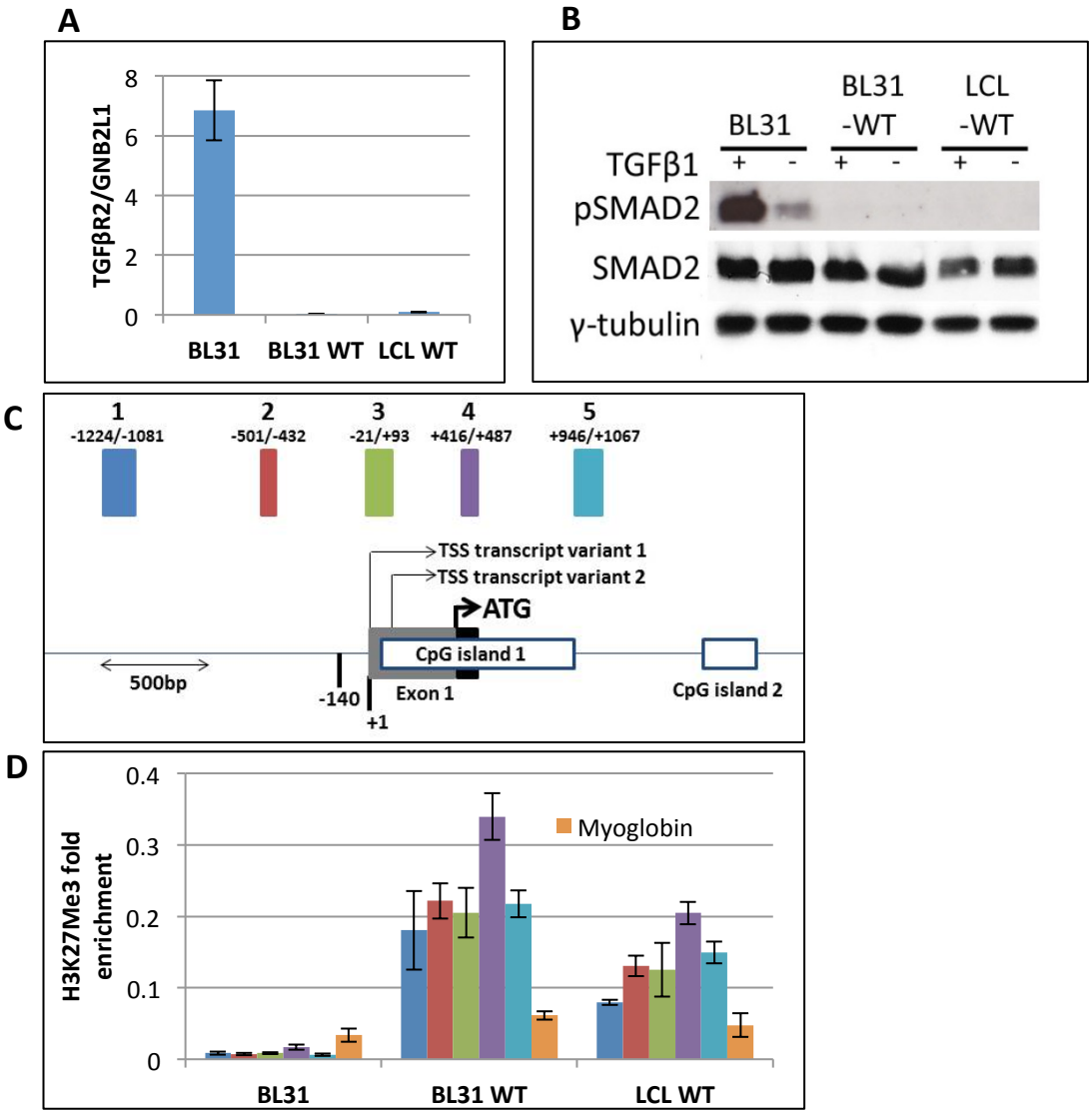



**Figure 6.1: TGFβR2 is epigenetically repressed in LCLs established with wild-type EBV, leading to suppression of TGFβ signalling**

**(A)** qRT-PCR for TGFβR2 in BL31, BL31-WT and a wild-type LCL. Values are expressed as a ratio to the endogenous control gene *GNB2L1,* with error bars representing standard deviation of triplicate qPCR reactions. This is representative data of experiments performed several times in at least three different wild-type LCLs. **(B)** Western blot for pSMAD2 in BL31, BL31-WT and LCL-WT one hour after treatment with TGFβ1 5ng/ml or vehicle (2mg/ml BSA in 1xPBS, labelled '-TGFβ1'). Total SMAD2 is shown for comparison, with γ-tubulin as a loading control. This is representative data from experiments performed at least three times using different LCLs. **(C)** Schematic diagram of the TGFβR2 promoter region showing the location of products of primer sets used, the putative transcription start site (TSS) and CpG islands. The coloured blocks represent the primer pairs 1-5, shown as bars from left to right for each cell line in figure D. **(D)** ChIP followed by qPCR showing ratio of H3K27Me3 to input DNA at the TGFβR2 promoter using the five primer sets shown in (C), and control promoter primers myoglobin (repressed) and GAPDH (active). The values for GAPDH were negligible and so are not shown in the chart. The error bars represent standard deviations from triplicate qPCR reactions for both input and IP. The LCL data is representative from experiments performed three times.

### 6.2.2 TGFβR2 is down-regulated by EBV upon infection of primary B cells

In order to investigate further the repression of TGFβR2 in normal B cells infected by EBV, purified primary B cells were infected with wild-type EBV and TGFβR2 expression measured by qRT-PCR with time after infection, as LCLs became established (arbitrarily considered to occur at 40 days post-infection). This showed that TGFβR2 expression was high in uninfected primary resting B cells but was gradually repressed after EBV infection, with near maximal repression occurring by around 21 days post-infection and persisting after this time (fig. 6.2). This confirms the down-regulation of TGFβR2 by EBV and is consistent with LCLs having little or no detectable TGFβR2 mRNA (fig. 6.1A).

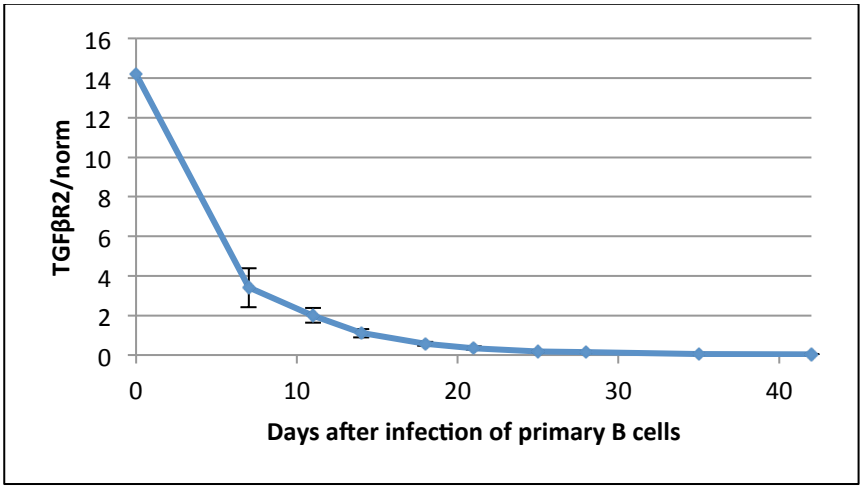



**Figure 6.2: EBV down-regulates TGFβR2 expression after infection of primary B cells**

qRT-PCR for TGFβR2 with time after infection of purified primary B cells with wild-type EBV. The values represent the ratio of expression to the mean of values of two endogenous control gene *GNB2L1* and *RPLPO* ('norm'). Data shown are from one of two separate infection experiments, in which the pattern of variation of values is representative. The error bars represent standard deviations from triplicate qPCR reactions.

### 6.2.3 EBNA3B deletion leads to de-repression of TGFβR2 and an increase in TGFβ signalling in LCLs

Since EBNA3B, EBNA3C, LMP1 and LMP2A appear to cooperate in the down-regulation of TGFβR2 in BL31 cells, it was hypothesised that the same latent proteins may cooperate to down-regulate TGFβR2 in LCLs. In LCLs, the effect of deletion of certain individual latent proteins cannot be directly assessed as some (EBNA1, EBNA2, LMP1 and EBNA3C) are absolutely necessary for the transformation of B cells by EBV into LCLs (Cohen et al. 1989, Kaye et al. 1993, Tomkinson et al. 1993, Lee et al. 1999). Since EBNA3B is dispensable for

transformation of B cells to LCLs, it is possible to establish EBNA3B KO LCLs, and thus the effect of EBNA3B deletion on TGFβR2 expression was investigated in newly infected B cells and established LCLs.

Initially, purified primary B cells were infected with wild-type, EBNA3B KO and EBNA3B revertant viruses and samples harvested at various time points for RNA extraction and qRT-PCR for TGFβR2 mRNA (fig. 6.3A). Although the repression of TGFβR2 was slower initially for wild-type than EBNA3B KO or revertant EBV, similar levels of TGFβR2 repression were seen by around 25 days after infection for all three viruses (fig 6.3A). This initial difference was likely to be due to a difference in titres of virus used to infect the primary B cells, as the titre of wild-type virus was approximately 20-fold lower than either EBNA3B KO or revertant viruses. However, the titres of EBNA3B KO and revertant were more comparable, but with that for EBNA3B KO being slightly higher than for the revertant (WT 4 $x10^4$ GRU/ml, EBNA3B KO 1.0 x $10^6$ GRU/ml; EBNA3B revertant 0.75 x $10^6$ GRU/ml). It was observed that, from days 10-28 post-infection, there was slightly less repression of TGFβR2 in EBNA3B KO than revertant despite EBNA3B KO being of slightly higher titre (fig. 6.3A). After day 28, however, TGFβR2 appeared to be profoundly repressed by all three viruses (fig. 6.3A).

mRNA was extracted from the LCLs established from primary B cells infected by wild-type, EBNA3B KO and EBNA3B revertant viruses at six weeks post-infection and analysed by qRT-PCR. This showed that although TGFβR2 expression was low for all three viruses, there was some relaxation of repression in EBNA3B KO compared to wild-type and revertant – infected cells, suggesting that EBNA3B may contribute to the repression of TGFβR2 in LCLs, although this contribution appears to be minor (fig. 6.3B).

The effect of EBNA3B deletion on TGFβ signalling was also investigated at around six weeks post-infection using the wild-type, EBNA3B KO and revertant LCLs established from the infections of primary B cells. This showed that signalling, as detected by pSMAD2, was increased in EBNA3B KO compared to wild-type or revertant LCLs, consistent with the de-repression of TGFβR2 in these cells (fig. 6.3C). The level of pSMAD2 was somewhat surprising as, despite the level of TGFβR2 appearing to be markedly repressed overall, the

de-repression seen in EBN3B KO LCLs compared to wild-type was sufficient to restore signalling. This will be discussed further in section 6.5.

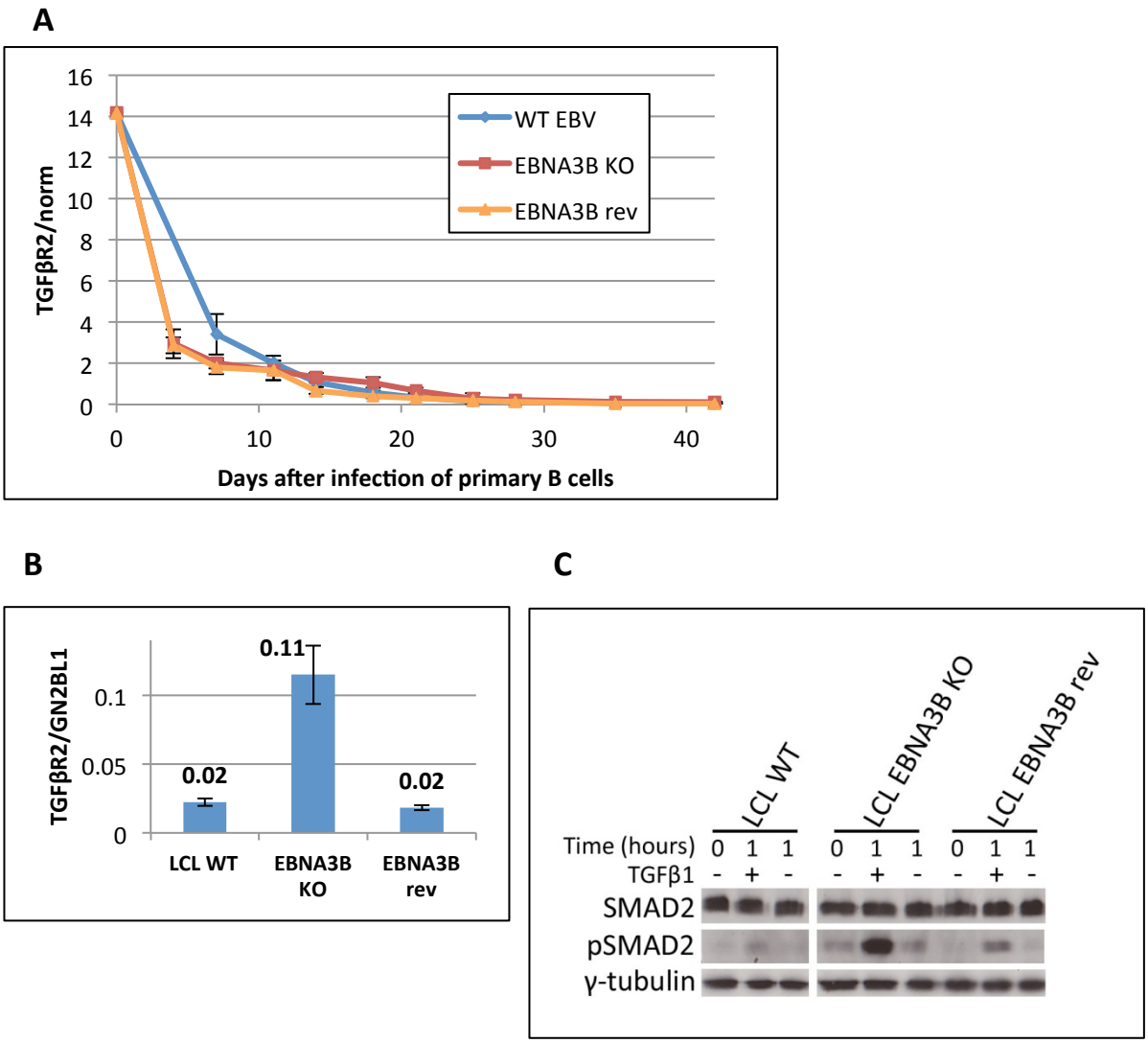



**Figure 6.3: EBNA3B deletion leads to a modest de-repression of TGFβR2 and increased TGFβ signalling via pSMAD2**

**(A)** qRT-PCR for TGFβR2 with time after infection of purified primary B cells with wild-type (WT), EBNA3B KO and EBNA3B revertant virus. The values represent the ratio of expression to the mean of values of two endogenous control gene *GNB2L1* and *RPLPO* ('norm'). The error bars represent standard deviations from triplicate qPCR reactions. **(B)** qRT-PCR for TGFβR2 in WT, EBNA3B KO and EBNA3B revertant LCLs at 6 weeks post-infection. The values represent the ratio of expression to the endogenous control gene *GNB2L1.* The error bars represent standard deviations from triplicate qPCR reactions. **(C)** Western blot for pSMAD2 in the cell lines as in (B) before treatment (time 0) and one hour after treatment with TGFβ1 10ng/ml or vehicle (2mg/ml BSA in 1xPBS), at 6 weeks post-infection. Total SMAD2 is shown for comparison, with γ-tubulin as a loading control. All data shown are from one experiment, in which the pattern of variation of values for the cell lines is representative of at least two separate experiments.

### 6.2.4 TGFβR2 remains repressed after withdrawal of 4HT in 3CHT-LCLs established in the presence of 4HT

Next, the effect of non-functional EBNA3C on TGFβR2 expression was investigated in LCLs, since EBNA3C is necessary for the repression of TGFβR2 in BL31 cells (fig. 3.1A). Because EBNA3C is required for transformation of normal B cells into LCLs, and therefore when B cells are infected with EBNA3C KO virus they are unable to survive or grow out to become LCLs (Tomkinson et al. 1993), a recombinant EBV conditional for EBNA3C function was used. A conditional system has been developed in which EBNA3C is fused to a modified, 4-hydroxytamoxifen (4HT)-dependent murine oestrogen receptor. This 3CHT virus, created from a B95.8 virus in the BAC system, has been used to infect primary B cells in order to establish 3CHT-LCLs (Skalska et al. 2010). These LCLs express EBNA3C-HT, a modified form of EBNA3C, which is functional only when 4HT is present in the growth medium. When 4HT is withdrawn, EBNA3C-HT is sequestered and degraded, and the LCLs gradually stop proliferating due to the absence of functional EBNA3C and concomitant increase in $p16^{INK4A}$ (Maruo et al. 2006, Skalska et al. 2010).

The effect of deactivating EBNA3C on TGFβR2 expression was investigated using this system. A 3CHT-LCL, which had been established from infection of normal donor B cells, was grown continuously with 4HT, thus expressing functional EBNA3C. The cells were washed and grown subsequently in medium with or without 4HT for 21 days, with samples harvested at day 0 and 21 days post withdrawal or continuation of 4HT (cell treatments, harvesting and RNA extraction performed by Lenka Skalska). qRT-PCR for TGFβR2 expression in these samples, along with BL31 and BL31-WT for comparison, showed that TGFβR2 remained profoundly repressed, with values similar to those at day 0, even 21 days after withdrawal of 4HT (fig. 6.4). The degree of repression was also similar to that seen in BL31-WT.

Since functional EBNA3C had been shown to be withdrawn by day 21 after 4HT withdrawal in these cells (Skalska et al. 2010), the findings would suggest that withdrawal of 4HT and hence functional EBNA3C does not lead to de-repression of TGFβR2, and thus that EBNA3C is not necessary for the repression of TGFβR2 in LCLs. However, EBNA3C has been shown to repress BIM and $p16^{INK4A}$ by polycomb-mediated repression and to bind to multiple cellular promoters suggesting direct repression (Paschos et al. 2009, Skalska et al. 2010, McClellan

et al. 2012, Paschos et al. 2012, Skalska et al. 2013). Thus it remains possible that prior exposure to EBNA3C (when the cells were previously grown in the presence of 4HT) could have led to epigenetic repression of TGFβR2, with TGFβR2 then remaining repressed even after functional EBNA3C was removed.

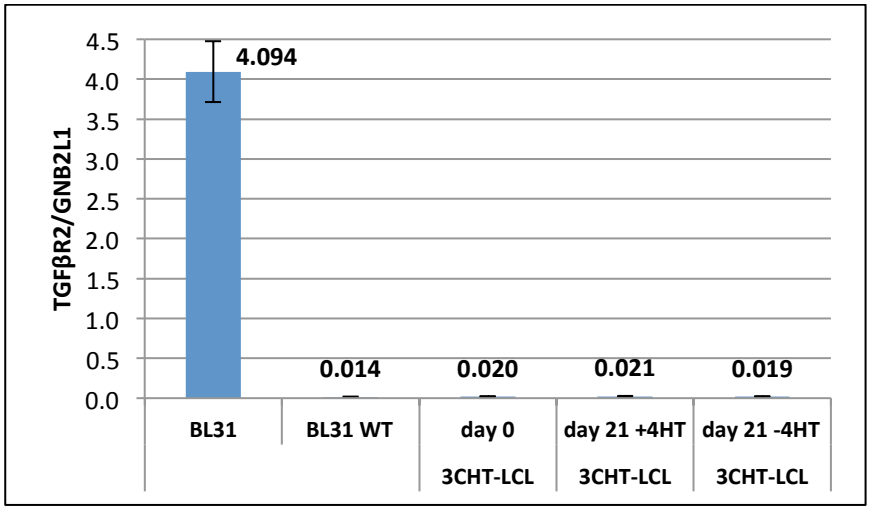



**Figure 6.4: In 3CHT-LCLs established with 4HT, TGFβR2 remains repressed despite withdrawal of 4HT**

LCLs were established from a donor with wild-type p16, in the presence of 4HT. Cells were harvested at day zero and then half of the cells were grown continuously in 4HT whereas the other half were washed and then subsequently grown in medium without 4HT. After 21 days, samples were harvested from each. RNA was extracted and then qRT-PCR for TGFβR2 was performed with these samples as well as uninfected and wild-type infected BL31. Values (shown above the bars) are expressed as a ratio to the endogenous control gene *GNB2L1*, with error bars representing standard deviation of triplicate qPCR reactions (LCL samples grown, harvested and RNA extracted by Lenka Skalska).

### 6.2.5 In 3CHT-LCLs established in a p16-null background, without ever being exposed to 4HT and thus EBNA3C, TGFβR2 is moderately de-repressed, leading to restoration of TGFβ signalling

In order to test the hypothesis that in LCLs, TGFβR2 remains epigenetically repressed following previous exposure to EBNA3C, it would be necessary to obtain an LCL that had never been exposed to EBNA3C. As previously mentioned, EBNA3C is absolutely required for B cell transformation by EBV (Tomkinson et al. 1993) and thus it would theoretically be impossible to obtain an LCL in which EBNA3C had never been expressed. However, at the time this work was being undertaken, colleagues in the laboratory were investigating the effects of EBNA3C on repression of $p16^{INK4A}$ and had hypothesised that the repression of

$p16^{INK4A}$ may be the reason why EBNA3C is required for B cell transformation. Therefore they had obtained B cells from an individual with a homozygous deletion in *CDKN2A*, the gene encoding $p16^{INK4A}$, and infected these B cells with the 3CHT virus, both in the presence and absence of 4HT. As predicted, in the p16-null background they were able to successfully establish LCLs even in the absence of 4HT, and thus the resultant p16-null 3CHT-LCLs (established without 4HT) would have never expressed functional EBNA3C (Skalska et al. 2013).

Therefore, TGFβR2 expression was investigated in these cells, based on the hypothesis that EBNA3C represses TGFβR2 epigenetically, and this would be maintained in progeny cells even if 4HT is withdrawn. If this hypothesis were correct, then in the p16-null 3CHT-LCLs TGFβR2 would be de-repressed in those cells which had never been exposed to 4HT and hence EBNA3C. Samples were harvested at 42 days after infection of p16-null B cells with 3CHT virus, and subsequently cultured with or without 4HT in the medium (infections and harvesting of samples performed by Rob White). RNA was extracted from these samples, followed by qRT-PCR for TGFβR2. In those cells which had never been exposed to EBNA3C, there was at least a two-fold de-repression of TGFβR2, although compared to uninfected BL31s the expression remained low (fig. 6.5A-B). This suggests that EBNA3C does contribute to the repression of TGFβR2 in LCLs although, similarly to EBNA3B, the effect is small.

In EBNA3B KO LCLs, the de-repression of TGFβR2 led to an increase in pSMAD2 after TGFβ1 treatment compared to that seen in wild-type or revertant LCLs (fig. 6.3B-C), even though the overall expression of TGFβR2 was very low relative to uninfected primary B cells (fig. 6.3A). Therefore, the effects of EBNA3C expression on TGFβ signalling were investigated. Using the p16-null 3CHT-LCLs that had been established with or without 4HT (as shown in figs. 6.5A-B), a crossover experiment was performed by Rob White in which the two established LCLs (p16-null 3CHT established with and without 4HT) were then each divided into two and subsequently grown with or without 4HT in the medium. This created four different conditions: (1) never exposed to 4HT, (2) grown out for 3 months without 4HT but then 4HT added, (3) grown out continuously with 4HT, or (4) grown out with 4HT but then 4HT withdrawn. Samples harvested from these at 30 days after changeover of the medium were investigated by qRT-PCR for TGFβR2 (fig. 6.5C). This showed that TGFβR2 expression

was again generally low in all conditions, but in those LCLs established initially without 4HT, TGFβR2 was slightly higher than those established with 4HT. However, the addition or withdrawal of 4HT after the LCLs had been established did not really alter TGFβR2 expression (fig. 6.5C), i.e. values were similar for both 'never exposed' conditions and also for both conditions grown out with 4HT. This suggests that the absence of EBNA3C as LCLs are being established may lead to less repression of TGFβR2, but once the LCLs are established the addition or withdrawal of EBNA3C does not alter TGFβR2 expression. This is consistent with the findings in 3CHT-LCLs established in a p16-wild-type background, where withdrawal of 4HT did not alter TGFβR2 expression (fig. 6.4).

p16-null 3CHT-LCLs grown under the four conditions as described above were investigated for TGFβ signalling, by treatment with TGFβ1 or vehicle for one hour, followed by harvesting, protein extraction, SDS-PAGE and Western blot for pSMAD2 (fig. 6.5D). This showed that in cells grown out without 4HT, there was greater induction of pSMAD2 by TGFβ1 than those LCLs grown out in the presence of 4HT. However, the addition or withdrawal of 4HT subsequent to establishment of the LCLs seemed to have no effect. These findings were consistent with the slight de-repression of TGFβR2 seen in cells established in the absence of 4HT (fig. 6.5C).

Taken together, these results suggest that initial exposure to EBNA3C in LCLs leads to a more profound repression of TGFβR2, with consequent suppression of TGFβ signalling, than in LCLs which have never been exposed to functional EBNA3C. This effect persists despite subsequent exposure to, or removal of, EBNA3C. This could be consistent with the repression of TGFβR2 by EBNA3C being epigenetically mediated as discussed in section 6.2.4.

The expression of TGFβR2 in p16-null cells even without EBNA3C exposure, as well as in EBNA3B KO LCLs, is low in comparison to uninfected primary B cells (figs. 6.3A and 6.5A), suggesting that the contributions of both EBNA3B and EBNA3C to the initial repression are minimal in LCLs. Thus it is likely that, in LCLs, other latent protein(s) may contribute more to the repression of TGFβR2 than EBNA3B or EBNA3C. Since in BL31 cells, LMP1 and LMP2A also both appear to contribute (see chapter 4), one or both of these may also contribute in

LCLs. However, it is interesting to note that although the expression level of TGFβR2 remains generally low even in the absence of EBNA3B or functional EBNA3C, this level of TGFβR2 expression is nevertheless sufficient to allow TGFβ signalling via pSMAD2. Furthermore, what appears to be only a slight de-repression of TGFβR2 in the absence of EBNA3B or functional EBNA3C leads to a definite increase in TGFβ-induced pSMAD2 (figs. 6.3C and 6.5D).

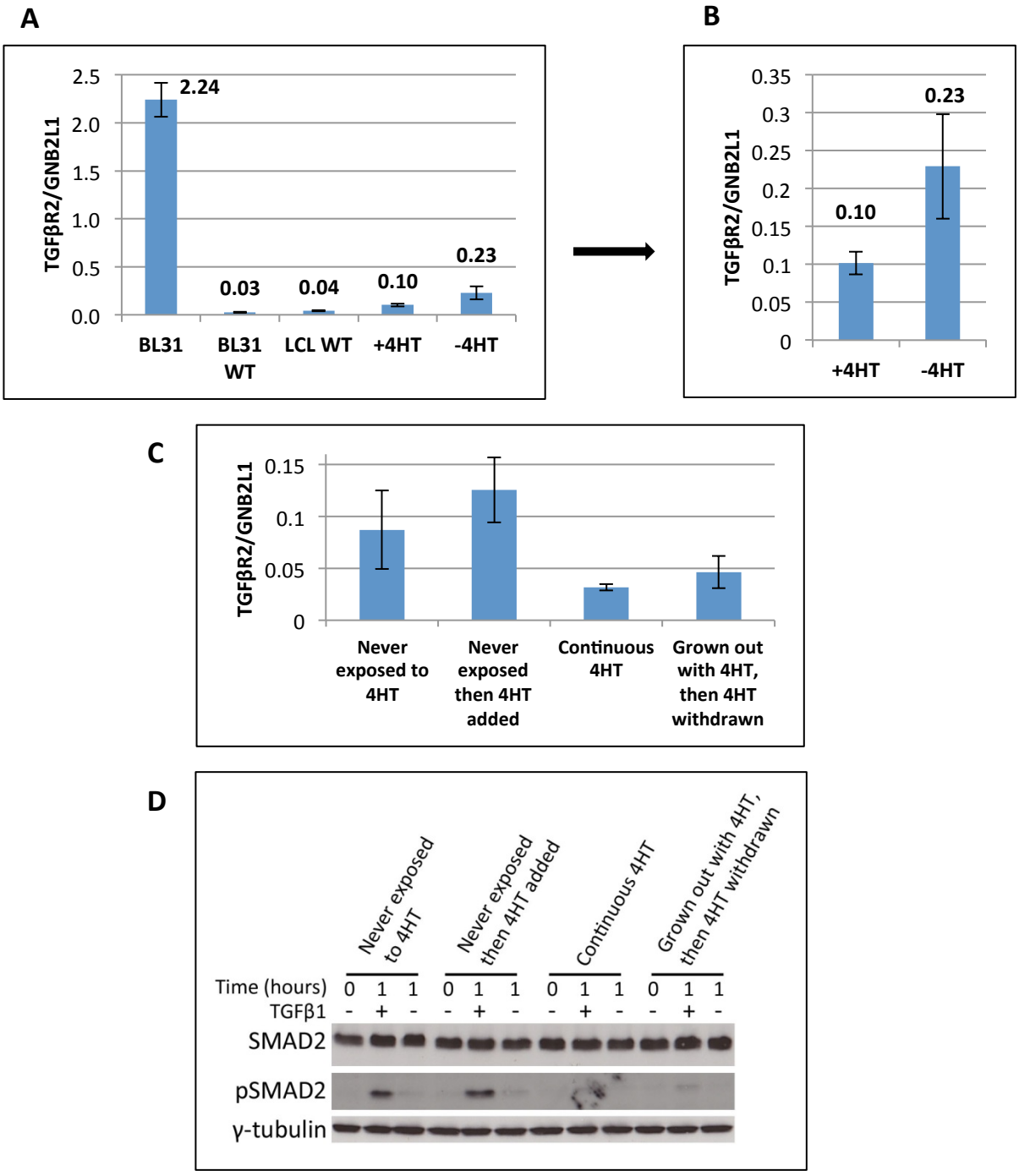

A
TGFβR2/GNB2L1
2.5
2.0
1.5
1.0
0.5
0.0
2.24
0.03
0.04
0.10
0.23
BL31
BL31 WT
LCL WT
+4HT
-4HT
B
TGFβR2/GNB2L1
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.23
0.10
+4HT
-4HT
C
TGFβR2/GNB2L1
0.15
0.1
0.05
0
Never exposed to 4HT
Never exposed then 4HT added
Continuous 4HT
Grown out with 4HT, then 4HT withdrawn
D
Never exposed to 4HT
Never exposed then 4HT added
Continuous 4HT
Grown out with 4HT, then 4HT withdrawn
Time (hours) 0 1 1 0 1 1 0 1 1 0 1 1
TGFβ1 - + - - + - - + - - + -
SMAD2
pSMAD2
γ-tubulin

**Figure 6.5: In 3CHT-LCLs established in a p16-null background, which have never been exposed to 4HT and therefore never expressed functional EBNA3C, there is a modest de-repression of TGFβR2 leading to increased TGFβ signalling**

**(A-B)** Primary B cells from a donor homozygous for a deletion of p16 ('p16-null') were infected with 3CHT virus, half with and half without 4HT in the medium. 3CHT-LCL samples were harvested 6 weeks after infection (infections and harvesting performed by Rob White). RNA extraction, reverse transcription and qPCR for TGFβR2 were performed along with samples from BL31, BL31-WT and a wild-type LCL for comparison. **(B)** shows an enlarged version of the data shown in (A) for the p16-null 3CHT-LCL cell lines established with and without 4HT. **(C)** The p16-null 3CHT-LCLs established with 4HT for 3 months were then divided into two samples in which 4HT was continued ('continuous 4HT') or withdrawn ('grown out with 4HT, then 4HT withdrawn'). At the same time, the cells that were established without 4HT then had it added ('never exposed then 4HT added') or growth was continued in medium without 4HT ('never exposed to 4HT'). 30 days after these changes samples were harvested (cells grown and harvested by Rob White). RNA was extracted and qRT-PCR performed for TGFβR2. Values are expressed as a ratio to the endogenous control gene *GNB2L1*, with error bars representing standard deviation of triplicate qPCR reactions. **(D)** The four sets of cells as in (C) were frozen down then re-established in the appropriate media. Once established, equal numbers of each were harvested at time zero then treated for one hour with TGFβ1 10ng/ml or vehicle (2mg/ml BSA in PBS) before harvesting. Protein was extracted followed by SDS-PAGE and western blot for SMAD2 and pSMAD2 in parallel. γ-tubulin was used as a loading control.

### 6.2.6 Effects of EBNA3A KO, EBNA3C KO and total EBNA3 KO on TGFβR2 expression early after infection of primary B cells

Having investigated the effects of EBNA3B and EBNA3C on TGFβR2 expression in LCLs, the effect of EBNA3A was also investigated. Although EBNA3A was originally thought to be required for B cell transformation to LCLs, more recent work had shown that it is possible to establish EBNA3A KO LCLs when feeder cells are present (Tomkinson et al. 1993, Hertle et al. 2009, Skalska et al. 2010). Colleagues in the laboratory had made several attempts to establish EBNA3A KO LCLs, and the success of this was rather variable, such that in many cases it was not possible to establish wild-type, EBNA3A KO and revertant LCLs all in the same donor background. Therefore the EBNA3A KO LCLs obtained were very heterogeneous. Preliminary investigations of expression of TGFβR2 in EBNA3A KO compared to wild-type/revertant LCLs in several sets of LCLs by qRT-PCR showed very heterogeneous results and therefore these were not investigated further.

Primary B cell infections were also performed (with the help of Rob White) using EBNA3A KO and revertant, EBNA3C KO and revertant, total EBNA3 KO and revertant, and 3CHT with and without 4HT, viruses in mixed random donor (i.e. p16 wild-type) B cells. Samples were harvested at various time points after infection and RNA extraction and qRT-PCR were performed for TGFβR2 (fig. 6.6). As expected, the cells infected with EBNA3C KO, EBNA3 KO and 3CHT virus (without 4HT), and also those infected with EBNA3A KO virus in this case, did not survive beyond days 14-18 and thus it is not possible to see the effect of these KOs on TGFβR2 repression at the time at which TGFβR2 is fully repressed by wild-type/revertant viruses, i.e. around day 21 (fig. 6.6). Nevertheless, up to the point when these cells die the TGFβR2 expression generally seemed to be similar to that in the wild-types and revertants, supporting the earlier findings that none of the EBNA3 proteins appear to have a large effect on TGFβR2 expression in LCLs. The cells infected with 3CHT virus (without 4HT) also died by 14 days post-infection, as expected. In these cells, TGFβR2 expression appeared to follow a similar pattern to EBNA3B KO (i.e. slightly de-repressed compared to wild-type/revertants up to this time point), possibly suggesting a small effect of EBNA3C on the repression of TGFβR2. However, these data should be interpreted with caution since the virus titres used were not all equivalent. In addition, the 3CHT virus tends to be slightly less efficient than other wild-type/revertant viruses, presumably due to the fusion altering the efficiency of

EBNA3C [data not shown plus (Skalska et al. 2010, Skalska et al. 2013)], and hence this could explain why TGFβR2 appears to be slightly less repressed in these cells than in the wild-types and revertants; indeed, the graph for 3CHT virus with 4HT also shows slightly less repression of TGFβR2 than the other wild-types/revertants at all time points (pale green line, fig. 6.6).

Since TGFβR2 is repressed by all the recombinant viruses used, it is possible that this repression could be due to B cell activation per se rather than to EBV itself. However, in an experiment to compare EBV infection of PBMCs with activation by CD40L and IL-4 (to activate the B cells), TGFβR2 mRNA levels were similar with EBV infection and CD40L/IL-4 treatment at 2 days post-infection/activation but by day 4, TGFβR2 mRNA started to increase again in the CD40L-activated samples, and continued to increase up to day 7, whereas TGFβR2 expression continued to decrease as shown here (Lenka Skalska, unpublished data). Therefore the continued repression of TGFβR2 appears to be an effect of EBV itself rather than of B cell activation.

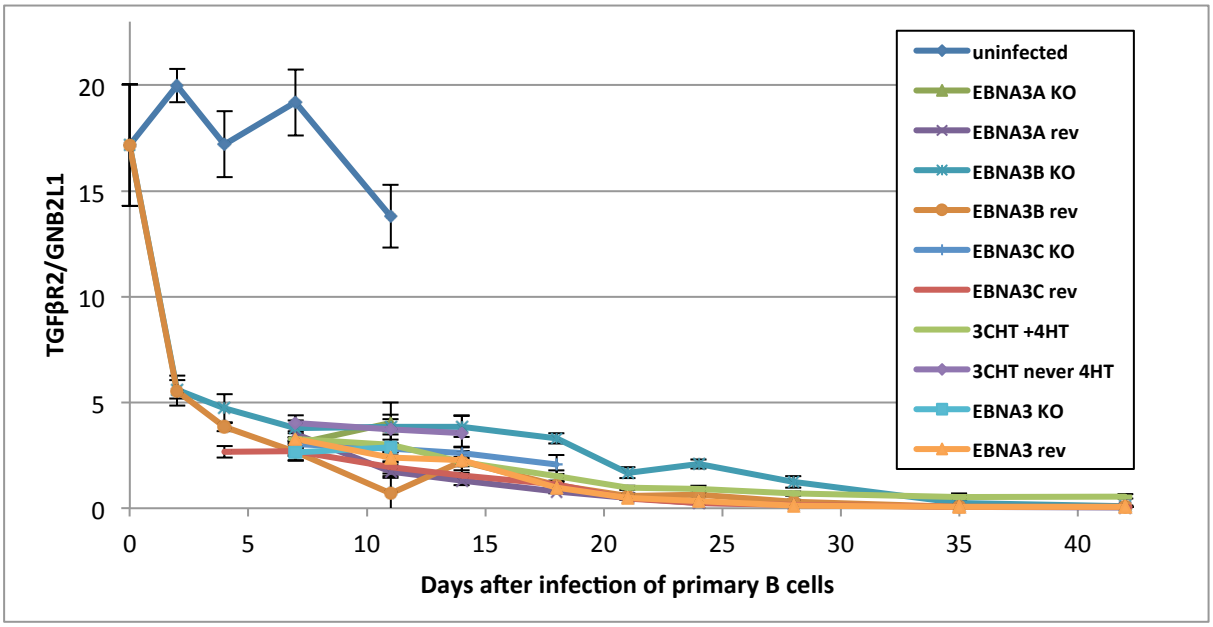



**Figure 6.6: EBNA3A and EBNA3C are not necessary for the down-regulation of TGFβR2 by EBV in newly infected primary B cells**

Purified primary B cells were obtained from random donors (p16 wild-type) and equal numbers infected with each of the recombinant viruses shown (EBNA3A, EBNA3B, EBNA3C and total EBNA3 locus deletions and their revertants, as well as conditional 3CHT with/without 4HT), followed by harvesting at intervals, RNA extraction, and qRT-PCR for TGFβR2. Values are expressed as ratios to the endogenous control gene *GNB2L1*, with error bars representing standard deviation of triplicate qPCR reactions.

### 6.2.7 In EREB2.5 cells, TGFβR2 is de-repressed upon withdrawal of β-estradiol

Since LMP1 and LMP2A cooperate in the down-regulation of TGFβR2 in BL31 cells, the effect of these in LCLs was investigated, to the extent that this was possible. EBNA2 is absolutely required for B cell immortalisation, and thus it is not possible to make EBNA2 KO LCLs (Cohen et al. 1989). Although LMP1 was originally thought to be absolutely required for B cell transformation (Kaye et al. 1993), subsequent work has shown that LMP1 expression is needed for cellular proliferation, and in fact not absolutely required for transformation, but that transformation efficiency is severely reduced without its expression (Dirmeier et al. 2003); therefore it is extremely difficult to make LMP1 KO LCLs.

In order to try to investigate the effects of EBNA2 expression in LCLs, Kempkes *et al* developed the EREB2.5 cell line, an LCL in which EBNA2 is fused to an oestrogen receptor such that expression of EBNA2 is conditional upon the presence of oestrogen in the medium (Kempkes et al. 1995). When oestrogen is withdrawn from these cells, they stop proliferating and a significant proportion become apoptotic. However, approximately 50% remain viable for up to five days, arrested in G1, during which time re-addition of oestrogen can re-induce DNA synthesis. In EREB2.5 cells, the amount of EBNA2-oestrogen receptor (EBNA2-ER) fusion protein is also reduced after withdrawal of oestrogen, again through sequestration and degradation. Kempkes *et al* demonstrated that withdrawal of oestrogen from EREB2.5 cells also led to a reduction in LMP1, as predicted, since EBNA2 transactivates LMP1 (Kempkes et al. 1995). Presumably LMP2A would be reduced upon withdrawal of oestrogen, in a similar manner.

EREB2.5 cells were used to investigate the effects of oestrogen withdrawal on the expression of TGFβR2. Equal numbers of proliferating EREB2.5 cells were either seeded into a new flask containing medium with β-estradiol (an oestrogen), or washed twice in PBS and then re-suspended in RPMI medium without β-estradiol. Samples were harvested for RNA and protein extraction on day 0, after washing and re-suspension, and subsequently at 24 hour intervals to day 4. SDS-PAGE followed by western blot showed that as β-estradiol was withdrawn, expression of EBNA2-ER gradually decreased from day 2 onwards, although the expression of EBNA2-ER was also notably lower in the cells grown with β-estradiol at day 0; the reason for this is not clear although possibly reflects the difference in treatment in

terms of washing the 'without estradiol' cells (fig. 6.7). The loading was the same for both, as the level of γ-tubulin shown is on the western blot performed for EBNA2. As expected, since EBNA2 transactivates LMP1 and LMP2A, the expression of these were reduced by 1-2 days after β-estradiol withdrawal. Expression of LMP1 and LMP2A was similar on day zero in the samples both with and without β-estradiol (fig. 6.7).

RNA was extracted and then qRT-PCR performed for TGFβR2 expression (fig. 6.7). Since upon withdrawal of oestrogen, many of the EREB2.5 cells die, primer sets for several endogenous control (normalisation) genes (GAPDH, ALAS1, TUBB, RPLPO and GNB2L1) were used. The values for all normalisation genes varied between samples: for ALAS1, TUBB and GAPDH the values all gradually decreased from days 1-4, whereas the values for RPLPO and GNB2L1 varied between samples but did not show a gradual decline with time (data not shown). The data were therefore analysed first using each normalisation gene separately, and in all cases TGFβR2 was seen to gradually increase with time after oestrogen withdrawal. Therefore, for each sample the mean of the values for all normalisation genes was calculated and the values shown in the histogram are therefore expressed as a ratio to this mean normalisation value ('norm') (fig. 6.7).

With this caveat in mind, TGFβR2 expression appeared to gradually increase with time after β-estradiol withdrawal. In this case the expression at RNA level was similar at day 0 in the cells with and without β-estradiol (in contrast to the apparent EBNA2-ER expression by western blot; fig. 6.7). The gradual de-repression of TGFβR2 could be due to the decreasing expression of EBNA2, LMP1 and/or LMP2A in these cells; it is not possible to determine which of the latent proteins is responsible. Indeed, it is also possible that the withdrawal of β-estradiol alters the expression of EBNA3 proteins; however this cannot be accurately determined due to the relatively long half-life of the EBNA3 proteins, over 24 hours (Touitou et al. 2005). In addition, since there were significant changes in expression of all normalisation genes used, likely to be as a result of significant cell death, it is also possible that the de-repression of TGFβR2 could be due to other processes occurring in the cells as a result of death or apoptosis. In addition, the gradual decline of values for some of the normalisation genes could cause an artificial increase in TGFβR2 expression, since the values are expressed as the ratio of TGFβR2 value to endogenous control.

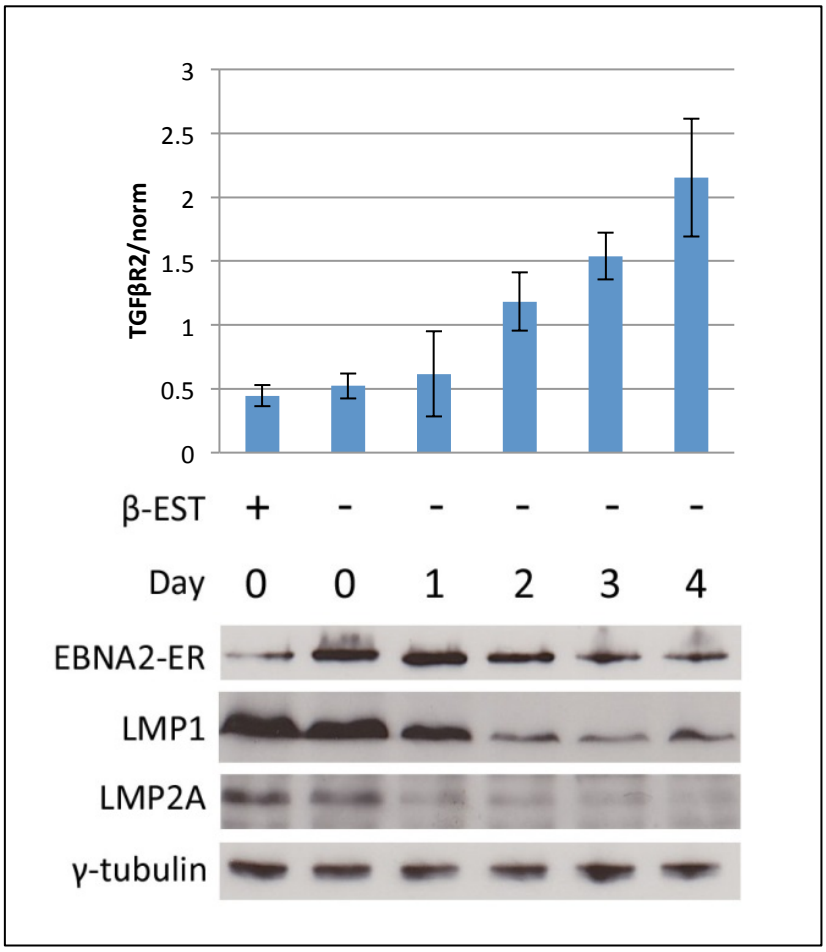



**Figure 6.7: TGFβR2 is de-repressed upon withdrawal of β-estradiol in EREB2.5 cells**

EREB2.5 cells were grown in the presence of β-estradiol and then equal numbers taken on day zero. One set was washed twice with PBS and then re-suspended in fresh medium without β-estradiol (-β-EST) and the others were re-suspended in fresh medium containing β-estradiol (+β-EST). Samples were harvested for protein and RNA on day 0 (after washing) and then at 24-hour intervals for four days. RNA was extracted, reverse transcribed and then qPCR performed for TGFβR2. The values are expressed as a ratio to the mean of five endogenous control genes (*GAPDH, ALAS1, TUBB, RPLPO* and *GNB2L1*), with error bars representing standard deviation of triplicate qPCR reactions. SDS-PAGE and western blot was performed for EBNA2 (in this case detected as the EBNA2-ER fusion protein at a higher molecular weight than standard EBNA2), LMP1 and LMP2A. γ-tubulin was used as a loading control.

Since LMP1 and LMP2A were shown to cooperate with EBNA3B and EBNA3C in causing the repression of TGFβR2 in BL31 cells (chapter 4), an attempt was also made to produce LMP2A KO LCLs in order to investigate the specific effect of LMP2A deletion. It should theoretically be possible to produce LMP2A KO LCLs because LMP2A is not strictly necessary for cell transformation/immortalisation (Longnecker et al. 1993a, Longnecker et al. 1993b), and other groups have successfully made LMP2A KO LCLs. However, there is some discrepancy as to whether lack of LMP2A reduces the efficiency of immortalisation, with some groups showing that LMP2A contributes to the efficiency (Brielmeier et al. 1996, Wasil et al. 2013), whereas others have shown it to be completely dispensable (Longnecker et al. 1992, Longnecker et al. 1993a, Longnecker et al. 1993b, Speck et al. 1999).

Unfortunately the attempted outgrowth of LMP2A KO LCLs by infection of primary B cells was unsuccessful in the current study and, due to time constraints, could not be repeated. Nevertheless this would be important to investigate in future work. Primary B cells were also infected with LMP1 KO virus, but outgrowth of these was unsuccessful; as mentioned in section 6.2.7, it is extremely difficult to make LMP1 KO LCLs, having been achieved only at very low frequency in the presence of a layer of fibroblasts as feeder cells (Dirmeier et al. 2003). It was also hypothesised that if LMP1 and/or LMP2A also contribute to the repression of TGFβR2 in LCLs, it could be that the full repression of TGFβR2 does not occur until around day 21 post-infection because LMP1 and/or LMP2A have to reach a certain expression level before they have these effects. It has been previously shown that the EBNA3 proteins are expressed fully early (2-3 days) after infection of B cells [(Nikitin et al. 2010) and unpublished data, Allday laboratory], whereas LMP1 is not fully expressed until around 21 days post-infection (Price et al. 2012).

## 6.3 The regulation of TGFβR3 by EBV in newly infected B cells and LCLs

### 6.3.1 EBV up-regulates TGFβR3 in LCLs; however, this occurs later than the down-regulation of TGFβR2 after infection of primary B cells with EBV

In BL31 cells, EBV was shown to up-regulate TGFβR3 in addition to repressing TGFβR2 (chapter 3). The results in BL31 cells suggested that TGFβR2 and TGFβR3 expression may be reciprocally regulated, although there were some cell lines in which this did not appear to be the case (particularly EBNA3A KOs 1-3, see section 4.6). Therefore the expression of TGFβR3 was also investigated in LCLs.

Since EBV had been shown to gradually repress TGFβR2 after infection of primary B cells, leading to repression and loss of TGFβ signalling in established LCLs, it was hypothesised that TGFβR3 would be up-regulated by EBV over a similar time period after infection of primary B cells. qRT-PCR for TGFβR3 was performed at various time points after infection of primary B cells (fig. 6.8B). Surprisingly, over the first few days after infection, TGFβR3 was actually down-regulated, reaching a nadir at day 11. However, from this time on TGFβR3 started to increase, and therefore harvesting of samples for RNA extraction and quantification of TGFβR2 and TGFβR3 expression was continued for an extended period, up to 11 weeks post-infection of primary B cells. This showed that TGFβR2 was completely repressed by around 30 days post-infection. However, TGFβR3 expression, after the initial decrease, increased very gradually, reaching a plateau after around 70 days post-infection (fig. 6.8B).

This suggests that although EBV (or possibly the process of B cell activation) initially down-regulates TGFβR3, the subsequent up-regulation may be due to a clonal selection process rather than due to a direct effect of EBV. Although this effect was seen in most of the wild-type and revertant viruses used, there was one revertant cell line, in one experiment, in which TGFβR3 was not up-regulated by day 42 (see chapter 7, fig. 7.10C). However, since that experiment was not continued for an extended period, it remains possible that up-regulation of TGFβR3 could have occurred later. Nevertheless, this lack of up-regulation of TGFβR3 by 42 days post-infection could support the hypothesis that this occurs indirectly, possibly via a clonal selection process. Since up-regulation of TGFβR3 did occur in the

majority of wild-type and revertant LCLs it seems likely that, if it occurs via clonal selection, EBV infection has created conditions which are highly favourable for this to occur.

It is also possible that the down-regulation of TGFβR2 itself somehow favours the up-regulation of TGFβR3, especially in view of the reciprocal nature of expression seen in the majority of BL31 cell lines. However, after primary B cell infections TGFβR3 starts to increase before TGFβR2 is fully repressed; nevertheless it could be that once TGFβR2 is repressed to a certain level then TGFβR3 can increase. This could be via the mechanism discussed in section 3.4.3, i.e. that TGFβ1 represses TGFβR3. This will be discussed further in section 6.3.5.

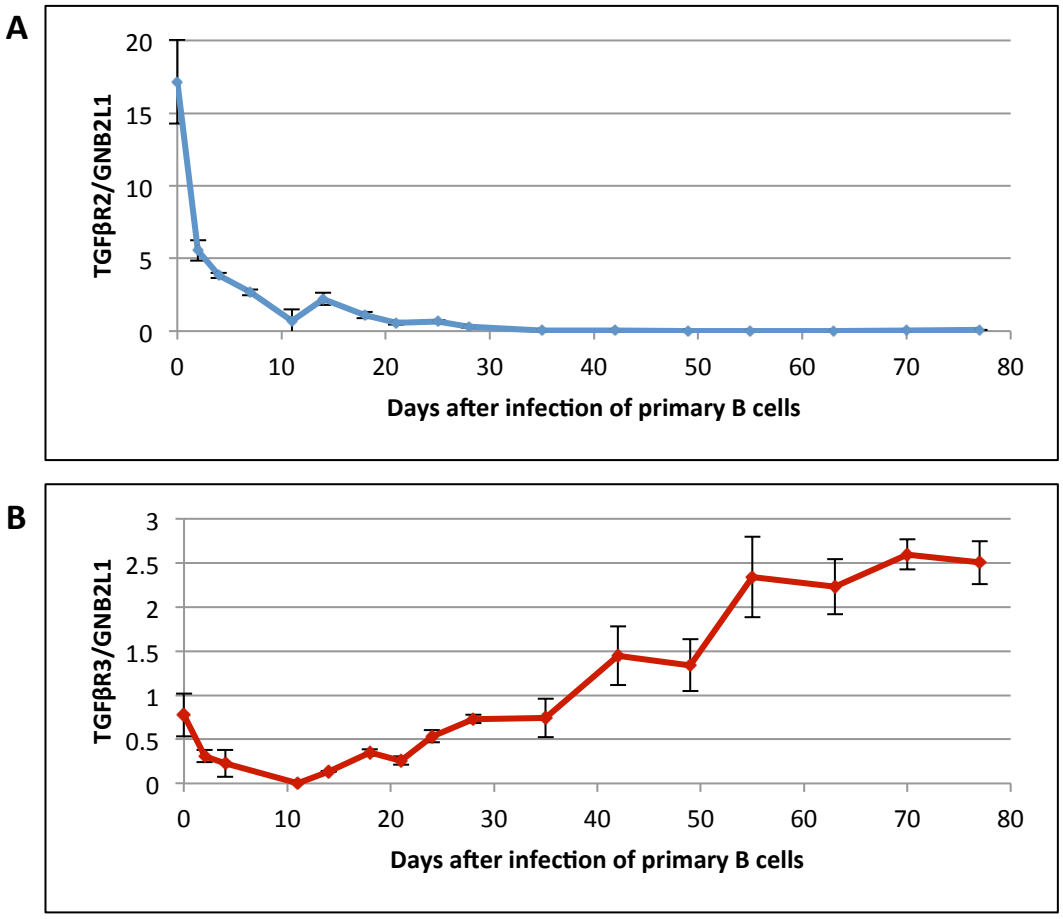



**Figure 6.8: The up-regulation of TGFβR3 occurs later than the down-regulation of TGFβR2 after infection of primary B cells with wild-type EBV**

qRT-PCR for **(A)** TGFβR2 and **(B)** TGFβR3 expression with time after infection of primary B cells with wild-type EBV. The values represent the ratio to the endogenous control gene *GNB2L1*. Error bars represent standard deviation of triplicate qPCR reactions. Data shown are from one of two separate infection experiments, in which the pattern of variation of values is representative.

### 6.3.2 EBNA3B is necessary for the up-regulation of TGFβR3 in LCLs

Since EBNA3B is necessary for the up-regulation of TGFβR3 in BL31 cells (fig. 3.11A-B), the effect of EBNA3B deletion on TGFβR3 expression was investigated in LCLs. qRT-PCR for TGFβR3 in established wild-type, EBNA3B KO and revertant LCLs showed that in both EBNA3B KO lines, TGFβR3 expression is low, whereas it is up-regulated in the wild-type and revertant LCLs (fig. 6.9A). This suggests that EBNA3B is necessary for the up-regulation of TGFβR3 in LCLs.

qRT-PCR for TGFβR3 in primary B cells infected with wild-type and EBNA3B KO viruses showed that after around day 11 TGFβR3 was gradually up-regulated by wild-type EBV; however, this up-regulation did not occur in EBNA3B KO cells, with expression remaining at a steady low level (fig. 6.9B). These findings suggest that EBNA3B is necessary for the up-regulation of TGFβR3 in LCLs, as it is in BL31 cells.

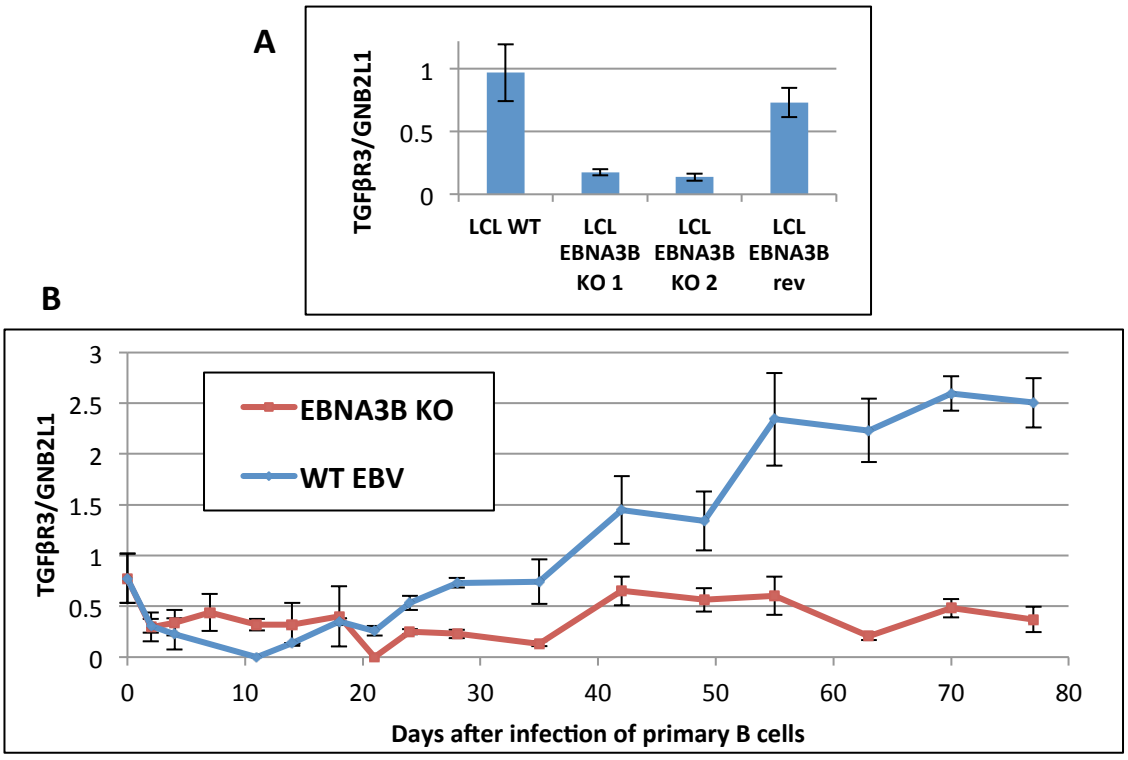



**Figure 6.9: EBNA3B is necessary for the up-regulation of TGFβR3 in LCLs**

**(A)** qRT-PCR for TGFβR3 in a set of established wild-type, EBNA3B KO and EBNA3B revertant LCLs. **(B)** qRT-PCR for TGFβR3 expression with time after infection of primary B cells by wild-type or EBNA3B KO LCLs. The values represent the ratio to the endogenous control gene *GNB2L1*. Error bars represent standard deviation of triplicate qPCR reactions. The data shown are representative of experiments performed at least three times (A) and twice (B).

### 6.3.3 EBNA3C also contributes to the up-regulation of TGFβR3 in LCLs

In order to determine the effect of EBNA3C deletion on TGFβR3 expression in LCLs, this was initially investigated in 3CHT-LCLs established by infection of p16-null donor B cells with 3CHT virus in the presence of 4HT and thus established with EBNA3C present (see section 6.2.5 for explanation). Once these p16 wild-type 3CHT-LCLs were established (at approximately three months post-infection), 4HT was withdrawn from some of the cells for a period of 30 days, then re-added; at the same time those in which growth had been continued in 4HT then had 4HT withdrawn – see figure 6.10A for the outline of this experiment, performed by Rob White, showing the timings at which samples were harvested. RNA was then extracted and qRT-PCR performed for TGFβR3 on these samples. This showed that TGFβR3 expression was relatively high in the established 3CHT-LCLs with 4HT (at day 0 of this experiment, see fig. 6.10A-B), and then expression decreased significantly by 5 days after 4HT was withdrawn, with slight further decreases to day 37 (fig. 6.10B). When 4HT was re-added, there was a slight increase in TGFβR3 expression by day 5 but a more marked increase by day 33. Repeated withdrawal led to a similar reduction in TGFβR3 expression by 33 days post-withdrawal (fig. 6.10B). Similar findings were seen in the array data using the same samples (fig. 6.10C) [www.epstein-barrvirus.org.uk and (Skalska et al. 2013)]. Thus, in contrast to the findings for TGFβR2, TGFβR3 expression appears to vary directly according to EBNA3C expression and continues to be regulated thus even after exposure to EBNA3C has occurred. In addition, the results suggest that the decrease in TGFβR3 expression after 4HT withdrawal is quicker than the increase after 4HT exposure.

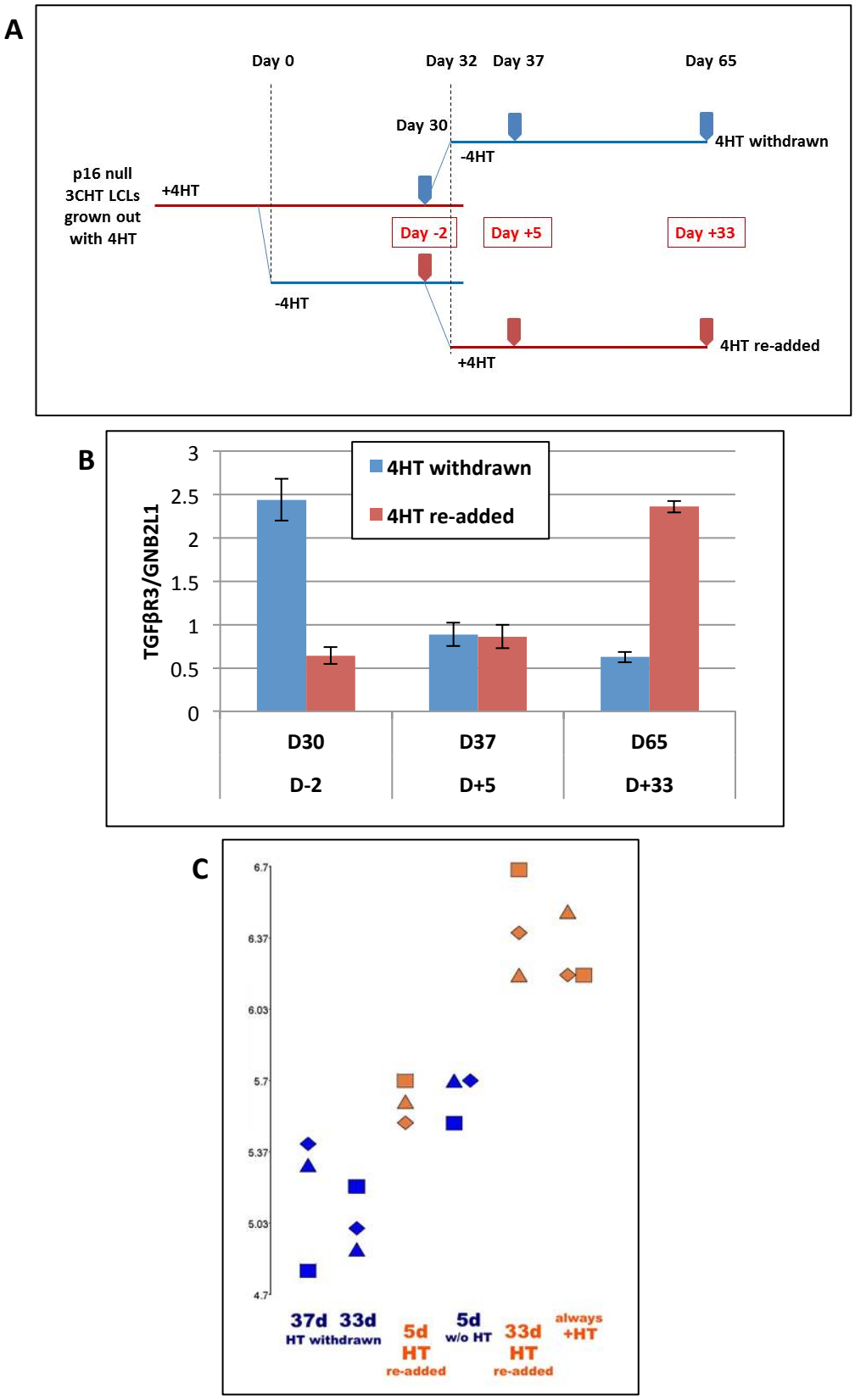

A
Day 0
Day 32
Day 37
Day 65
Day 30
4HT withdrawn
-4HT
p16 null
3CHT LCLs
grown out
with 4HT
+4HT
Day -2
Day +5
Day +33
-4HT
4HT re-added
+4HT
B
4HT withdrawn
4HT re-added
TGFβR3/GNB2L1
3
2.5
2
1.5
1
0.5
0
D30
D-2
D37
D+5
D65
D+33
C
6.7
6.37
6.03
5.7
5.37
5.03
4.7
37d
HT withdrawn
33d
5d
HT
re-added
5d
w/o HT
33d
HT
re-added
always
+HT

**Figure 6.10: In p16-null 3CHT-LCLs established in the presence of 4HT, TGFβR3 expression varies with subsequent 4HT exposure**

**(A)** Schematic diagram of experimental procedure (as performed by Rob White). Peripheral blood lymphocytes (PBLs) from a donor with a homozygous mutation of p16 (p16-null PBLs) were infected with 3CHT virus in the presence of 4HT. Once LCLs were established (after three months grown with 4HT), the cells were divided into two: half were washed and then grown in medium without 4HT (day 0, 4HT withdrawn), and the remainder continued to be grown with 4HT. 30 days after this change samples were harvested for RNA from both sets (4HT withdrawn or 4HT continued), then two days later (i.e. day 32) the media were changed again so that those previously growing with 4HT were washed and then grown without it (upper blue line, 4HT withdrawn) and those growing without it for the past 32 days had 4HT re-added (4HT re-added, lower red line). Samples were then harvested for RNA at day +5 and day +33 of these changes, i.e. days 37 and 65 of the whole experiment. Block arrows show the timing of samples for RNA, with the colours of the arrows represented in the chart in (B). Days shown in red relate to the timing of the second change in conditions, i.e. the re-addition of 4HT. Figure adapted from (Skalska et al. 2013). **(B)** qRT-PCR for TGFβR3 expression showing variation as 4HT is withdrawn (blue) or re-added (red), with the timings as shown in (A). The values are expressed as a ratio to the endogenous control gene *GNB2L1*, with error bars representing standard deviation of triplicate qPCR reactions. This experiment was performed in parallel in three separate LCLs established using two independently derived 3CHT viruses to infect PBLs from a single p16-null donor, with representative data shown here. cDNA samples were obtained from Rob White and qPCRs performed by the author. **(C)** Microarray data showing the results for all cell lines, from the experiment as shown in (A) and (B). Each point represents a single cell line, with the values on the y axis representing mRNA expression. From www.epstein-barrvirus.org.uk and (Skalska et al. 2013).

The effect of EBNA3C on TGFβR3 expression was also investigated in 3CHT-LCLs established without 4HT in a p16-null background, so that the effect of having never been exposed to EBNA3C could be seen. Samples harvested at three months post-infection of PBL with 3CHT virus showed that TGFβR3 expression was higher in those cells grown out in the presence of 4HT, and thus expressing functional EBNA3C, than those which had never expressed functional EBNA3C (fig. 6.11A).

qRT-PCR for TGFβR3 was then performed in samples from a time course experiment in which p16-null 3CHT-LCLs had been established initially in the presence or absence of 4HT, as above. Once the LCLs were established (at around three months post-infection), at day zero in this experiment, the growth conditions were then changed such that each of the two cell lines (grown out with versus without 4HT) were divided into two, and one continued to be grown with 4HT but the other was washed and then grown without 4HT. Thus there were four conditions (as also described in section 6.2.5): (1) never exposed to 4HT, (2) never exposed to 4HT but then exposed once LCLs were established, (3) grown out initially with 4HT but then 4HT withdrawn upon establishment of LCLs, and lastly (4) continuously grown out with 4HT. This experiment was performed by Rob White, but the RNA extraction and qRT-PCR were all performed by the author.

This showed that, as before, TGFβR3 expression was increased in established LCLs grown out with 4HT, i.e. with EBNA3C, compared to those without. Then after the media were changed over, those newly or continuously expressing functional EBNA3C showed a gradual increase in TGFβR3 expression, whereas those no longer or never exposed to EBNA3C showed declining TGFβR3 expression (fig. 6.11B). These findings suggest that EBNA3C is necessary for the up-regulation of TGFβR3 seen in LCLs but that, in contrast to TGFβR2, TGFβR3 expression continues to vary directly with the presence or absence of EBNA3C. The regulation of TGFβR3 by EBNA3C in LCLs is consistent with the findings of Skalska *et al*, where in p16-null 3CHT-LCLs established in the presence of 4HT and in which 4HT was then withdrawn for four weeks, TGFβR3 was shown to be down-regulated over two-fold, thus confirming the induction of TGFβR3 by EBNA3C (Skalska et al. 2013).

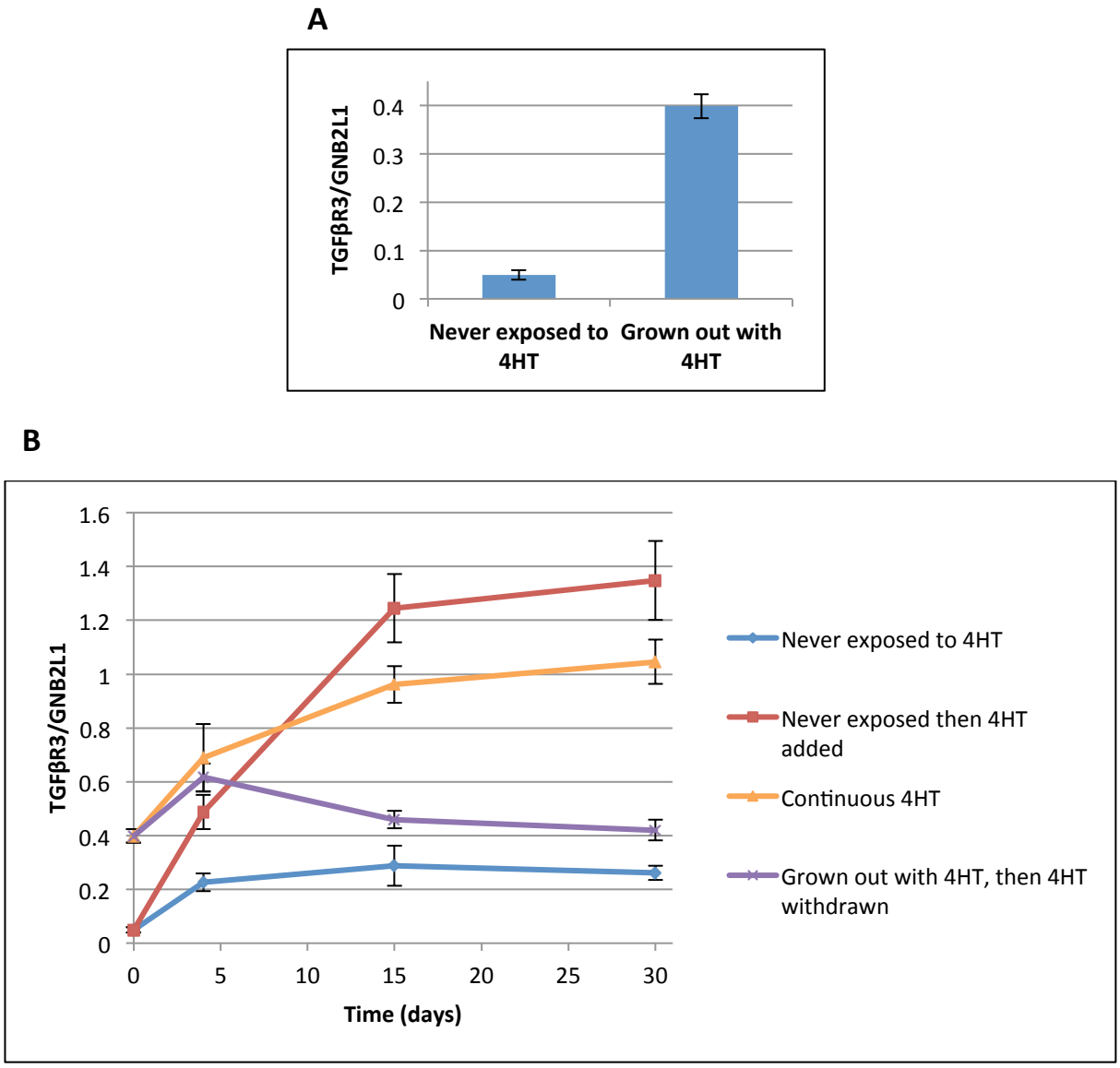



**Figure 6.11: TGFβR3 expression is increased in p16-null 3CHT-LCLs established in the presence of 4HT, and is continuously regulated by 4HT exposure**

**(A)** qRT-PCR for TGFβR3 expression approximately three months post-infection of p16-null PBLs with 3CHT virus either without (never exposed) or with 4HT in the medium. **(B)** Following establishment of p16-null 3CHT-LCLs in the presence or absence of 4HT at around three months post-infection, 4HT was then withdrawn or continued in those established with it, or added or not to those established without it. Samples were harvested at time points after these changes for RNA extraction, reverse transcription and qPCR for TGFβR3 expression. All values are expressed as ratio to the endogenous control gene *GNB2L1*. Error bars represent standard deviation of triplicate qPCR reactions. Cells established, time course performed and samples harvested by Rob White; RNA extractions and qRT-PCR done by the author.

Experiments to investigate the expression of TGFβR3 in EBNA3A KO LCLs showed no consistent effect (data not shown), as has been previously described for TGFβR2 (section 6.2.6). The effect of EBNA3A KO, EBNA3 KO, EBNA3C KO and EBNA3CHT (without 4HT) viruses on TGFβR3 expression after infection of purified primary B cells were also investigated, but as expected the cells infected with these viruses all died before the time at which TGFβR3 expression increased above baseline in wild-type LCLs, hence the data are not shown here.

### 6.3.4 EBNA2, LMP1 and/or LMP2A may also contribute to the up-regulation of TGFβR3 in LCLs

Since LMP1 and LMP2A also appeared to cooperate with EBNA3B and EBNA3C in the up-regulation of TGFβR3 in BL31 cells, the effect of this was also investigated in LCLs. The expression of TGFβR3 was also investigated in EREB2.5 cells by qRT-PCR, in the same experiment as described in section 6.2.7 (see fig. 6.7). Bearing in mind the constraints of the EREB2.5 cell system, including the variability in endogenous control expression, as discussed previously (section 6.2.7), this showed that TGFβR3 expression appeared to be reduced by withdrawal of β-estradiol, correlating with the reduction in functional EBNA2 and LMP1/LMP2A expression (fig. 6.12). However, TGFβR3 was slightly reduced in the cells without β-estradiol compared to with estradiol even on day 0, the reason for which is unclear. TGFβR3 expression decreased markedly even by day one post withdrawal of β-estradiol. This could be consistent with the expression of LMP2A, which appeared to reduce sooner than the reduction of LMP1 or EBNA2. It is not clear why at day 4 the expression of TGFβR3 was seen to increase again (fig. 6.12). Nevertheless, as discussed previously, the findings in EREB2.5 cells are difficult to interpret for several reasons (see section 6.2.7), but could be consistent with a role for LMP2A, LMP1 and/or possibly EBNA2 in the up-regulation of TGFβR3 in LCLs.

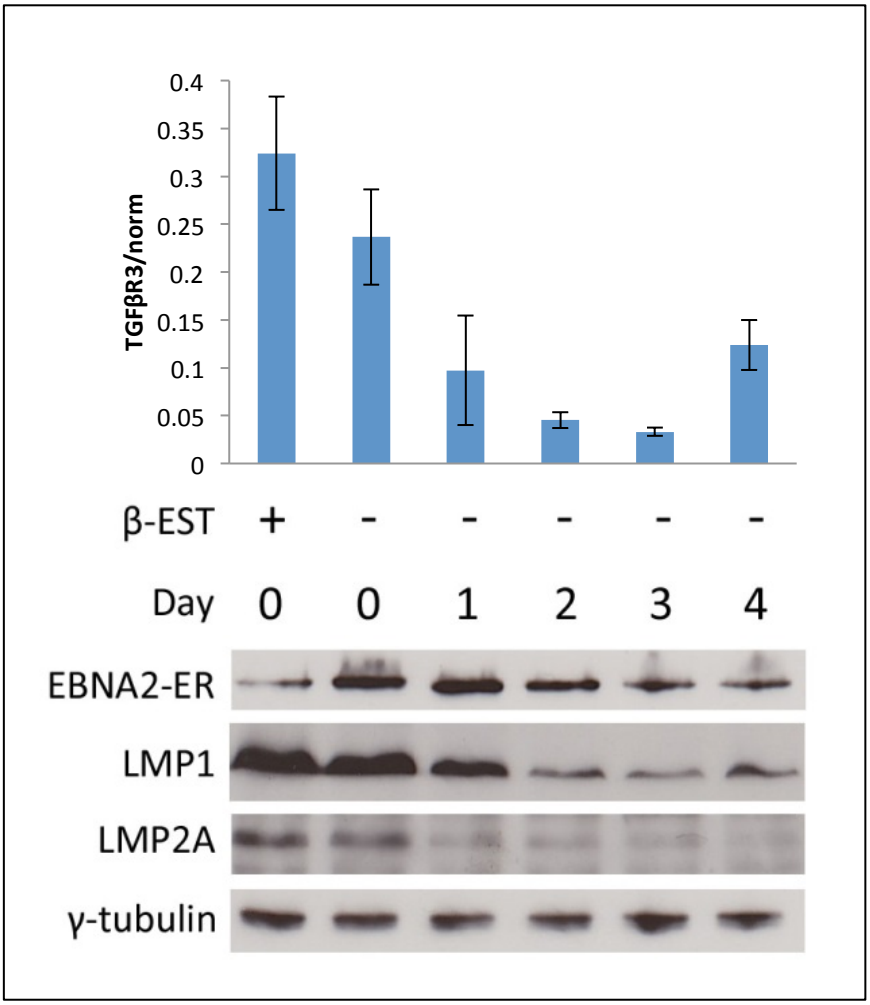



**Figure 6.12: EBNA2, LMP1 and/or LMP2A contribute to the up-regulation of TGFβR3 in LCLs**

qRT-PCR for TGFβR3 in EREB2.5 cells in which β-estradiol has been withdrawn for 4 days (see figure 6.7 for details of experimental procedures). The values are expressed as a ratio to the mean of values of 5 endogenous control genes. Western blot for EBNA2, LMP1 and LMP2A in samples harvested at the same time as the RNA used for the qRT-PCR. γ-tubulin is shown as a loading control.

### 6.3.5 EBV does not significantly alter expression of TGFβ1 or TGFβR1 after primary B cell infection

In BL31 cells, TGFβR2 and TGFβR3 appeared to be reciprocally regulated (figs. 3.1A and 3.11A), although some differences were noted, especially for EBNA3A KO lines (section 4.6, fig. 4.6A-D). In BL31 cells, a possible mechanism for the reciprocal expression was demonstrated by a two-fold de-repression of TGFβR3 in the presence of the TGFβR1 inhibitor SB431542 (fig. 3.13), suggesting that TGFβ1 may repress TGFβR3 when signalling is active, as has been reported in breast and ovarian cancer cells (Hempel et al. 2008). In BL31 cells infected with wild-type EBV, TGFβR2 is repressed and signalling suppressed; this could lead to the relief of TGFβ1-mediated TGFβR3 repression and hence to the up-regulation of TGFβR3 whenever TGFβR2 is repressed to the point where signalling is suppressed (see section 3.4.3).

In view of this it was important to investigate whether the expression of TGFβ1 or TGFβR1 vary after EBV infection of primary B cells, as significant changes in these could also alter TGFβR3 expression if this TGFβ1-mediated repression of TGFβR3 does occur in B cells. qRT-PCR for TGFβR1 and TGFβ1 were performed in the samples harvested after primary B cell infection with wild-type EBV, as previously shown for TGFβR2 and TGFβR3 (for example figs. 6.2 and 6.8). This showed that, although there is some minor variation in TGFβ1 and TGFβR1 expression in the first 14 days or so (which could be explained by the variation in expression of the normalisation controls), overall EBV infection does not have a significant effect on the expression of TGFβ1 or TGFβR1 (fig. 6.13). Since the medium used for these experiments was from the same batch throughout the duration of the experiment, there is also unlikely to have been significant variation in the amount of exogenous TGFβ1 to which the cells were exposed, although this was not formally checked.

Nevertheless, it remains possible that once TGFβR2 is repressed below a critical level, at which signalling via pSMAD2 is suppressed, the repression of TGFβR3 by exogenous and autocrine TGFβ1 could be relieved and hence this is why TGFβR3 subsequently increases. In EBNA3B KO LCLs and p16-null 3CHT-LCLs, even though TGFβR2 remains relatively repressed, TGFβ signalling via pSMAD2 still occurs and thus TGFβ1 would still repress TGFβR3.

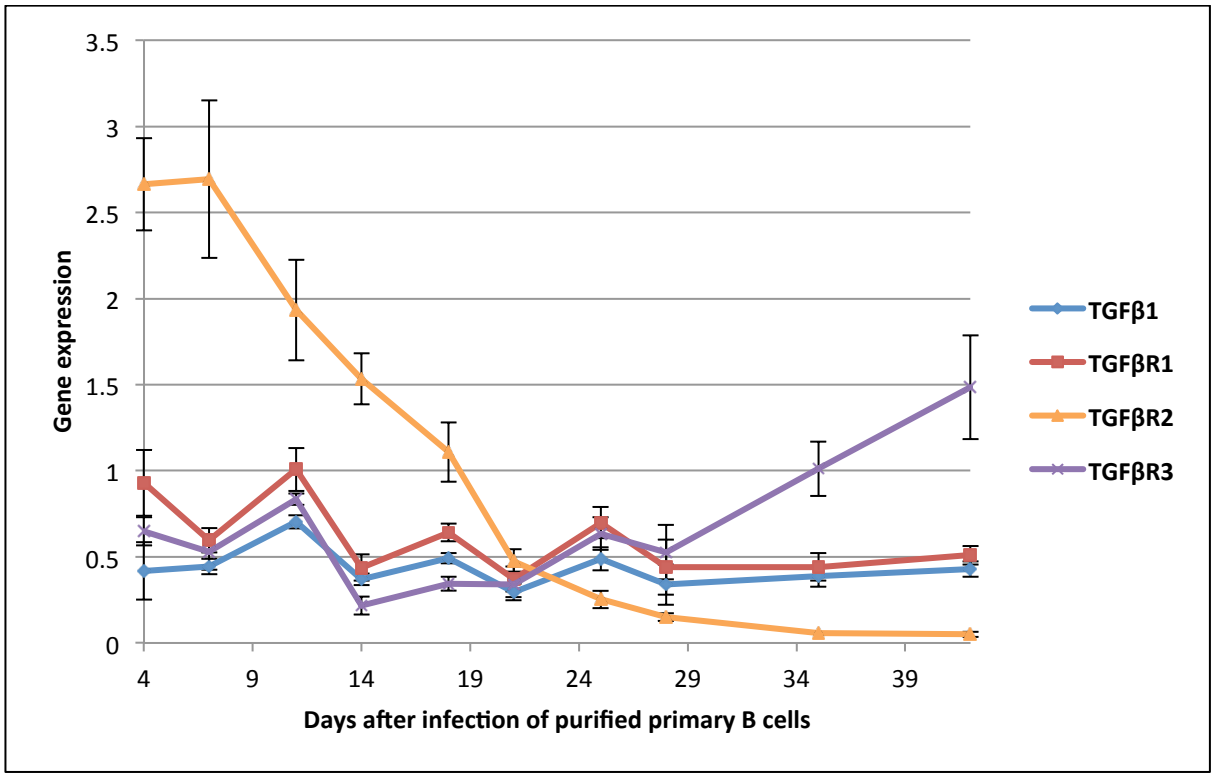



**Figure 6.13: TGFβ1 and TGFβR1 expression do not vary significantly after EBV infection of primary B cells**

qRT-PCR for TGFβ1, TGFβR1, TGFβR2 and TGFβR3 mRNA expression with time after infection of purified primary B cells with wild-type EBV. Values are all expressed as a ratio to the mean of the normalisation genes *GNB2L1* and *RPLPO*. Error bars represent standard deviation of triplicate qPCR reactions. Values are shown starting from day 4 post-infection rather than day 0 so that all values are within a similar range for comparison.

## 6.4 The effects of TGFβ on apoptosis/arrest and p15$^{INK4B}$ expression in LCLs

### 6.4.1 Wild-type LCLs are resistant to TGFβ-induced apoptosis and show no change in the cell cycle with TGFβ treatment

It has previously been shown that LCLs are resistant to the anti-proliferative and pro-apoptotic effects of TGFβ1 (Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Inman and Allday 2000b, Kenney et al. 2001, Horndasch et al. 2002). Therefore, in order to confirm this, wild-type LCLs were treated with TGFβ1 or vehicle for 48 hours and the samples analysed for PARP cleavage by SDS-PAGE and western blot as well as by propidium iodide (PI) stained FC for cell cycle analysis. As expected, this showed no induction of apoptosis, as detected by increased PARP cleavage or an increased sub-G1 component on cell cycle analysis, nor of G1 arrest, by TGFβ in wild-type LCLs (figs. 6.14A-C).

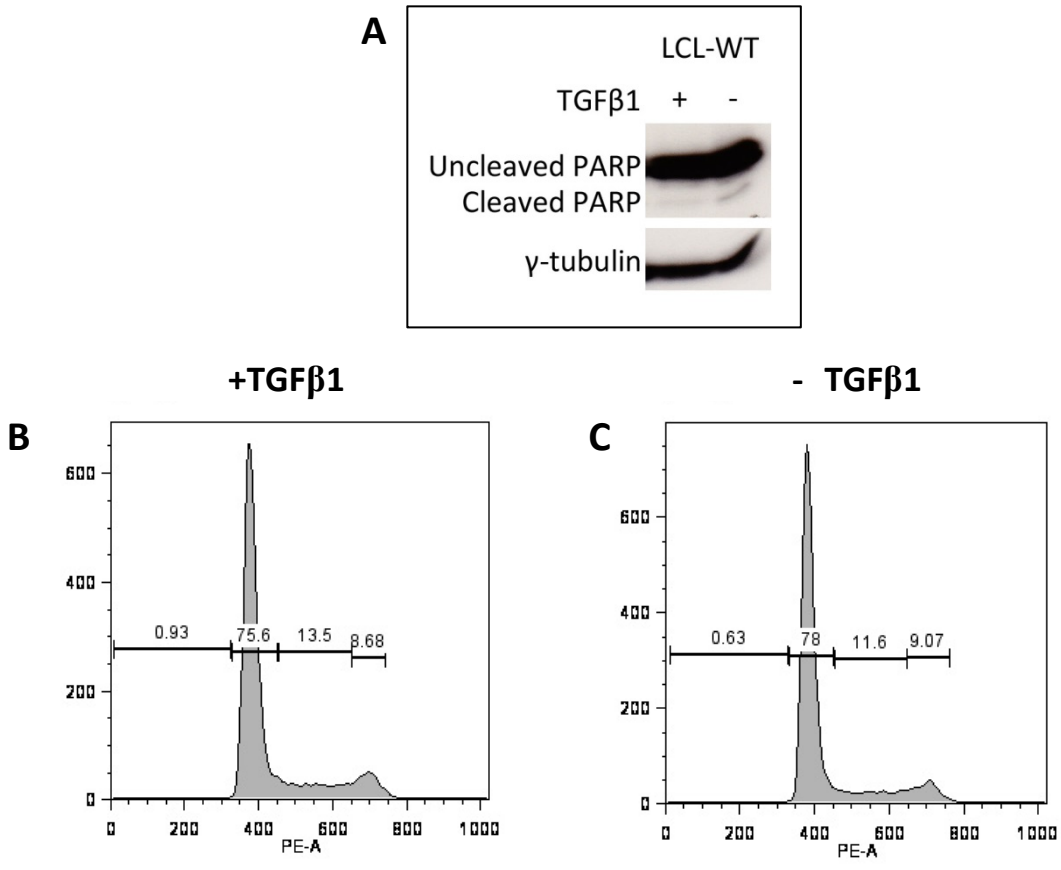



**Figure 6.14: TGFβ1 does not induce apoptosis or G1 arrest in wild-type LCLs**

**(A)** Western blot for PARP after 48 hours' treatment with TGFβ1 5ng/ml or vehicle, with γ-tubulin shown as a loading control. **(B)** Cell cycle analysis by propidium iodide staining and FC in LCL-WT after 48 hours' treatment with TGFβ1 5ng/ml or vehicle.

### 6.4.2 $p15^{INK4B}$ expression is repressed by EBNA3C, but TGFβ does not induce $p15^{INK4B}$ in LCLs

Investigation of binding of EBNA3C to regions around the $p14^{ARF}$/$p15^{INK4B}$/$p16^{INK4A}$ locus had shown binding of EBNA3C proximal to the TSS of *CDKN2B*, the gene encoding $p15^{INK4B}$, suggesting that EBNA3C or EBNA3A may coordinately repress $p15^{INK4B}$ and $p16^{INK4A}$ (hereafter referred to as p15 and p16 respectively) (Skalska et al. 2013). Therefore it was hypothesised that if EBV represses p15, this may also be an additional means of altering the response to TGFβ, since p15 is a known target of TGFβ whose induction in epithelial cells results in growth arrest (Hannon and Beach 1994, Reynisdottir et al. 1995).

qRT-PCR was performed for CDKN2B (p15) mRNA expression in a panel of LCLs including wild-type, EBNA3B KO and revertant, and in the set of p16-null 3CHT-LCLs under four conditions as described in section 6.2.5. This showed that in wild-type, EBNA3B KO and EBNA3B revertant LCLs, p15 was repressed (fig. 6.15A). However, in p16-null 3CHT-LCL grown with continued 4HT, the repression was slightly less than for the wild-type/EBNA3B KO/revertant. For the other conditions in p16-null 3CHT-LCLs, a marked de-repression was seen in the cells which had never been exposed to 4HT and therefore EBNA3C. Cells which had been initially exposed to 4HT but it was then withdrawn, as well as those in which 4HT was not present initially but was subsequently added, had an intermediate level of de-repression (fig. 6.15A). These findings suggest that EBNA3C does indeed repress p15. The effects of TGFβ on induction of p15 was then investigated in these same cell lines, by treating with TGFβ1 10ng/ml or vehicle alone for two hours and then harvesting for RNA and protein. qRT-PCR was performed for p15 (CDKN2B), and in each case the results were expressed as the fold change in p15 expression, i.e. the ratio of the value after two hours' treatment with TGFβ1 or vehicle to that at time zero, untreated (fig. 6.15B). This showed no induction of p15 by TGFβ treatment in any cell line.

Hence even though EBNA3C represses p15, and thus p15 is de-repressed when cells have never been exposed to EBNA3C, TGFβ does not induce p15 expression even though in this case it does induce pSMAD2. This suggests that, although induction of p15 is an important means of TGFβ-induced growth arrest in epithelial cells (Reynisdottir et al. 1995, Iavarone and Massague 1997), this does not appear to be a mechanism used in B cells. TGFβ does not

induce p15 in BL cells [(Spender and Inman 2009b) and fig. 3.9]. Furthermore, in LCLs conditional for EBNA2 and Myc, in the absence of EBNA2 TGFβ induced Id3 and junB and caused growth arrest, without induction of p15 or p21, suggesting that growth arrest also occurs independently of p15 in LCLs (Horndasch et al. 2002). p15 acts by inhibiting the cyclin dependent kinase inhibitors CDK4 and CDK6, leading to cell cycle arrest. However, mutations of p15 are found in many malignancies and in several cell types CDK4/CDK6 inhibition can occur via other mechanisms, hence induction of p15 does not always occur in response to TGFβ (Iavarone and Massague 1997).

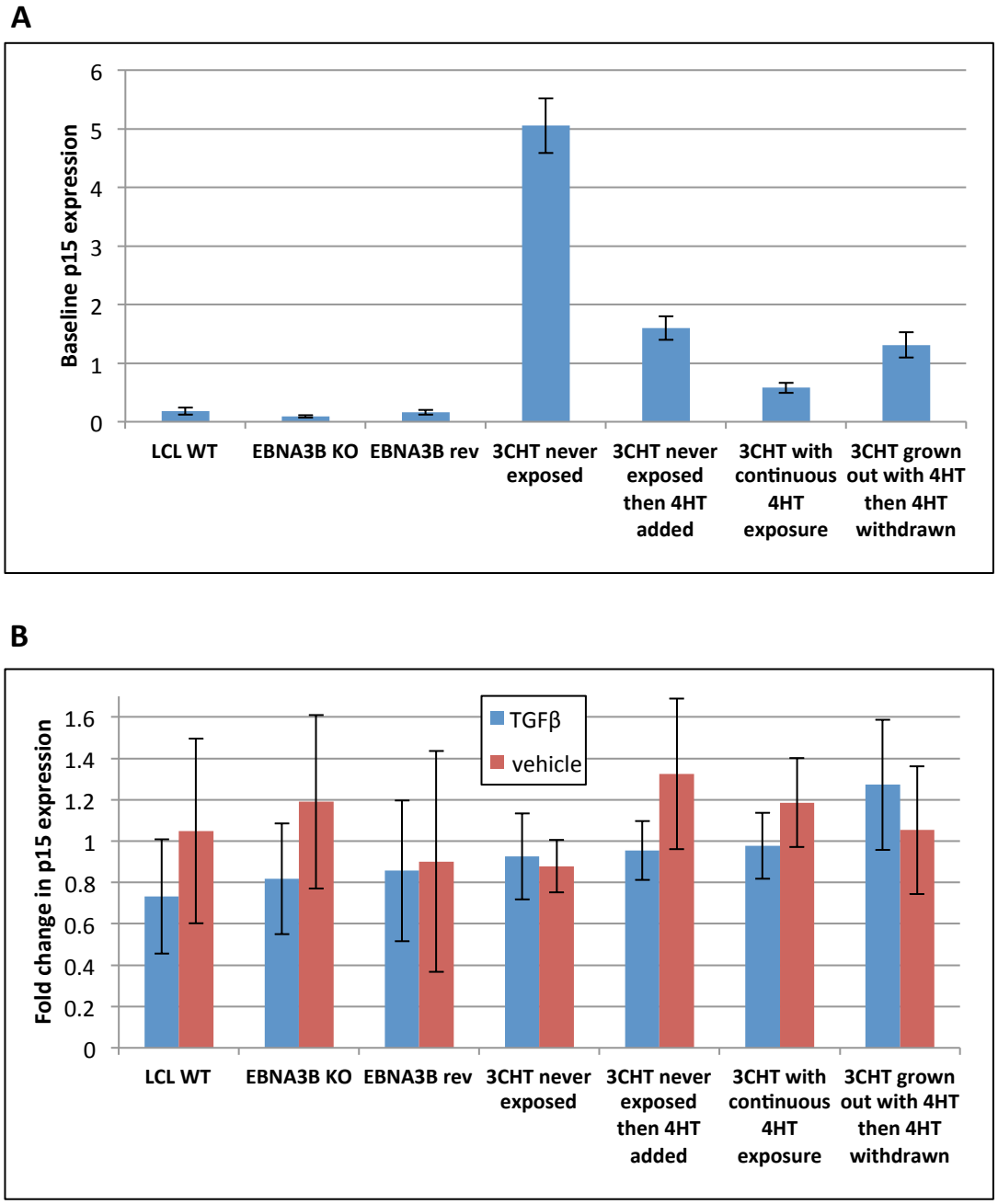



**Figure 6.15: p15$^{INK4B}$ is repressed by EBNA3C but is not induced by TGFβ in LCLs**

**(A)** Baseline expression, without any TGFβ1 treatment, of CDKN2B (p15$^{INK4B}$) mRNA measured by qRT-PCR in all the LCLs as shown. **(B)** Expression of p15$^{INK4B}$ after treatment with TGFβ1 10ng/ml or vehicle alone for two hours in the set of LCLs shown, as in (A). Values are expressed as a ratio of the value after two hours' treatment with TGFβ (blue) or vehicle (2mg/ml BSA in PBS, shown in red) to that at time zero, untreated, with each value calculated as the ratio to the endogenous control gene *GNB2L1*. Error bars represent standard deviations of triplicate qPCR reactions.

## 6.5 Discussion

### EBV represses TGFβR2 in LCLs, leading to suppression of TGFβ signalling via pSMAD2

It was previously known that LCLs are resistant to some of the obvious effects of TGFβ (Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Inman and Allday 2000b, Kenney et al. 2001, Horndasch et al. 2002), and that TGFβR2 is down-regulated in LCLs (Kumar et al. 1991, Inman and Allday 2000b), although other studies had not shown a reduction in TGFβR2 (Horndasch et al. 2002) or did not specifically investigate TGFβR2 expression (Altiok et al. 1991, Altiok et al. 1993). The current study has confirmed the repression of TGFβR2 at the mRNA level in established LCLs (fig. 6.1A) and also shown that EBV represses TGFβR2 after infection of primary B cells with EBV (fig. 6.2), as well as showing that the repression leads to suppression of TGFβ signalling via pSMAD2 in LCLs (fig. 6.1B).

This study has also investigated which latent proteins are responsible for the repression of TGFβR2. When EBNA3B is deleted, some de-repression of TGFβR2 is seen, both in stable LCLs and after primary B cell infections (figs. 6.3A-B). However, the effect of EBNA3B deletion on TGFβR2 mRNA levels appears only moderate, in that TGFβR2 expression in EBNA3B KO LCLs remains very low compared to that in uninfected primary B cells (figs. 6.3A-B). Nevertheless, deletion of EBNA3B leads to a clear increase in TGFβ-induced pSMAD2, suggesting that this apparently modest de-repression of TGFβR2 is sufficient to restore signalling (fig. 6.3C).

Although EBNA3C is required to transform B cells to LCLs, and hence it is not possible to establish EBNA3C KO LCLs in 'normal' B cells, the effect of EBNA3C expression on TGFβR2 expression in LCLs could be investigated using a conditional EBNA3C-modified oestrogen receptor fusion protein system that depends on the presence of 4HT in the medium for EBNA3C function. When these cells (in a wild-type p16 B cell background) were established with 4HT, i.e. with EBNA3C present, TGFβR2 was repressed, as expected since these cells should be equivalent to wild-type LCLs. However, surprisingly, when 4HT was withdrawn from these cells, after such time that EBNA3C would no longer be functional, the expression of TGFβR2 was unchanged (fig. 6.4). There were two possible explanations for this: either that EBNA3C does not contribute to the repression of TGFβR2 in LCLs, unlike in BL31s, or

alternatively that the previous exposure to EBNA3C as the cells were becoming established was able to maintain repression of TGFβR2 even after EBNA3C was removed.

Fortunately, at this time, colleagues in the laboratory had developed a similar conditional system in which LCLs could be established in the absence of EBNA3C, by using B cells from an individual deficient in p16. Using this system, the p16-null 3CHT-LCL, it was possible to demonstrate some de-repression of TGFβR2 in those cells which had never been exposed to 4HT, thus to functional EBNA3C, compared to those cells established with 4HT (fig. 6.5A-B). This de-repression again appeared to be minimal, with overall expression of TGFβR2 remaining markedly lower than in primary B cells. However, this minimal level of de-repression again appeared to be sufficient to restore TGFβ signalling via pSMAD2 in those cells which had been established without 4HT compared to those established with it (fig. 6.5D). Furthermore, it was observed that once the p16-null 3CHT-LCL cells were established with or without 4HT, subsequent changing of growth conditions by addition or withdrawal of 4HT did not significantly alter TGFβR2 expression or TGFβ signalling (fig. 6.5C-D).

Taken together, these findings suggest that the presence of functional EBNA3C as LCLs are established contributes to the repression of TGFβR2 but, once established, the presence of EBNA3C is no longer required to maintain the repression and consequent suppression of signalling. EBNA3C has been shown to bind to the promoter regions of several repressed genes, in association with repressive chromatin modifications (Skalska et al. 2010, McClellan et al. 2012, Paschos et al. 2012, Skalska et al. 2013). Hence similar epigenetic mechanisms of repression of TGFβR2, which are heritable to daughter cells, could explain why the continued presence of EBNA3C is no longer required for maintenance of the repression. Although H3K27Me3 was increased on the TGFβR2 promoter in wild-type LCLs (fig. 6.1D), due to time constraints this was not investigated in the 3CHT-LCLs. However, investigation of chromatin modifications at the TGFβR2 could be performed in future in the p16-null 3CHT-LCLs established with 4HT, compared to those established without 4HT, in order to confirm this epigenetic repression of TGFβR2 by EBNA3C.

Hence EBNA3B and EBNA3C both contribute to the repression of TGFβR2, which may occur via epigenetic histone modifications, in LCLs as they do in BL31 cells. This in turn leads to

suppression of TGFβ signalling. However, in contrast to the marked level of repression of TGFβR2 in wild-type LCLs and BL31-WT compared to uninfected BL31 or primary B cells, the contributions of EBNA3B and EBNA3C are very small in LCLs. This is in contrast to the findings in BL31, where individual deletions of EBNA3B or EBNA3C lead to increases in TGFβR2 expression to approximately 25% of the level seen in uninfected BL31 cells (fig. 3.1A). Thus the contribution of both EBNA3B and EBNA3C to the repression of TGFβR2 in LCLs appears to be much more modest than in BL31 cells. This also suggests that, in LCLs, one or more other latent proteins (or possibly miRNAs) may be responsible for the repression, probably having a greater effect than EBNA3B or EBNA3C.

Since in BL31 cells, LMP1 and LMP2A appear to cooperate with EBNA3B and EBNA3C to cause repression of TGFβR2, it is likely that one or both of these proteins also contributes to repression of TGFβR2 in LCLs. Unfortunately, it was not possible to produce viable LMP2A KO LCLs in order to investigate this, although this should be possible and has been achieved by other groups. This should be a priority for future work leading on from this project. Nevertheless, investigation of TGFβR2 after withdrawal of oestrogen in the conditional oestrogen-dependent EREB2.5 cell line was suggestive of de-repression of TGFβR2 occurring with decreasing expression of EBNA2, LMP1 and LMP2A (fig. 6.7). However, since in these cells expression of all three latent proteins was reduced after oestrogen withdrawal, it is not possible to conclude which specific latent protein(s) is/are responsible. In addition, due to the nature of the EREB2.5 cell line's response to oestrogen withdrawal, it is difficult to ascribe the changes in TGFβR2 expression purely to changes in latent gene expression as the cells undergo multiple changes after oestrogen withdrawal, reflected in the significant variation of all endogenous control genes used. Also, the effect of oestrogen withdrawal on expression/function of the EBNA3s could not be investigated in these cells, because available antibodies do not recognise the EBNA3 proteins (being derived from type 2 P3HR1 virus), hence it cannot be excluded that the de-repression of TGFβR2 was due to decreases in expression of EBNA3B or EBNA3C.

After primary B cell infection, TGFβR2 expression was gradually reduced, reaching its nadir at around 21 days post-infection (fig. 6.2). This timing is consistent with similar studies performed by other laboratory members for BIM, Notch2, COBLL1, ADAM28 and

ADAMDEC1 expression after primary B cell infection [Lenka Skalska PhD thesis, Kostas Paschos and Jens Kalchschmidt, unpublished data, and (Skalska et al. 2013)]. Furthermore, in a microarray analysis comparing gene expression in uninfected B cells, proliferating blasts at day 6 post-infection and LCLs, Price *et al* found that TGFβR2 was significantly down-regulated between the proliferating blasts and LCL stages, rather than from uninfected to proliferating stages (Price et al. 2012).

A recent study has shown that LMP1 expression at both mRNA and protein levels did not reach steady state until day 21 but then was stable at that level up to day 35, when the experiment was terminated (Price et al. 2012). In the current study, preliminary data on mRNA expression of both LMP1 and LMP2A showed a similar delay in reaching maximum steady state levels. The expression of LMP1 is therefore considerably delayed after primary B cell infection, in comparison to EBNA2 and the EBNA3s which are fully expressed within the first few days after infection [Allday laboratory unpublished data and (Nikitin et al. 2010)]. Although the onset of expression of LMP2A during B cell transformation does not appear to have been previously investigated, it is conceivable that this has a similar delay in reaching full expression, as found in preliminary data in the current study.

Therefore the delay in expression of LMP1 and LMP2A, if either or both do also contribute to the repression of TGFβR2 in LCLs, may partly explain why the repression is not maximal until around 21 days post-infection. A recent microarray analysis in splenic B cells also suggested that LMP2A down-regulates TGFβR2 (Shair and Raab-Traub 2012). To further investigate the contributions of LMP1 and LMP2A to TGFβR2 expression in LCLs, this could in future be investigated using LCLs established by the Kempkes group which are conditional for both EBNA2 expression and c-Myc expression, so that cell proliferation can be maintained even when EBNA2 is withdrawn (Horndasch et al. 2002). Other possible means of investigation could include using siRNA or anti-sense to LMP1 or LMP2A, such as the anti-sense to LMP1 used by Kenney *et al* (Kenney et al. 1998, Kenney et al. 2001).

**The effects of down-regulation of TGFβR2 in LCLs**

The down-regulation of TGFβR2 in wild-type LCLs results in suppression of TGFβ signalling via pSMAD2 (figs. 6.1A-B). In the EBNA3B KO LCLs and p16-null 3CHT-LCLs established

without 4HT, TGFβR2 was only slightly de-repressed, with expression remaining generally low compared to the expression level in uninfected B cells or BL31 cells (figs. 6.3A-B and 6.5A-C). However, this apparent slight de-repression of TGFβR2 led to a definite increase in TGFβ signalling via pSMAD2 (figs. 6.3C and 6.5D). The reason for this apparent discrepancy between the expression of TGFβR2 and the degree of pSMAD2 is not clear, but it may be that a particular threshold of TGFβR2 expression has to be reached for signalling to occur. It was considered whether the concomitant up-regulation of TGFβR3 could itself augment TGFβ signalling, so that TGFβ signalling can occur even with low expression of TGFβR2. However, in the EBNA3B KO LCLs and the 3CHT-LCLs established without 4HT, where slight de-repression of TGFβR2 was seen but signalling was restored, TGFβR3 was not up-regulated, excluding this as a possibility.

In addition, wild-type B95.8-derived LCLs are resistant to apoptosis and cell cycle arrest with TGFβ treatment (fig. 6.14), as had been previously shown (Blomhoff et al. 1987, Janssen et al. 1990, Altiok et al. 1991, Kumar et al. 1991, Altiok et al. 1992, Altiok et al. 1993, Inman and Allday 2000b, Kenney et al. 2001, Horndasch et al. 2002). The effects of TGFβ on the cell cycle were not investigated in the EBNA3B KO and 3CHT-LCLs, but it would be interesting see whether the restoration of signalling seen in these cells would result in TGFβ-induced apoptosis or growth inhibition. However, as discussed for BL31s in chapter 5, the link between pSMAD2 and the effects of TGFβ (i.e. apoptosis/growth arrest) appear to be indirect, and it is possible that pSMAD3 is more important for apoptosis even in B cells, as has been shown for epithelial cells; unfortunately pSMAD3 was not investigated in this study.

The effects of EBNA2 expression on TGFβ responses have been previously investigated in LCLs using an LCL with conditional expression of both EBNA2 and Myc; in this way the cells are able to continue growing even in the absence of EBNA2. When EBNA2 was expressed, the cells became resistant to TGFβ-induced growth inhibition, although no difference was seen in the cell cycle profiles between those with and without EBNA2, so the mechanism of the resistance to TGFβ–induced growth inhibition was not clear. The effect of TGFβ on several target genes was also investigated, and although neither p15 nor p21 were induced in these cells under any condition, Id3 was induced by TGFβ. However, there was no effect

of the presence or absence of EBNA2 on the level of Id3 induction by TGFβ. The authors concluded that the presence of EBNA2 made cells resistant to the growth-inhibitory effects of TGFβ, although signalling (as detected by Id3 induction) still occurred (Horndasch et al. 2002). This suggests that TGFβ signalling is not directly linked to its growth inhibitory effects, as discussed for BL31s in chapter 5.

**EBV also up-regulates TGFβR3 in LCLs**

This study demonstrated that EBV also up-regulates TGFβR3 in BL31 cells, and that in many cases the expression of TGFβR3 and TGFβR2 appears to be reciprocally regulated (see chapters 3 and 4). Therefore the expression of TGFβR3, and the relationship between expression of TGFβR2 and TGFβR3, were investigated in LCLs. This confirmed that EBV does up-regulate TGFβR3 also in LCLs (fig. 6.8B). Although after primary B infections, TGFβR2 is repressed and TGFβR3 is up-regulated by EBV, the timing of these changes is different, and it was surprising to see that TGFβR3 was in fact initially down-regulated before being up-regulated later, and much more slowly, after infection (fig. 6.8B). Thus the up-regulation of TGFβR3 occurs later than the down-regulation of TGFβR2.

The role of individual latent proteins in the up-regulation of TGFβR3 was also investigated. EBNA3B and EBNA3C apparently cooperate in this, to a greater extent for the up-regulation of TGFβR3 than for the down-regulation of TGFβR2 (figs. 6.9, 6.10 and 6.11). The involvement of LMP1, LMP2A or indeed EBNA2 in these changes in LCLs could not be fully ascertained due to the limitations described above (section 6.3.4). Since the up-regulation of TGFβR3 occurred later than the down-regulation of TGFβR2, this suggests that TGFβR2 and TGFβR3 are not directly reciprocally regulated, at least in LCLs. It is possible that once TGFβR2 expression falls below a threshold, then TGFβR3 is up-regulated.

In the p16-null 3CHT-LCLs, the up-regulation of TGFβR3 depends on the presence of 4HT in the medium, i.e. the continued presence of EBNA3C is required for the maintenance of TGFβR3 up-regulation (figs. 6.10 and 6.11). In addition, after 4HT is withdrawn from these cells, TGFβR3 expression decreases quickly, with expression similar 5 days after withdrawal to that at 33 days, whereas following addition of 4HT, the expression is only slightly increased by 5 days but more so at 33 days (fig. 6.10B). Therefore, 4HT is slower to induce

TGFβR3 than its withdrawal is to decrease it. In 3CHT-LCLs, EBNA3C expression was not significantly decreased until 7 days after withdrawal, but significant amounts were again expressed just one day after re-addition (Skalska et al. 2010), and thus the difference in timings of TGFβR3 expression upon addition or withdrawal of 4HT are not due to variations in time to alter EBNA3C expression.

Once LCLs were established in the presence of 4HT, and thus EBNA3C, this led to repression of TGFβR2, which was not reversed even if 4HT (functional EBNA3C) was subsequently withdrawn (figs. 6.4 and 6.5A-C). This contrasts with the regulation of TGFβR3 in these cells, where TGFβR3 expression is altered fairly rapidly according to the presence or absence of 4HT, with the withdrawal of 4HT in particular leading to a fairly rapid, near maximal, decrease in TGFβR3 expression even before EBNA3C expression has been completely lost (fig. 6.10).

There are several possible mechanisms for the up-regulation of TGFβR3 in LCLs; these will be discussed in turn. Firstly, this could be a clonal selection process. This may be suggested by the very gradual increase over a ten-week period after initial infection of primary B cells, and the fact that down-regulation of TGFβR3 is seen over the first few days post-infection. In further support of this clonal selection, a lack of up-regulation of TGFβR3, despite TGFβR2 down-regulation, was seen by day 42 in one particular experiment in which primary B cells were infected with EBNA3B revertant (see figure 7.10C). However, the fact that TGFβR3 up-regulation occurred consistently in the majority of experiments in LCLs, and was consistent in BL31s, suggests that if this is a clonal selection process then there must be highly favourable conditions for this to develop.

The functions of TGFβR3 in B cells are not completely understood, and although in certain contexts it can inhibit TGFβ signalling, it generally serves to enhance signalling. It has tumour suppressor properties, with loss of expression occurring in many non-haematopoietic malignancies (Florio et al. 2005, Dong et al. 2007, Hempel et al. 2007, Turley et al. 2007, Finger et al. 2008b, Gordon et al. 2008, Margulis et al. 2008, Cooper et al. 2010), and has more recently been shown to be tumour suppressive in multiple myeloma cell lines (Lambert et al. 2011); however, its expression is increased in CLL cells compared to normal B

cells, although the reason for this is not known (Klein et al. 2001, Jelinek et al. 2003). In addition, it is essential for haematopoiesis, although its exact functions in this are not known (Stenvers et al. 2003). Since it is required for signalling by TGFβ2 (Stenvers et al. 2003), and also enhances signalling by activins/inhibins, it is possible that its up-regulation may provide a selective advantage due to the effects of one of these ligands.

EBNA3B and EBNA3C seem to be very clearly necessary for the up-regulation of TGFβR3 so this does not entirely fit with a clonal selection process – unless they are necessary, instead, for an unknown intermediate event which itself strongly predisposes to TGFβR3 up-regulation. Furthermore, TGFβR3 expression continues to vary with the addition and removal of 4HT in p16-null 3CHT-LCLs (figs. 6.10 and 6.11), suggesting a direct effect of EBNA3C on TGFβR3 expression, and hence not entirely consistent with the hypothesis that this is a clonal selection process.

The second possibility is the relief of TGFβ1-mediated repression of TGFβR3, as suggested by the de-repression of TGFβR3 upon treatment of EBV-negative BL31 cells with the TGFβR1 inhibitor SB431542 (see chapter 3, fig. 3.13). If this were the case in LCLs, then the repression of TGFβR2 in wild-type LCLs, leading to suppression of TGFβ signalling via pSMAD2 (fig. 6.1A-B), would block the TGFβ1-mediated repression of TGFβR3 and hence lead to up-regulation of TGFβR3. Thus, after primary B cell infection, TGFβR3 would not be de-repressed until TGFβR2 repression reaches a level at which TGFβ signalling is suppressed enough to block the TGFβ1-mediated repression of TGFβR3. This could explain why the up-regulation of TGFβR3 does not occur until after the down-regulation of TGFβR2 (fig. 6.8). In addition, in EBNA3B KO LCLs and p16-null 3CHT-LCLs established without 4HT, in which TGFβR2 is slightly de-repressed compared to wild-type, and in which this is sufficient to restore TGFβ signalling via pSMAD2 (figs. 6.3 and 6.5), TGFβ1-mediated repression of TGFβR3 would still occur and hence TGFβR3 remains repressed. Furthermore, since TGFβ1 and TGFβR1 expression were not significantly altered after primary B cell infection (fig. 6.13), the main variable affecting the TGFβR3 expression after primary B cell infection would be the presence of TGFβ signalling through TGFβR2. This could also be an explanation for why EBNA3B and EBNA3C seem to be more clearly involved in the up-regulation of TGFβR3

than the down-regulation of TGFβR2, i.e. that the TGFβR3 expression is related to the degree of TGFβ signalling, rather than to the expression of TGFβR2 itself.

However, in p16-null 3CHT-LCLs established with 4HT, TGFβR2 remains repressed, with suppression of TGFβ signalling, even when 4HT is subsequently withdrawn (fig. 6.4C). This would be expected to produce continued de-repression of TGFβR3, i.e. TGFβR3 would be up-regulated in these cells regardless of whether 4HT was continued or withdrawn. However, this is not the case, as TGFβR3 expression continues to vary with the presence or absence of 4HT, and thus EBNA3C, even though TGFβR2 does not vary in this way. Therefore the findings in the p16-null 3CHT-LCLs established without 4HT do not fit with the hypothesis of TGFβ1-mediated repression of TGFβR3.

A third mechanism is that the up-regulation of TGFβR3 by EBV occurs directly, i.e. is not dependent on TGFβR2 expression. This is suggested by the fairly rapid and continued variation of TGFβR3 expression in p16-null 3CHT-LCLs as 4HT is added or withdrawn from the medium (figs. 6.10 and 6.11). However, if this were a direct effect of EBV (EBNA3C) on TGFβR3 transcription, then it is not clear why the process of TGFβR3 up-regulation occurs so slowly after primary B cell infection (fig. 6.8). It is also possible that EBV up-regulates TGFβR3 directly via epigenetic mechanisms in LCLs, in a similar way to that suggested in BL31 cells by the variation in histone modifications at the proximal TGFβR3 promoter (fig. 3.12), although this has not been investigated in 3CHT-LCLs. TGFβR3 has been shown to be de-repressed by treatment with TSA and azacytidine, suggesting it is epigenetically down-regulated, in prostate and ovarian cancer (Hempel et al. 2007, Turley et al. 2007).

A fourth possible mechanism for the up-regulation of TGFβR3 is that the suppression of TGFβ signalling leads the cell to increase TGFβR3 in order to try to restore signalling. This could occur if there was an advantage to the cells having intact TGFβ signalling *in vitro,* and thus the cellular response to lack of TGFβR2 and consequent lack of signalling would be to up-regulate its TGFβR3 in order to try to augment signalling from any remaining TGFβR2 receptors. However, there is no evidence that this occurs in other cell types, for instance in solid tumours/epithelial cells where TGFβR2 is mutated or repressed there are no reports of up-regulation of TGFβR3. Furthermore, it is likely to be an advantage to cells in culture to

become resistant to TGFβ signalling which could otherwise limit their proliferation. Also, this mechanism again does not really fit with the findings of variation of TGFβR3 expression with presence/absence of 4HT in 3CHT-LCLs.

Therefore, no single mechanism for the up-regulation of TGFβR3 by EBV appears to fit with all the findings in the current study. Nevertheless, it is possible that a combination of these mechanisms may occur, for instance the TGFβ1-mediated repression of TGFβR3 occurs but there is an additional direct effect of EBNA3C on TGFβR3 transcription.

As discussed in chapter 3 and above, the consequences of the up-regulation of TGFβR3 by EBV are not known; however, one possibility is that this promotes or inhibits BMP signalling. This will be investigated in the next chapter, chapter 7.

**In LCLs, EBNA3C represses $p15^{INK4B}$; however, TGFβ does not induce its expression in LCLs**

In this study, the expression of $p15^{INK4B}$ (referred to hereafter as p15) was also investigated in LCLs, since preliminary investigations by colleagues in the laboratory had revealed that EBNA3C binds to the promoter region of p15 (Skalska et al. 2013). It was therefore hypothesised that by repressing p15 in LCLs, EBV would prevent TGFβ-mediated induction of p15, which is a mechanism for TGFβ-induced growth arrest in epithelial cells (Hannon and Beach 1994). This study confirmed that p15 is repressed in wild-type LCLs, with de-repression in p16-null 3CHT-LCLs which have never been exposed to 4HT and hence EBNA3C (fig. 6.15A). However, even in the cell lines where total p15 was de-repressed, there was no induction of p15 by TGFβ treatment (fig. 6.15B). p15 was similarly not induced by TGFβ in BL31 cells, when pSMAD2, Id1 and Id2 were all induced (fig. 3.9). This is consistent with previous findings in B cells, in which TGFβ generally does not induce p15, and the mechanism of TGFβ-induced growth arrest is different from that in epithelial cells (Horndasch et al. 2002, Spender and Inman 2009b). Thus the TGFβ-mediated induction of p15 may be specific to epithelial cells.

In EBV-negative BL31 cells, p15 expression was completely repressed, but was up-regulated by EBV (fig. 3.9). Despite the up-regulation by EBV in BL31 cells, no induction of p15 was seen in response to TGFβ (fig. 3.9); this would be consistent with the repression of TGFβR2

and suppression of TGFβ signalling by EBV. It has been reported that the p15 promoter often shows CpG methylation, consistent with gene silencing, in BLs (Klangby et al. 1998). Therefore EBV infection seems to de-repress p15 in BL31 cells, but the effect of this is not known.

The effect of EBV on p15 expression is therefore completely different in BL31 cells and LCLs, since EBV up-regulates (or de-represses) p15 in BL31 cells, yet represses it in LCLs. Nevertheless, future work could investigate the apparent repression of p15 by EBNA3A and EBNA3C in LCLs, since these same latent proteins are already known to repress p16 and thus this could be another mechanism by which EBV promotes lymphomagenesis.

# Chapter 7 The effects of EBV on BMP signalling and its canonical receptor-mediated SMADs in B cells

## 7.1 Introduction

BMP signalling pathways are considerably more diverse than TGFβ signalling, involving four type I and three type II receptors, in contrast to one of each in the TGFβ signalling pathway. There are also at least 20 different ligands for BMP receptors. At the start of this project, relatively little was known about the effects of BMPs in lymphoid cells. It had been shown that BMP6 inhibits proliferation of human peripheral blood naïve and memory B cells that had been stimulated to proliferate by treatment with anti-IgM alone or with CD40L, as well as in the EBV-negative BL line Ramos (Kersten et al. 2005). BMP6 also inhibits proliferation of bone marrow normal human B cell progenitor cells *in vitro* (Kersten et al. 2006). In addition, several BMP ligands are pro-apoptotic or anti-proliferative in myeloma cells (Kawamura et al. 2000, Hjertner et al. 2001, Ro et al. 2004, Fukuda et al. 2006b, Seckinger et al. 2009). However, the effects of BMPs in other malignant B cell disorders or EBV-infected B cells were not known.

BMP signalling is closely related to TGFβ signalling, and the current study has shown that EBV up-regulates TGFβR3, which is also a co-receptor for BMP signalling. Thus it was hypothesised that the up-regulation of TGFβR3 by EBV may alter BMP signalling, and that this may in turn lead to tumour-promoting effects. Therefore, in this chapter, the effects of EBV on BMP signalling and expression of some of its components were investigated.

## 7.2 The effects of EBV on BMP signalling and its effectors in BL31 cells

### 7.2.1 In BL31 cells, latent EBV leads to increased signalling in response to BMP2, BMP4 and BMP6 but not BMP7 or BMP9

TGFβR3 directly binds multiple ligands, including BMP2, BMP4 and BMP7, and facilitates their binding to the relevant type II receptors, therefore enhancing BMP signalling (Gatza et al. 2010). It has also been shown that TGFβR3 presents BMP2 to the type I receptors BMPRIA or BMPRIB, increasing signalling by BMP2 (Kirkbride et al. 2008). Conversely, inhibin, an inhibitor of BMP signalling, requires TGFβR3 for binding to its type II receptors

activin receptor II and BMPR2 (Gatza et al. 2010). Hence theoretically the up-regulation of TGFβR3 could also result in enhanced inhibition of BMP signalling. It was therefore hypothesised that, as a consequence of up-regulating TGFβR3 expression, EBV might alter BMP signalling.

Uninfected and wild-type EBV infected BL31 cells were treated with a range of BMP ligands. The BMPs used were selected to include BMP2, BMP4 and BMP7 because they have been shown to bind to TGFβR3, as well as BMP6 as it has been shown to have effects in both normal and malignant B cells. BMP2 and BMP4 are from the same subgroup of ligands; similarly BMP6 and BMP7 are from the same subgroup (Derynck and Miyazono 2008). BMP9 was also investigated as it was predicted to behave somewhat differently from the others: it is from a third subgroup, has been shown to be pro-proliferative in certain cell types and conditions (Herrera et al. 2009, Herrera et al. 2013), but anti-proliferative in others (David et al. 2007), and had not been shown to have effects in lymphoid cells.

A preliminary experiment was performed using BMP4 at two different concentrations (50ng/ml and 100ng/ml); the induction of C-terminal phosphorylation of SMAD1/5/8 (pSMAD1/5/8) was similar with both doses, so 50ng/ml was used in subsequent experiments (data not shown). For the other ligands, a single dose was selected based on previous literature and if this induced pSMAD1/5/8 in preliminary experiments, the same dose was used for any further experiments. The canonical signalling response to ligand was assessed after one hour of treatment with the relevant BMP or an equivalent volume of vehicle (0.5% BSA in 4mM HCl), by performing western blot for total SMAD1, SMAD5, SMAD1 linker phosphorylation (pSMAD1), SMAD5 C-terminal phosphorylation (pSMAD5) and pSMAD1/5/8 in parallel (fig. 7.1). No commercially available antibody could be identified for SMAD8 or pSMAD8 and the antibody to SMAD5 proved variable in its successful use.

Although total SMAD1 expression was increased in EBV-infected, relative to EBV-negative, BL31 cells (see section 7.2.5 for further details), there was a more marked increase in the induction of pSMAD1 with BMP2, BMP4 and BMP6 in EBV-infected compared to EBV-negative BL31 (fig. 7.1A-C). pSMAD5 and pSMAD1/5/8 were also increased more in

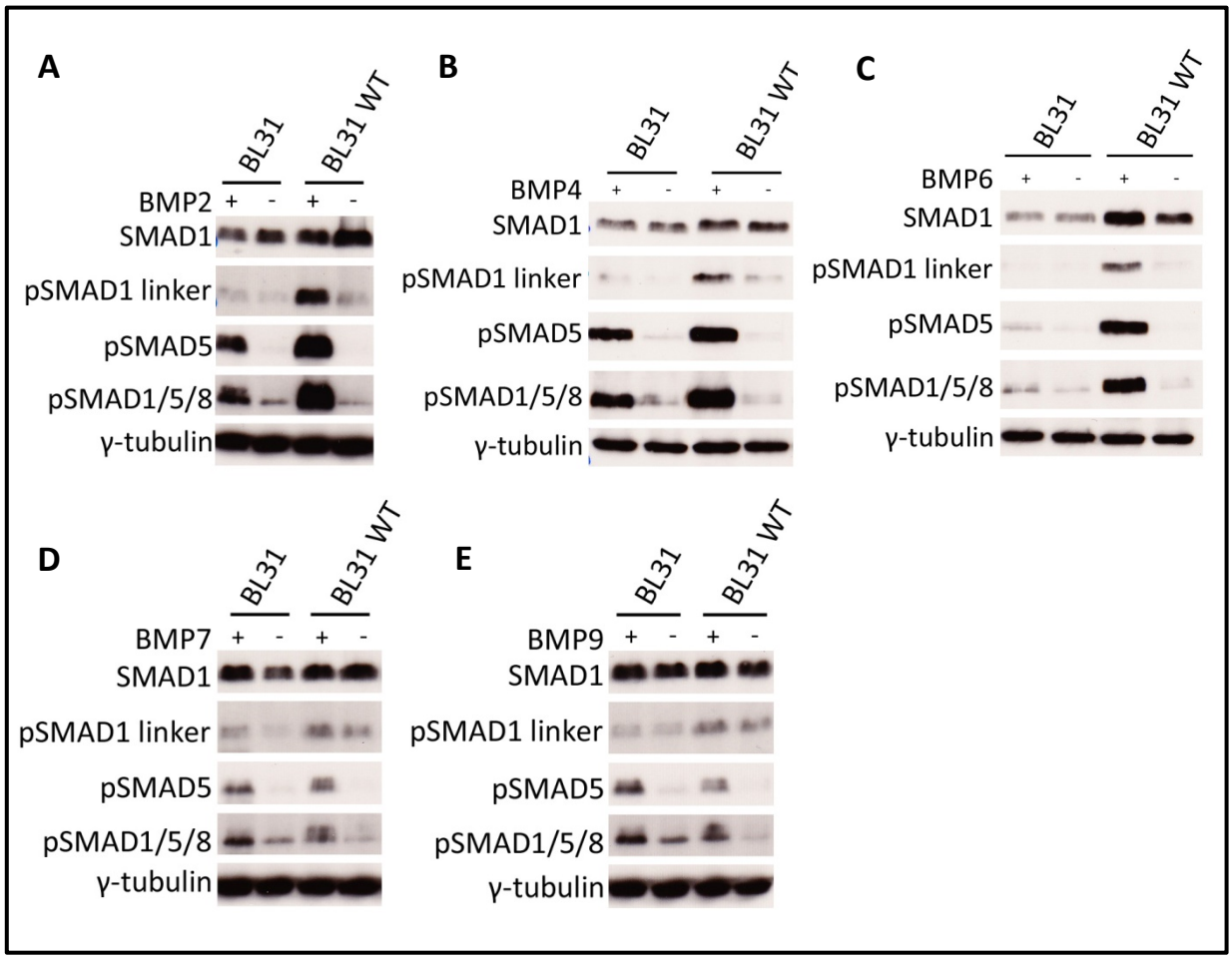
response to BMP treatment in EBV-infected than uninfected BL31 cells, although the difference between these was less marked than for pSMAD1, even taking into account the increase in total SMAD1 in EBV-infected compared to uninfected cells. These findings were consistent for BMP2, BMP4 and BMP6 (fig. 7.1A-C). However, with BMP7 and BMP9, although there was a slight increase in pSMAD1 in the presence of EBV, this was commensurate with the increase in total SMAD1 (fig. 7.1D-E). With BMP7 and BMP9, no difference was seen in the induction of pSMAD5 whether or not EBV was present, and for pSMAD1/5/8 there was only a slight increase in the presence of EBV, reflecting the increase in pSMAD1 as a result of increased total SMAD1.

It was noted in these experiments that generally the amount of pSMAD1/5/8 reflected that of pSMAD1 plus pSMAD5 (fig. 7.1), suggesting that SMAD8 phosphorylation is not prominent in this cell type, as has been found previously (Bakkebo et al. 2010, Yin et al. 2010). Haematopoietic stem cells also express SMADs 1 and 5 but not SMAD8 (Bhatia et al. 1999), suggesting that SMAD8 may not be important in lymphoid cells; the EBV-negative BL cell line Ramos has recently been shown to lack expression of SMAD8 (Kawabata et al. 2013).

SMAD1 and SMAD5 are more similar to each other (at amino acid level) than to SMAD8. BMP2 and BMP4 have been shown to activate SMAD1, SMAD5 and SMAD8 whereas BMP6 and BMP7 only activate SMAD1 and SMAD5 (Ebisawa et al. 1999, Aoki et al. 2001). In the current study, for BMP2 and BMP4 the effect of EBV was particularly marked for induction of pSMAD1 rather than pSMAD5 (fig. 7.1A-B). For BMP6, the increased induction with EBV infection was marked for both pSMAD1 and pSMAD5 (fig. 7.1C), whereas for BMP7 there was no increase in induction of pSMAD1/5 with EBV infection compared to uninfected (fig. 7.1D). Generally pSMAD1 was found to be constitutively present, even in the absence of exogenous BMPs, whereas pSMAD5 was only induced with BMP (fig. 7.1). Furthermore, it appears that in BL31 cells, BMP treatment does not induce pSMAD1, i.e. there is no increase in pSMAD1 with BMP compared to vehicle alone (fig. 7.1). However, in the presence of EBV infection, BMP2, BMP4 and BMP6 do induce pSMAD1. Although the constitutive pSMAD1 is likely to reflect background linker phosphorylation by MAP kinases, SMAD1 linker phosphorylation can also be induced by BMP treatment (Sapkota et al. 2007). The induction

seen in EBV-infected cells therefore suggests that EBV infection enables signalling by BMP2, BMP4 and BMP6.

On the basis of these results, as well as the known binding of BMP2 to TGFβR3 and because much previously published work is based on BMP2, it was decided to focus mainly on BMP2 in subsequent experiments. In order to confirm that EBV increases BMP signalling in response to BMP2, as suggested by the increased induction of pSMAD1/5, the response of a known BMP target gene *ID1* was assessed by qRT-PCR (fig. 7.1F). ID1 expression remained extremely low in both EBV-infected and EBV-negative BL31 cells in the absence of BMP2. However, in the presence of BMP2, ID1 mRNA was induced, peaking at two hours but persisting for at least 24 hours. It should be noted that no time points were measured between 2 and 24 hours, so the peak reached may have been higher and occurred at any time up to 24 hours; however, BMP treatment has previously been shown to induce ID1 mRNA maximally at 2 hours in resting mature human B cells, with levels remaining elevated for up to 24 hours (Kersten et al. 2005). In the current study, the peak levels of ID1 were higher in both wild-type EBV-infected cell lines than in the uninfected cell line, supporting the increased responsiveness to BMP2 in EBV-infected compared to uninfected BL31.



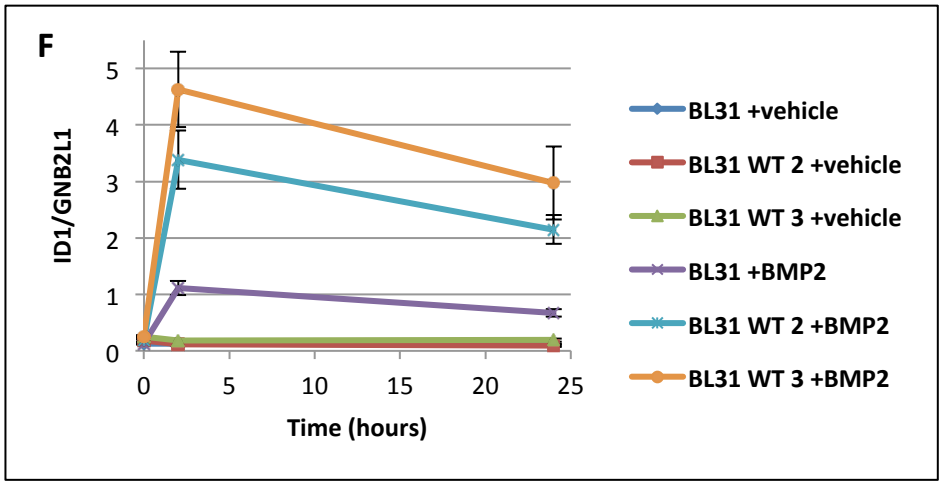




**Figure 7.1: EBV infection leads to increased signalling in response to BMP2, BMP4 and BMP6, but not BMP7 or BMP9, in BL31 cells**

**(A-E):** Western blot analysis showing phosphorylated SMAD1 linker region, SMAD5 and SMAD1/5/8 in response to treatment with BMP ligands or vehicle (0.5% BSA in 4mM HCl) for one hour. Total SMAD1 is shown for comparison, with γ-tubulin as a loading control. **(A)** BMP2 100ng/ml, **(B)** BMP4 50ng/ml, **(C)** BMP6 100ng/ml, **(D)** BMP7 100ng/ml and **(E)** BMP9 10ng/ml. **(F):** qRT-PCR for mRNA expression of ID1, a known BMP target gene, with time after treatment with BMP2 or vehicle in uninfected and wild-type EBV-infected BL31 cells. ID1 is expressed relative to the endogenous control gene *GNB2L1*.

### 7.2.2 EBNA2, EBNA3B and EBNA3C are unnecessary for the increased signalling in response to BMP2 in EBV-infected compared to uninfected BL31 cells

The current study has shown that EBV up-regulates TGFβR3 in BL31 cells, with reduced up-regulation when EBNA2, EBNA3B or EBNA3C are deleted (fig. 3.11A-B). Signalling in response to BMP2 is increased in EBV-infected relative to uninfected BL31 (fig. 7.1A). Therefore, to investigate whether the increase in BMP signalling occurs as a result of the increased TGFβR3 expression, the effect of deletion of the relevant latent proteins on BMP signalling was investigated. A panel of BL31 cell lines were treated with BMP2 or vehicle for one hour and then western blot performed for SMAD1, SMAD5, pSMAD1 linker, pSMAD5 and pSMAD1/5/8. These included BL31 infected with viruses with deletions of EBNA2, EBNA3B and EBNA3C as well as their respective revertants (fig. 7.2). Increases in BMP2-induced pSMAD1 linker, pSMAD5 and pSMAD1/5/8 were seen in the revertants (which should behave as wild-type) compared to uninfected BL31. However, when EBNA2, EBNA3B or EBNA3C were deleted, signalling remained similar to that seen in the revertants (fig. 7.2A-C). This suggests that these latent proteins are not necessary for the increased responsiveness to BMP signalling as a result of EBV infection. Therefore, the increased signalling in EBV-infected compared to uninfected cells is unlikely to occur as a direct result of the increased TGFβR3 expression.

It was also noted that there was a significant reduction in SMAD5 protein level in EBV-infected (wild-type and revertant) BL31 compared to uninfected BL31 (fig. 7.2); this will be discussed further in section 7.2.6.

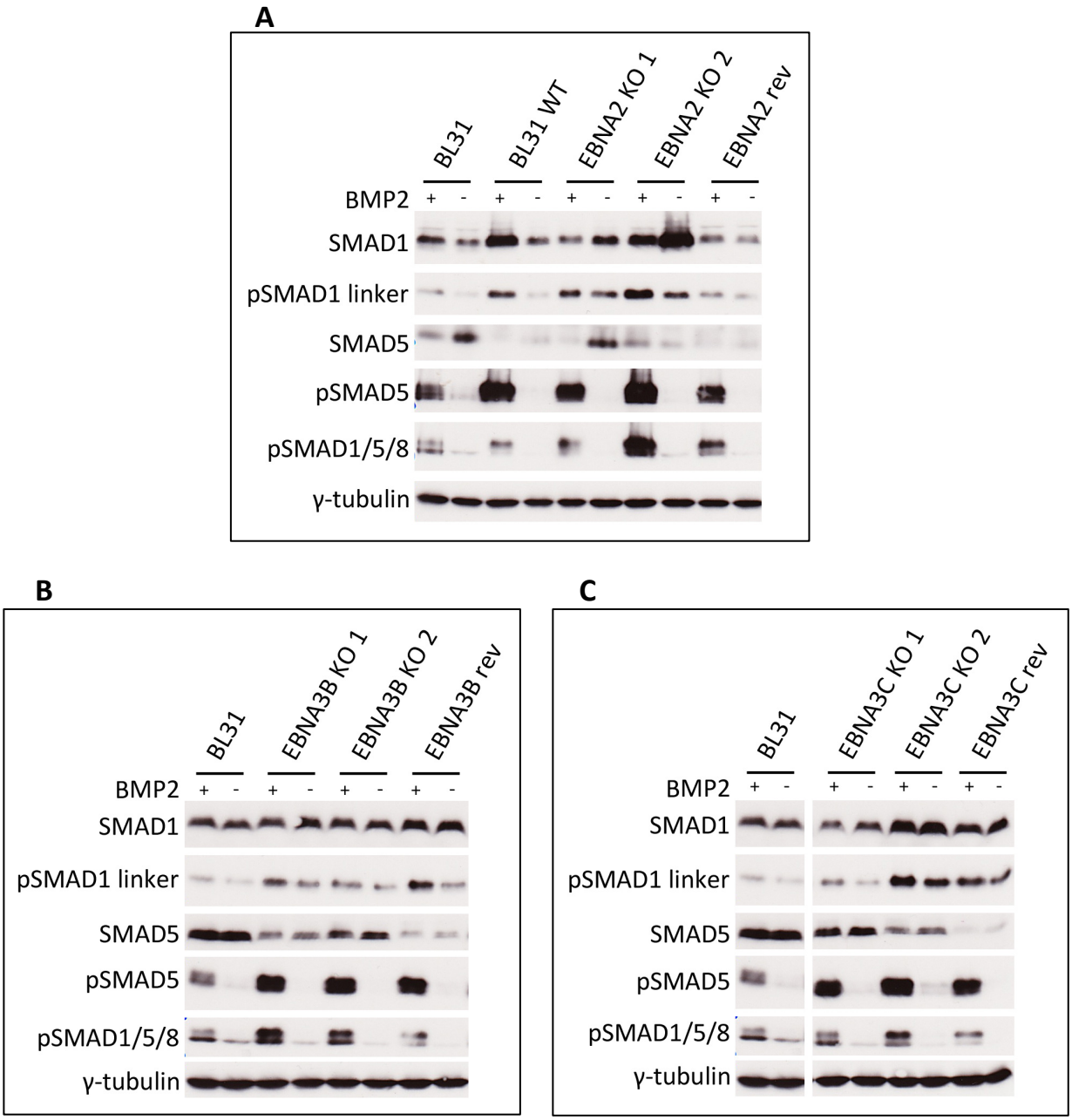



**Figure 7.2: EBNA2, EBNA3B and EBNA3C are not necessary for the increased signalling in response to BMP2 in EBV-infected compared to uninfected BL31 cells**

Western blot analysis showing the effect of treatment with exogenous BMP2 (100 ng/ml) or vehicle (0.5% BSA in 4mM HCl) for one hour on levels of phosphorylated SMAD1 linker (pSMAD1), pSMAD5 and pSMAD1/5/8 in a panel of BL31 cells infected with recombinant virus, showing the effect of: **(A)** EBNA2 KO, **(B)** EBNA3B KO and **(C)** EBNA3C KO. Total SMAD1 and SMAD5 levels are shown for comparison and γ-tubulin was used as a loading control.

### 7.2.3 The increased BMP signalling occurring in EBV-infected BL31 may be due to up-regulation of BMPR1A

Since the increase in BMP signalling is unlikely to be via the up-regulation of TGFβR3, it was hypothesised that EBV may alter one of the other components of the BMP signalling pathway. This could occur via several mechanisms, including up-regulation of type I or type II BMP receptors. Review of microarray data suggested that EBV moderately up-regulates BMPRIA, with EBNA3A and EBNA3C contributing to the up-regulation (fig. 7.3B) [www.epstein-barrvirus.org.uk and (White et al. 2010)]. qRT-PCR for BMPRIA in a panel of BL31 cell lines showed up-regulation by EBV, with the possible exception of EBNA3A KO 1 (fig. 7.3A). However, due to time constraints it was not possible to further investigate the mechanism of increased BMP signalling by EBV.

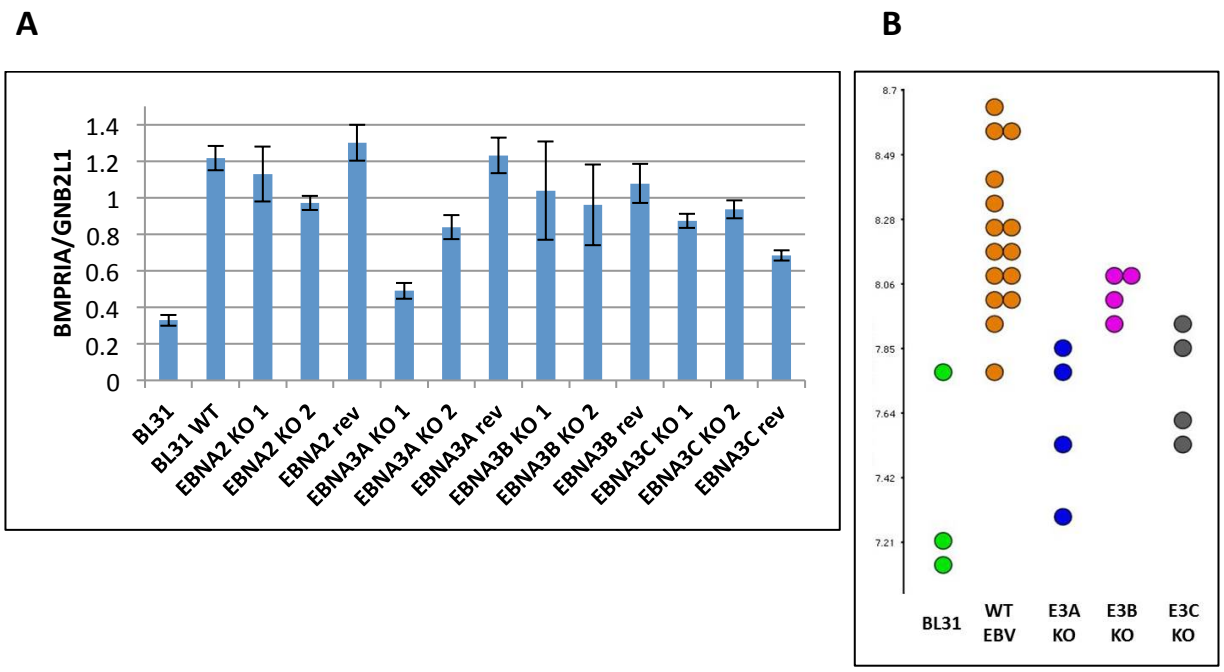



**Figure 7.3: BMPRIA (ALK3) is up-regulated by EBV in BL31 cells**

**(A)** qRT-PCR for BMPRIA mRNA in panel of BL31 cell lines. The values are expressed relative to the endogenous gene *GNB2L1*. Error bars represent standard deviations from triplicate qPCR reactions. **(B)** Microarray data for BMPRIA expression in uninfected BL31 cells, plus those infected with wild-type, EBNA3A KO, EBNA3B KO, or EBNA3C KO EBV (labelled WT EBV, E3A KO, E3B KO and E3C KO respectively). Each point represents a single cell line, with the degree of expression shown on the y axis. From [www.epstein-barrvirus.org.uk and (White et al. 2010)].

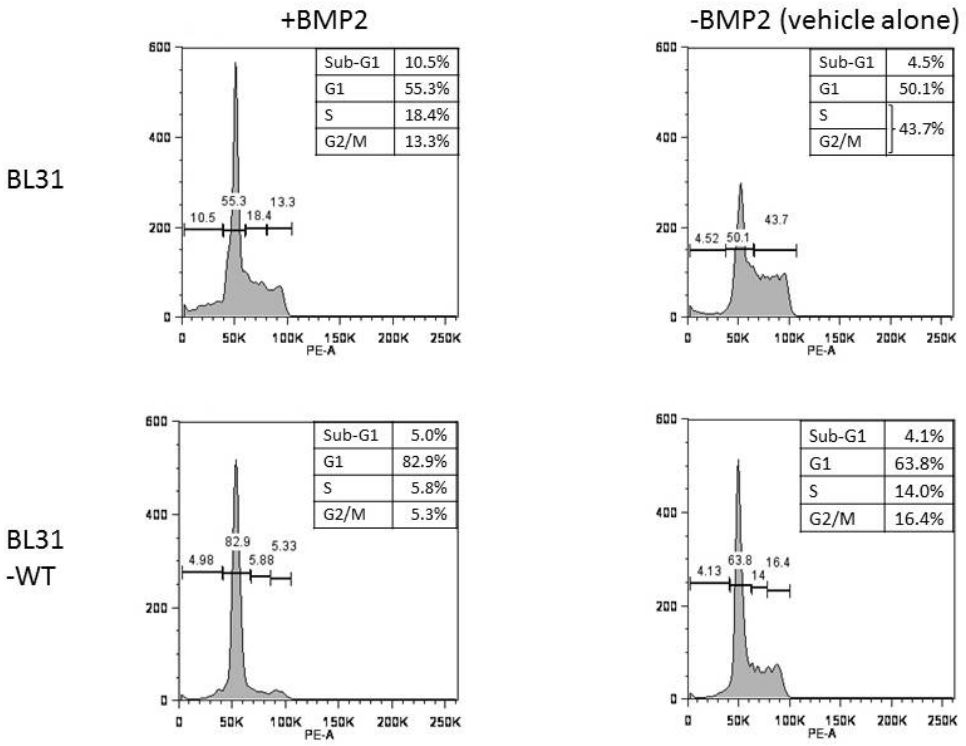
### 7.2.4 BMP2 and BMP4, but not BMP6, cause G1 growth arrest in both uninfected and EBV-infected BL31 cells

In view of the observation that EBV enhances BMP signalling, for BMP2, BMP4 and BMP6, the consequences of this were considered. It had been shown that BMP6 is anti-proliferative in naïve or memory B cells (stimulated to proliferate by anti-IgM) and CD10+ve B progenitor cells (Kersten et al. 2005, Kersten et al. 2006), that BMP6 is anti-proliferative in the EBV-negative BL line Ramos (Kersten et al. 2005) and that BMP2, BMP4, BMP6 and BMP7 are anti-proliferative or pro-apoptotic in myeloma cells (Kawamura et al. 2000, Hjertner et al. 2001, Ro et al. 2004, Fukuda et al. 2006b, Seckinger et al. 2009). However, little was known about the effects of BMP ligands on B-lymphoma, including EBV-positive BL, cells at the time these investigations were performed. In order to investigate the functional effects of BMPs in BL cells, and to see whether the increase in BMP signalling by EBV may alter this, uninfected and EBV-infected BL31 cells were treated with BMP2, BMP4 and BMP6, or vehicle, for 48 hours, stained with propidium iodide and analysed by FC (fig. 7.4). This showed that, in uninfected BL31 cells, treatment with BMP2 and BMP4 results in G1 arrest, as indicated by an increase in the G1 phase with reductions in S and G2/M phases relative to untreated cells (fig. 7.4A-B, upper figures). For BMP6 treatment (100ng/ml) there was no difference between the profiles for treated versus untreated BL31 (fig. 7.4C, upper figure), even though this dose induced pSMAD5 and pSMAD1/5/8 in BL31 (see fig. 7.1C).

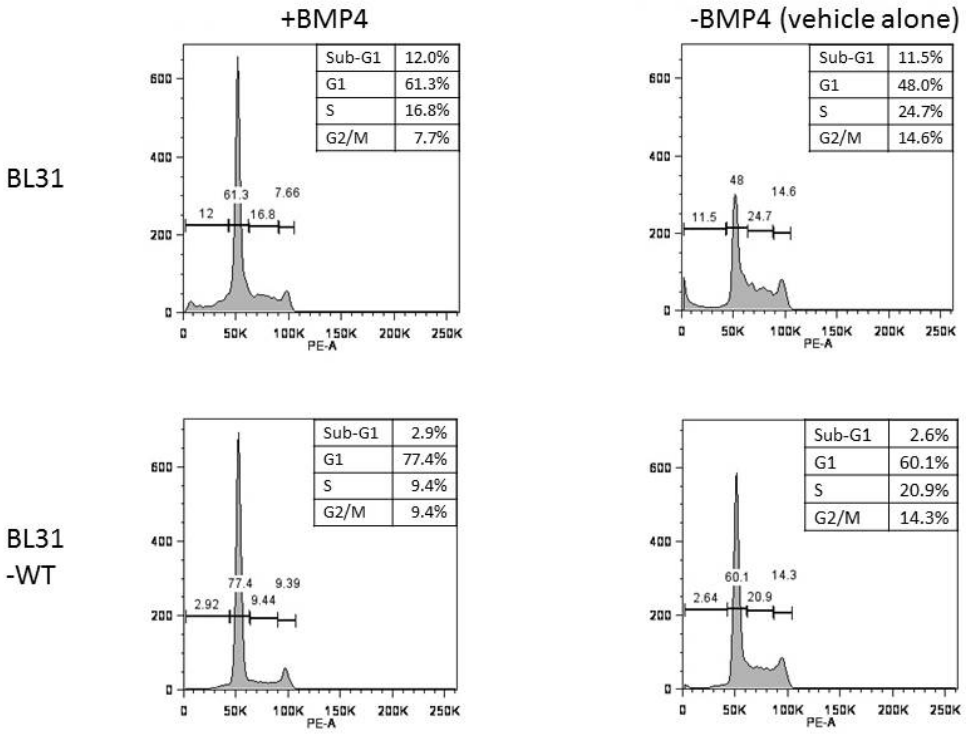
When EBV-infected BL31 cells were treated with BMP2 and BMP4, G1 arrest again occurred, to a similar degree as in uninfected BL31 cells (fig. 7.4A-B, lower figures). With BMP6 treatment, there was a moderate increase in the G1 and decreases in the S and G2/M components, suggesting that BMP6 at this dose (100ng/ml) may cause G1 arrest in EBV-infected but not in EBV-negative BL31 (fig. 7.4C, lower figures), although it did induce strong phosphorylation of pSMAD1, pSMAD5 and pSMAD1/5/8 even in EBV-negative BL31 (see fig. 7.1C).

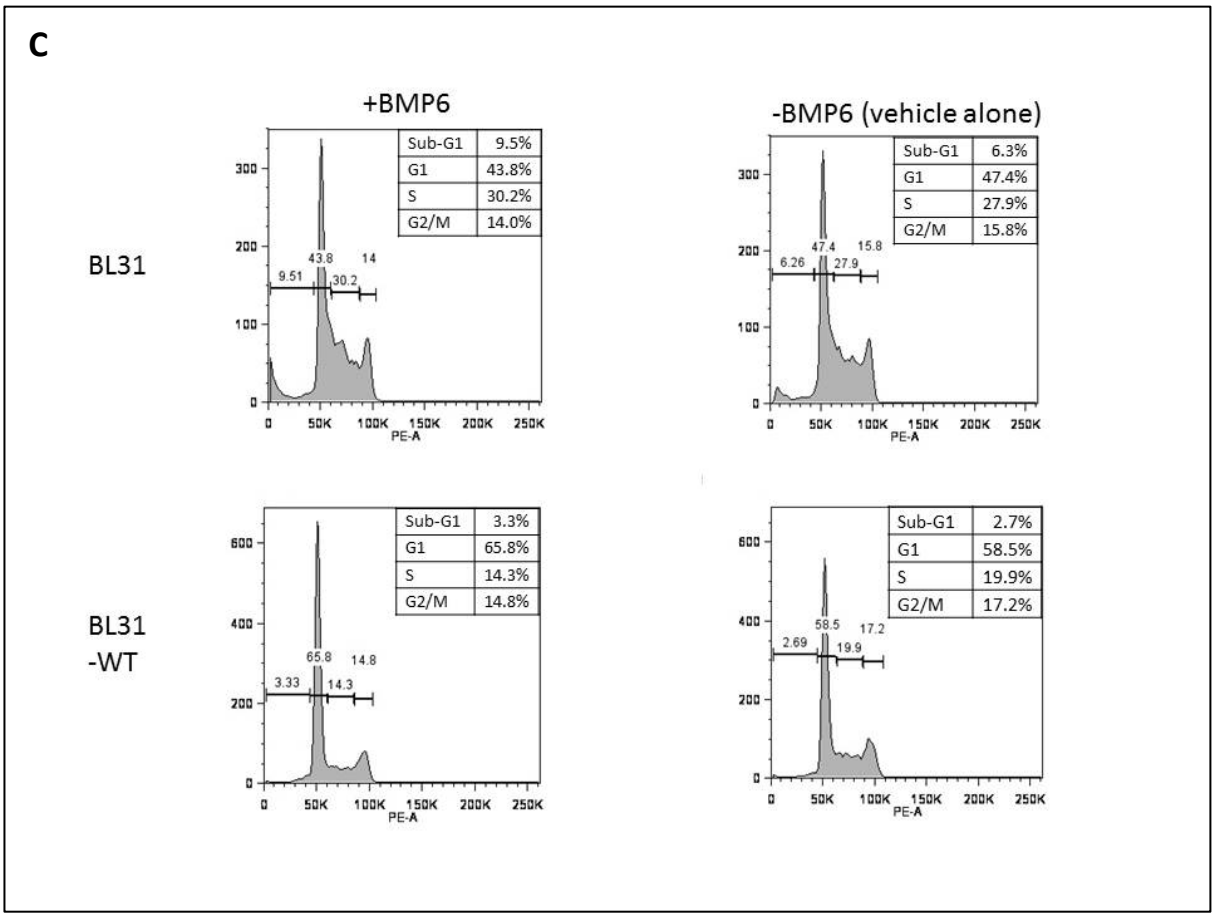
In EBV-negative BL31, there was also noted to be an increase in the sub-G1 component with BMP2 in treated compared to untreated cells (figs. 7.4A). However, western blot for PARP cleavage as a measure of apoptosis did not show any difference in PARP cleavage between BMP2-treated and untreated BL31 cells (fig. 7.4D).

**A**


+BMP2
-BMP2 (vehicle alone)
BL31
BL31
-WT
Sub-G1 10.5%
G1 55.3%
S 18.4%
G2/M 13.3%
Sub-G1 4.5%
G1 50.1%
S
G2/M
43.7%
Sub-G1 5.0%
G1 82.9%
S 5.8%
G2/M 5.3%
Sub-G1 4.1%
G1 63.8%
S 14.0%
G2/M 16.4%
PE-A

**B**


+BMP4
-BMP4 (vehicle alone)
BL31
BL31
-WT
Sub-G1 12.0%
G1 61.3%
S 16.8%
G2/M 7.7%
Sub-G1 11.5%
G1 48.0%
S 24.7%
G2/M 14.6%
Sub-G1 2.9%
G1 77.4%
S 9.4%
G2/M 9.4%
Sub-G1 2.6%
G1 60.1%
S 20.9%
G2/M 14.3%
PE-A



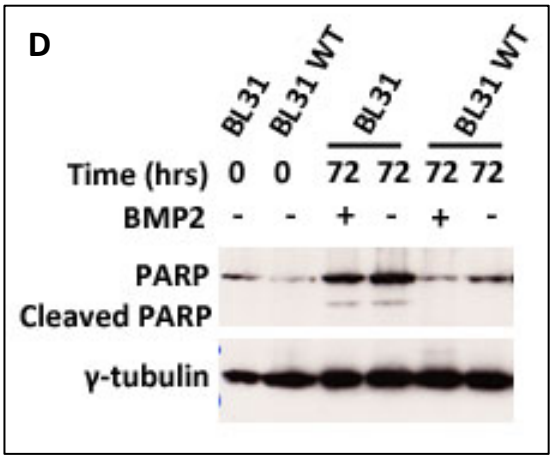




**Figure 7.4: The effects of BMP2, BMP4 and BMP6 on cell cycle arrest and apoptosis in uninfected and EBV-infected BL31 cells**

**(A-C)** Equal numbers of BL31 and wild-type EBV-infected (BL31 WT) cells were treated with BMP ligand (left-hand figures) or vehicle (0.5% BSA in 4mM HCl; right-hand figures) for 48 hours and then cells harvested, fixed and stained with propidium iodide before performing FC analysis to quantify the proportion of cells in each phase of the cell cycle. **(A)** BMP2 (100ng/ml), **(B)** BMP4 (50ng/ml) and **(C)** BMP6 (100ng/ml). **(D)** The effect of BMP2 treatment on PARP cleavage in uninfected and EBV-infected BL31 cells. BL31 and BL31 WT were treated for 72 hours with BMP2 100ng/ml or vehicle, followed by protein extraction and western blot for total and cleaved PARP. γ-tubulin was used as a loading control.

### 7.2.5 EBV moderately up-regulates SMAD1 in BL31 cells, due to the combined effects of EBNA3 proteins and LMP2A

The previous experiments on the effect of EBV on BMP signalling in BL31 cells had suggested, in some cases, that SMAD1 is up-regulated by EBV (see figs. 7.1 and 7.2), hence this was further investigated, including the effect of the individual latent proteins. qRT-PCR for SMAD1 in a panel of BL31 cell lines showed a relatively high baseline expression in uninfected BL31 with a moderate up-regulation by EBV (fig. 7.5A). These findings were consistent with microarray data in BL31 [www.epstein-barrvirus.org.uk and (White et al. 2010)]. SMAD1 expression was reduced when either LMP2A, or the entire EBNA3 locus, was deleted (fig. 7.5A). EBNA2 deletion had no effect, EBNA3B deletion had a minimal effect and EBNA3C deletion had an intermediate effect. EBNA3A deletion had an effect in only in two out of four cell lines (EBNA3A KOs 1 and 2) (fig. 7.5A). Thus it appears that the up-regulation of SMAD1 involves cooperation between the EBNA3 proteins and LMP2A.

Western blotting for SMAD1 confirmed the up-regulation of SMAD1 in EBV-infected relative to uninfected BL31s (fig. 7.5B). SMAD1 was again reduced when the whole EBNA3 locus was deleted, or when LMP2A was deleted. However, the effects for individual EBNA3s were less marked. In particular, EBNA3C deletion had a greater effect than EBNA3B deletion in reduction of SMAD1 expression compared to wild-type/revertant infected BL31s (fig. 7.5B).

In order to investigate whether the lack of up-regulation of SMAD1 in EBNA3 KO BL31 could in fact be due to lower LMP2A expression, qRT-PCR was performed for LMP2A in these cell lines. Western blot for LMP2A was also attempted for these cell lines; however, the performance of the antibody was too poor to interpret the result. Expression of LMP2A by qRT-PCR was markedly lower in the EBNA3 KO than wild-type or revertant BL31 (fig. 7.5C), hence the low LMP2A expression may be contributing to the reduced SMAD1 expression in EBNA3 KO. Nevertheless, since SMAD1 expression is lower in EBNA3 KO (which express low levels of LMP2A) than the LMP2A KO (which completely lack LMP2A), it is likely that there is some additional contribution from the EBNA3 proteins as well as LMP2A to the up-regulation of SMAD1 in BL31 (fig. 7.5B).

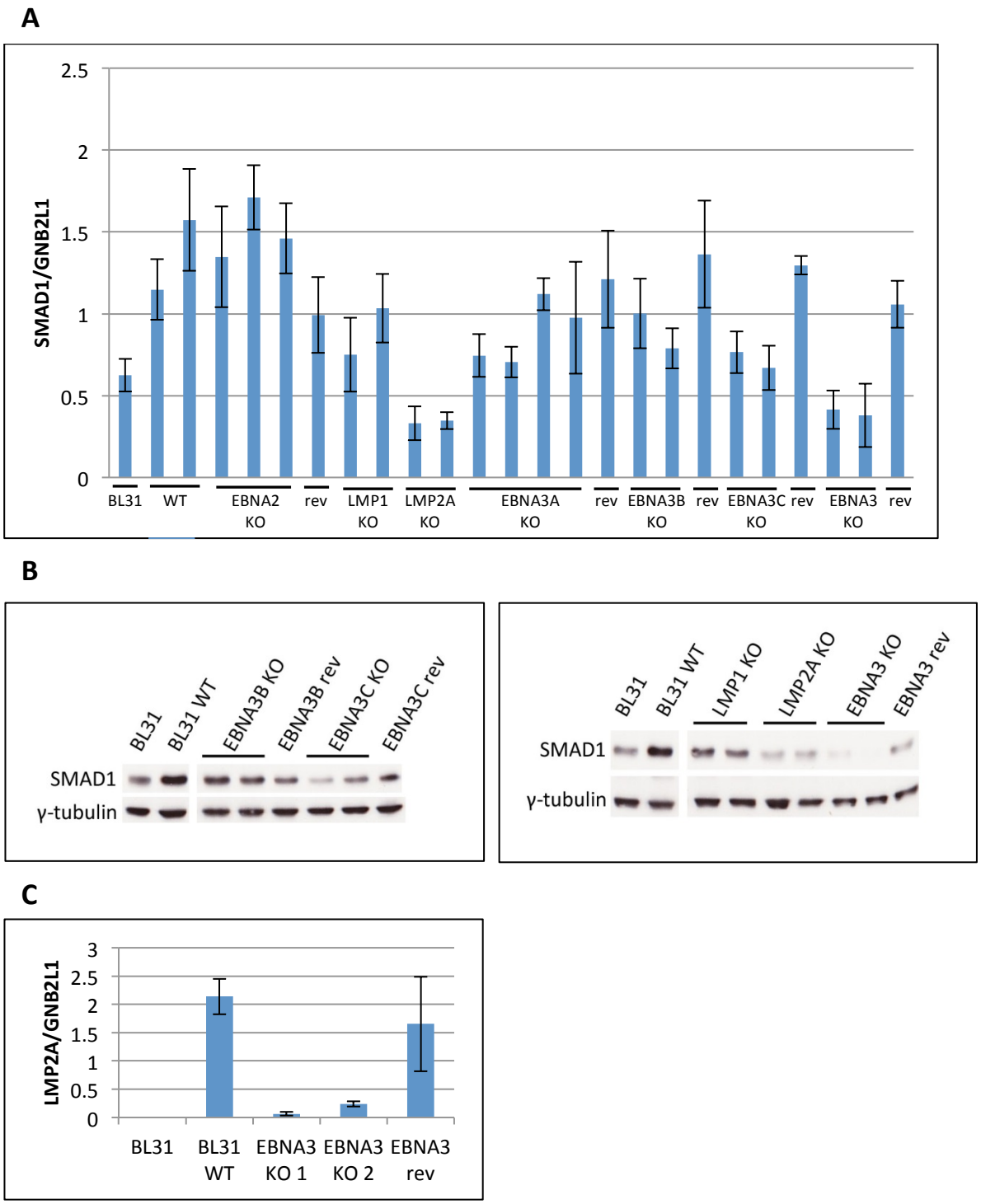



**Figure 7.5: EBV infection up-regulates expression of SMAD1 in BL31 cells**

**(A)** qRT-PCR for SMAD1 in a panel of BL31 cell lines, including those infected with virus with KO of the entire EBNA3 locus (EBNA3 KO) and its revertant (EBNA3 rev). The values are expressed relative to the endogenous gene *GNB2L1*. Error bars represent standard deviations from triplicate qPCR reactions. **(B)** Western blots for SMAD1 expression in a panel of BL31 cell lines. γ-tubulin is shown as a loading control. **(C)** qRT-PCR for LMP2A mRNA in EBNA3 KO and revertant BL31s. The values are expressed relative to the endogenous gene *GNB2L1*. Error bars represent standard deviations from triplicate qPCR reactions.

### 7.2.6 Latent EBV down-regulates SMAD5 in BL31 cells

Whilst examining the effects of EBV infection on BMP-induced signalling, it was observed that EBV down-regulates SMAD5 expression in BL31 cells, with reduced protein expression shown in all wild-type and revertant infected lines (figs. 7.2A-C).

To investigate whether the down-regulation of SMAD5 by EBV occurs at the transcriptional level in BL31, qRT-PCR for SMAD5 was performed, showing a reduction in SMAD5 mRNA with EBV infection (fig. 7.6A). Western blot for SMAD5 expression in the BL31 cell line panel confirmed that SMAD5 protein expression is reduced in wild-type EBV-infected cells compared to uninfected BL31 (fig. 7.6B).

It has been reported that microRNA 155 (miR-155) (encoded by B cell integration cluster or BIC) targets SMAD5 (Rai et al. 2010, Yin et al. 2010), and that LMP1 up-regulates miR-155 expression (Rahadiani et al. 2008). Indeed the microarray data shows a significant up-regulation of miR-155 by EBV (fig. 7.6C) [www.epstein-barrvirus.org.uk and (White et al. 2010)]. It is therefore possible that the up-regulation of BIC/miR-155 by EBV leads to the reduction in SMAD5 expression.

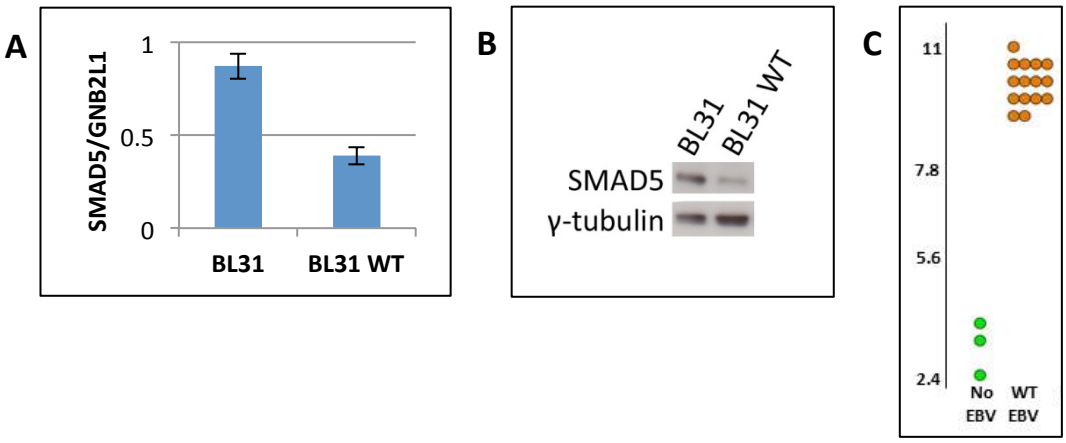



**Figure 7.6: EBV down-regulates SMAD5 and up-regulates miR-155 expression in BL31 cells**

**(A)** qRT-PCR for SMAD5 mRNA in BL31 and BL31 WT. The values are expressed relative to the endogenous gene *GNB2L1*. Error bars represent standard deviations from triplicate qPCR reactions. **(B)** Western blot for SMAD5, with γ-tubulin as a loading control. **(C)** MiR-155 expression in BL31 cell lines in microarray analysis**.** Expression of miR-155 in BL31 cells uninfected or infected with wild-type (including revertant) EBV. The vertical axis represents quantity of RNA on a $\log_2$ scale. A value of <3 generally indicates that a gene is not detectably expressed. Each point represents a single cell line. From [www.epstein-barrvirus.org.uk (White et al. 2010)].

Since EBV up-regulates SMAD1 (section 7.2.5) and down-regulates SMAD5 in BL31, it was hypothesised that EBV may alter the downstream effectors of BMP signalling in favour of SMAD1 rather than SMAD5. In this way, the target gene profile of BMPs could theoretically be altered, thus changing the ultimate effect of BMP signalling. In response to BMP2 and BMP4, pSMAD1 induction is increased more by EBV than pSMAD5 induction (see figs. 7.1A-B), which could be consistent with EBV altering the SMAD usage in favour of SMAD1 rather than SMAD5. However, for BMP6, both pSMAD1 and pSMAD5 are markedly increased in EBV-infected compared to uninfected cells (fig. 7.1C). Moreover, even though total SMAD5 is reduced by wild-type/revertant EBV, the induction of pSMAD5 by BMP2 is actually increased in EBV-infected compared to uninfected BL31 cells (figs 7.2A-C), suggesting that actually EBV is not altering the pattern of SMADs phosphorylated upon BMP2 treatment.

### 7.2.7 EBV infection leads to abrogation of non-canonical TGFβ-induced phosphorylation of SMAD5 in BL31 cells

It has been reported that TGFβ ligands can induce phosphorylation of SMAD1/5 via a non-canonical pathway in B cells including B-lymphoma cell lines (Munoz et al. 2004, Bakkebo et al. 2010, Rai et al. 2010, Kawabata et al. 2013). More recently it has been demonstrated that this pathway is important for TGFβ-induced growth arrest in B cells, enhancing the induction of p15 and p21 (Jiang and Aguiar 2014). Therefore the existence of such a pathway was investigated in BL31 cells, in order to see whether, by up-regulating SMAD1 and down-regulating SMAD5 expression, EBV may alter this non-canonical pathway. Uninfected and wild-type EBV-infected BL31 were treated with TGFβ1 or vehicle alone and samples were collected at several time points up to 24 hours. This showed transient induction of pSMAD5 by TGFβ1 at 2 hours in BL31, no longer seen at later time points, hence only the data for up to 6 hours are shown here. This effect was abrogated in the presence of EBV infection (fig. 7.7).

As shown previously, EBV infection increased total SMAD1 and decreased total SMAD5. There was a constitutive increase in SMAD1 linker phosphorylation (pSMAD1) in EBV-infected cells, not induced by TGFβ1, commensurate with the increase in total SMAD1. There was also constitutive expression of pSMAD5 in BL31, generally reduced by EBV

infection, commensurate with the decrease in total SMAD5. There was a general decrease in pSMAD1/5/8 with EBV infection, reflecting the more marked decrease in total SMAD5 than the increase in total SMAD1 by EBV (fig. 7.7).

Thus, although a non-canonical signalling response does occur with TGFβ at 2 hours, increasing pSMAD5, the down-regulation of SMAD5 and up-regulation of SMAD1 by EBV does not appear to result in a switch from SMAD5 to SMAD1 usage in this pathway; rather, EBV infection appears to abrogate this pathway. EBV does, however, alter constitutive signalling in favour of pSMAD1 rather than pSMAD5 as a result of the changes in levels of total SMAD1 and SMAD5, and it is possible that this alters its downstream effects.

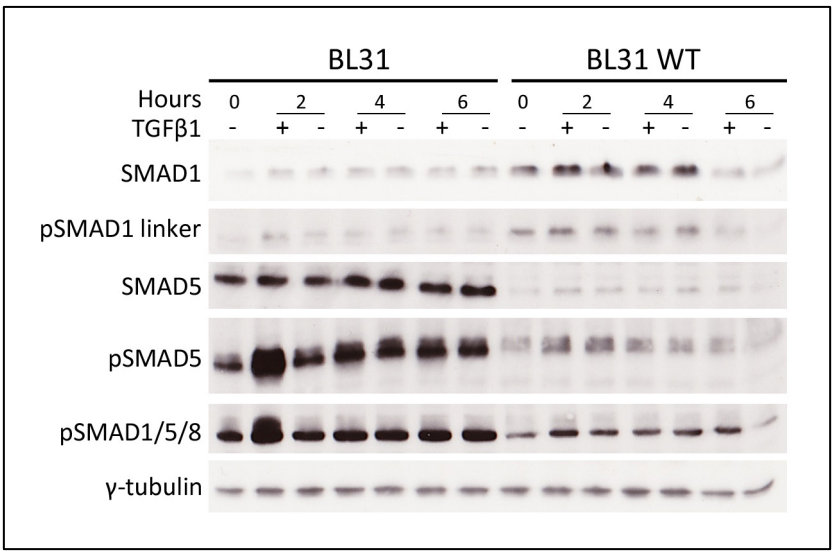



**Figure 7.7: A non-canonical pathway, in which TGFβ induces phosphorylation of SMAD5, is active in BL31 cells and is abrogated by EBV infection**

Equal numbers of BL31 and BL31 WT were treated with TGFβ1 10ng/ml or vehicle (2mg/ml BSA in sterile PBS) for up to 6 hours and samples harvested at the time points shown. Protein was extracted and then equal amounts loaded into gels for SDS-PAGE followed by western blot for SMAD1, pSMAD1 linker, SMAD5, pSMAD5 and pSMAD1/5/8. γ-tubulin was used as a loading control.

## 7.3 The effects of EBV on BMP signalling and its effectors in LCLs

### 7.3.1 SMAD1 expression is increased in wild-type compared to EBNA3B KO LCLs

The effects of EBV on BMP signalling and its components were also investigated in LCLs. Microarray analysis had suggested that SMAD1 expression was markedly lower in EBNA3B KO compared to wild-type LCLs [www.epstein-barrvirus.org.uk (White et al. 2010)]. qRT-PCR and western blot analysis in several sets of established EBNA3B KO compared to their counterpart wild-type LCLs confirmed that SMAD1 expression is markedly reduced in EBNA3B KO LCLs (see fig. 7.8 for representative data). This suggested that EBV up-regulates SMAD1, with EBNA3B being necessary.

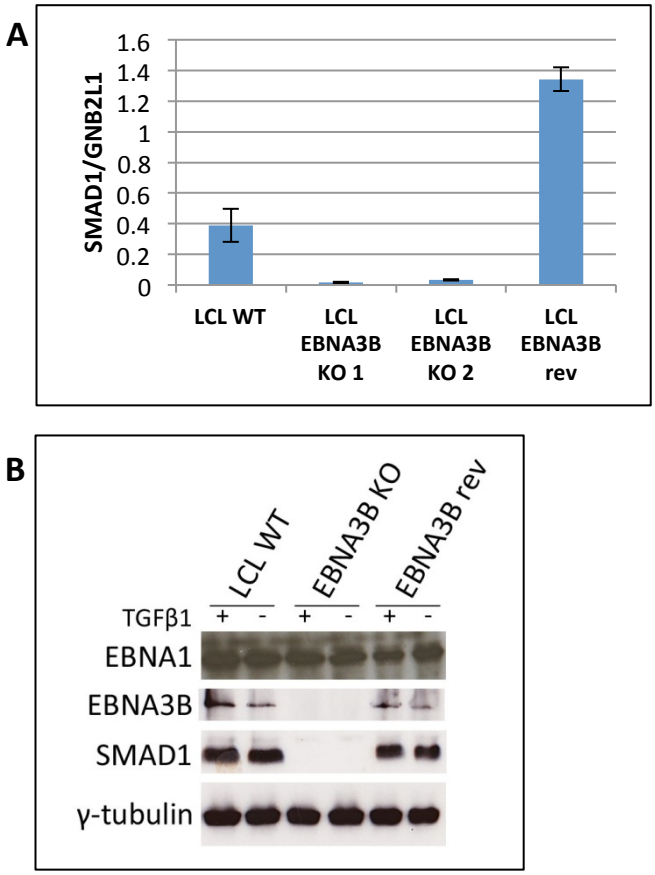


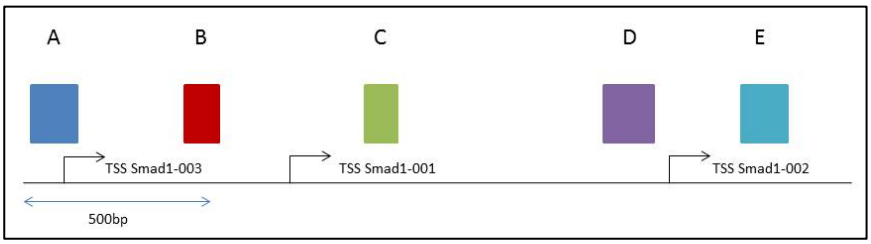

**Figure 7.8: SMAD1 expression is markedly reduced in EBNA3B KO compared to wild-type LCLs**

**(A)** qRT-PCR for SMAD1 in WT, EBNA3B KO and EBNA3B revertant LCLs. The values are expressed relative to the endogenous gene *GNB2L1*. Error bars represent standard deviations from triplicate qPCR reactions. **(B)** Western blot for SMAD1 in WT, EBNA3B KO and EBNA3B revertant LCLs, also showing EBNA1 and EBNA3B expression plus γ-tubulin as a loading control. The blot shown is from an experiment in which the cells had been treated with TGFβ1 5ng/ml or vehicle for one hour before harvesting cells for protein extraction; however, the results are representative of at least three experiments using different sets of LCLs.

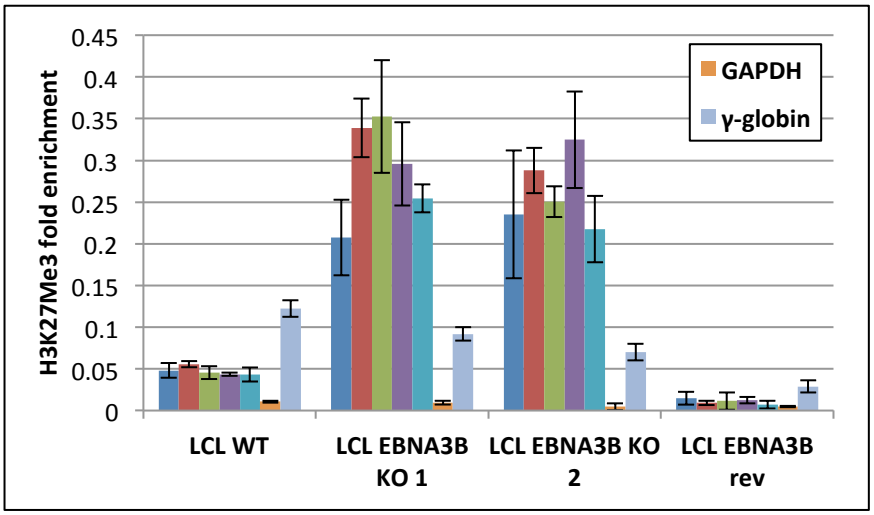
### 7.3.2 The up-regulation of SMAD1 by EBNA3B in LCLs is associated with chromatin modifications at the SMAD1 promoter

Having previously shown that EBV regulates both TGFβR2 and TGFβR3 by epigenetic mechanisms, and since the up-regulation by wild-type compared to EBNA3B KO LCLs was significant, the question was addressed of whether SMAD1 is regulated by similar epigenetic mechanisms in LCLs. Therefore several set of primers were designed covering the region including the transcription start sites of the majority of SMAD1 transcripts. Five primer sets were ultimately selected from those designed (see fig. 7.9A). Chromatin immunoprecipitation analysis was performed in EBNA3B KO and wild-type/revertant LCLs for H3K27Me3, H3K9Ac and H3K4Me3 (figs. 7.9B-D). These showed an increase in the repressive mark H3K27Me3 in EBNA3B KO compared to wild-type LCLs, with reductions in the activation marks H3K9Ac and H3K4Me3. For H3K27Me3, values were lower for LCL-EBNA3B revertant than wild-type; however, the value for globin was also reduced in this sample compared to the others suggesting that this reflects a reduction in binding in this particular sample (fig. 7.9B). For H3K27Me3 a broad peak was seen covering the region tested, whereas for H3K9Ac and H3K4Me3, a peak was seen at the site of primer set C, near the transcription start site of transcript 1 (figs. 7.9C-D). Taken together, these findings confirm that SMAD1 expression is also epigenetically regulated in LCLs.

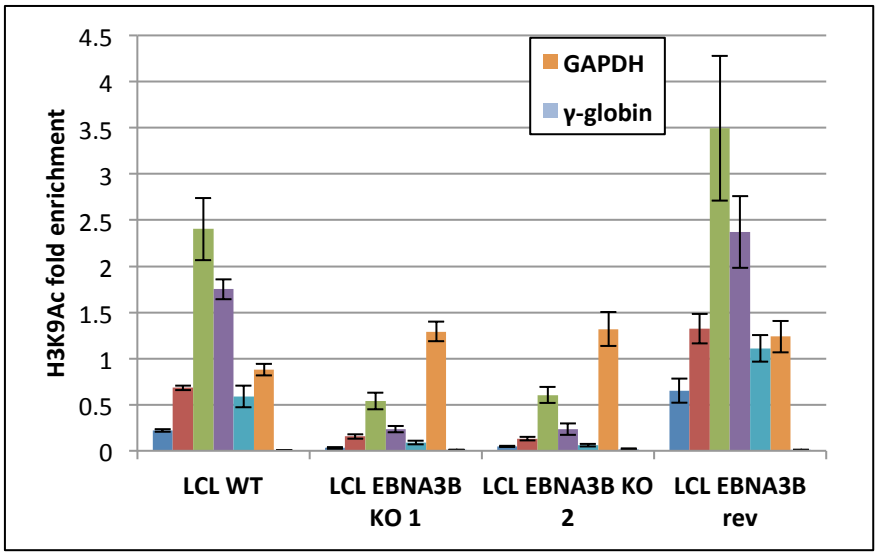
**A**


A
B
C
D
E
TSS Smad1-003
TSS Smad1-001
TSS Smad1-002
500bp

**B**


H3K27Me3 fold enrichment
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
GAPDH
γ-globin
LCL WT
LCL EBNA3B KO 1
LCL EBNA3B KO 2
LCL EBNA3B rev

**C**


H3K9Ac fold enrichment
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
GAPDH
γ-globin
LCL WT
LCL EBNA3B KO 1
LCL EBNA3B KO 2
LCL EBNA3B rev

**D**

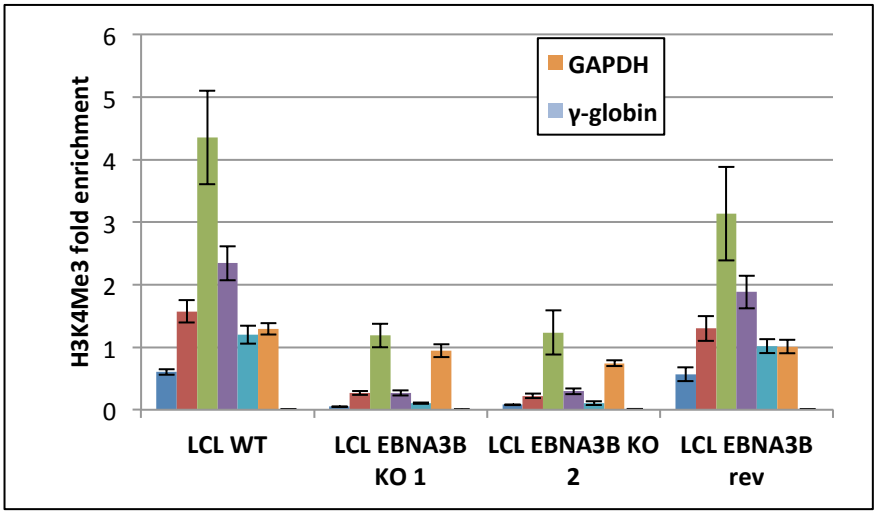

H3K4Me3 fold enrichment
6
5
4
3
2
1
0
GAPDH
γ-globin
LCL WT
LCL EBNA3B KO 1
LCL EBNA3B KO 2
LCL EBNA3B rev

**Figure 7.9: The up-regulation of SMAD1 by EBNA3B in LCLs is epigenetically mediated**

**(A)** Schematic diagram of the SMAD1 promoter showing the location of products of primer sets used and the putative transcription start sites (TSS) of three transcripts; the region shown covers the TSS of the majority of transcript variants (www.ensembl.org). The coloured blocks represent the primer pairs A-E, shown as bars from left to right for each cell line in the remaining figures. **(B-D)** qPCRs showing ratio of histone modification to input DNA at the SMAD1 promoter for **(B)** H3K27Me3, **(C)** H3K9Ac and **(D)** H3K4Me3. The results are compared to those for the control promoter primers globin (repressed) and GAPDH (active), whose colours are shown in the legends on each chart. The error bars represent standard deviations from triplicate qPCR reactions for both input and immunoprecipitation.

### 7.3.3 EBNA3B up-regulates SMAD1 expression after infection of primary B cells with EBV

In order to confirm that EBV up-regulates SMAD1, with EBNA3B being necessary, purified primary B cells were infected with wild-type, EBNA3B KO and EBNA3B revertant virus and the expression of SMAD1 measured with time after infection by qRT-PCR. SMAD1 expression was low until around 14 days after infection, when levels began to increase in the wild-type and revertant infected cells. SMAD1 expression peaked at 28 days and then appeared to reach a stable level of expression. In EBNA3B KO, SMAD1 remained extremely low (fig. 7.10A). In contrast, CD23, a marker of B cell activation, was up-regulated by day two post-infection (fig. 7.10B).

EBV up-regulates both SMAD1 and TGFβR3. In BL31 cells, EBNA3s (3B/3C) and LMP2A appear to be necessary for up-regulation of both. This led to the hypothesis that SMAD1 and TGFβR3 may be co-regulated in some way. However, the correlation in BL31s is difficult to assess since the expression of SMAD1 is relatively high in uninfected BL31 cells, and is increased only moderately by EBV infection (fig. 7.5), whereas TGFβR3 expression is very low in uninfected BL31 and therefore the effect of EBV to increase it is much greater than for SMAD1 (fig. 3.11).

The same question was therefore addressed in LCLs, where SMAD1 and TGFβR3 expression are both low in uninfected primary B cells. However, in one of the experiments in which primary B cells were infected with wild-type, EBNA3B KO or revertant EBV, there was noted to be discordance between SMAD1 and TGFβR3 up-regulation for EBNA3B revertant: SMAD1 was up-regulated by both wild-type and revertant EBV, whereas TGFβR3 was not up-regulated by revertant EBV in this case (figs. 7.10A&C). This suggests that SMAD1 and TGFβR3 are not directly co-regulated. Nevertheless it remains possible that SMAD1 up-regulation may result in favourable conditions for TGFβR3 up-regulation, for instance by clonal selection.

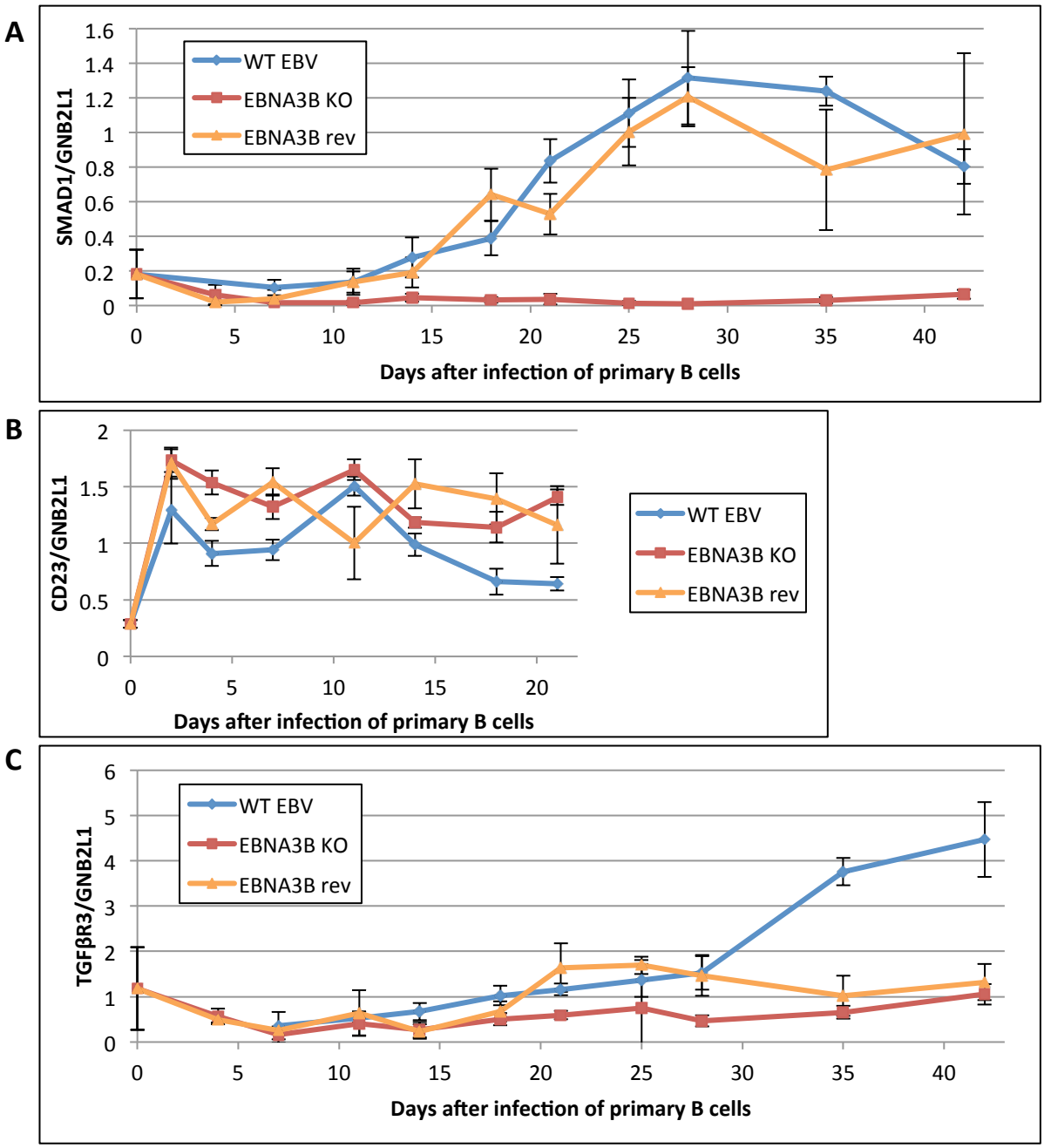



**Figure 7.10: SMAD1 compared with CD23 and TGFβR3 expression after infection of purified primary B cells with wild-type, EBNA3B KO and revertant EBV**

**(A-B)** qRT-PCR measuring expression over time of **(A)** SMAD1 and **(B)** CD23, a marker of B cell activation which is known to be up-regulated by EBV infection, in primary B cells infected with wild-type, EBNA3B KO or EBNA3B revertant virus. **(C)** SMAD1 and TGFβR3 expression are not directly co-regulated after infection of primary B cells. qRT-PCR for TGFβR3 expression with time after infection of primary B cells with wild-type, EBNA3B KO and EBNA3B revertant EBV, showing that in this particular experiment TGFβR3 expression remained lower than for wild-type EBV. All values are expressed as ratio to the endogenous control gene *GNB2L1.* Error bars represent the standard deviation of triplicate qPCR reactions.

### 7.3.4 BMP signalling still occurs in EBNA3B KO LCLs despite the lack of SMAD1 expression

Since in EBNA3B KO LCLs, expression of SMAD1 is very low, it seemed likely that this could alter the response to BMP signalling. Therefore the effect of EBNA3B KO on BMP signalling in LCLs was investigated. In particular, it was again hypothesised that EBV may promote a switch from use of SMAD5 in favour of SMAD1 as downstream effectors of signalling. EBNA3B KO and WT LCLs were treated with BMP2 and BMP4, followed by protein extraction and western blotting for SMADs 1 and 5, pSMAD1 linker, pSMAD5 and pSMAD1/5/8 (fig. 7.11). As expected, this showed that in EBNA3B KO LCLs, there was no SMAD1 present and therefore no pSMAD1 was detected after BMP treatment. In EBNA3B KO LCLs, total SMAD5 did not appear to be altered compared to wild-type LCLs. However, there was less pSMAD5 induced by both BMP2 and BMP4 in EBNA3B KO than in wild-type LCLs. The total pSMAD1/5/8 was also reduced for EBNA3B KO compared to wild-type LCLs (fig. 7.11). Hence BMP signalling is generally reduced in EBNA3B KO compared to wild-type LCLs, disproving the hypothesis that EBV promotes a switch from SMAD5 to SMAD1 in BMP signalling, at least at the level of SMAD phosphorylation.

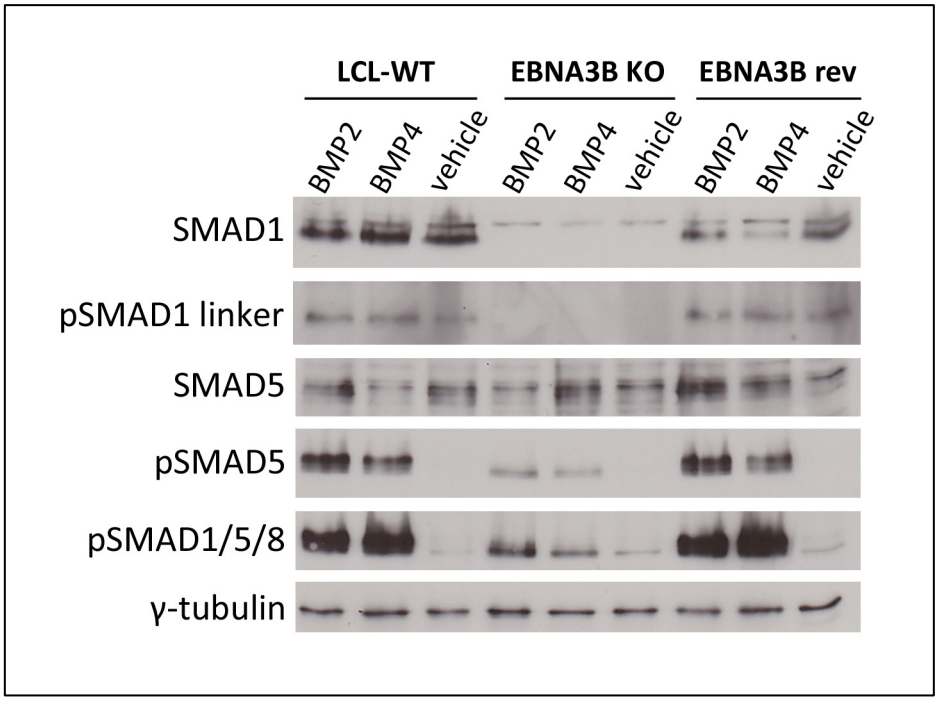



**Figure 7.11: BMP signalling via SMADs 1, 5 and 8 is reduced in EBNA3B KO compared to wild-type LCLs**

Western blot analysis showing the effect of exogenous BMP2 (100 ng/ml), BMP4 (50 ng/ml) or vehicle (0.5% BSA in 4mM HCl) alone for 1 hour on levels of phosphorylated SMAD1 linker (pSMAD1), pSMAD5 and pSMAD1/5/8. Total SMAD1 and SMAD5 levels are shown for comparison and γ-tubulin was used as a loading control.

## 7.4 Discussion

### EBV increases signalling in response to BMP2, BMP4 and BMP6 in BL31 cells, but not via up-regulation of TGFβR3

Since EBV up-regulates TGFβR3, and TGFβR3 is a co-receptor for BMP signalling, the effect of EBV on BMP signalling was investigated. This showed that EBV increased signalling, as detected by phosphorylation of SMAD1, 5 and 1/5/8, in response to BMP2, BMP4 and BMP6 (fig. 7.1A-C). However, although BMP7 and BMP9 also induced phosphorylation of SMAD1/5, EBV did not appear to increase signalling in response to these ligands (fig. 7.1D-E). When the effect of deletion of the individual latent proteins which regulate TGFβR3 was investigated, however, signalling in response to BMP2 remained at the same levels as for wild-type EBV when EBNA2, EBNA3B or EBNA3C were deleted (fig. 7.2). Hence the increased BMP signalling in EBV infection is not a direct consequence of the up-regulation of TGFβR3.

BMPs can be divided into subgroups based on structural similarity and their affinity for type I receptors. BMP2 and BMP4 form one subgroup, binding preferentially to ALK3 (BMPRIA) and ALK6 (BMPRIB). BMP6 and BMP7 are structurally related members of a second subgroup, whose members bind preferentially to ALK2 and ALK6 but only weakly to ALK3. BMP9 belongs to a third subgroup, binding to ALK1 and ALK2. Normal B cells have been variably shown to express ALK2, ALK3 and ALK6, suggesting that they can respond to BMP2, BMP4, BMP6 and BMP7 (Kersten et al. 2005, Seckinger et al. 2009).

As expected, BMP2 and BMP4 showed similar effects, with EBV infection resulting in a greater induction of phosphorylation of both SMAD1 and SMAD5 than in uninfected cells, but with a more pronounced effect on phosphorylation of SMAD1 than of SMAD5 (figs. 7.1A-B). The increased effects by EBV could be explained by, for example, up-regulation of ALK3 or ALK6, as these are the type I receptors used by BMP2 and BMP4. A preliminary investigation of effects of EBV on BMP receptors suggested that EBV does indeed up-regulate ALK3 (BMPRIA) in BL31 (fig. 7.3), therefore this may be a contributing factor to increased BMP signalling.

EBV infection markedly increased the induction of both SMAD1 linker phosphorylation and C-terminal SMAD5 phosphorylation in response to BMP6 (fig. 7.1C). Although BMP7, which

is part of the same subgroup, induced phosphorylation of SMAD1 and SMAD5, EBV did not appear to increase this induction (fig. 7.1D). Members of this ligand subgroup bind preferentially to ALK2 and ALK6 but only weakly to ALK3. Naïve B cells stimulated to differentiate into Ig-producing plasmablasts by treatment with CD40 ligand and IL-21 showed induction of ALK2 expression, which would enhance response to BMP6 and BMP7, but the inhibition of this induced Ig production was greater with BMP6 than with BMP7 (Huse et al. 2011). In the current study only BMP6, but not BMP7, signalling was enhanced by EBV (fig. 7.1C-D). Despite their structural similarity and use of the same type I receptors, BMP6 and BMP7 can have different effects in B cells. In naïve and memory B cells that had been stimulated to proliferate by anti-IgM treatment, BMP6 inhibited proliferation and induced only a minimal increase in apoptosis, whereas BMP7 strongly induced apoptosis (Kersten et al. 2005, Huse et al. 2011) and in B-lymphoma cell lines BMP6, but not BMP7, inhibits growth (Huse et al. 2012).

BMP9 was also tested as a member of a third subgroup. It uses ALK1 and ALK2 as type I receptors; however, ALK1 is expressed on endothelial cells and fibroblasts but not in haematopoietic cells. It is not known to have any effects in lymphoid cells, and can be pro-proliferative in some circumstances (Herrera et al. 2009, Herrera et al. 2013). In the current study, it induced phosphorylation of SMAD1 and of SMAD5, but EBV infection had no effect on these (fig. 7.1E). Hence although BMP9 can induce signalling in BL cells, EBV does not alter this.

As well as changes in expression of type I or type II BMP receptors, possible mechanisms for the enhancement of BMP signalling by EBV could include: reduced availability or activity of extracellular BMP antagonists such as noggin and chordin, altered availability or activity of intracellular accessory proteins which control access of R-SMADs to the internal portion of receptors, decreased activity of inhibitory SMADs (SMAD6 and SMAD7) which compete with R-SMADs for binding to type I receptors, or increased availability of R-SMADs. However, for the purposes of this study the exact mechanism was not investigated; this could be investigated further in future work.

**BMP2 and BMP4, but not BMP6, induce G1 arrest in both uninfected and EBV-infected BL31 cells**

At the start of this project, there was little published data available on the effects of BMPs in B cells. BMP6 had been shown to be anti-proliferative in lymphoid progenitors and mature B cells (Kersten et al. 2005, Kersten et al. 2006), and several studies had shown BMPs to be pro-apoptotic and anti-proliferative in myeloma cells (Kawamura et al. 2000, Hjertner et al. 2001, Ro et al. 2004, Fukuda et al. 2006b, Seckinger et al. 2009). Since EBV infection had been shown to increase BMP signalling in BL31 cells, the effect of this on the cell cycle was investigated. BMP2 and BMP4 caused G1 arrest in BL31 cells, and EBV infection possibly slightly enhanced this (figs. 7.4A-B). Since this work was done, BMP2 and BMP4 have been shown to inhibit growth of several B-lymphoma-derived cell lines (Huse et al. 2012).

On the other hand, BMP6 did not induce growth arrest or significant apoptosis in uninfected BL31 cells (fig. 7.4C). This is in contrast to the findings of BMP6-induced growth arrest seen in studies on normal B cells and, more recently, other B-lymphoma lines (Kersten et al. 2005, Huse et al. 2011, Huse et al. 2012), as well as BMP6-induced apoptosis in the EBV-negative BL Ramos (Kersten et al. 2005). However, in the current study a dose of 100ng/ml of BMP6 was used, which was nevertheless sufficient to induce pSMAD1/5/8. It is possible that higher doses would be needed to induce growth arrest and/or apoptosis; a dose-response curve showed that at 100ng/ml inhibition of proliferation in Ramos cells was only modest whereas 1000ng/ml had a much more marked effect (Kersten et al. 2005), and in the later studies by Huse et al a dose of 500ng/ml was used (Huse et al. 2011, Huse et al. 2012).

In addition, these findings may reflect something specific to BL31, as the effects were variable between different cell lines used in the previous studies. In the current study, although BMP6 did not cause growth arrest or apoptosis in EBV-negative BL31, signalling in response to BMP6 was enhanced by EBV, which then appeared to enable BMP6 to cause growth arrest (figs. 7.1C and 7.4C). It is also possible that the enhancement of BMP6 signalling by EBV also has an alternative effect, such as enhancement of the inhibitory effect of BMP6 on plasmacytic differentiation, since BMP2, BMP4 and in particular BMP6 have been shown to inhibit differentiation of mature B cells into plasma cells (Huse et al. 2011).

Since plasmacytic differentiation would favour lytic infection, this would be a means of promoting EBV latency, rather than lytic infection, as is normally the case in memory B cells.

The finding that EBV-infected as well as uninfected BL31 cells undergo G1 arrest in response to BMP2 and BMP4 was initially surprising, since the effect of EBV on BMP signalling is growth-inhibitory whereas the effects of EBV on TGFβ signalling is anti-apoptotic or growth-promoting, i.e. EBV appears to have opposing effects on TGFβ and BMP signalling. However, the significance of these findings presumably depends on the local environment *in vivo*. Resistance to TGFβ signalling is likely to be important in the germinal centre reaction, where TGFβ-induced apoptosis is a prominent mechanism of removing B cells which lack survival signals (Spender et al. 2009), in order to enable the EBV-infected B cells to survive and thus maintain latent infection in memory B cells. It is possible that BMP-induced growth arrest could be favourable in another part of the lymphoid system, as it is thought that infected B cells continuously circulate around the blood and lymphoid organs. For instance, BMP levels could be increased in the peripheral blood, where infected B cells would not be proliferating. The BMP milieu of various components of the lymphoid system is generally unknown, although several recent papers by a Norwegian group have suggested that BMP signalling in lymphoid cells is likely to be important (Bakkebo et al. 2010, Huse et al. 2011, Huse et al. 2012). Since normal B cells and lymphoma cells express BMP receptors and respond to BMP signalling, it is likely that BMP signalling in B cells, and the effects of EBV on it, are biologically relevant.

**EBV up-regulates SMAD1 in BL31 and LCLs; however, the mechanism for this appears to be different in the two cell types**

In BL31 cells and after infection of primary B cells with wild-type EBV to produce LCLs, SMAD1 is up-regulated by EBV (figs. 7.5A-B, 7.10A). However, in BL31s, the baseline SMAD1 expression is relatively high (fig. 7.5A-B), whereas in primary B cells it is low (fig. 7.10A). The degree of up-regulation by wild-type EBV in LCLs is therefore much greater than in BL31 cells.

SMAD1 expression has been shown to be relatively low in naïve and memory B cells from peripheral blood, whereas centroblasts and centrocytes have higher levels, although it should be noted that in that study only five samples per cell type were investigated

(Bakkebo et al. 2010). Nevertheless, in the current study SMAD1 was low in all uninfected primary B cell samples (fig. 7.10A), in each case combined from several healthy individuals. SMAD1 has been shown to be overexpressed in some BL (Maesako et al. 2003, Bakkebo et al. 2010) and in some cases of follicular lymphoma (FL), although in these cases expression was highly variable between different tumours (Husson et al. 2002, Munoz et al. 2004). C-terminal phosphorylation of SMAD1 has been identified in clinical biopsy samples of FL, but not in normal lymphoid tissue (Munoz et al. 2004). BL cell lines, such as BL31, have already acquired several genetic/epigenetic changes *in vivo* in order to become malignant, and have probably undergone further clonal changes *in vitro* during repeated passage. Since SMAD1 is overexpressed in several B cell malignancies, this suggests that up-regulation of SMAD1 may confer an advantage on tumour cells; the worse prognosis seen in clinical FL cases with SMAD1 overexpression would also support this (Harjunpaa et al. 2006). It is therefore possible that the high baseline SMAD1 expression in BL31 (and certain other lymphoma cell lines) has occurred via a clonal selective process.

The up-regulation of SMAD1 by EBV would be expected to increase BMP signalling via pSMAD1. In the current study, SMAD1 appears to be constitutively phosphorylated at the linker region in BL31 cells and LCLs, possibly via action of MAP kinases (Sapkota et al. 2007), whereas it is not induced by the BMP ligands tested in EBV-negative BL31s (fig. 7.1) or wild-type/revertant LCLs (fig. 7.11). However, in BL31 cells infected with wild-type EBV, BMP2, BMP4 and BMP6 do increase induction of SMAD1 linker phosphorylation compared to vehicle alone (figs. 7.1A-C). Thus EBV appears to enable these BMPs to induce SMAD1 linker phosphorylation. C-terminal phosphorylation of SMAD1 alone was not investigated in this study; however, it is possible that EBV may also enable or enhance this. This may have a specific effect, for instance if pSMAD1 targets specific genes not targeted by pSMAD5. The differential effects of C-terminal phosphorylation of SMAD1 versus SMAD5 on target genes are not known, but this could be investigated in future work.

Another possibility is that by increasing SMAD1, EBV increases the amount of SMAD1 available for non-canonical TGFβ signalling. However, TGFβ1 did not increase induction of SMAD1 linker phosphorylation compared with vehicle whether or not EBV infection was present (fig. 7.7), although TGFβ1 has been shown to induce linker phosphorylation

(Sapkota et al. 2007). Furthermore, in BL31 cells EBV infection led to a decrease in constitutive pSMAD1/5/8 consistent with the decrease in SMAD5 expression, with no induction of pSMAD1/5/8 by TGFβ seen in EBV-infected cells (fig. 7.7). Hence EBV infection probably does not lead to TGFβ-mediated induction of C-terminal phosphorylation of SMAD1 in BL31 cells. The effect of TGFβ1 on pSMAD1/5 in LCLs was not investigated, but it would be interesting to see whether non-canonical induction of pSMAD1/5 with TGFβ occurs in LCLs. Moreover, if this non-canonical signalling occurs via TGFβR2, this would not be expected to occur in wild-type LCLs since TGFβR2 is repressed and canonical TGFβ signalling suppressed (see figs. 6.1A-B).

A third possibility is that by increasing SMAD1, EBV may increase competition with canonical TGFβ-responsive SMAD2 and/or SMAD3 for binding to SMAD4. This would be relevant if both BMPs and TGFβ were acting at same time, in which case excess pSMAD1 would reduce TGFβ signalling via pSMAD2/3 and hence enhance resistance to TGFβ; however, a recent study has suggested that the opposite occurs if cells are co-stimulated with TGFβ and BMP ligands, with TGFβ repressing BMP-induced target gene transcription (Gronroos et al. 2012). Nevertheless, as the converse was not specifically investigated, it remains possible that the up-regulation of SMAD1, and enhancement of BMP-induced pSMAD1, by EBV may be another mechanism for reducing canonical TGFβ signalling, in addition to down-regulation of TGFβR2.

It has been shown that SMAD1 is necessary for the anti-proliferative effects of TGFβ1 and enhances the anti-proliferative effect of BMP2, without being absolutely necessary for this (Munoz et al. 2004). In this case SMAD1 would be acting as a tumour suppressor and therefore it would be advantageous to the cells to lose SMAD1 expression rather than increasing it, so it is unclear why there would be advantage to tumour cells or EBV in increasing SMAD1 expression. In the current study, BMP2 caused growth arrest in both uninfected and EBV-infected BL31 cells (fig. 7.4A), consistent with the expression of SMAD1. When the effects of individual latent proteins on SMAD1 expression were investigated, the extent to which EBNA3B and EBNA3C were involved in the up-regulation were different in BL31 and LCLs: in LCLs, there was a marked requirement for EBNA3B for the up-regulation, whereas in BL31, although a loss of up-regulation was seen when the entire EBNA3 locus

was deleted, there was only a minimal effect for EBNA3B, and a modest effect for EBNA3C (fig. 7.5A-B); in addition, LMP2A was required for the up-regulation of SMAD1. Hence in BL31 cells, EBNA3B, EBNA3C and LMP2A apparently cooperate to up-regulate SMAD1. This is consistent with the recent finding that SMAD1 expression is increased in EBNA3-expressing, Wp-restricted versus latency I Awia-BL (Kelly et al. 2013), i.e. up-regulated by the EBNA3 proteins.

After infection of primary B cells with EBV, it was EBNA3B that had the major effect on SMAD1 expression; in the absence of EBNA3B, SMAD1 expression remained extremely low (fig. 7.10A). In stable LCLs, EBNA3B KO again have extremely low SMAD1 expression compared to wild-type or revertant LCLs (fig. 7.8A-B). Unfortunately the effect of LMP2A deletion on the expression of SMAD1 after infection of primary B cells could not be investigated, but this should be done in future work. The involvement of specific latent proteins in the up-regulation of SMAD1 is therefore different between BL31 cells and LCLs. In BL31s, EBNA3B is much less clearly involved than in LCLs, and the other EBNA3 proteins do not appear to be involved in LCLs.

The up-regulation of SMAD1 by EBV after infection of primary B cells does not occur until around 11-14 days after infection (fig. 7.10A). The timing of this is consistent between experiments and, although delayed in comparison to other genes up-regulated by EBV (fig. 7.10B), it occurs too soon after infection to represent a clonal selection process; the up-regulation of TGFβR3 in LCLs, however, occurs more gradually over a longer time period, suggesting that this may be due to clonal selection (see chapter 6).

It was hypothesised that the up-regulation of SMAD1 by EBV may lead to increased use of pSMAD1 and reduced use of pSMAD5 in response to BMP signalling. However, in EBNA3B KO LCLs, as expected BMPs do not induce pSMAD1 (since SMAD1 expression is extremely low in the absence of EBNA3B), but surprisingly, the induction of pSMAD5 was also less in EBNA3B KO than wild-type/revertant LCLs (fig. 7.11). Hence BMP signalling was generally reduced when SMAD1 was poorly expressed; the reason for this is not clear.

**EBV down-regulates SMAD5 in BL31 cells**

In the experiments investigating the effects of EBV on BMP signalling in BL31 cells, it was observed that the expression of SMAD5 was reduced by EBV (figs 7.2). Therefore SMAD5 expression was investigated by qRT-PCR and western blot (fig. 7.6A-B). This confirmed that SMAD5 is down-regulated by wild-type/revertant EBV in BL31 cells.

MicroRNA 155 (miR-155) has been shown to be overexpressed in DLBCL, particularly the activated B cell type, which has a poorer prognosis (Eis et al. 2005) and it has been shown to bind to the 3'UTR of SMAD5, repressing SMAD5 protein expression (Rai et al. 2010, Yin et al. 2010). LMP1 up-regulates miR-155 (Rahadiani et al. 2008). Microarray analysis showed marked up-regulation of miR-155 by EBV in BL31 (fig. 7.6C), consistent with up-regulation of miR-155 by LMP1 [www.epstein-barrvirus.org.uk and (White et al. 2010)].

Repression of SMAD5 by expression of microRNAs which target it or by shRNA-mediated knockdown rendered Ramos and DLBCL cell lines resistant to the growth-inhibitory effects of TGFβ (Rai et al. 2010, Liu et al. 2012), suggesting that SMAD5, and TGFβ-induced phosphorylation of it, is necessary for TGFβ-induced growth inhibition, recently confirmed by the same group (Jiang and Aguiar 2014). SMAD5 knockdown also resulted in resistance to the growth-inhibitory effects of BMP2/BMP4, suggesting that SMAD5 may have a tumour suppressive function in lymphoid cells (Rai et al. 2010). Thus EBV-mediated repression of SMAD5 would result in resistance to the growth-inhibitory effects of TGFβ and BMP2/4. However, in the current study, BMP2 and BMP4 resulted in growth arrest in both EBV-negative and –positive BL31 (figs. 7.4A-B). This is consistent with the fact that, although in BL31-WT SMAD5 was markedly repressed, pSMAD5 was still strongly induced by BMP2 (fig. 7.2).

**A non-canonical signalling pathway is seen in BL31s, in which TGFβ induces phosphorylation of SMAD5; this pathway is abrogated by EBV, suggesting that it may occur via TGFβR2**

It has been previously shown that SMAD1 phosphorylation can occur in response to TGFβ1 in various epithelial and endothelial cell lines. In most cases this requires the combination of TGFβR1, one of the BMP type I receptors and TGFβR2 (Goumans et al. 2002, Goumans et al.

2003, Liu et al. 2009, Wrighton et al. 2009). This alternative pathway appears to require a higher dose of TGFβ1, be activated later after TGFβ1 stimulation and be sustained for a shorter time than canonical TGFβ1 signalling via SMAD2/3; hence it has been proposed that this pathway has a distinct function from either the canonical TGFβ pathway via SMAD2/3 or the canonical BMP pathway via SMAD1/5/8 (Wrighton et al. 2009). Distinct functions have been shown for the non-canonical pathway in endothelial and epithelial cells (Goumans et al. 2002, Daly et al. 2008).

TGFβ-induced phosphorylation of SMAD1 and/or SMAD5 has also been demonstrated in several different B cell lymphoma cell lines (Munoz et al. 2004, Bakkebo et al. 2010, Rai et al. 2010) as well as primary human B cells (Bakkebo et al. 2010). TGFβ1-induced phosphorylation of SMAD1 and/or SMAD5 has been shown to occur from 30 minutes after treatment, peaking at one hour but lasting for between 2 and 6 hours post-treatment (Munoz et al. 2004, Bakkebo et al. 2010).

TGFβR2 was found to be necessary for this pathway in B-lymphoma cells (Munoz et al. 2004), whereas the findings of Rai *et al* suggested that both TGFβR1 and TGFβR2 were important (Rai et al. 2010). The pathway was also partially blocked by inhibition of type I BMPRs, supporting the proposition of Daly *et al* that heteromultimeric receptor complexes are involved (Daly et al. 2008). In lymphoma cell lines in which TGFβ-induced pSMAD1/5 did not occur, reduced levels of TGFβR1 and/or TGFβR2 expression were demonstrated. The cell lines in which TGFβ induced pSMAD1/5 also showed induction of pSMAD2, and all were sensitive to TGFβ-induced growth inhibition (Bakkebo et al. 2010).

Since this pathway involves SMAD1 and SMAD5, it was hypothesised that this pathway may also occur in BL31 cells and that, if so, the up-regulation of SMAD1 and down-regulation of SMAD5 by EBV may alter this. Treatment of BL31 cells with TGFβ induced transient phosphorylation of SMAD5 at two hours, with pSMAD5 levels returning to baseline at 4 hours and beyond (fig. 7.7). Thus the non-canonical pathway occurs in EBV-negative BL31 cells as has been seen in other B cell lines. Although Rai *et al* (2010) used an antibody to pSMAD1 and/or 5, they went on to demonstrate by co-immunoprecipitation experiments that the effect occurred predominantly through SMAD5, whereas Munoz *et al* (2004)

showed induction of pSMAD1, but did not investigate pSMAD5, and Bakkebo *et al* (2010) did not distinguish between pSMAD1 and pSMAD5.

In EBV-infected BL31, no induction of pSMAD5 was seen with TGFβ (fig. 7.7), suggesting that EBV abrogates this non-canonical pathway in a similar way to its abrogation of canonical TGFβ signalling. This suggests that the non-canonical pathway may also be mediated by TGFβR2, as EBV down-regulates TGFβR2 (see chapter 3). Due to time constraints, the effect of deletion of the individual latent proteins on this was not investigated, but if the same proteins appeared to be involved as for the down-regulation of TGFβR2, that would be consistent with the hypothesis that this is mediated by TGFβR2. Within this pathway, however, EBV increased the constitutive phosphorylation of the SMAD1 linker and reduced the constitutive C-terminal phosphorylation of SMAD5, as well as that of SMAD1/5/8, generally correlating with the increases in total SMAD1 and total SMAD5 respectively. It remains possible that EBV alters constitutive signalling in favour of SMAD1 rather than SMAD5 C-terminal phosphorylation and activation, as a result of the changes in levels of total SMAD1 and SMAD5; however it is not known what the downstream effects of this would be.

# Chapter 8 Discussion

## 8.1 Latent EBV leads to PRC2-mediated repression of TGFβR2 and suppression of TGFβ signalling via SMAD2

This study has shown that latent EBV infection leads to down-regulation of TGFβR2 in both BL31 cells and established LCLs, and leads to near-complete repression by approximately 21 days after infection of purified primary B cells. TGFβR2 binding to TGFβR1 is necessary to propagate signals from TGFβ ligands. Accordingly, the down-regulation of TGFβR2 in these cell types leads to suppression of TGFβ signalling via phosphorylation of SMAD2.

TGFβ signalling is generally tumour suppressive, being pro-apoptotic and anti-proliferative in B cells and most other cell types. EBV has been shown to repress other tumour suppressor genes, including BIM and $p16^{INK4A}$, via epigenetic mechanisms including polycomb-mediated repression (Paschos et al. 2009, Skalska et al. 2010, Paschos et al. 2012). The transcriptional repression of TGFβR2 by EBV was accompanied by increased H3K27Me3 deposition and binding of SUZ12, a component of PRC2, to the TGFβR2 promoter. Therefore this study has demonstrated polycomb-mediated, epigenetic, repression by EBV of another TSG in addition to $p16^{INK4A}$ and BIM.

H3K27Me3 can increase the likelihood of the more stable epigenetic repression by CpG methylation of DNA. In certain cell lines DNA hypermethylation of the TGFβR2 promoter occurred, in some cases replacing H3K27Me3, causing repression of TGFβR2. In future, it would be interesting to investigate for DNA methylation at this locus in tumour biopsy samples.

## 8.2 The cooperation between EBNA3B, EBNA3C, LMP1 and LMP2A in the repression of TGFβR2

By using a panel of EBV-negative BL31 cells infected with BAC-derived recombinant EBVs, this study aimed to identify which latent proteins are responsible for the repression of TGFβR2. In initial experiments, when EBNA2, EBNA3B or EBNA3C were deleted, de-repression of TGFβR2 was seen, as well as restoration of TGFβ signalling via phosphorylation

of SMAD2, suggesting cooperation between these three latent proteins in the repression of TGFβR2.

The EBNA3 proteins are a closely related set of proteins that regulate transcription of multiple cellular genes, by targeting DNA via interaction with RBP-Jκ and probably other transcription factors. Several recent studies have demonstrated binding sites of EBNA3C to target gene promoters and distal regulatory elements (e.g. enhancers) (Skalska et al. 2010, McClellan et al. 2012, Paschos et al. 2012, McClellan et al. 2013). The EBNA3 proteins often cooperate in regulation of target genes, with the extent of cooperation revealed by an exome microarray analysis (Paschos et al. 2009, Skalska et al. 2010, White et al. 2010, Paschos et al. 2012, Skalska et al. 2013). An attempt to show binding of EBNA3C to the TGFβR2 promoter was unsuccessful, however this has been shown for other genes repressed by EBNA3C (Skalska et al. 2010, McClellan et al. 2012, Paschos et al. 2012, McClellan et al. 2013, Skalska et al. 2013) and other members of the laboratory are currently investigating binding of EBNA3B to promoters.

The effect of EBNA3B deletion was also investigated In LCLs and primary B cell infections with EBNA3B KO virus, since EBNA3B is dispensable for transformation (Tomkinson et al. 1993). Initial experiments suggested an extremely moderate de-repression of TGFβR2 in EBNA3B KO compared to wild-type LCLs. However, even though the degree of de-repression was small, this led a to a definite increase in the amount of pSMAD2, suggesting that the de-repression was enough to bring TGFβR2 expression above a threshold required for TGFβ signalling.

Investigation of the effect of EBNA3C in LCLs was more difficult, since EBNA3C was previously thought to be absolutely required for transformation to LCLs (Tomkinson et al. 1993). Therefore initial experiments were done using 3CHT-LCLs, which have conditional expression of functional EBNA3C, established from donor B cells with wild-type $p16^{INK4A}$. This showed that TGFβR2 remained repressed even after withdrawal of functional EBNA3C, suggesting that EBNA3C was not necessary for the repression in LCLs, unlike in BL31 cells. However, the known epigenetic mechanism of repression by EBNA3C in BL31 cells led to the alternative hypothesis that previous exposure to functional EBNA3C would repress TGFβR2

and that since this is epigenetic, the repression would persist even after withdrawal of EBNA3C function. Fortuitously, at this time other colleagues in the laboratory were investigating the effect of EBNA3C on $p16^{INK4A}$ and had therefore obtained B cells from an individual with a homozygous mutation of $p16^{INK4A}$ (Skalska et al. 2013). Infection of these cells with 3CHT-EBV was able to successfully produce LCLs even in the absence of 4HT, and thus which had never expressed functional EBNA3C. By investigating these cells, and other sets which had been established with 4HT but then had 4HT withdrawn, the current study was able to confirm the hypothesis of prior exposure to EBNA3C leading to persistent repression of TGFβR2. Similarly to the case with EBNA3B KO, the degree of de-repression of TGFβR2 in those cells which had never been exposed to 4HT, and therefore EBNA3C, was very moderate; however, this was again sufficient to lead to a definite increase in signalling via phosphorylation of SMAD2. In future, investigation of chromatin modifications at the TGFβR2 promoter could be performed in the p16-null 3CHT-LCLs established with 4HT compared to without 4HT, in order to confirm this epigenetic repression of TGFβR2 by EBNA3C.

The apparent involvement of EBNA2 in repression of TGFβR2 and suppression of signalling in BL31 cells was surprising, as EBNA2 generally up-regulates cellular genes and has opposing effects to the EBNA3 proteins, which are transcriptional repressors. This led to the hypothesis that in fact LMP1 and/or LMP2A were involved in the repression, since EBNA2 transactivates their expression. Therefore, towards the end of the project, LMP1 and LMP2A KO viruses were obtained and used to produce BL31 LMP1 KO and BL31 LMP2A KO cell lines, established in a similar manner to the other recombinant-infected BL31 cell lines used in this project. Investigation of latent protein expression in these cell lines, however, showed that the LMP2A KO lines also lacked expression of LMP1, and the two different LMP1 KO lines expressed LMP2A in different amounts.

Investigation of TGFβR2 expression and TGFβ signalling showed de-repression of TGFβR2 and restoration of signalling in both LMP1 KO and LMP2A KO lines. Although an effect of EBNA2 itself also could not entirely be excluded, due to the nature of expression of other latent proteins in all these cell lines (see chapter 4 for full explanation), it therefore seems

likely that both LMP1 and LMP2A cooperate, with EBNA3B and EBNA3C, in the repression of TGFβR2 and suppression of TGFβ signalling.

LMP1 and LMP2A are both membrane proteins and have many similar effects, both mimicking signalling processes (those of CD40L and BCR respectively) that promote survival of infected B cells within germinal centres, thus enabling infected cells to survive and reach the memory B cell stage. This is necessary according to the Thorley-Lawson model, in which EBV uses normal B cell developmental pathways to establish latency (Thorley-Lawson 2001, Thorley-Lawson and Gross 2004, Thorley-Lawson et al. 2013). LMP1 and LMP2A are expressed in latency II, found in HL and NPC, as well as latency III, found in tumours in the setting of immunosuppression. They both activate other cellular signalling pathways, including the generally pro-survival NFκB, and they both have several different anti-apoptotic effects. Recent microarray studies in transgenic mice have shown that they cooperate to regulate many cellular genes, and together cooperate to enhance carcinogenesis (Shair et al. 2012); however, in lymphoid cells their combined effects can be synergistic or in some cases antagonistic (Shair and Raab-Traub 2012). Unlike the EBNA3s, they do not alter transcription through DNA binding factors, as they are found on the cell membrane. However, since they do alter transcription of multiple cellular genes, this is likely to occur via their effects on other cellular pathways. Thus it is feasible that they could cooperate to alter transcription of TGFβR2.

Few studies, if any, have previously demonstrated the combined effects of EBNA3s and LMPs in regulation of gene expression, but it seems likely that many more genes may be regulated in this way. Previous studies have tended to focus either on the effects of individual latent proteins, expressed in isolation in EBV-negative cells, or have investigated the effects of deletion of latent proteins on cellular gene expression. Since these studies have shown cooperation between EBNA3 proteins (Anderton et al. 2008, Skalska et al. 2010, White et al. 2010, Maruo et al. 2011, Skalska et al. 2013), between LMP1 and LMP2A (Shair et al. 2012, Shair and Raab-Traub 2012), or between EBNA2 and EBNA3C (Zhao et al. 2011), it seems entirely feasible that more cooperation could be found between LMPs and EBNA3s. Now that the LMP1 and LMP2A KO BL31 cell lines have been established, the cooperation between these latent proteins and EBNA3 proteins in regulation of cellular gene expression

could be used in a similar microarray-based approach as used by White and colleagues (White et al. 2010). Ideally such a study would also investigate the revertants to wild-type from each knockout, in order to confirm that nothing else is altered apart from the gene of interest; this was done for the EBNA3s study, but revertants for the LMP1 KO and LMP2A KO BACs used in the current study were not available. A limitation of such experiments, however, is that often deletion of one latent gene seems to affect expression of others, for instance as shown here for the EBNA3 KO cell lines which also lacked LMP2A expression, and the LMP2A KO lines that lack LMP1 expression.

Unfortunately in the current study an attempt to infect primary B cells with the LMP1 KO and LMP2A KO viruses, to investigate their involvement in repression of TGFβR2 also in LCLs, was unsuccessful, but this experiment could be re-attempted. Using the EREB2.5 cell line, withdrawal of β-estradiol from the medium, and hence withdrawal of functional EBNA2 as well as LMP1 and LMP2A, resulted in de-repression of TGFβR2, suggesting that one or more of these latent proteins may also repress TGFβR2 in LCLs. However, this approach had many limitations, since the cells undergo arrest, and approximately half die by apoptosis, upon withdrawal of estradiol (Kempkes et al. 1995), and the EBNA3 expression could not be investigated in them due to the lack of appropriate (type 2 EBV-specific) antibodies as well as the relatively long half-life of the EBNA3 proteins.

### 8.3 The up-regulation of TGFβR3 by EBV

This study has confirmed that EBV, in addition to down-regulating TGFβR2, up-regulates TGFβR3 expression in BL31 cells and LCLs. In BL31 cells the same latent proteins, EBNA3B, EBNA3C, LMP1 and LMP2A are also responsible for the up-regulation of TGFβR3. In most cases the expression of TGFβR2 and TGFβR3 appeared to be reciprocal, and the ChIP for H3K27me3 and H3K9Ac were generally also reciprocal, although there were some exceptions in the EBNA3A KO lines, which as described in section 4.6 were rather heterogeneous. This apparently reciprocal nature of expression led to the hypothesis that TGFβR2 and TGFβR3 were somehow co-regulated. TGFβ1–mediated transcriptional repression of TGFβR3, via the TGFβR3 proximal promoter, had been shown in breast/ovarian carcinoma cell lines (Hempel et al. 2008). Treatment of BL31 cells with the

specific TGFβR1 inhibitor SB431542 led to a 2.5-fold increase in transcription of TGFβR3, suggesting that a similar mechanism may be present in BL31 cells. This could explain the reciprocal expression of TGFβR2 and TGFβR3, as once TGFβR2 expression falls below a threshold, so that signalling is repressed, this TGFβ1-mediated repression of TGFβR3 would no longer occur, allowing de-repression of TGFβR3.

After infection of primary B cells there was an initial down-regulation of TGFβR3 in the first 7-10 days. TGFβR3 expression then increased very gradually, and this gradual increase appeared to start only after the repression of TGFβR2 had reached a certain level. This may also be consistent with de-repression of TGFβR3 as a result of loss of TGFβ signalling once TGFβR2 is repressed below a threshold, supported by the relatively stable expression of TGFβ1 mRNA after B cell infection. However, the very slow increase in TGFβR3 also suggested a possible clonal selection process, especially as in one particular experiment TGFβR3 was not up-regulated by six weeks in one revertant cell line, even though TGFβR2 was near maximally repressed by four weeks post-infection in that experiment.

The consequences of TGFβR3 up-regulation are not known. A recent study showed low TGFβR2 and high TGFβR3 expression in several B cell lymphoma lines, although in the small number of primary B-NHL tumour samples tested, TGFβR3 expression was low (Yang et al. 2013). This again could be due to clonal selection within culture of the cell lines and may not occur *in vivo*. However, increased TGFβR3 has also been shown in CLL, possibly suggesting a tumour promoting effect, in two studies (Klein et al. 2001, Jelinek et al. 2003).

In non-haematopoietic malignancies TGFβR3 is generally tumour suppressive and has been shown to be down-regulated in many different epithelial malignancies (Florio et al. 2005, Dong et al. 2007, Hempel et al. 2007, Turley et al. 2007, Finger et al. 2008b, Gordon et al. 2008, Margulis et al. 2008, Cooper et al. 2010). It is possible that the cellular response to loss of TGFβR2 is to increase TGFβR3 in order to try to augment remaining TGFβ signalling; however there is no evidence that this occurs in other cell types such as epithelial cells and so seems an unlikely explanation. TGFβR3 is a co-receptor for BMP signalling as well as activin/inhibin signalling and is specifically required for TGFβ2 signalling. Therefore it is possible that by up-regulating TGFβR3, EBV is altering one of these pathways, but there was

not sufficient time to investigate any of them in this study. Only the possibility that it would alter BMP signalling was investigated (see chapter 7), but although EBV increased BMP signalling, the mechanism was not via the up-regulation of TGFβR3. The significance of TGFβR3 up-regulation is therefore still not known. Nevertheless, it would be interesting to investigate whether increased TGFβR3 expression is seen in tumours, especially EBV-positive lymphomas, as this could possibly represent a marker for EBV-positivity or even a target for therapy.

### 8.4 The effects of EBV on BMP signalling and SMAD1/5 expression

In chapter 7, the effects of EBV on BMP signalling were investigated. At the start of the project very little was known about the effects of BMPs in B cells, but during the course of the project, several papers were published investigating expression of BMPs and BMP receptors in B cells and lymphomas (Bakkebo et al. 2010, Huse et al. 2011, Huse et al. 2012), suggesting that BMP signalling may be important in the B cell system.

Treatment with BMP2 and BMP4, which act preferentially via ALK3 (BMPRIA) and ALK6, induced phosphorylation of SMAD1/5 and G1 arrest in BL31 cells. EBV infection enhanced SMAD1 linker phosphorylation, but not SMAD5 phosphorylation, in response to BMP2/4. Preliminary investigations also suggested that EBV up-regulates expression of BMPRIA/ALK3, which would be expected to enhance signalling and the effects of BMP2/4, as was seen.

BMP6 also induced pSMAD1/5 in BL31 cells but did not induce G1 arrest at the dose used. In the presence of EBV infection, the induction of both SMAD1 linker phosphorylation and SMAD5 C-terminal phosphorylation by BMP6 was increased, and BMP6 did induce cell cycle arrest. BMP6 acts preferentially via ALK2 and ALK6 and binds only weakly to ALK3.

EBV infection was also found to robustly up-regulate SMAD1 expression in both LCLs and BL31 cells, although the latent proteins involved seem to be different between the two cell types. In BL31 cells LMP2A, EBNA3C and to a lesser extent EBNA3B were required for the up-regulation. In LCLs and primary B cells, however, this was clearly an effect of EBNA3B, as SMAD1 expression remained extremely low in EBNA3B KO LCLs. In LCLs the up-regulation of

SMAD1 by EBNA3B also appears to be via epigenetic regulation of transcription. Interestingly, the up-regulation of SMAD1 expression after primary B cell infection does not occur until around 14 days post-infection, whereas EBNA3B would be fully expressed in the first few days. LMP1 is generally not fully expressed until much later than the EBNA3 proteins after primary B cell infection, with maximal expression seen at around 3-4 weeks. LMP1 and LMP2A mRNA expression were both slow to increase in the current study, reaching maximum at around 25-30 days post-infection (data not shown). Thus it is possible that LMP2A also contributes to the up-regulation of SMAD1, hence why this is delayed until around 14 days post-infection. This could be investigated in future by infecting primary B cells with LMP2A KO virus and thus hopefully establishing LMP2A KO LCLs. The other latent proteins have a very minimal contribution, if any, to the up-regulation of SMAD1 in LCLs. However, in BL31 cells, the overall up-regulation of SMAD1 by EBV is less marked than in LCLs, although this may be partly because BL31 have a relatively high baseline expression.

EBV infection was also found to down-regulate SMAD5 in BL31 in preliminary experiments. This could be via the up-regulation of BIC/miR-155 by LMP1 (Rahadiani et al. 2008), which would lead to reduced SMAD5 expression (Rai et al. 2010), but the mechanism was not investigated in the current study because of time constraints. The up-regulation of SMAD1 and down-regulation of SMAD5 by EBV led to the hypothesis that EBV may alter expression of BMP target genes by altering SMAD usage. In addition, a non-canonical pathway involving TGFβ-mediated induction of phosphorylated SMAD1/5 had been shown in many cell types, more recently including B cells (Munoz et al. 2004, Bakkebo et al. 2010, Rai et al. 2010, Jiang and Aguiar 2014), and it was hypothesised that EBV may alter this; this will be discussed further below.

In EBV-negative BL31 cells, very little phosphorylation of the SMAD1 linker was seen, whereas in the presence of EBV infection this phosphorylation was induced by BMP2, BMP4 and BMP6, but not BMP7 or BMP9. Phosphorylation of SMAD5 was strongly induced by BMP2 and BMP4 in EBV-negative BL31, and only slightly increased with EBV infection (fig. 7.1). With BMP6, however, there was very little phosphorylation of SMAD5 in uninfected BL31, but this was markedly increased in EBV-infected cells. BMP6-induced G1 arrest was seen in EBV-infected but not in EBV-negative BL31, whereas both EBV-infected and –

uninfected BL31 cells underwent G1 arrest, in conjunction with strong induction of SMAD5 phosphorylation, after BMP2/4 treatment. In those experiments the levels of pSMAD5 were generally similar to those for pSMAD1/5/8, suggesting that SMAD5 is the predominant R-SMAD used in these cells. Taken together, these findings suggest that phosphorylation of SMAD5 is important for BMP-induced growth arrest.

### 8.5 TGFβ-induced phosphorylation of SMAD1/5 in B cells

TGFβ-induced phosphorylation of SMAD1/5 has recently been shown to be important for TGFβ-induced cell cycle arrest, in murine B cells and B lymphoma cell lines (Bakkebo et al. 2010, Rai et al. 2010, Jiang and Aguiar 2014), although a role for SMAD1 in BMP and TGFβ induced arrest had also been suggested previously (Munoz et al. 2004). Although Rai *et al* (2010) used an antibody to phosphorylated SMAD1 and/or SMAD5, they went on to demonstrate by co-immunoprecipitation experiments that TGFβ-induced pSMAD1/5 occurred predominantly through SMAD5, whereas Munoz *et al* (2004) showed induction of phosphorylated SMAD1, but did not investigate phosphorylation of SMAD5. Furthermore, Bakkebo *et al* (2010) did not distinguish between phosphorylation of SMAD1 and SMAD5. Jiang and Aguiar (2014) showed that knockdown of SMAD5 by miR-155 or by siRNA led to loss of TGFβ-induced $p15^{INK4B}$/$p21^{CIP1/WAF1}$ induction and concomitant cell cycle arrest; this also occurred in one cell line which completely lacked SMAD1 expression, suggesting that SMAD1 was not necessary. Thus it is not entirely clear whether TGFβ-induced arrest occurs only via SMAD5 (as suggested by the Aguiar group studies) or whether SMAD1 can also contribute, as suggested by the Munoz study (Munoz et al. 2004).

In the current study, TGFβ-induced phosphorylation of SMAD5 was confirmed in BL31, at two hours post treatment, but was not sustained beyond this time point. Previous studies had shown that the TGFβ-induced phosphorylation of SMAD5 was similarly short-lived. In EBV-infected BL31, no induction of pSMAD5 was seen with TGFβ, suggesting that EBV abrogates this non-canonical pathway in a similar way to its abrogation of canonical TGFβ signalling. This suggests that the non-canonical pathway may also be mediated by TGFβR2, as EBV down-regulates TGFβR2.

Although EBV infection increased constitutive SMAD1 linker phosphorylation and decreased constitutive SMAD5 C-terminal phosphorylation, these seemed to be commensurate with the total SMAD1/5 levels in BL31 and so it did not appear that EBV was promoting a switch from use of SMAD5 to SMAD1 in this non-canonical TGFβ pathway. In EBNA3B KO LCLs, treatment with BMPs surprisingly led to a general reduction in BMP signalling in EBNA3B KO compared to wild-type LCLs, with reductions of phosphorylated SMAD5 as well as SMAD1 linker phosphorylation. Again the consequence of this is not clear; it may alter the responsiveness to TGFβ or BMPs in EBNA3B KO LCLs, although a preliminary experiment did not show any difference in cell cycle profile between EBNA3B KO and wild-type LCLs with TGFβ or BMP treatment (data not shown). However, due to time constraints this experiment was only attempted on one occasion and should be repeated, ideally in conjunction with an array analysis to investigate the BMP and TGFβ target gene profile of wild-type versus EBNA3B KO LCLs when treated with TGFβ and BMP ligands. In addition, siRNA could be used to knock down expression of SMAD1 and SMAD5 separately, in order to investigate the hypothesis that EBV changes the target gene profile by altering SMAD1/5 expression. Even if this were confirmed not to alter the cell cycle profile in response to TGFβ/BMPs, it is possible that the change in SMAD expression alters other cellular processes such as differentiation.

### 8.6 The effects of EBV on TGFβ-induced arrest and apoptosis

Prior to this work, the majority of studies on the effects of EBV on TGFβ signalling had investigated the effects on cellular responses to TGFβ. TGFβ generally promotes apoptosis and/or is anti-proliferative, and multiple studies had suggested that cells expressing full latency III EBV were resistant to the anti-proliferative effects of EBV. However, the mechanisms for this were not entirely understood, with only a few studies investigating expression of TGFβR1 and/or TGFβR2, often showing discrepant results. These studies had been done in several different cell types, largely tumour cell lines which may have altered cell cycle/apoptotic pathways anyway as part of the tumour development, and which may have acquired further changes by clonal selection in culture.

Several studies had shown that LCLs were resistant to the effects of TGFβ. Although LCLs are more reflective of the situation *in vivo*, they are still subject to changes during culture (Lee et al. 2010). However, investigation of the EBV-negative counterpart to LCLs is more problematic; most previous studies used murine B cells isolated from spleen, murine B cell lymphoma cell lines, again subject to the problems of tumour cells and selection in culture, or used human B cells isolated from peripheral blood or from tonsillar extracts. However, there appear to be some differences in TGFβ responsiveness and apoptotic pathways between human and murine B cells (Spender et al. 2013). Furthermore, the mature B cells used in these studies would have been quiescent and therefore some means was required of artificially inducing their proliferation before investigating the effects of TGFβ. In some cases B lymphoma cell lines were used, including BL, DLBCL, unspecified B cell lymphomas, or CLL cells, but these are all subject to the biases of tumours and cell lines as already described.

The current study, having shown that EBNA3B, EBNA3C, LMP1 and LMP2A cooperate to repress TGFβR2 and suppress signalling in BL cells, investigated the effects of knockout of these latent proteins on TGFβ-induced apoptosis and/or cell cycle arrest, since it was hypothesised that the same latent proteins may be protective against the effects of TGFβ. However, TGFβ treatment of the panel of BL31 cell lines showed that although BL31 cells underwent apoptosis with TGFβ, none of the KO cell lines showed apoptosis to the same degree. TGFβ-induced apoptosis may occur via SMAD3, which was not investigated in this study, or via SMAD-independent pathways involving cross-talk with other cellular pathways; the mechanism could be investigated further in future. This study has similar limitations to previous studies, as it used a tumour cell line that already has alterations in cell cycle regulation. Furthermore, it is very important to remember that EBV has multiple effects on apoptotic pathways (Allday 2009, Spender and Inman 2011, Allday 2013) and these may also alter the final responses of B cells to TGFβ. It is likely that anti-apoptotic effects of other EBV latent proteins can protect B cells from TGFβ-induced apoptosis even when individual latent genes are deleted such that TGFβ signalling is restored.

In agreement with previous studies, BL31-WT and LCL-WT showed no apoptosis or arrest with TGFβ treatment. Several of the BL31 recombinant cell lines did, however, undergo G1

cell cycle arrest with TGFβ, in contrast to the apoptosis seen in uninfected cells. The cell lines that underwent G1 arrest all lacked LMP2A expression, suggesting that LMP2A may be required to inhibit the anti-proliferative effects of TGFβ. LMP2A is known to have anti-apoptotic effects, but little is known of its effects on cell cycle progression.

As described above, a recent study in murine B cells and human DLBCL cell lines had demonstrated that TGFβ induced $p15^{INK4B}$ and $p21^{CIP1/WAF1}$, as well as reduction in phosphorylation of Rb, leading to arrest (Jiang and Aguiar 2014). Knockdown of SMAD5 by siRNA or via overexpression of miR-155, which the same group had previously shown to target SMAD5 (Rai et al. 2010), led to interruption of the TGFβ-induced induction of $p15^{INK4B}$/ $p21^{CIP1/WAF1}$ and cell cycle arrest, suggesting that SMAD5 was required for TGFβ-induced arrest. However, in the EBV-negative BL line CA46, which is unable to undergo apoptosis due to lack of BAX expression, Spender and Inman had shown that the mechanism of TGFβ-induced arrest occurred independently of $p15^{INK4B}$/ $p21^{CIP1/WAF1}$ induction, instead occurring via repression of E2F1 (Spender and Inman 2009b). In another study, TGFβ did induce $p21^{CIP1/WAF1}$ expression in the EBV-negative BL cell line Ramos (Di Bartolo et al. 2008). In the current study, TGFβ treatment did not induce $p15^{INK4B}$ in either BL31 or LCLs. The differences between studies may reflect different arrest mechanisms between BL (as used in the current study and in (Spender and Inman 2009b) and DLBCL lines or murine B cells (as used in Jiang and Aguiar 2014).

### 8.7 The significance of BMP signalling in B cell biology and B cell malignancies

The significance of the increased BMP signalling and concomitant cell cycle arrest by EBV is not known, and there is limited information available about the expression and activity of BMPs within the peripheral lymphoid system. Initially BMPs were thought to be produced only in bone, by osteoclasts, but it has since been shown that they are expressed by various other cell types, including within the bone marrow where they are important for controlling haematopoiesis (Bhatia et al. 1999, Passa et al. 2011). BMP7 is expressed in normal B cells (Detmer et al. 1999), and tonsillar centroblasts/centrocytes also showed high expression of BMP7, with low BMP6 expression (Huse et al. 2012). In several B lymphoma cell lines BMP2 expression was undetectable, whereas BMP4 expression varied between cell lines (Huse et

al. 2012). Naïve and memory B cells express ALK2, ALK3 and ALK6 (Kersten et al. 2005, Seckinger et al. 2009) and so would be expected to respond to BMP2, BMP4, BMP6 and BMP7.

The studies described above have investigated BMP expression by B cells themselves, which would be important for autocrine/paracrine signalling. However, it is also possible that secretion of BMPs by other cell types, such as stromal cells and other immune cells, could occur in different compartments of the lymphoid system. Since EBV enhances the anti-proliferative effects of BMP2, BMP4 and BMP6, signalling by these ligands is probably not prominent in GCs otherwise this would inhibit proliferation of EBV-infected cells. However, it is possible that in another part of the lymphoid system, it may be advantageous for EBV to enhance the anti-proliferative effects of these BMPs. BMP2, BMP4 and BMP6 inhibit differentiation of mature B cells into plasma cells (Huse et al. 2011), so perhaps EBV enhances this inhibition, thus maintaining latency by preventing differentiation to plasma cells which would result in lytic infection. Alternatively, BMP2, BMP6 and BMP7 can induce EBV lytic replication in latency I but not latency III infected Mutu (BL) cells *in vitro* (Yin et al. 2010), so it is possible that BMPs may be more active in the oropharynx thus enhancing lytic replication where it is desired.

A few studies have investigated the expression of BMPs in tumour samples from patients with B cell malignancies. BMP4 and BMP6 are secreted by the malignant plasma cells of myeloma patients (Grcevic et al. 2010), with another study showing BMP6 expression by both normal plasma cells and myeloma cell lines (Seckinger et al. 2009). BMP6 inhibits proliferation of myeloma cells, and high BMP6 expression was associated with a better prognosis (Seckinger et al. 2009), suggesting that autocrine/paracrine BMP signalling may limit proliferation in myeloma cells. However, BMP2, BMP4 and BMP6 were all able to partially protect myeloma cells from apoptosis induced by the proteasome inhibitor bortezomib, which is used as part of the treatment for myeloma (Grcevic et al. 2010). Furthermore, increased BMP7 expression has been found at relapse in mantle cell lymphoma (MCL), suggesting selective survival of BMP7-expressing cells after initial chemotherapy. The increased BMP7 expression correlated with resistance to both bortezomib and cytarabine, although the numbers were small in this study (Camara-Clayette

et al. 2013). Thus it is possible that BMPs may limit proliferation, but also by inducing cell cycle arrest may render cells relatively resistant to chemotherapy.

It would be interesting to further investigate the expression of BMPs and their receptors in different lymphoid tissues, for example by immunohistochemistry of human tonsillar tissue and lymphoma biopsy samples. In addition, ELISA for BMPs could be performed on peripheral blood of healthy volunteers, compared with patients with active lymphoma, in order to investigate the relevance of BMP signalling in B cell malignancies.

### 8.8 The importance of suppressing TGFβ signalling in lymphoma development

For a tumour to develop, it needs to switch off TSGs within the cell itself, but also needs to alter its microenvironment to favour tumour growth, for instance by evading immune responses. EBV, along with other gamma-herpesviruses and other tumour viruses, has evolved multiple mechanisms to suppress TSGs, allowing infected cells to survive in order to establish latency. In addition EBV has evolved several means of avoiding host cell immune responses, again important to allow survival of the infected cell. By switching off TGFβ signalling EBV is achieving both of these aims, as this allows the infected cell to survive the GC environment in order to get into the memory B cell pool and thereby establish latency, from where it can infect other hosts, and also inhibits T cell immune responses. Successful viruses aim to transmit virus widely between hosts, ideally without threatening the life of the host. Herpesviruses, including EBV, are highly successful at this, as demonstrated by the fact that most people are latently infected with EBV, in a worldwide distribution, and most are asymptomatic and yet continue to shed virus so that new hosts can become infected.

In order to establish latency, however, EBV must stimulate B cells to differentiate into hyper-proliferating lymphoblasts, within the GC, requiring expressing of its latent proteins, including pro-survival LMP1 and LMP2A, in order to survive in the pro-apoptotic GC environment. Expression of the EBNA3 proteins is then switched off, once the cell is proliferating, as EBNA3A and EBNA3C are highly oncogenic and immunogenic. However, the physiological GC environment is high-risk since double strand breaks (DSBs) and mutations are induced as part of SHM; it is no coincidence that so many different lymphomas arise

from cells within the GC. Under normal circumstances, the majority of cells with abnormal DNA would quickly be destroyed by pro-apoptotic mechanisms within the GC. However, expression of EBV latent proteins might enable cells to tolerate certain mutations, which would otherwise result in destruction of the affected cell. Therefore EBV infection can substitute for some additional mutations which occur in the development of tumours, thus increasing the likelihood of tumour development, even though this is not the 'intention' of the virus.

TGFβ signalling is an important component of apoptotic mechanisms within GC B cells, in the process of 'death by neglect'. Therefore switching off TGFβ signalling helps the infected cells to survive the GC environment. In addition, TGFβ signalling is generally immuno-suppressive, by suppressing T cell responses. Tumour cells, including lymphoma cells, often continue to secrete TGFβ, which can inhibit T cells thus inhibiting the immune response to tumour. Meanwhile the tumour itself has become resistant, by switching off TGFβ signalling, so is able to proliferate without being destroyed by this arm of the immune system.

Herpesviruses have developed multiple mechanisms for targeting TGFβ signalling, including via epigenetic regulation and miRNAs. KSHV LANA epigenetically silences TGFβR2 in PEL cell lines (Di Bartolo et al. 2008) and the virus also secretes a miRNA which targets TGFβR2 (Lei et al. 2012). In addition, another miRNA produced by KSHV targets SMAD5 and thus suppresses the non-canonical TGFβ signalling pathway when ectopically expressed in Ramos (Liu et al. 2012). MiR-155 is induced by EBV, and KSHV and Marek's disease virus (MDV, an oncogenic herpesvirus found in chickens), encode homologues of miR-155 (Yin et al. 2010). miR-155 is a known oncogenic miRNA (oncomiR), with expression leading to development of high grade lymphoma in a transgenic mouse model (Costinean et al. 2006), and has been shown to be overexpressed in DLBCL (Eis et al. 2005). Thus epigenetic and/or miRNA-induced changes to TGFβ signalling are commonly used by oncogenic herpesviruses to enable them to reach latency - but in doing so, these changes may promote tumour development.

Furthermore, other oncogenic viruses alter TGFβ signalling, for example overexpression of adenovirus E1A leads to down-regulation of TGFβR2 (Tarakanova and Wold 2003) and

inhibits TGFβ-mediated induction of p15 and p21 (Datto et al. 1997). The Tax protein of HTLV1 represses TGFβ signalling by interfering with recruitment of the co-activator p300 to SMAD-responsive elements (Mori et al. 2001) and a viral protein of hepatitis C inhibits the DNA-binding ability of SMAD3 interfering with SMAD3-mediated transcriptional activation (Cheng et al. 2004).

### 8.9 Clinical implications of the findings in this project

Transfection of the EBV-negative BL cell line Ramos with dominant-negative TGFβR2, leading to suppression of SMAD2 phosphorylation, resulted in increased tumour growth and decreased apoptosis *in vivo*, demonstrating the importance of inhibition of TGFβ-induced apoptosis in promoting tumour development (Kawabata et al. 2013). Therefore, inhibition of TGFβ signalling, which is tumour suppressive, is a mechanism for promoting lymphoma development. It could be hypothesised that suppression of TGFβ signalling might also be also found in non-EBV-associated B cell lymphomas.

In a gene expression profiling study of DLBCL, TGFβR2 expression was higher in the activated B cell-like, which have a worse prognosis, than the GC-like subtype (Alizadeh et al. 2000). Tumours may use several alternative mechanisms to evade apoptosis, for example suppressing TGFβ signalling in one particular tumour, or mutating p53 in another. The mechanism of disruption of apoptosis may alter the tumour's responsiveness to chemotherapy. For many chemotherapy agents, the exact mechanism by which they induce apoptosis are not entirely understood.

The treatment of lymphomas, and other malignancies, is moving more towards targeted therapies, amidst recognition that even within a particular type of tumour there are differences in the acquired abnormalities between individual tumours, and even between different sub-clones within the same tumour. Treatment of lymphomas was previously based on chemotherapy, which has non-specific cytotoxic effects; however, the introduction of the anti-CD20 monoclonal antibody Rituximab revolutionised treatment of B cell lymphomas, which commonly express CD20. Several drugs now in development or in early clinical trials inhibit specific signalling pathways within B cells, such as PI3K inhibitors and

BCR antagonists. The suppression of TGFβ signalling by EBV suggests that another means of targeting EBV-positive tumours could involve activation of TGFβR2 and thus restoration of TGFβ signalling. Several compounds targeting TGFβ signalling are currently in development, but unfortunately all are aimed at inhibiting TGFβ signalling, due to the tumour-promoting effects of TGFβ in several carcinomas (Smith et al. 2012). Nevertheless, if suppression of TGFβ signalling were found to be a mechanism used by many B cell malignancies, it should theoretically be possible to develop a pharmaceutical means of restoring signalling. The importance of TGFβ signalling disruption in development of lymphomas has not been systematically investigated, thus it would be interesting to investigate tumour biopsies of EBV-positive and negative lymphomas to see whether this occurs.

Since this study has shown that EBV induces epigenetic repression of TGFβR2, associated with decreased H3K9Ac and increased H3K27Me3, this suggest that drugs targeting epigenetic modifications may be beneficial in EBV-associated lymphomas. Such drugs include HDAC inhibitors, DNMT inhibitors and histone methyltransferase inhibitors. Several such drugs have been investigated in early clinical trials in lymphomas and other B cell malignancies [reviewed in (Hassler et al. 2013)]. These drugs would have the additional benefit of stimulating lytic viral infection and so could be combined with anti-viral therapy. DNMT inhibitors, such as azacytidine, are already in clinical use for the treatment of myelodysplastic syndrome (Fenaux et al. 2009) and the HDAC inhibitor vorinostat is licensed in the USA for treatment of cutaneous T-cell lymphoma (Hassler et al. 2013).

### 8.10 Directions for future work

This study has raised several questions for further investigation:

1. What are the consequences of the up-regulation of TGFβR3? In particular, does this alter cellular responses to TGFβ2, activins and/or inhibins?
2. How and to what extent do the EBNA3 proteins and LMPs together cooperate in regulating gene expression? Do LMP1 and LMP2A cooperate via modulation of other cellular signalling cascades such as NFκB or PI3K/Akt?
3. Are LMP1 and LMP2A also required for TGFβR2 repression and/or TGFβR3/SMAD1 up-regulation in LCLs?

4. What are the consequences of EBV up-regulating SMAD1 and down-regulating SMAD5 – does this alter responses to BMPs and/or non-canonical TGFβ signalling, particularly in terms of cell cycle arrest? Does this alter the BMP target gene profile?
5. What are the mechanisms of TGFβ-induced arrest and apoptosis in B cells - are these mediated via SMAD3, or by non-SMAD pathways?
6. Do biopsy samples of lymphoma (especially EBV-positive, but also EBV-negative) show DNA methylation of TGFβR2? Are TGFβR3 and SMAD1 expression increased in EBV-positive tumours? Does TGFβR2 repression replace deregulation of other tumour suppressor pathways, e.g. p53 mutations, and if so is this of prognostic relevance?
7. Does EBV, via EBNA3C, repress p15 in a similar way to p16, and what are the consequences of this?

## 8.11 Summary of the main findings of this study

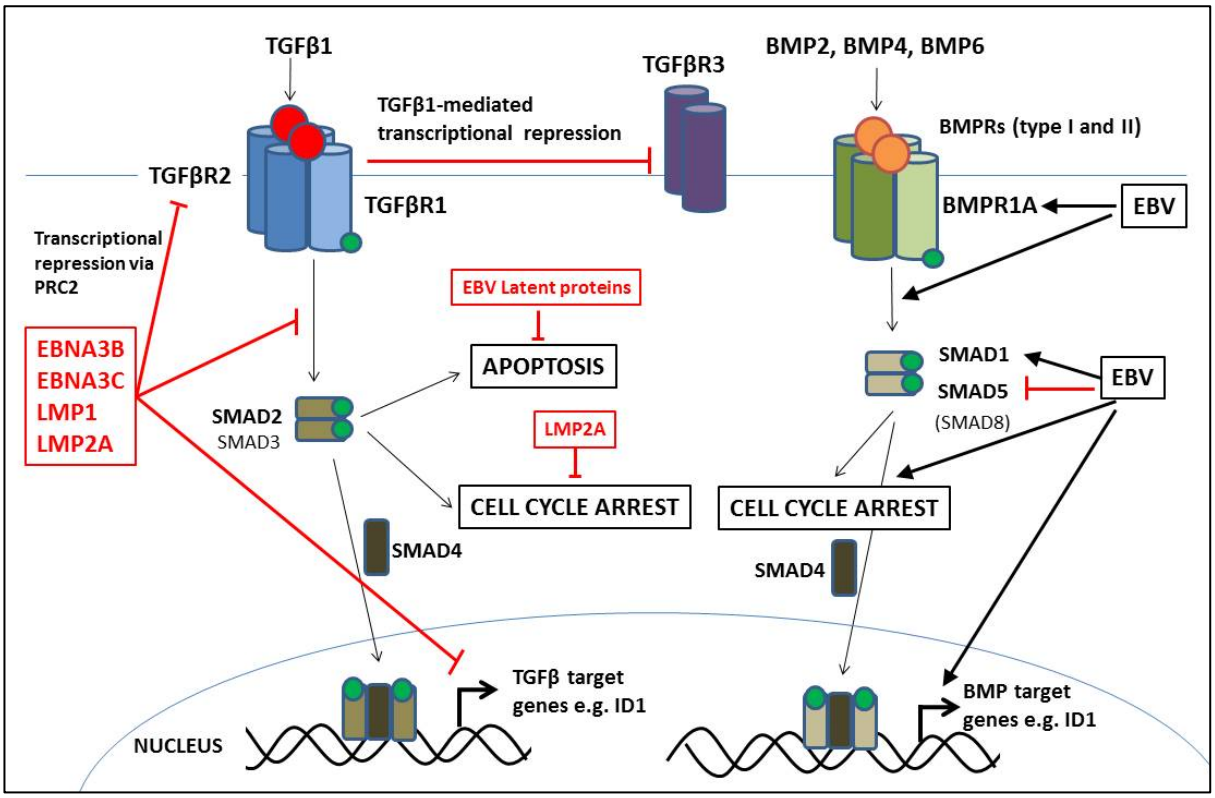



**Figure 8.1: The effects of latent EBV infection on TGFβ and BMP signalling**

The effects of latent EBV infection on TGFβ signalling (left side of figure) and on BMP signalling (right side), as demonstrated in this study, are shown. The effects of EBV on TGFβ signalling are generally inhibitory, shown in red, whereas the effects of EBV on BMP signalling components are generally enhancing (black arrows). The latent proteins EBNA3B, EBNA3C, LMP1 and LMP2A cooperate to repress transcription of TGFβR2, leading to suppression of TGFβ-induced phosphorylation of SMAD2 (pSMAD2) and hence inhibition of TGFβ-mediated induction of target genes ID1 and ID2. However, even though knockout of EBNA3B, EBNA3C, LMP1 and/or LMP2A leads to restoration of TGFβR2 expression and TGFβ-induced pSMAD2, this does not result in TGFβ-induced apoptosis. This is likely to be because EBV has multiple mechanisms to protect the cell from apoptosis. Knockout of LMP2A, but not the other latent genes, results in TGFβ-induced cell cycle arrest, suggesting that LMP2A protects against this. EBNA3B, EBNA3C, LMP1 and LMP2A also cooperate in transcriptional up-regulation of TGFβR3, possibly via relief of TGFβ1-mediated transcriptional repression of TGFβR3.

The effects of EBV on BMP signalling are generally opposite to those for BMP signalling, as seen in other cellular systems. EBV infection enhances phosphorylation of SMAD1/5/8 by BMP2, BMP4 and BMP6, leading to enhancement of BMP-induced cell cycle arrest and enhanced induction of ID1 expression. This enhancement of BMP signalling by EBV may be due to up-regulation of BMPRIA, but is not due to the up-regulation of TGFβR3. EBV infection also leads to up-regulation of SMAD1 but down-regulation of SMAD5 expression. The effects of EBV on TGFβ signalling via SMAD3 were not investigated in this study, and SMAD8 is probably not important/expressed in B cells.

## 8.12 Final conclusions

In summary, this thesis has shown that EBV represses TGFβ signalling via polycomb-mediated repression of TGFβR2, and has indicated which latent proteins are responsible. This repression involves cooperation between two EBNA3 proteins (EBNA3B and EBNA3C) as well as two latent membrane proteins, LMP1 and LMP2A, suggesting there may be more cooperation between these different types of latent protein than has previously been shown. The suppression of TGFβ signalling is presumably a mechanism used by EBV to promote survival of infected B cells in order to establish latency and long-term persistence; however, in doing this EBV also increases the risk of lymphoma development.

Furthermore, this thesis has demonstrated that EBV up-regulates expression of the co-receptor TGFβR3, although the significance of this is not yet known. EBV also increases signalling in response to BMP2, BMP4 and BMP6, although not by the up-regulation of TGFβR3. It also up-regulates SMAD1 expression, although it is not yet clear how this alters BMP signalling responses or non-canonical TGFβ signalling.

The repression of TGFβR2 and suppression of TGFβ signalling, as well as up-regulation of TGFβR3 and SMAD1, has been demonstrated in an EBV-negative BL cell line infected with BAC-derived recombinant viruses, after infection of purified primary B cells and in established LCLs. The findings on BMP responses in B cells suggest that BMP signalling is likely to be important in B cell biology, a field that has been hitherto overlooked, but is gradually attracting attention.

# References

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter (2008). *Molecular Biology of the Cell*, Garland Science.

Alexandrow, M. G., M. Kawabata, M. Aakre and H. L. Moses (1995). Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta 1. *Proc Natl Acad Sci U S A* **92**(8): 3239-3243.

Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown and L. M. Staudt (2000). Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* **403**(6769): 503-511.

Allday, M. J. (2009). How does Epstein-Barr virus (EBV) complement the activation of Myc in the pathogenesis of Burkitt's lymphoma? *Semin Cancer Biol* **19**(6): 366-376.

Allday, M. J. (2013). EBV finds a polycomb-mediated, epigenetic solution to the problem of oncogenic stress responses triggered by infection. *Front Genet* **4**: 212.

Allday, M. J., D. H. Crawford and J. A. Thomas (1993). Epstein-Barr virus (EBV) nuclear antigen 6 induces expression of the EBV latent membrane protein and an activated phenotype in Raji cells. *J Gen Virol* **74 ( Pt 3)**: 361-369.

Allday, M. J. and P. J. Farrell (1994). Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G1-arrested cells. *J Virol* **68**(6): 3491-3498.

Altiok, A., M. T. Bejarano, G. Klein and E. Klein (1992). Effect of TGF-beta 1 on the EBV-induced transformation of human lymphocyte cultures. *Int J Cancer* **50**(5): 772-776.

Altiok, A., M. T. Bejarano, F. Ruscetti, E. Altiok, G. Klein and E. Klein (1991). Effect of transforming growth factor-beta 1 and -beta 2 on the proliferation of Burkitt lymphoma and lymphoblastoid cell lines. *Growth Factors* **4**(2): 117-128.

Altiok, A., L. Di Renzo and E. Altiok (1994). Influence of transforming growth factor-beta (TGF-beta) on the immunoglobulin production by EBV-infected B cell cultures. *Immunol Lett* **43**(3): 199-202.

Altiok, A., B. Ehlin-Henriksson and E. Klein (1993). Correlation between the growth-inhibitory effect of TGF-beta 1 and phenotypic characteristics in a panel of B-cell lines. *Int J Cancer* **55**(1): 137-140.

Anderson, L. J. and R. Longnecker (2008). EBV LMP2A provides a surrogate pre-B cell receptor signal through constitutive activation of the ERK/MAPK pathway. *J Gen Virol* **89**(Pt 7): 1563-1568.

Anderton, E., J. Yee, P. Smith, T. Crook, R. E. White and M. J. Allday (2008). Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma. *Oncogene* **27**(4): 421-433.

Andres, J. L., K. Stanley, S. Cheifetz and J. Massague (1989). Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. *J Cell Biol* **109**(6 Pt 1): 3137-3145.

Anzano, M. A., A. B. Roberts, J. M. Smith, M. B. Sporn and J. E. De Larco (1983). Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. *Proc Natl Acad Sci U S A* **80**(20): 6264-6268.

Aoki, H., M. Fujii, T. Imamura, K. Yagi, K. Takehara, M. Kato and K. Miyazono (2001). Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. *J Cell Sci* **114**(Pt 8): 1483-1489.

Arsura, M., M. Wu and G. E. Sonenshein (1996). TGF beta 1 inhibits NF-kappa B/Rel activity inducing apoptosis of B cells: transcriptional activation of I kappa B alpha. *Immunity* **5**(1): 31-40.

Arvanitakis, L., N. Yaseen and S. Sharma (1995). Latent membrane protein-1 induces cyclin D2 expression, pRb hyperphosphorylation, and loss of TGF-beta 1-mediated growth inhibition in EBV-positive B cells. *J Immunol* **155**(3): 1047-1056.

Attisano, L. and J. L. Wrana (2000). Smads as transcriptional co-modulators. *Curr Opin Cell Biol* **12**(2): 235-243.

Babcock, G. J., L. L. Decker, R. B. Freeman and D. A. Thorley-Lawson (1999). Epstein-barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. *J Exp Med* **190**(4): 567-576.

Bae, H. W., A. G. Geiser, D. H. Kim, M. T. Chung, J. K. Burmester, M. B. Sporn, A. B. Roberts and S. J. Kim (1995). Characterization of the promoter region of the human transforming growth factor-beta type II receptor gene. *J Biol Chem* **270**(49): 29460-29468.

Bakkebo, M., K. Huse, V. I. Hilden, E. B. Smeland and M. P. Oksvold (2010). TGF-beta-induced growth inhibition in B-cell lymphoma correlates with Smad1/5 signalling and constitutively active p38 MAPK. *BMC Immunol* **11**: 57.

Balfour, H. H., Jr., O. A. Odumade, D. O. Schmeling, B. D. Mullan, J. A. Ed, J. A. Knight, H. E. Vezina, W. Thomas and K. A. Hogquist (2013). Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. *J Infect Dis* **207**(1): 80-88.

Bell, A. I., K. Groves, G. L. Kelly, D. Croom-Carter, E. Hui, A. T. Chan and A. B. Rickinson (2006). Analysis of Epstein-Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays. *J Gen Virol* **87**(Pt 10): 2885-2890.

Ben-Sasson, S. A. and G. Klein (1981). Activation of the Epstein-Barr virus genome by 5-aza-cytidine in latently infected human lymphoid lines. *Int J Cancer* **28**(2): 131-135.

Berger, S. L., T. Kouzarides, R. Shiekhattar and A. Shilatifard (2009). An operational definition of epigenetics. *Genes Dev* **23**(7): 781-783.

Bernabeu, C., J. M. Lopez-Novoa and M. Quintanilla (2009). The emerging role of TGF-beta superfamily coreceptors in cancer. *Biochim Biophys Acta* **1792**(10): 954-973.

Bernstein, B. E., T. S. Mikkelsen, X. Xie, M. Kamal, D. J. Huebert, J. Cuff, B. Fry, A. Meissner, M. Wernig, K. Plath, R. Jaenisch, A. Wagschal, R. Feil, S. L. Schreiber and E. S. Lander (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**(2): 315-326.

Bhatia, M., D. Bonnet, D. Wu, B. Murdoch, J. Wrana, L. Gallacher and J. E. Dick (1999). Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J Exp Med* **189**(7): 1139-1148.

Blobe, G. C., X. Liu, S. J. Fang, T. How and H. F. Lodish (2001a). A novel mechanism for regulating transforming growth factor beta (TGF-beta) signaling. Functional modulation of type III TGF-beta receptor expression through interaction with the PDZ domain protein, GIPC. *J Biol Chem* **276**(43): 39608-39617.

Blobe, G. C., W. P. Schiemann, M. C. Pepin, M. Beauchemin, A. Moustakas, H. F. Lodish and M. D. O'Connor-McCourt (2001b). Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. *J Biol Chem* **276**(27): 24627-24637.

Blomhoff, H. K., E. Smeland, A. S. Mustafa, T. Godal and R. Ohlsson (1987). Epstein-Barr virus mediates a switch in responsiveness to transforming growth factor, type beta, in cells of the B cell lineage. *Eur J Immunol* **17**(2): 299-301.

Bodescot, M. and M. Perricaudet (1986). Epstein-Barr virus mRNAs produced by alternative splicing. *Nucleic Acids Res* **14**(17): 7103-7114.

Boos, H., M. Stoehr, M. Sauter and N. Mueller-Lantzsch (1990). Flow cytometric analysis of Epstein-Barr virus (EBV) latent membrane protein expression in EBV-infected Raji cells. *J Gen Virol* **71 ( Pt 8)**: 1811-1815.

Bornkamm, G. W. and W. Hammerschmidt (2001). Molecular virology of Epstein-Barr virus. *Philos Trans R Soc Lond B Biol Sci* **356**(1408): 437-459.

Bottardi, S., A. Aumont, F. Grosveld and E. Milot (2003). Developmental stage-specific epigenetic control of human beta-globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation. *Blood* **102**(12): 3989-3997.

Brenet, F., P. Kermani, R. Spektor, S. Rafii and J. M. Scandura (2013). TGFbeta restores hematopoietic homeostasis after myelosuppressive chemotherapy. *J Exp Med* **210**(3): 623-639.

Brielmeier, M., J. Mautner, G. Laux and W. Hammerschmidt (1996). The latent membrane protein 2 gene of Epstein-Barr virus is important for efficient B cell immortalization. *J Gen Virol* **77 ( Pt 11)**: 2807-2818.

Bristow, R. E., R. L. Baldwin, S. D. Yamada, M. Korc and B. Y. Karlan (1999). Altered expression of transforming growth factor-beta ligands and receptors in primary and recurrent ovarian carcinoma. *Cancer* **85**(3): 658-668.

Brooks, J. M., S. P. Lee, A. M. Leese, W. A. Thomas, M. Rowe and A. B. Rickinson (2009). Cyclical expression of EBV latent membrane protein 1 in EBV-transformed B cells underpins heterogeneity of epitope presentation and CD8+ T cell recognition. *J Immunol* **182**(4): 1919-1928.

Brown, K. A., J. A. Pietenpol and H. L. Moses (2007). A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. *J Cell Biochem* **101**(1): 9-33.

Bruno, E., S. K. Horrigan, D. Van Den Berg, E. Rozler, P. R. Fitting, S. T. Moss, C. Westbrook and R. Hoffman (1998). The Smad5 gene is involved in the intracellular signaling pathways that mediate the

inhibitory effects of transforming growth factor-beta on human hematopoiesis. *Blood* **91**(6): 1917-1923.

Bultema, R., R. Longnecker and M. Swanson-Mungerson (2009). Epstein-Barr virus LMP2A accelerates MYC-induced lymphomagenesis. *Oncogene* **28**(11): 1471-1476.

Burkitt, D. (1958). A sarcoma involving the jaws in African children. *Br J Surg* **46**(197): 218-223.

Buske, C., D. Becker, M. Feuring-Buske, H. Hannig, F. Griesinger, W. Hiddemann and B. Wormann (1998). TGF-beta and its receptor complex in leukemic B-cell precursors. *Exp Hematol* **26**(12): 1155-1161.

Cai, X., A. Schafer, S. Lu, J. P. Bilello, R. C. Desrosiers, R. Edwards, N. Raab-Traub and B. R. Cullen (2006). Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. *PLoS Pathog* **2**(3): e23.

Calender, A., M. Billaud, J. P. Aubry, J. Banchereau, M. Vuillaume and G. M. Lenoir (1987). Epstein-Barr virus (EBV) induces expression of B-cell activation markers on in vitro infection of EBV-negative B-lymphoma cells. *Proc Natl Acad Sci U S A* **84**(22): 8060-8064.

Callan, M. F., L. Tan, N. Annels, G. S. Ogg, J. D. Wilson, C. A. O'Callaghan, N. Steven, A. J. McMichael and A. B. Rickinson (1998). Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. *J Exp Med* **187**(9): 1395-1402.

Camara-Clayette, V., S. Koscielny, S. Roux, T. Lamy, J. Bosq, M. Bernard, T. Fest, V. Lazar, G. Lenoir and V. Ribrag (2013). BMP7 Expression Correlates with Secondary Drug Resistance in Mantle Cell Lymphoma. *PLoS One* **8**(9): e73993.

Cancian, L., R. Bosshard, W. Lucchesi, C. E. Karstegl and P. J. Farrell (2011). C-terminal region of EBNA-2 determines the superior transforming ability of type 1 Epstein-Barr virus by enhanced gene regulation of LMP-1 and CXCR7. *PLoS Pathog* **7**(7): e1002164.

Cao, R., Y. Tsukada and Y. Zhang (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol Cell* **20**(6): 845-854.

Cao, R., L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R. S. Jones and Y. Zhang (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**(5595): 1039-1043.

Chadwick, K., L. Wang, L. Li, P. Menendez, B. Murdoch, A. Rouleau and M. Bhatia (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* **102**(3): 906-915.

Chang, L. K. and S. T. Liu (2000). Activation of the BRLF1 promoter and lytic cycle of Epstein-Barr virus by histone acetylation. *Nucleic Acids Res* **28**(20): 3918-3925.

Chaouchi, N., L. Arvanitakis, M. T. Auffredou, D. A. Blanchard, A. Vazquez and S. Sharma (1995). Characterization of transforming growth factor-beta 1 induced apoptosis in normal human B cells and lymphoma B cell lines. *Oncogene* **11**(8): 1615-1622.

Cheifetz, S., H. Hernandez, M. Laiho, P. ten Dijke, K. K. Iwata and J. Massague (1990). Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J Biol Chem* **265**(33): 20533-20538.

Chen, G., P. Ghosh, T. O'Farrell, R. Munk, L. J. Rezanka, C. Y. Sasaki and D. L. Longo (2012). Transforming growth factor beta1 (TGF-beta1) suppresses growth of B-cell lymphoma cells by p14(ARF)-dependent regulation of mutant p53. *J Biol Chem* **287**(27): 23184-23195.

Chen, G., P. Ghosh, H. Osawa, C. Y. Sasaki, L. Rezanka, J. Yang, T. J. O'Farrell and D. L. Longo (2007). Resistance to TGF-beta 1 correlates with aberrant expression of TGF-beta receptor II in human B-cell lymphoma cell lines. *Blood* **109**(12): 5301-5307.

Chen, W., K. C. Kirkbride, T. How, C. D. Nelson, J. Mo, J. P. Frederick, X. F. Wang, R. J. Lefkowitz and G. C. Blobe (2003). Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling. *Science* **301**(5638): 1394-1397.

Chen, Y. G., A. Hata, R. S. Lo, D. Wotton, Y. Shi, N. Pavletich and J. Massague (1998). Determinants of specificity in TGF-beta signal transduction. *Genes Dev* **12**(14): 2144-2152.

Cheng, P. L., M. H. Chang, C. H. Chao and Y. H. Lee (2004). Hepatitis C viral proteins interact with Smad3 and differentially regulate TGF-beta/Smad3-mediated transcriptional activation. *Oncogene* **23**(47): 7821-7838.

Chijioke, O., T. Azzi, D. Nadal and C. Munz (2013). Innate immune responses against Epstein Barr virus infection. *J Leukoc Biol* **94**(6): 1185-1190.

Choy, E. Y., K. L. Siu, K. H. Kok, R. W. Lung, C. M. Tsang, K. F. To, D. L. Kwong, S. W. Tsao and D. Y. Jin (2008). An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival. *J Exp Med* **205**(11): 2551-2560.

Clybouw, C., B. McHichi, S. Mouhamad, M. T. Auffredou, M. F. Bourgeade, S. Sharma, G. Leca and A. Vazquez (2005). EBV infection of human B lymphocytes leads to down-regulation of Bim expression: relationship to resistance to apoptosis. *J Immunol* **175**(5): 2968-2973.

Clybouw, C., B. E. McHichi, A. Hadji, A. Portier, M. T. Auffredou, D. Arnoult, G. Leca and A. Vazquez (2008). TGFbeta-mediated apoptosis of Burkitt's lymphoma BL41 cells is associated with the relocation of mitochondrial BimEL. *Oncogene* **27**(24): 3446-3456.

Cohen, J. I., F. Wang, J. Mannick and E. Kieff (1989). Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proc Natl Acad Sci U S A* **86**(23): 9558-9562.

Conery, A. R., Y. Cao, E. A. Thompson, C. M. Townsend, Jr., T. C. Ko and K. Luo (2004). Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* **6**(4): 366-372.

Cooper, S. J., H. Zou, S. N. Legrand, L. A. Marlow, C. A. von Roemeling, D. C. Radisky, K. J. Wu, N. Hempel, V. Margulis, H. W. Tun, G. C. Blobe, C. G. Wood and J. A. Copland (2010). Loss of type III transforming growth factor-beta receptor expression is due to methylation silencing of the transcription factor GATA3 in renal cell carcinoma. *Oncogene* **29**(20): 2905-2915.

Cordier, M., A. Calender, M. Billaud, U. Zimber, G. Rousselet, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm and G. M. Lenoir (1990). Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *J Virol* **64**(3): 1002-1013.

Costinean, S., N. Zanesi, Y. Pekarsky, E. Tili, S. Volinia, N. Heerema and C. M. Croce (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci U S A* **103**(18): 7024-7029.

Countryman, J. and G. Miller (1985). Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proc Natl Acad Sci U S A* **82**(12): 4085-4089.

Countryman, J. K., L. Gradoville and G. Miller (2008). Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors. *J Virol* **82**(10): 4706-4719.

Crawford, D. H. (2001). Biology and disease associations of Epstein-Barr virus. *Philos Trans R Soc Lond B Biol Sci* **356**(1408): 461-473.

D'Souza, B. N., L. C. Edelstein, P. M. Pegman, S. M. Smith, S. T. Loughran, A. Clarke, A. Mehl, M. Rowe, C. Gelinas and D. Walls (2004). Nuclear factor kappa B-dependent activation of the antiapoptotic bfl-1 gene by the Epstein-Barr virus latent membrane protein 1 and activated CD40 receptor. *J Virol* **78**(4): 1800-1816.

Daly, A. C., R. A. Randall and C. S. Hill (2008). Transforming growth factor beta-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth. *Mol Cell Biol* **28**(22): 6889-6902.

Dambaugh, T., K. Hennessy, L. Chamnankit and E. Kieff (1984). U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. *Proc Natl Acad Sci U S A* **81**(23): 7632-7636.

Danial, N. N. and S. J. Korsmeyer (2004). Cell death: critical control points. *Cell* **116**(2): 205-219.

Datto, M. B., P. P. Hu, T. F. Kowalik, J. Yingling and X. F. Wang (1997). The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B. *Mol Cell Biol* **17**(4): 2030-2037.

Datto, M. B., Y. Li, J. F. Panus, D. J. Howe, Y. Xiong and X. F. Wang (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci U S A* **92**(12): 5545-5549.

Dave, S. S., K. Fu, G. W. Wright, L. T. Lam, P. Kluin, E. J. Boerma, T. C. Greiner, D. D. Weisenburger, A. Rosenwald, G. Ott, H. K. Muller-Hermelink, R. D. Gascoyne, J. Delabie, L. M. Rimsza, R. M. Braziel, T. M. Grogan, E. Campo, E. S. Jaffe, B. J. Dave, W. Sanger, M. Bast, J. M. Vose, J. O. Armitage, J. M. Connors, E. B. Smeland, S. Kvaloy, H. Holte, R. I. Fisher, T. P. Miller, E. Montserrat, W. H. Wilson, M. Bahl, H. Zhao, L. Yang, J. Powell, R. Simon, W. C. Chan and L. M. Staudt (2006). Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* **354**(23): 2431-2442.

David, L., C. Mallet, S. Mazerbourg, J. J. Feige and S. Bailly (2007). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* **109**(5): 1953-1961.

Dawson, C. W., J. H. George, S. M. Blake, R. Longnecker and L. S. Young (2001). The Epstein-Barr virus encoded latent membrane protein 2A augments signaling from latent membrane protein 1. *Virology* **289**(2): 192-207.

de Caestecker, M. (2004). The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev* **15**(1): 1-11.

de Caestecker, M. P., W. T. Parks, C. J. Frank, P. Castagnino, D. P. Bottaro, A. B. Roberts and R. J. Lechleider (1998). Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. *Genes Dev* **12**(11): 1587-1592.

de Carvalho, F., G. W. Colleoni, M. S. Almeida, A. L. Carvalho and A. L. Vettore (2009). TGFbetaR2 aberrant methylation is a potential prognostic marker and therapeutic target in multiple myeloma. *Int J Cancer* **125**(8): 1985-1991.

De Leo, A., G. Arena, C. Stecca, M. Raciti and E. Mattia (2011). Resveratrol Inhibits Proliferation and Survival of Epstein Barr Virus-Infected Burkitt's Lymphoma Cells Depending on Viral Latency Program. *Mol Cancer Res* **9**(10): 1346-1355.

DeCoteau, J. F., P. I. Knaus, H. Yankelev, M. D. Reis, R. Lowsky, H. F. Lodish and M. E. Kadin (1997). Loss of functional cell surface transforming growth factor beta (TGF-beta) type 1 receptor correlates with insensitivity to TGF-beta in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* **94**(11): 5877-5881.

Dehay, C. and H. Kennedy (2007). Cell-cycle control and cortical development. *Nat Rev Neurosci* **8**(6): 438-450.

Delecluse, H. J., T. Hilsendegen, D. Pich, R. Zeidler and W. Hammerschmidt (1998). Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. *Proc Natl Acad Sci U S A* **95**(14): 8245-8250.

Dennler, S., S. Itoh, D. Vivien, P. ten Dijke, S. Huet and J. M. Gauthier (1998). Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J* **17**(11): 3091-3100.

Derynck, R. and K. Miyazono (2008). *The TGF-β Family*, Cold Spring Harbor Laboratory Press.

Derynck, R. and Y. E. Zhang (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**(6958): 577-584.

Desruisseau, S., J. Palmari, C. Giusti, S. Romain, P. M. Martin and Y. Berthois (2006). Determination of TGFbeta1 protein level in human primary breast cancers and its relationship with survival. *Br J Cancer* **94**(2): 239-246.

Detmer, K., T. A. Steele, M. A. Shoop and H. Dannawi (1999). Lineage-restricted expression of bone morphogenetic protein genes in human hematopoietic cell lines. *Blood Cells Mol Dis* **25**(5-6): 310-323.

Detmer, K. and A. N. Walker (2002). Bone morphogenetic proteins act synergistically with haematopoietic cytokines in the differentiation of haematopoietic progenitors. *Cytokine* **17**(1): 36-42.

Deveraux, Q. L., N. Roy, H. R. Stennicke, T. Van Arsdale, Q. Zhou, S. M. Srinivasula, E. S. Alnemri, G. S. Salvesen and J. C. Reed (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* **17**(8): 2215-2223.

Deveraux, Q. L., R. Takahashi, G. S. Salvesen and J. C. Reed (1997). X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388**(6639): 300-304.

Di Bartolo, D. L., M. Cannon, Y. F. Liu, R. Renne, A. Chadburn, C. Boshoff and E. Cesarman (2008). KSHV LANA inhibits TGF-beta signaling through epigenetic silencing of the TGF-beta type II receptor. *Blood* **111**(9): 4731-4740.

di Renzo, L., A. Altiok, G. Klein and E. Klein (1994). Endogenous TGF-beta contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines. *Int J Cancer* **57**(6): 914-919.

Dirmeier, U., B. Neuhierl, E. Kilger, G. Reisbach, M. L. Sandberg and W. Hammerschmidt (2003). Latent membrane protein 1 is critical for efficient growth transformation of human B cells by epstein-barr virus. *Cancer Res* **63**(11): 2982-2989.

Doetzlhofer, A., H. Rotheneder, G. Lagger, M. Koranda, V. Kurtev, G. Brosch, E. Wintersberger and C. Seiser (1999). Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol Cell Biol* **19**(8): 5504-5511.

Dominguez-Sola, D., G. D. Victora, C. Y. Ying, R. T. Phan, M. Saito, M. C. Nussenzweig and R. Dalla-Favera (2012). The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry. *Nat Immunol* **13**(11): 1083-1091.

Dong, M. and G. C. Blobe (2006). Role of transforming growth factor-beta in hematologic malignancies. *Blood* **107**(12): 4589-4596.

Dong, M., T. How, K. C. Kirkbride, K. J. Gordon, J. D. Lee, N. Hempel, P. Kelly, B. J. Moeller, J. R. Marks and G. C. Blobe (2007). The type III TGF-beta receptor suppresses breast cancer progression. *J Clin Invest* **117**(1): 206-217.

Dong, Z., W. Guo, Y. Guo, G. Kuang and Z. Yang (2012). Concordant promoter methylation of transforming growth factor-beta receptor types I and II occurs early in esophageal squamous cell carcinoma. *Am J Med Sci* **343**(5): 375-381.

Douglas, R. S., R. J. Capocasale, R. J. Lamb, P. C. Nowell and J. S. Moore (1997). Chronic lymphocytic leukemia B cells are resistant to the apoptotic effects of transforming growth factor-beta. *Blood* **89**(3): 941-947.

Du, Z. M., L. F. Hu, H. Y. Wang, L. X. Yan, Y. X. Zeng, J. Y. Shao and I. Ernberg (2011). Upregulation of MiR-155 in nasopharyngeal carcinoma is partly driven by LMP1 and LMP2A and downregulates a negative prognostic marker JMJD1A. *PLoS One* **6**(4): e19137.

Ebisawa, T., M. Fukuchi, G. Murakami, T. Chiba, K. Tanaka, T. Imamura and K. Miyazono (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* **276**(16): 12477-12480.

Ebisawa, T., K. Tada, I. Kitajima, K. Tojo, T. K. Sampath, M. Kawabata, K. Miyazono and T. Imamura (1999). Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. *J Cell Sci* **112 ( Pt 20)**: 3519-3527.

Egle, A., A. W. Harris, P. Bouillet and S. Cory (2004). Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci U S A* **101**(16): 6164-6169.

Eis, P. S., W. Tam, L. Sun, A. Chadburn, Z. Li, M. F. Gomez, E. Lund and J. E. Dahlberg (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* **102**(10): 3627-3632.

Elliott, R. L. and G. C. Blobe (2005). Role of transforming growth factor Beta in human cancer. *J Clin Oncol* **23**(9): 2078-2093.

Epstein, M. A., B. G. Achong and Y. M. Barr (1964). VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA. *Lancet* **1**(7335): 702-703.

Epstein, M. A. and Y. M. Barr (1964). CULTIVATION IN VITRO OF HUMAN LYMPHOBLASTS FROM BURKITT'S MALIGNANT LYMPHOMA. *Lancet* **1**(7327): 252-253.

Esteller, M. (2007). Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* **8**(4): 286-298.

Evans, A. S., J. C. Niederman and R. W. McCollum (1968). Seroepidemiologic studies of infectious mononucleosis with EB virus. *N Engl J Med* **279**(21): 1121-1127.

Everett, R. D., J. Murray, A. Orr and C. M. Preston (2007). Herpes simplex virus type 1 genomes are associated with ND10 nuclear substructures in quiescently infected human fibroblasts. *J Virol* **81**(20): 10991-11004.

Fahmi, H., C. Cochet, Z. Hmama, P. Opolon and I. Joab (2000). Transforming growth factor beta 1 stimulates expression of the Epstein-Barr virus BZLF1 immediate-early gene product ZEBRA by an indirect mechanism which requires the MAPK kinase pathway. *J Virol* **74**(13): 5810-5818.

Faqing, T., H. Zhi, Y. Liqun, T. Min, G. Huanhua, D. Xiyun and C. Ya (2005). Epstein-Barr virus LMP1 initiates cell proliferation and apoptosis inhibition via regulating expression of Survivin in nasopharyngeal carcinoma. *Exp Oncol* **27**(2): 96-101.

Fear, D. J. (2013). Mechanisms regulating the targeting and activity of activation induced cytidine deaminase. *Curr Opin Immunol* **25**(5): 619-628.

Feederle, R., E. J. Bartlett and H. J. Delecluse (2010). Epstein-Barr virus genetics: talking about the BAC generation. *Herpesviridae* **1**(1): 6.

Feeley, B. T., S. C. Gamradt, W. K. Hsu, N. Liu, L. Krenek, P. Robbins, J. Huard and J. R. Lieberman (2005). Influence of BMPs on the formation of osteoblastic lesions in metastatic prostate cancer. *J Bone Miner Res* **20**(12): 2189-2199.

Fenaux, P., G. J. Mufti, E. Hellstrom-Lindberg, V. Santini, C. Finelli, A. Giagounidis, R. Schoch, N. Gattermann, G. Sanz, A. List, S. D. Gore, J. F. Seymour, J. M. Bennett, J. Byrd, J. Backstrom, L. Zimmerman, D. McKenzie, C. Beach and L. R. Silverman (2009). Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol* **10**(3): 223-232.

Feng, X. H. and R. Derynck (1997). A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. *EMBO J* **16**(13): 3912-3923.

Feng, X. H. and R. Derynck (2005). Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol* **21**: 659-693.

Finger, E. C., N. Y. Lee, H. J. You and G. C. Blobe (2008a). Endocytosis of the type III transforming growth factor-beta (TGF-beta) receptor through the clathrin-independent/lipid raft pathway regulates TGF-beta signaling and receptor down-regulation. *J Biol Chem* **283**(50): 34808-34818.

Finger, E. C., R. S. Turley, M. Dong, T. How, T. A. Fields and G. C. Blobe (2008b). TbetaRIII suppresses non-small cell lung cancer invasiveness and tumorigenicity. *Carcinogenesis* **29**(3): 528-535.

Florio, P., P. Ciarmela, F. M. Reis, P. Toti, L. Galleri, R. Santopietro, E. Tiso, P. Tosi and F. Petraglia (2005). Inhibin alpha-subunit and the inhibin coreceptor betaglycan are downregulated in endometrial carcinoma. *Eur J Endocrinol* **152**(2): 277-284.

Frederick, J. P., N. T. Liberati, D. S. Waddell, Y. Shi and X. F. Wang (2004). Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. *Mol Cell Biol* **24**(6): 2546-2559.

Friedenberg, W. R., S. A. Salzman, S. M. Phan and J. K. Burmester (1999). Transforming growth factor-beta and multidrug resistance in chronic lymphocytic leukemia. *Med Oncol* **16**(2): 110-118.

Fruehling, S. and R. Longnecker (1997). The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. *Virology* **235**(2): 241-251.

Fukuda, M., K. Ikuta, K. Yanagihara, M. Tajima, H. Kuratsune, T. Kurata and T. Sairenji (2001). Effect of transforming growth factor-beta1 on the cell growth and Epstein-Barr virus reactivation in EBV-infected epithelial cell lines. *Virology* **288**(1): 109-118.

Fukuda, M., H. Kurosaki and T. Sairenji (2006a). Loss of functional transforming growth factor (TGF)-beta type II receptor results in insensitivity to TGF-beta1-mediated apoptosis and Epstein-Barr virus reactivation. *J Med Virol* **78**(11): 1456-1464.

Fukuda, M., W. Kurosaki, K. Yanagihara, H. Kuratsune and T. Sairenji (2002). A mechanism in Epstein-Barr virus oncogenesis: inhibition of transforming growth factor-beta 1-mediated induction of MAPK/p21 by LMP1. *Virology* **302**(2): 310-320.

Fukuda, M. and R. Longnecker (2004). Latent membrane protein 2A inhibits transforming growth factor-beta 1-induced apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. *J Virol* **78**(4): 1697-1705.

Fukuda, N., M. Saitoh, N. Kobayashi and K. Miyazono (2006b). Execution of BMP-4-induced apoptosis by p53-dependent ER dysfunction in myeloma and B-cell hybridoma cells. *Oncogene* **25**(25): 3509-3517.

Fukushima, D., R. Butzow, A. Hildebrand and E. Ruoslahti (1993). Localization of transforming growth factor beta binding site in betaglycan. Comparison with small extracellular matrix proteoglycans. *J Biol Chem* **268**(30): 22710-22715.

Gatza, C. E., S. Y. Oh and G. C. Blobe (2010). Roles for the type III TGF-beta receptor in human cancer. *Cell Signal* **22**(8): 1163-1174.

Ghosh, S. K., S. P. Perrine, R. M. Williams and D. V. Faller (2012). Histone deacetylase inhibitors are potent inducers of gene expression in latent EBV and sensitize lymphoma cells to nucleoside antiviral agents. *Blood* **119**(4): 1008-1017.

Gloghini, A., R. Dolcetti and A. Carbone (2013). Lymphomas occurring specifically in HIV-infected patients: From pathogenesis to pathology. *Semin Cancer Biol* **23**(6): 457-467.

Gordon, K. J., M. Dong, E. M. Chislock, T. A. Fields and G. C. Blobe (2008). Loss of type III transforming growth factor beta receptor expression increases motility and invasiveness associated with epithelial to mesenchymal transition during pancreatic cancer progression. *Carcinogenesis* **29**(2): 252-262.

Gottschalk, S., H. E. Heslop and C. M. Rooney (2005). Adoptive immunotherapy for EBV-associated malignancies. *Leuk Lymphoma* **46**(1): 1-10.

Gottschalk, S., C. Y. Ng, M. Perez, C. A. Smith, C. Sample, M. K. Brenner, H. E. Heslop and C. M. Rooney (2001). An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs. *Blood* **97**(4): 835-843.

Goumans, M. J., G. Valdimarsdottir, S. Itoh, F. Lebrin, J. Larsson, C. Mummery, S. Karlsson and P. ten Dijke (2003). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell* **12**(4): 817-828.

Goumans, M. J., G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras and P. ten Dijke (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* **21**(7): 1743-1753.

Grcevic, D., R. Kusec, N. Kovacic, A. Lukic, I. K. Lukic, S. Ivcevic, D. Nemet, R. S. Seiwerth, S. K. Ostojic, P. I. Croucher and A. Marusic (2010). Bone morphogenetic proteins and receptors are over-expressed in bone-marrow cells of multiple myeloma patients and support myeloma cells by inducing ID genes. *Leuk Res* **34**(6): 742-751.

Grimm, T., S. Schneider, E. Naschberger, J. Huber, E. Guenzi, A. Kieser, P. Reitmeir, T. F. Schulz, C. A. Morris and M. Sturzl (2005). EBV latent membrane protein-1 protects B cells from apoptosis by inhibition of BAX. *Blood* **105**(8): 3263-3269.

Gronroos, E., I. J. Kingston, A. Ramachandran, R. A. Randall, P. Vizan and C. S. Hill (2012). Transforming growth factor beta inhibits bone morphogenetic protein-induced transcription through novel phosphorylated Smad1/5-Smad3 complexes. *Mol Cell Biol* **32**(14): 2904-2916.

Grundhoff, A. and C. S. Sullivan (2011). Virus-encoded microRNAs. *Virology* **411**(2): 325-343.

Guasparri, I., D. Bubman and E. Cesarman (2008). EBV LMP2A affects LMP1-mediated NF-kappaB signaling and survival of lymphoma cells by regulating TRAF2 expression. *Blood* **111**(7): 3813-3820.

Guo, W., Z. Dong, Y. Guo, G. Kuang, Z. Yang and B. Shan (2012). Concordant repression and aberrant methylation of transforming growth factor-beta signaling pathway genes occurs early in gastric cardia adenocarcinoma. *Mol Biol Rep* **39**(10): 9453-9462.

Hadinoto, V., M. Shapiro, C. C. Sun and D. A. Thorley-Lawson (2009). The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. *PLoS Pathog* **5**(7): e1000496.

Hammerschmidt, W. and B. Sugden (1988). Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. *Cell* **55**(3): 427-433.

Hammerschmidt, W. and B. Sugden (1989). Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature* **340**(6232): 393-397.

Hannigan, A., P. Smith, G. Kalna, C. Lo Nigro, C. Orange, D. I. O'Brien, R. Shah, N. Syed, L. C. Spender, B. Herrera, J. K. Thurlow, L. Lattanzio, M. Monteverde, M. E. Maurer, F. M. Buffa, J. Mann, D. C. Chu, C. M. West, M. Patridge, K. A. Oien, J. A. Cooper, M. C. Frame, A. L. Harris, L. Hiller, L. J. Nicholson, M. Gasco, T. Crook and G. J. Inman (2010). Epigenetic downregulation of human disabled homolog 2 switches TGF-beta from a tumor suppressor to a tumor promoter. *J Clin Invest* **120**(8): 2842-2857.

Hannon, G. J. and D. Beach (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371**(6494): 257-261.

Harada, S. and E. Kieff (1997). Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation. *J Virol* **71**(9): 6611-6618.

Harjunpaa, A., M. Taskinen, M. Nykter, M. L. Karjalainen-Lindsberg, H. Nyman, O. Monni, S. Hemmer, O. Yli-Harja, S. Hautaniemi, S. Meri and S. Leppa (2006). Differential gene expression in non-malignant tumour microenvironment is associated with outcome in follicular lymphoma patients treated with rituximab and CHOP. *Br J Haematol* **135**(1): 33-42.

Hart, P. J., S. Deep, A. B. Taylor, Z. Shu, C. S. Hinck and A. P. Hinck (2002). Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex. *Nat Struct Biol* **9**(3): 203-208.

Hart, S. L., C. V. Arancibia-Carcamo, M. A. Wolfert, C. Mailhos, N. J. O'Reilly, R. R. Ali, C. Coutelle, A. J. George, R. P. Harbottle, A. M. Knight, D. F. Larkin, R. J. Levinsky, L. W. Seymour, A. J. Thrasher and C. Kinnon (1998). Lipid-mediated enhancement of transfection by a nonviral integrin-targeting vector. *Hum Gene Ther* **9**(4): 575-585.

Hassler, M. R., A. I. Schiefer and G. Egger (2013). Combating the epigenome: epigenetic drugs against non-Hodgkin's lymphoma. *Epigenomics* **5**(4): 397-415.

Hawkins, J. B., E. Delgado-Eckert, D. A. Thorley-Lawson and M. Shapiro (2013). The Cycle of EBV Infection Explains Persistence, the Sizes of the Infected Cell Populations and Which Come under CTL Regulation. *PLoS Pathog* **9**(10): e1003685.

He, G. and D. M. Margolis (2002). Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat. *Mol Cell Biol* **22**(9): 2965-2973.

Heath, E., N. Begue-Pastor, S. Chaganti, D. Croom-Carter, C. Shannon-Lowe, D. Kube, R. Feederle, H. J. Delecluse, A. B. Rickinson and A. I. Bell (2012). Epstein-Barr virus infection of naive B cells in vitro frequently selects clones with mutated immunoglobulin genotypes: implications for virus biology. *PLoS Pathog* **8**(5): e1002697.

Heldin, C. H. and A. Moustakas (2012). Role of Smads in TGFbeta signaling. *Cell Tissue Res* **347**(1): 21-36.

Hemann, M. T., A. Bric, J. Teruya-Feldstein, A. Herbst, J. A. Nilsson, C. Cordon-Cardo, J. L. Cleveland, W. P. Tansey and S. W. Lowe (2005). Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. *Nature* **436**(7052): 807-811.

Hempel, N., T. How, S. J. Cooper, T. R. Green, M. Dong, J. A. Copland, C. G. Wood and G. C. Blobe (2008). Expression of the type III TGF-beta receptor is negatively regulated by TGF-beta. *Carcinogenesis* **29**(5): 905-912.

Hempel, N., T. How, M. Dong, S. K. Murphy, T. A. Fields and G. C. Blobe (2007). Loss of betaglycan expression in ovarian cancer: role in motility and invasion. *Cancer Res* **67**(11): 5231-5238.

Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff and A. Rickinson (1991). Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* **65**(7): 1107-1115.

Henle, G., W. Henle and V. Diehl (1968). Relation of Burkitt's tumor-associated herpes-ytpe virus to infectious mononucleosis. *Proc Natl Acad Sci U S A* **59**(1): 94-101.

Hennino, A., M. Berard, P. H. Krammer and T. Defrance (2001). FLICE-inhibitory protein is a key regulator of germinal center B cell apoptosis. *J Exp Med* **193**(4): 447-458.

Herrera, B., M. Garcia-Alvaro, S. Cruz, P. Walsh, M. Fernandez, C. Roncero, I. Fabregat, A. Sanchez and G. J. Inman (2013). BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells. *PLoS One* **8**(7): e69535.

Herrera, B., M. van Dinther, P. Ten Dijke and G. J. Inman (2009). Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation. *Cancer Res* **69**(24): 9254-9262.

Hertle, M. L., C. Popp, S. Petermann, S. Maier, E. Kremmer, R. Lang, J. Mages and B. Kempkes (2009). Differential gene expression patterns of EBV infected EBNA-3A positive and negative human B lymphocytes. *PLoS Pathog* **5**(7): e1000506.

Hickabottom, M., G. A. Parker, P. Freemont, T. Crook and M. J. Allday (2002). Two nonconsensus sites in the Epstein-Barr virus oncoprotein EBNA3A cooperate to bind the co-repressor carboxyl-terminal-binding protein (CtBP). *J Biol Chem* **277**(49): 47197-47204.

Hislop, A. D., M. Kuo, A. B. Drake-Lee, A. N. Akbar, W. Bergler, N. Hammerschmitt, N. Khan, U. Palendira, A. M. Leese, J. M. Timms, A. I. Bell, C. D. Buckley and A. B. Rickinson (2005). Tonsillar homing of Epstein-Barr virus-specific CD8+ T cells and the virus-host balance. *J Clin Invest* **115**(9): 2546-2555.

Hislop, A. D., G. S. Taylor, D. Sauce and A. B. Rickinson (2007). Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* **25**: 587-617.

Hjertner, O., H. Hjorth-Hansen, M. Borset, C. Seidel, A. Waage and A. Sundan (2001). Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells. *Blood* **97**(2): 516-522.

Holien, T., T. K. Vatsveen, H. Hella, C. Rampa, G. Brede, L. A. Groseth, M. Rekvig, M. Borset, T. Standal, A. Waage and A. Sundan (2012). Bone morphogenetic proteins induce apoptosis in multiple myeloma cells by Smad-dependent repression of MYC. *Leukemia* **26**(5): 1073-1080.

Holley-Guthrie, E. A., E. B. Quinlivan, E. C. Mar and S. Kenney (1990). The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. *J Virol* **64**(8): 3753-3759.

Horndasch, M., E. E. Raschke, G. Bommer, M. Schuhmacher, E. Dumont, C. Kuklik-Roos, D. Eick and B. Kempkes (2002). Epstein-Barr virus antagonizes the antiproliferative activity of transforming growth factor-beta but does not abolish its signaling. *Int J Cancer* **101**(5): 442-447.

Huen, D. S., S. A. Henderson, D. Croom-Carter and M. Rowe (1995). The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* **10**(3): 549-560.

Hummel, M., S. Bentink, H. Berger, W. Klapper, S. Wessendorf, T. F. Barth, H. W. Bernd, S. B. Cogliatti, J. Dierlamm, A. C. Feller, M. L. Hansmann, E. Haralambieva, L. Harder, D. Hasenclever, M. Kuhn, D. Lenze, P. Lichter, J. I. Martin-Subero, P. Moller, H. K. Muller-Hermelink, G. Ott, R. M. Parwaresch, C. Pott, A. Rosenwald, M. Rosolowski, C. Schwaenen, B. Sturzenhofecker, M. Szczepanowski, H. Trautmann, H. H. Wacker, R. Spang, M. Loeffler, L. Trumper, H. Stein and R. Siebert (2006). A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N Engl J Med* **354**(23): 2419-2430.

Humphries, D. E., B. B. Bloom, A. Fine and R. H. Goldstein (1994). Structure and expression of the promoter for the human type II transforming growth factor-beta receptor. *Biochem Biophys Res Commun* **203**(2): 1020-1027.

Huse, K., M. Bakkebo, M. P. Oksvold, L. Forfang, V. I. Hilden, T. Stokke, E. B. Smeland and J. H. Myklebust (2011). Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: differential effects of BMP-6 and BMP-7. *Eur J Immunol* **41**(11): 3135-3145.

Huse, K., M. Bakkebo, S. Walchli, M. P. Oksvold, V. I. Hilden, L. Forfang, M. L. Bredahl, K. Liestol, A. A. Alizadeh, E. B. Smeland and J. H. Myklebust (2012). Role of Smad Proteins in Resistance to BMP-Induced Growth Inhibition in B-Cell Lymphoma. *PLoS One* **7**(10): e46117.

Huse, M., T. W. Muir, L. Xu, Y. G. Chen, J. Kuriyan and J. Massague (2001). The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* **8**(3): 671-682.

Husson, H., E. G. Carideo, D. Neuberg, J. Schultze, O. Munoz, P. W. Marks, J. W. Donovan, A. C. Chillemi, P. O'Connell and A. S. Freedman (2002). Gene expression profiling of follicular lymphoma and normal germinal center B cells using cDNA arrays. *Blood* **99**(1): 282-289.

Iavarone, A. and J. Massague (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature* **387**(6631): 417-422.

Inman, G. J. and M. J. Allday (2000a). Apoptosis induced by TGF-beta 1 in Burkitt's lymphoma cells is caspase 8 dependent but is death receptor independent. *J Immunol* **165**(5): 2500-2510.

Inman, G. J. and M. J. Allday (2000b). Resistance to TGF-beta1 correlates with a reduction of TGF-beta type II receptor expression in Burkitt's lymphoma and Epstein-Barr virus-transformed B lymphoblastoid cell lines. *J Gen Virol* **81**(Pt 6): 1567-1578.

Inman, G. J., U. K. Binne, G. A. Parker, P. J. Farrell and M. J. Allday (2001). Activators of the Epstein-Barr virus lytic program concomitantly induce apoptosis, but lytic gene expression protects from cell death. *J Virol* **75**(5): 2400-2410.

Inman, G. J. and C. S. Hill (2002). Stoichiometry of active smad-transcription factor complexes on DNA. *J Biol Chem* **277**(52): 51008-51016.

Inman, G. J., F. J. Nicolas and C. S. Hill (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* **10**(2): 283-294.

Ishida, W., T. Hamamoto, K. Kusanagi, K. Yagi, M. Kawabata, K. Takehara, T. K. Sampath, M. Kato and K. Miyazono (2000). Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone

morphogenetic protein-responsive element in the mouse Smad6 promoter. *J Biol Chem* **275**(9): 6075-6079.

Itoh, S. and P. ten Dijke (2007). Negative regulation of TGF-beta receptor/Smad signal transduction. *Curr Opin Cell Biol* **19**(2): 176-184.

Janssen, O., S. Gillis and D. Kabelitz (1990). In vitro transformation by Epstein-Barr virus induces a switch in growth factor and anti-IgM responsiveness in a human leukemic B cell clone. *Eur J Immunol* **20**(1): 7-14.

Janz, S., M. Potter and C. S. Rabkin (2003). Lymphoma- and leukemia-associated chromosomal translocations in healthy individuals. *Genes Chromosomes Cancer* **36**(3): 211-223.

Jelinek, D. F., R. C. Tschumper, G. A. Stolovitzky, S. J. Iturria, Y. Tu, J. Lepre, N. Shah and N. E. Kay (2003). Identification of a global gene expression signature of B-chronic lymphocytic leukemia. *Mol Cancer Res* **1**(5): 346-361.

Jenkins, P. J., U. K. Binne and P. J. Farrell (2000). Histone acetylation and reactivation of Epstein-Barr virus from latency. *J Virol* **74**(2): 710-720.

Jennings, R., M. Alsarraj, K. L. Wright and T. Munoz-Antonia (2001). Regulation of the human transforming growth factor beta type II receptor gene promoter by novel Sp1 sites. *Oncogene* **20**(47): 6899-6909.

Ji, C., Y. Chen, T. L. McCarthy and M. Centrella (1999). Cloning the promoter for transforming growth factor-beta type III receptor. Basal and conditional expression in fetal rat osteoblasts. *J Biol Chem* **274**(43): 30487-30494.

Jiang, D. and R. C. Aguiar (2014). MicroRNA-155 controls RB phosphorylation in normal and malignant B lymphocytes via the noncanonical TGF-beta1/SMAD5 signaling module. *Blood* **123**(1): 86-93.

Jiang, R., X. Gu, C. O. Nathan and L. Hutt-Fletcher (2008). Laser-capture microdissection of oropharyngeal epithelium indicates restriction of Epstein-Barr virus receptor/CD21 mRNA to tonsil epithelial cells. *J Oral Pathol Med* **37**(10): 626-633.

Jimenez-Ramirez, C., A. J. Brooks, L. P. Forshell, K. Yakimchuk, B. Zhao, T. Z. Fulgham and C. E. Sample (2006). Epstein-Barr virus EBNA-3C is targeted to and regulates expression from the bidirectional LMP-1/2B promoter. *J Virol* **80**(22): 11200-11208.

Jonk, L. J., S. Itoh, C. H. Heldin, P. ten Dijke and W. Kruijer (1998). Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. *J Biol Chem* **273**(33): 21145-21152.

Kaiser, C., G. Laux, D. Eick, N. Jochner, G. W. Bornkamm and B. Kempkes (1999). The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2. *J Virol* **73**(5): 4481-4484.

Kalla, M. and W. Hammerschmidt (2012). Human B cells on their route to latent infection--early but transient expression of lytic genes of Epstein-Barr virus. *Eur J Cell Biol* **91**(1): 65-69.

Kalla, M., A. Schmeinck, M. Bergbauer, D. Pich and W. Hammerschmidt (2010). AP-1 homolog BZLF1 of Epstein-Barr virus has two essential functions dependent on the epigenetic state of the viral genome. *Proc Natl Acad Sci U S A* **107**(2): 850-855.

Kanda, T., M. Otter and G. M. Wahl (2001). Coupling of mitotic chromosome tethering and replication competence in epstein-barr virus-based plasmids. *Mol Cell Biol* **21**(10): 3576-3588.

Kang, Y., C. R. Chen and J. Massague (2003). A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* **11**(4): 915-926.

Kanzler, H., R. Kuppers, M. L. Hansmann and K. Rajewsky (1996). Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. *J Exp Med* **184**(4): 1495-1505.

Kaptein, J. S., C. K. Lin, C. L. Wang, T. T. Nguyen, C. I. Kalunta, E. Park, F. S. Chen and P. M. Lad (1996). Anti-IgM-mediated regulation of c-myc and its possible relationship to apoptosis. *J Biol Chem* **271**(31): 18875-18884.

Katagiri, T., M. Imada, T. Yanai, T. Suda, N. Takahashi and R. Kamijo (2002). Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* **7**(9): 949-960.

Kawabata, K. C., S. Ehata, A. Komuro, K. Takeuchi and K. Miyazono (2013). TGF-beta-induced apoptosis of B-cell lymphoma Ramos cells through reduction of MS4A1/CD20. *Oncogene* **32**(16): 2096-2106.

Kawamura, C., M. Kizaki, K. Yamato, H. Uchida, Y. Fukuchi, Y. Hattori, T. Koseki, T. Nishihara and Y. Ikeda (2000). Bone morphogenetic protein-2 induces apoptosis in human myeloma cells with modulation of STAT3. *Blood* **96**(6): 2005-2011.

Kaye, K. M., K. M. Izumi and E. Kieff (1993). Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc Natl Acad Sci U S A* **90**(19): 9150-9154.

Kee, B. L., R. R. Rivera and C. Murre (2001). Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta. *Nat Immunol* **2**(3): 242-247.

Kehrl, J. H., A. B. Roberts, L. M. Wakefield, S. Jakowlew, M. B. Sporn and A. S. Fauci (1986). Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. *J Immunol* **137**(12): 3855-3860.

Kehrl, J. H., A. S. Taylor, G. A. Delsing, A. B. Roberts, M. B. Sporn and A. S. Fauci (1989). Further studies of the role of transforming growth factor-beta in human B cell function. *J Immunol* **143**(6): 1868-1874.

Kelly, G., A. Bell and A. Rickinson (2002). Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. *Nat Med* **8**(10): 1098-1104.

Kelly, G. L., A. E. Milner, R. J. Tierney, D. S. Croom-Carter, M. Altmann, W. Hammerschmidt, A. I. Bell and A. B. Rickinson (2005). Epstein-Barr virus nuclear antigen 2 (EBNA2) gene deletion is consistently linked with EBNA3A, -3B, and -3C expression in Burkitt's lymphoma cells and with increased resistance to apoptosis. *J Virol* **79**(16): 10709-10717.

Kelly, G. L., J. Stylianou, J. Rasaiyaah, W. Wei, W. Thomas, D. Croom-Carter, C. Kohler, R. Spang, C. Woodman, P. Kellam, A. B. Rickinson and A. I. Bell (2013). Different Patterns of Epstein-Barr Virus Latency in Endemic Burkitt Lymphoma (BL) Lead to Distinct Variants within the BL-Associated Gene Expression Signature. *J Virol* **87**(5): 2882-2894.

Kempkes, B., D. Spitkovsky, P. Jansen-Durr, J. W. Ellwart, E. Kremmer, H. J. Delecluse, C. Rottenberger, G. W. Bornkamm and W. Hammerschmidt (1995). B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2. *EMBO J* **14**(1): 88-96.

Kennedy, G., J. Komano and B. Sugden (2003). Epstein-Barr virus provides a survival factor to Burkitt's lymphomas. *Proc Natl Acad Sci U S A* **100**(24): 14269-14274.

Kenney, J. L., M. E. Guinness, T. Curiel and J. Lacy (1998). Antisense to the epstein-barr virus (EBV)-encoded latent membrane protein 1 (LMP-1) suppresses LMP-1 and bcl-2 expression and promotes apoptosis in EBV-immortalized B cells. *Blood* **92**(5): 1721-1727.

Kenney, J. L., M. E. Guinness, M. Reiss and J. Lacy (2001). Antisense to the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP-1) sensitizes EBV-immortalized B cells to transforming growth factor-beta and chemotherapeutic agents. *Int J Cancer* **91**(1): 89-98.

Kerr, J. F., A. H. Wyllie and A. R. Currie (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**(4): 239-257.

Kersten, C., G. Dosen, J. H. Myklebust, E. A. Sivertsen, M. E. Hystad, E. B. Smeland and E. Rian (2006). BMP-6 inhibits human bone marrow B lymphopoiesis--upregulation of Id1 and Id3. *Exp Hematol* **34**(1): 72-81.

Kersten, C., E. A. Sivertsen, M. E. Hystad, L. Forfang, E. B. Smeland and J. H. Myklebust (2005). BMP-6 inhibits growth of mature human B cells; induction of Smad phosphorylation and upregulation of Id1. *BMC Immunol* **6**(1): 9.

Khan, G., E. M. Miyashita, B. Yang, G. J. Babcock and D. A. Thorley-Lawson (1996). Is EBV persistence in vivo a model for B cell homeostasis? *Immunity* **5**(2): 173-179.

Khan, H., C. Vale, T. Bhagat and A. Verma (2013). Role of DNA methylation in the pathogenesis and treatment of myelodysplastic syndromes. *Semin Hematol* **50**(1): 16-37.

Kilger, E., A. Kieser, M. Baumann and W. Hammerschmidt (1998). Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J* **17**(6): 1700-1709.

Kim, B. C., M. Mamura, K. S. Choi, B. Calabretta and S. J. Kim (2002). Transforming growth factor beta 1 induces apoptosis through cleavage of BAD in a Smad3-dependent mechanism in FaO hepatoma cells. *Mol Cell Biol* **22**(5): 1369-1378.

Kim, J. H., W. S. Kim and C. Park (2012). Epstein-Barr virus latent membrane protein-1 protects B-cell lymphoma from rituximab-induced apoptosis through miR-155-mediated Akt activation and up-regulation of Mcl-1. *Leuk Lymphoma* **53**(8): 1586-1591.

Kim, S. G., H. A. Kim, H. S. Jong, J. H. Park, N. K. Kim, S. H. Hong, T. Y. Kim and Y. J. Bang (2005). The endogenous ratio of Smad2 and Smad3 influences the cytostatic function of Smad3. *Mol Biol Cell* **16**(10): 4672-4683.

Kim, W. Y. and N. E. Sharpless (2006). The regulation of INK4/ARF in cancer and aging. *Cell* **127**(2): 265-275.

King, I. L. and M. Mohrs (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med* **206**(5): 1001-1007.

Kirk, O., C. Pedersen, A. Cozzi-Lepri, F. Antunes, V. Miller, J. M. Gatell, C. Katlama, A. Lazzarin, P. Skinhoj and S. E. Barton (2001). Non-Hodgkin lymphoma in HIV-infected patients in the era of highly active antiretroviral therapy. *Blood* **98**(12): 3406-3412.

Kirkbride, K. C., T. A. Townsend, M. W. Bruinsma, J. V. Barnett and G. C. Blobe (2008). Bone morphogenetic proteins signal through the transforming growth factor-beta type III receptor. *J Biol Chem* **283**(12): 7628-7637.

Kirmizis, A., S. M. Bartley, A. Kuzmichev, R. Margueron, D. Reinberg, R. Green and P. J. Farnham (2004). Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. *Genes Dev* **18**(13): 1592-1605.

Kis, L. L., N. Gerasimcik, D. Salamon, E. K. Persson, N. Nagy, G. Klein, E. Severinson and E. Klein (2011). STAT6 signaling pathway activated by the cytokines IL-4 and IL-13 induces expression of the Epstein-Barr virus-encoded protein LMP-1 in absence of EBNA-2: implications for the type II EBV latent gene expression in Hodgkin lymphoma. *Blood* **117**(1): 165-174.

Kis, L. L., J. Nishikawa, M. Takahara, N. Nagy, L. Matskova, K. Takada, P. G. Elmberger, A. Ohlsson, G. Klein and E. Klein (2005). In vitro EBV-infected subline of KMH2, derived from Hodgkin lymphoma, expresses only EBNA-1, while CD40 ligand and IL-4 induce LMP-1 but not EBNA-2. *Int J Cancer* **113**(6): 937-945.

Kis, L. L., D. Salamon, E. K. Persson, N. Nagy, F. A. Scheeren, H. Spits, G. Klein and E. Klein (2010). IL-21 imposes a type II EBV gene expression on type III and type I B cells by the repression of C- and activation of LMP-1-promoter. *Proc Natl Acad Sci U S A* **107**(2): 872-877.

Kis, L. L., M. Takahara, N. Nagy, G. Klein and E. Klein (2006). IL-10 can induce the expression of EBV-encoded latent membrane protein-1 (LMP-1) in the absence of EBNA-2 in B lymphocytes and in Burkitt lymphoma- and NK lymphoma-derived cell lines. *Blood* **107**(7): 2928-2935.

Klangby, U., I. Okan, K. P. Magnusson, M. Wendland, P. Lind and K. G. Wiman (1998). p16/INK4a and p15/INK4b gene methylation and absence of p16/INK4a mRNA and protein expression in Burkitt's lymphoma. *Blood* **91**(5): 1680-1687.

Klein, U. and R. Dalla-Favera (2008). Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol* **8**(1): 22-33.

Klein, U., Y. Tu, G. A. Stolovitzky, M. Mattioli, G. Cattoretti, H. Husson, A. Freedman, G. Inghirami, L. Cro, L. Baldini, A. Neri, A. Califano and R. Dalla-Favera (2001). Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* **194**(11): 1625-1638.

Komano, J., S. Maruo, K. Kurozumi, T. Oda and K. Takada (1999). Oncogenic role of Epstein-Barr virus-encoded RNAs in Burkitt's lymphoma cell line Akata. *J Virol* **73**(12): 9827-9831.

Konrad, L., J. A. Scheiber, E. Volck-Badouin, M. M. Keilani, L. Laible, H. Brandt, A. Schmidt, G. Aumuller and R. Hofmann (2007). Alternative splicing of TGF-betas and their high-affinity receptors T

beta RI, T beta RII and T beta RIII (betaglycan) reveal new variants in human prostatic cells. *BMC Genomics* **8**: 318.

Korchynskyi, O. and P. ten Dijke (2002). Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* **277**(7): 4883-4891.

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* **128**(4): 693-705.

Krauer, K. G., A. Burgess, M. Buck, J. Flanagan, T. B. Sculley and B. Gabrielli (2004). The EBNA-3 gene family proteins disrupt the G2/M checkpoint. *Oncogene* **23**(7): 1342-1353.

Kremer, J. P., G. Reisbach, C. Nerl and P. Dormer (1992). B-cell chronic lymphocytic leukaemia cells express and release transforming growth factor-beta. *Br J Haematol* **80**(4): 480-487.

Kretschmer, A., K. Moepert, S. Dames, M. Sternberger, J. Kaufmann and A. Klippel (2003). Differential regulation of TGF-beta signaling through Smad2, Smad3 and Smad4. *Oncogene* **22**(43): 6748-6763.

Kulwichit, W., R. H. Edwards, E. M. Davenport, J. F. Baskar, V. Godfrey and N. Raab-Traub (1998). Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. *Proc Natl Acad Sci U S A* **95**(20): 11963-11968.

Kumar, A., T. Rogers, A. Maizel and S. Sharma (1991). Loss of transforming growth factor beta 1 receptors and its effects on the growth of EBV-transformed human B cells. *J Immunol* **147**(3): 998-1006.

Kuzmichev, A., K. Nishioka, H. Erdjument-Bromage, P. Tempst and D. Reinberg (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* **16**(22): 2893-2905.

Lagneaux, L., A. Delforge, M. Bernier, P. Stryckmans and D. Bron (1998). TGF-beta activity and expression of its receptors in B-cell chronic lymphocytic leukemia. *Leuk Lymphoma* **31**(1-2): 99-106.

Lagneaux, L., A. Delforge, D. Bron, M. Massy, M. Bernier and P. Stryckmans (1997). Heterogenous response of B lymphocytes to transforming growth factor-beta in B-cell chronic lymphocytic leukaemia: correlation with the expression of TGF-beta receptors. *Br J Haematol* **97**(3): 612-620.

Laichalk, L. L. and D. A. Thorley-Lawson (2005). Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J Virol* **79**(2): 1296-1307.

Lambert, K. E., H. Huang, K. Mythreye and G. C. Blobe (2011). The type III transforming growth factor-beta receptor inhibits proliferation, migration, and adhesion in human myeloma cells. *Mol Biol Cell* **22**(9): 1463-1472.

Landstrom, M. (2010). The TAK1-TRAF6 signalling pathway. *Int J Biochem Cell Biol* **42**(5): 585-589.

Larsson, J. and S. Karlsson (2005). The role of Smad signaling in hematopoiesis. *Oncogene* **24**(37): 5676-5692.

Lee, J. E., H. Y. Nam, S. M. Shim, G. R. Bae, B. G. Han and J. P. Jeon (2010). Expression phenotype changes of EBV-transformed lymphoblastoid cell lines during long-term subculture and its clinical significance. *Cell Prolif* **43**(4): 378-384.

Lee, M. A., M. E. Diamond and J. L. Yates (1999). Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. *J Virol* **73**(4): 2974-2982.

Lee, N. Y., K. C. Kirkbride, R. D. Sheu and G. C. Blobe (2009). The transforming growth factor-beta type III receptor mediates distinct subcellular trafficking and downstream signaling of activin-like kinase (ALK)3 and ALK6 receptors. *Mol Biol Cell* **20**(20): 4362-4370.

Lei, X., Y. Zhu, T. Jones, Z. Bai, Y. Huang and S. J. Gao (2012). A Kaposi's sarcoma-associated herpesvirus microRNA and its variants target the transforming growth factor beta pathway to promote cell survival. *J Virol* **86**(21): 11698-11711.

Lerner, M. R., N. C. Andrews, G. Miller and J. A. Steitz (1981). Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. *Proc Natl Acad Sci U S A* **78**(2): 805-809.

Lewis, K. A., P. C. Gray, A. L. Blount, L. A. MacConell, E. Wiater, L. M. Bilezikjian and W. Vale (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404**(6776): 411-414.

Li, L. C. and R. Dahiya (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**(11): 1427-1431.

Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri and X. Wang (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**(4): 479-489.

Li, X., V. Placencio, J. M. Iturregui, C. Uwamariya, A. R. Sharif-Afshar, T. Koyama, S. W. Hayward and N. A. Bhowmick (2008). Prostate tumor progression is mediated by a paracrine TGF-beta/Wnt3a signaling axis. *Oncogene* **27**(56): 7118-7130.

Lin, H. Y., X. F. Wang, E. Ng-Eaton, R. A. Weinberg and H. F. Lodish (1992). Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. *Cell* **68**(4): 775-785.

Lin, J., E. Johannsen, E. Robertson and E. Kieff (2002). Epstein-Barr virus nuclear antigen 3C putative repression domain mediates coactivation of the LMP1 promoter with EBNA-2. *J Virol* **76**(1): 232-242.

Lin, P., T. J. Dickason, L. E. Fayad, P. A. Lennon, P. Hu, M. Garcia, M. J. Routbort, R. Miranda, X. Wang, W. Qiao and L. J. Medeiros (2012). Prognostic value of MYC rearrangement in cases of B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. *Cancer* **118**(6): 1566-1573.

Ling, M. T., X. Wang, S. W. Tsao and Y. C. Wong (2002). Down-regulation of Id-1 expression is associated with TGF beta 1-induced growth arrest in prostate epithelial cells. *Biochim Biophys Acta* **1570**(3): 145-152.

Liu, I. M., S. H. Schilling, K. A. Knouse, L. Choy, R. Derynck and X. F. Wang (2009). TGFbeta-stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGFbeta switch. *EMBO J* **28**(2): 88-98.

Liu, M., J. L. Duke, D. J. Richter, C. G. Vinuesa, C. C. Goodnow, S. H. Kleinstein and D. G. Schatz (2008). Two levels of protection for the B cell genome during somatic hypermutation. *Nature* **451**(7180): 841-845.

Liu, Y., R. Sun, X. Lin, D. Liang, Q. Deng and K. Lan (2012). Kaposi's sarcoma-associated herpesvirus-encoded microRNA miR-K12-11 attenuates transforming growth factor beta signaling through suppression of SMAD5. *J Virol* **86**(3): 1372-1381.

Lo, A. K., C. W. Dawson, K. W. Lo, Y. Yu and L. S. Young (2010). Upregulation of Id1 by Epstein-Barr Virus-encoded LMP1 confers resistance to TGFbeta-mediated growth inhibition. *Mol Cancer* **9**(1): 155.

Locklin, R. M., B. L. Riggs, K. C. Hicok, H. F. Horton, M. C. Byrne and S. Khosla (2001). Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology. *J Bone Miner Res* **16**(12): 2192-2204.

Longnecker, R., C. L. Miller, X. Q. Miao, A. Marchini and E. Kieff (1992). The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential. *J Virol* **66**(11): 6461-6469.

Longnecker, R., C. L. Miller, X. Q. Miao, B. Tomkinson and E. Kieff (1993a). The last seven transmembrane and carboxy-terminal cytoplasmic domains of Epstein-Barr virus latent membrane protein 2 (LMP2) are dispensable for lymphocyte infection and growth transformation in vitro. *J Virol* **67**(4): 2006-2013.

Longnecker, R., C. L. Miller, B. Tomkinson, X. Q. Miao and E. Kieff (1993b). Deletion of DNA encoding the first five transmembrane domains of Epstein-Barr virus latent membrane proteins 2A and 2B. *J Virol* **67**(8): 5068-5074.

Longnecker, R. M., E. Kieff and J. I. Cohen (2013). Epstein-Barr Virus. *Fields Virology*. D. M. Knipe and P. M. Howley, Wolters Kluwer Health/Lippincott Williams & Wilkins. **2:** 1898-1959.

Lopez-Casillas, F., S. Cheifetz, J. Doody, J. L. Andres, W. S. Lane and J. Massague (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* **67**(4): 785-795.

Lopez-Casillas, F., H. M. Payne, J. L. Andres and J. Massague (1994). Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *J Cell Biol* **124**(4): 557-568.

Lopez-Casillas, F., C. Riquelme, Y. Perez-Kato, M. V. Ponce-Castaneda, N. Osses, J. Esparza-Lopez, G. Gonzalez-Nunez, C. Cabello-Verrugio, V. Mendoza, V. Troncoso and E. Brandan (2003). Betaglycan expression is transcriptionally up-regulated during skeletal muscle differentiation. Cloning of murine betaglycan gene promoter and its modulation by MyoD, retinoic acid, and transforming growth factor-beta. *J Biol Chem* **278**(1): 382-390.

Lopez-Casillas, F., J. L. Wrana and J. Massague (1993). Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**(7): 1435-1444.

Lopez-Rovira, T., E. Chalaux, J. Massague, J. L. Rosa and F. Ventura (2002). Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem* **277**(5): 3176-3185.

Lotz, M., E. Ranheim and T. J. Kipps (1994). Transforming growth factor beta as endogenous growth inhibitor of chronic lymphocytic leukemia B cells. *J Exp Med* **179**(3): 999-1004.

Love, C., Z. Sun, D. Jima, G. Li, J. Zhang, R. Miles, K. L. Richards, C. H. Dunphy, W. W. Choi, G. Srivastava, P. L. Lugar, D. A. Rizzieri, A. S. Lagoo, L. Bernal-Mizrachi, K. P. Mann, C. R. Flowers, K. N. Naresh, A. M. Evens, A. Chadburn, L. I. Gordon, M. B. Czader, J. I. Gill, E. D. Hsi, A. Greenough, A. B. Moffitt, M. McKinney, A. Banerjee, V. Grubor, S. Levy, D. B. Dunson and S. S. Dave (2012). The genetic landscape of mutations in Burkitt lymphoma. *Nat Genet* **44**(12): 1321-1325.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**(6648): 251-260.

MacDonald, I., H. Wang, R. Grand, R. J. Armitage, W. C. Fanslow, C. D. Gregory and J. Gordon (1996). Transforming growth factor-beta 1 cooperates with anti-immunoglobulin for the induction of apoptosis in group I (biopsy-like) Burkitt lymphoma cell lines. *Blood* **87**(3): 1147-1154.

Machold, K. P., D. A. Carson and M. Lotz (1993). Transforming growth factor-beta (TGF beta) inhibition of Epstein-Barr virus (EBV)- and interleukin-4 (IL-4)-induced immunoglobulin production in human B lymphocytes. *J Clin Immunol* **13**(3): 219-227.

Maesako, Y., T. Uchiyama and H. Ohno (2003). Comparison of gene expression profiles of lymphoma cell lines from transformed follicular lymphoma, Burkitt's lymphoma and de novo diffuse large B-cell lymphoma. *Cancer Sci* **94**(9): 774-781.

Maier, S., G. Staffler, A. Hartmann, J. Hock, K. Henning, K. Grabusic, R. Mailhammer, R. Hoffmann, M. Wilmanns, R. Lang, J. Mages and B. Kempkes (2006). Cellular target genes of Epstein-Barr virus nuclear antigen 2. *J Virol* **80**(19): 9761-9771.

Mancao, C., M. Altmann, B. Jungnickel and W. Hammerschmidt (2005). Rescue of "crippled" germinal center B cells from apoptosis by Epstein-Barr virus. *Blood* **106**(13): 4339-4344.

Mancao, C. and W. Hammerschmidt (2007). Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival. *Blood* **110**(10): 3715-3721.

Margulis, V., T. Maity, X. Y. Zhang, S. J. Cooper, J. A. Copland and C. G. Wood (2008). Type III transforming growth factor-beta (TGF-beta) receptor mediates apoptosis in renal cell carcinoma independent of the canonical TGF-beta signaling pathway. *Clin Cancer Res* **14**(18): 5722-5730.

Markowitz, S., J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, B. Vogelstein and et al. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **268**(5215): 1336-1338.

Marshall, D. and C. Sample (1995). Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. *J Virol* **69**(6): 3624-3630.

Martinez-Valdez, H., C. Guret, O. de Bouteiller, I. Fugier, J. Banchereau and Y. J. Liu (1996). Human germinal center B cells express the apoptosis-inducing genes Fas, c-myc, P53, and Bax but not the survival gene bcl-2. *J Exp Med* **183**(3): 971-977.

Maruo, S., Y. Wu, S. Ishikawa, T. Kanda, D. Iwakiri and K. Takada (2006). Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression and growth maintenance of lymphoblastoid cells. *Proc Natl Acad Sci U S A* **103**(51): 19500-19505.

Maruo, S., B. Zhao, E. Johannsen, E. Kieff, J. Zou and K. Takada (2011). Epstein-Barr virus nuclear antigens 3C and 3A maintain lymphoblastoid cell growth by repressing p16INK4A and p14ARF expression. *Proc Natl Acad Sci U S A* **108**(5): 1919-1924.

Massague, J. (2012). TGFbeta signalling in context. *Nat Rev Mol Cell Biol* **13**(10): 616-630.

Mathew, S., V. V. Murty, S. Cheifetz, D. George, J. Massague and R. S. Chaganti (1994). Transforming growth factor receptor gene TGFBR2 maps to human chromosome band 3p22. *Genomics* **20**(1): 114-115.

Maunders, M. J., L. Petti and M. Rowe (1994). Precipitation of the Epstein-Barr virus protein EBNA 2 by an EBNA 3c-specific monoclonal antibody. *J Gen Virol* **75 ( Pt 4)**: 769-778.

McClellan, M. J., S. Khasnis, C. D. Wood, R. D. Palermo, S. N. Schlick, A. S. Kanhere, R. G. Jenner and M. J. West (2012). Downregulation of integrin receptor-signaling genes by Epstein-Barr virus EBNA 3C via promoter-proximal and -distal binding elements. *J Virol* **86**(9): 5165-5178.

McClellan, M. J., C. D. Wood, O. Ojeniyi, T. J. Cooper, A. Kanhere, A. Arvey, H. M. Webb, R. D. Palermo, M. L. Harth-Hertle, B. Kempkes, R. G. Jenner and M. J. West (2013). Modulation of enhancer looping and differential gene targeting by Epstein-Barr virus transcription factors directs cellular reprogramming. *PLoS Pathog* **9**(9): e1003636.

Molyneux, E. M., R. Rochford, B. Griffin, R. Newton, G. Jackson, G. Menon, C. J. Harrison, T. Israels and S. Bailey (2012). Burkitt's lymphoma. *Lancet* **379**(9822): 1234-1244.

Mori, N., M. Morishita, T. Tsukazaki, C. Z. Giam, A. Kumatori, Y. Tanaka and N. Yamamoto (2001). Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300. *Blood* **97**(7): 2137-2144.

Mori, N., M. Morishita, T. Tsukazaki and N. Yamamoto (2003). Repression of Smad-dependent transforming growth factor-beta signaling by Epstein-Barr virus latent membrane protein 1 through nuclear factor-kappaB. *Int J Cancer* **105**(5): 661-668.

Moustakas, A., S. Souchelnytskyi and C. H. Heldin (2001). Smad regulation in TGF-beta signal transduction. *J Cell Sci* **114**(Pt 24): 4359-4369.

Mu, Y., S. K. Gudey and M. Landstrom (2012). Non-Smad signaling pathways. *Cell Tissue Res* **347**(1): 11-20.

Mueller, T. D. and J. Nickel (2012). Promiscuity and specificity in BMP receptor activation. *FEBS Lett* **586**(14): 1846-1859.

Munoz, O., F. Fend, R. de Beaumont, H. Husson, A. Astier and A. S. Freedman (2004). TGFbeta-mediated activation of Smad1 in B-cell non-Hodgkin's lymphoma and effect on cell proliferation. *Leukemia* **18**(12): 2015-2025.

Murphy, K. (2011). *Janeway's Immunobiology*, Garland Science.

Murray, R. J., M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff and A. B. Rickinson (1992). Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. *J Exp Med* **176**(1): 157-168.

Nagarajan, R. P., J. Zhang, W. Li and Y. Chen (1999). Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *J Biol Chem* **274**(47): 33412-33418.

Nakao, A., M. Afrakhte, A. Moren, T. Nakayama, J. L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N. E. Heldin, C. H. Heldin and P. ten Dijke (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* **389**(6651): 631-635.

Neuhierl, B., R. Feederle, W. Hammerschmidt and H. J. Delecluse (2002). Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. *Proc Natl Acad Sci U S A* **99**(23): 15036-15041.

Nikitin, P. A., C. M. Yan, E. Forte, A. Bocedi, J. P. Tourigny, R. E. White, M. J. Allday, A. Patel, S. S. Dave, W. Kim, K. Hu, J. Guo, D. Tainter, E. Rusyn and M. A. Luftig (2010). An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. *Cell Host Microbe* **8**(6): 510-522.

Nitsche, F., A. Bell and A. Rickinson (1997). Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain. *J Virol* **71**(9): 6619-6628.

Noda, C., T. Murata, T. Kanda, H. Yoshiyama, A. Sugimoto, D. Kawashima, S. Saito, H. Isomura and T. Tsurumi (2011). Identification and characterization of CCAAT enhancer-binding protein (C/EBP) as a transcriptional activator for Epstein-Barr virus oncogene latent membrane protein 1. *J Biol Chem* **286**(49): 42524-42533.

Noer, A., L. C. Lindeman and P. Collas (2009). Histone H3 modifications associated with differentiation and long-term culture of mesenchymal adipose stem cells. *Stem Cells Dev* **18**(5): 725-736.

Ohm, J. E., K. M. McGarvey, X. Yu, L. Cheng, K. E. Schuebel, L. Cope, H. P. Mohammad, W. Chen, V. C. Daniel, W. Yu, D. M. Berman, T. Jenuwein, K. Pruitt, S. J. Sharkis, D. N. Watkins, J. G. Herman and S. B. Baylin (2007). A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet* **39**(2): 237-242.

Ohta, M., J. S. Greenberger, P. Anklesaria, A. Bassols and J. Massague (1987). Two forms of transforming growth factor-beta distinguished by multipotential haematopoietic progenitor cells. *Nature* **329**(6139): 539-541.

Osada, H., Y. Tatematsu, A. Masuda, T. Saito, M. Sugiyama, K. Yanagisawa and T. Takahashi (2001). Heterogeneous transforming growth factor (TGF)-beta unresponsiveness and loss of TGF-beta receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer Res* **61**(22): 8331-8339.

Oyama, T., K. Ichimura, R. Suzuki, J. Suzumiya, K. Ohshima, Y. Yatabe, T. Yokoi, M. Kojima, Y. Kamiya, H. Taji, Y. Kagami, M. Ogura, H. Saito, Y. Morishima and S. Nakamura (2003). Senile EBV+ B-cell lymphoproliferative disorders: a clinicopathologic study of 22 patients. *Am J Surg Pathol* **27**(1): 16-26.

Oyama, T., K. Yamamoto, N. Asano, A. Oshiro, R. Suzuki, Y. Kagami, Y. Morishima, K. Takeuchi, T. Izumo, S. Mori, K. Ohshima, J. Suzumiya, N. Nakamura, M. Abe, K. Ichimura, Y. Sato, T. Yoshino, T. Naoe, Y. Shimoyama, Y. Kamiya, T. Kinoshita and S. Nakamura (2007). Age-related EBV-associated B-cell lymphoproliferative disorders constitute a distinct clinicopathologic group: a study of 96 patients. *Clin Cancer Res* **13**(17): 5124-5132.

Park, K., S. J. Kim, Y. J. Bang, J. G. Park, N. K. Kim, A. B. Roberts and M. B. Sporn (1994). Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric

cancer cells: correlation with sensitivity to growth inhibition by TGF-beta. *Proc Natl Acad Sci U S A* **91**(19): 8772-8776.

Parker, G. A., T. Crook, M. Bain, E. A. Sara, P. J. Farrell and M. J. Allday (1996). Epstein-Barr virus nuclear antigen (EBNA)3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. *Oncogene* **13**(12): 2541-2549.

Parker, G. A., R. Touitou and M. J. Allday (2000). Epstein-Barr virus EBNA3C can disrupt multiple cell cycle checkpoints and induce nuclear division divorced from cytokinesis. *Oncogene* **19**(5): 700-709.

Paschos, K., G. A. Parker, E. Watanatanasup, R. E. White and M. J. Allday (2012). BIM promoter directly targeted by EBNA3C in polycomb-mediated repression by EBV. *Nucleic Acids Res* **40**(15): 7233-7246.

Paschos, K., P. Smith, E. Anderton, J. M. Middeldorp, R. E. White and M. J. Allday (2009). Epstein-Barr Virus Latency in B Cells Leads to Epigenetic Repression and CpG Methylation of the Tumour Suppressor Gene <italic>Bim</italic>. *PLoS Pathog* **5**(6): e1000492.

Passa, O., S. Tsalavos, N. N. Belyaev, A. Petryk, A. J. Potocnik and D. Graf (2011). Compartmentalization of bone morphogenetic proteins and their antagonists in lymphoid progenitors and supporting microenvironments and functional implications. *Immunology* **134**(3): 349-359.

Patil, S., G. M. Wildey, T. L. Brown, L. Choy, R. Derynck and P. H. Howe (2000). Smad7 is induced by CD40 and protects WEHI 231 B-lymphocytes from transforming growth factor-beta -induced growth inhibition and apoptosis. *J Biol Chem* **275**(49): 38363-38370.

Pattle, S. B. and P. J. Farrell (2006). The role of Epstein-Barr virus in cancer. *Expert Opin Biol Ther* **6**(11): 1193-1205.

Pegman, P. M., S. M. Smith, B. N. D'Souza, S. T. Loughran, S. Maier, B. Kempkes, P. A. Cahill, M. J. Simmons, C. Gelinas and D. Walls (2006). Epstein-Barr virus nuclear antigen 2 trans-activates the cellular antiapoptotic bfl-1 gene by a CBF1/RBPJ kappa-dependent pathway. *J Virol* **80**(16): 8133-8144.

Pellett, P. E. and B. Roizman (2013). Herpesviridae. *Fields Virology*. D. M. Knipe and P. M. Howley, Wolters Kluwer Health/Lippincott Williams & Wilkins. **2:** 1802-1823.

Phan, R. T. and R. Dalla-Favera (2004). The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432**(7017): 635-639.

Piek, E., W. J. Ju, J. Heyer, D. Escalante-Alcalde, C. L. Stewart, M. Weinstein, C. Deng, R. Kucherlapati, E. P. Bottinger and A. B. Roberts (2001). Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* **276**(23): 19945-19953.

Pieper, K., B. Grimbacher and H. Eibel (2013). B-cell biology and development. *J Allergy Clin Immunol* **131**(4): 959-971.

Pietersen, A. M. and M. van Lohuizen (2008). Stem cell regulation by polycomb repressors: postponing commitment. *Curr Opin Cell Biol* **20**(2): 201-207.

Polager, S. and D. Ginsberg (2008). E2F - at the crossroads of life and death. *Trends Cell Biol* **18**(11): 528-535.

Portal, D., A. Rosendorff and E. Kieff (2006). Epstein-Barr nuclear antigen leader protein coactivates transcription through interaction with histone deacetylase 4. *Proc Natl Acad Sci U S A* **103**(51): 19278-19283.

Portal, D., B. Zhao, M. A. Calderwood, T. Sommermann, E. Johannsen and E. Kieff (2011). EBV nuclear antigen EBNALP dismisses transcription repressors NCoR and RBPJ from enhancers and EBNA2 increases NCoR-deficient RBPJ DNA binding. *Proc Natl Acad Sci U S A* **108**(19): 7808-7813.

Portis, T., P. Dyck and R. Longnecker (2003). Epstein-Barr Virus (EBV) LMP2A induces alterations in gene transcription similar to those observed in Reed-Sternberg cells of Hodgkin lymphoma. *Blood* **102**(12): 4166-4178.

Portis, T. and R. Longnecker (2004). Epstein-Barr virus (EBV) LMP2A mediates B-lymphocyte survival through constitutive activation of the Ras/PI3K/Akt pathway. *Oncogene* **23**(53): 8619-8628.

Price, A. M., J. P. Tourigny, E. Forte, R. E. Salinas, S. S. Dave and M. A. Luftig (2012). Analysis of Epstein-Barr virus-regulated host gene expression changes through primary B-cell outgrowth reveals delayed kinetics of latent membrane protein 1-mediated NF-kappaB activation. *J Virol* **86**(20): 11096-11106.

Prokova, V., G. Mosialos and D. Kardassis (2002). Inhibition of transforming growth factor beta signaling and Smad-dependent activation of transcription by the Latent Membrane Protein 1 of Epstein-Barr virus. *J Biol Chem* **277**(11): 9342-9350.

Radkov, S. A., M. Bain, P. J. Farrell, M. West, M. Rowe and M. J. Allday (1997). Epstein-Barr virus EBNA3C represses Cp, the major promoter for EBNA expression, but has no effect on the promoter of the cell gene CD21. *J Virol* **71**(11): 8552-8562.

Radkov, S. A., R. Touitou, A. Brehm, M. Rowe, M. West, T. Kouzarides and M. J. Allday (1999). Epstein-Barr virus nuclear antigen 3C interacts with histone deacetylase to repress transcription. *J Virol* **73**(7): 5688-5697.

Rahadiani, N., T. Takakuwa, K. Tresnasari, E. Morii and K. Aozasa (2008). Latent membrane protein-1 of Epstein-Barr virus induces the expression of B-cell integration cluster, a precursor form of microRNA-155, in B lymphoma cell lines. *Biochem Biophys Res Commun* **377**(2): 579-583.

Rai, D., S. W. Kim, M. R. McKeller, P. L. Dahia and R. C. Aguiar (2010). Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. *Proc Natl Acad Sci U S A* **107**(7): 3111-3116.

Rauch, T. A. and G. P. Pfeifer (2010). DNA methylation profiling using the methylated-CpG island recovery assay (MIRA). *Methods* **52**(3): 213-217.

Rechsteiner, M. P., M. Bernasconi, C. Berger and D. Nadal (2008). Role of latent membrane protein 2 isoforms in Epstein-Barr virus latency. *Trends Microbiol* **16**(11): 520-527.

Remy, I., A. Montmarquette and S. W. Michnick (2004). PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol* **6**(4): 358-365.

Reynisdottir, I., K. Polyak, A. Iavarone and J. Massague (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* **9**(15): 1831-1845.

Richter, J., M. Schlesner, S. Hoffmann, M. Kreuz, E. Leich, B. Burkhardt, M. Rosolowski, O. Ammerpohl, R. Wagener, S. H. Bernhart, D. Lenze, M. Szczepanowski, M. Paulsen, S. Lipinski, R. B. Russell, S. Adam-Klages, G. Apic, A. Claviez, D. Hasenclever, V. Hovestadt, N. Hornig, J. O. Korbel, D. Kube, D. Langenberger, C. Lawerenz, J. Lisfeld, K. Meyer, S. Picelli, J. Pischimarov, B. Radlwimmer, T. Rausch, M. Rohde, M. Schilhabel, R. Scholtysik, R. Spang, H. Trautmann, T. Zenz, A. Borkhardt, H. G. Drexler, P. Moller, R. A. MacLeod, C. Pott, S. Schreiber, L. Trumper, M. Loeffler, P. F. Stadler, P. Lichter, R. Eils, R. Kuppers, M. Hummel, W. Klapper, P. Rosenstiel, A. Rosenwald, B. Brors and R. Siebert (2012). Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. *Nat Genet* **44**(12): 1316-1320.

Rickinson, A. B., L. S. Young and M. Rowe (1987). Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells. *J Virol* **61**(5): 1310-1317.

Ro, T. B., R. U. Holt, A. T. Brenne, H. Hjorth-Hansen, A. Waage, O. Hjertner, A. Sundan and M. Borset (2004). Bone morphogenetic protein-5, -6 and -7 inhibit growth and induce apoptosis in human myeloma cells. *Oncogene* **23**(17): 3024-3032.

Robbiani, D. F., A. Bothmer, E. Callen, B. Reina-San-Martin, Y. Dorsett, S. Difilippantonio, D. J. Bolland, H. T. Chen, A. E. Corcoran, A. Nussenzweig and M. C. Nussenzweig (2008). AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. *Cell* **135**(6): 1028-1038.

Robertson, E. S., S. Grossman, E. Johannsen, C. Miller, J. Lin, B. Tomkinson and E. Kieff (1995). Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein J kappa. *J Virol* **69**(5): 3108-3116.

Robinson, J., D. Smith and J. Niederman (1980). Mitotic EBNA-positive lymphocytes in peripheral blood during infectious mononucleosis. *Nature* **287**(5780): 334-335.

Ross, S. and C. S. Hill (2008). How the Smads regulate transcription. *Int J Biochem Cell Biol* **40**(3): 383-408.

Rovedo, M. and R. Longnecker (2007). Epstein-barr virus latent membrane protein 2B (LMP2B) modulates LMP2A activity. *J Virol* **81**(1): 84-94.

Rowe, M., R. Khanna, C. A. Jacob, V. Argaet, A. Kelly, S. Powis, M. Belich, D. Croom-Carter, S. Lee, S. R. Burrows and et al. (1995). Restoration of endogenous antigen processing in Burkitt's lymphoma cells by Epstein-Barr virus latent membrane protein-1: coordinate up-regulation of peptide transporters and HLA-class I antigen expression. *Eur J Immunol* **25**(5): 1374-1384.

Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani and A. B. Rickinson (1987). Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J* **6**(9): 2743-2751.

Rowe, M., L. S. Young, K. Cadwallader, L. Petti, E. Kieff and A. B. Rickinson (1989). Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. *J Virol* **63**(3): 1031-1039.

Ruzinova, M. B. and R. Benezra (2003). Id proteins in development, cell cycle and cancer. *Trends Cell Biol* **13**(8): 410-418.

Saito, M., U. Novak, E. Piovan, K. Basso, P. Sumazin, C. Schneider, M. Crespo, Q. Shen, G. Bhagat, A. Califano, A. Chadburn, L. Pasqualucci and R. Dalla-Favera (2009). BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A* **106**(27): 11294-11299.

Saltzman, A., R. Munro, G. Searfoss, C. Franks, M. Jaye and Y. Ivashchenko (1998). Transforming growth factor-beta-mediated apoptosis in the Ramos B-lymphoma cell line is accompanied by caspase activation and Bcl-XL downregulation. *Exp Cell Res* **242**(1): 244-254.

Sapkota, G., C. Alarcon, F. M. Spagnoli, A. H. Brivanlou and J. Massague (2007). Balancing BMP signaling through integrated inputs into the Smad1 linker. *Mol Cell* **25**(3): 441-454.

Saridakis, V., Y. Sheng, F. Sarkari, M. N. Holowaty, K. Shire, T. Nguyen, R. G. Zhang, J. Liao, W. Lee, A. M. Edwards, C. H. Arrowsmith and L. Frappier (2005). Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. *Mol Cell* **18**(1): 25-36.

Schiemann, W. P., D. Rotzer, W. M. Pfeifer, E. Levi, K. R. Rai, P. Knaus and M. E. Kadin (2004). Transforming growth factor-beta (TGF-beta)-resistant B cells from chronic lymphocytic leukemia patients contain recurrent mutations in the signal sequence of the type I TGF-beta receptor. *Cancer Detect Prev* **28**(1): 57-64.

Schlesinger, Y., R. Straussman, I. Keshet, S. Farkash, M. Hecht, J. Zimmerman, E. Eden, Z. Yakhini, E. Ben-Shushan, B. E. Reubinoff, Y. Bergman, I. Simon and H. Cedar (2007). Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet* **39**(2): 232-236.

Schmitz, R., R. M. Young, M. Ceribelli, S. Jhavar, W. Xiao, M. Zhang, G. Wright, A. L. Shaffer, D. J. Hodson, E. Buras, X. Liu, J. Powell, Y. Yang, W. Xu, H. Zhao, H. Kohlhammer, A. Rosenwald, P. Kluin, H. K. Muller-Hermelink, G. Ott, R. D. Gascoyne, J. M. Connors, L. M. Rimsza, E. Campo, E. S. Jaffe, J. Delabie, E. B. Smeland, M. D. Ogwang, S. J. Reynolds, R. I. Fisher, R. M. Braziel, R. R. Tubbs, J. R. Cook, D. D. Weisenburger, W. C. Chan, S. Pittaluga, W. Wilson, T. A. Waldmann, M. Rowe, S. M. Mbulaiteye, A. B. Rickinson and L. M. Staudt (2012). Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature* **490**(7418): 116-120.

Schrantz, N., D. A. Blanchard, M. T. Auffredou, S. Sharma, G. Leca and A. Vazquez (1999). Role of caspases and possible involvement of retinoblastoma protein during TGFbeta-mediated apoptosis of human B lymphocytes. *Oncogene* **18**(23): 3511-3519.

Seckinger, A., T. Meissner, J. Moreaux, H. Goldschmidt, G. M. Fuhler, A. Benner, M. Hundemer, T. Reme, J. D. Shaughnessy, Jr., B. Barlogie, U. Bertsch, J. Hillengass, A. D. Ho, V. Pantesco, A. Jauch, J. De Vos, J. F. Rossi, T. Mohler, B. Klein and D. Hose (2009). Bone morphogenic protein 6: a member of a novel class of prognostic factors expressed by normal and malignant plasma cells inhibiting proliferation and angiogenesis. *Oncogene* **28**(44): 3866-3879.

Seoane, J., C. Pouponnot, P. Staller, M. Schader, M. Eilers and J. Massague (2001). TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* **3**(4): 400-408.

Seto, E., A. Moosmann, S. Gromminger, N. Walz, A. Grundhoff and W. Hammerschmidt (2010). Micro RNAs of Epstein-Barr virus promote cell cycle progression and prevent apoptosis of primary human B cells. *PLoS Pathog* **6**(8).

Shair, K. H., K. M. Bendt, R. H. Edwards, J. N. Nielsen, D. T. Moore and N. Raab-Traub (2012). Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) and LMP2A function cooperatively to promote carcinoma development in a mouse carcinogenesis model. *J Virol* **86**(9): 5352-5365.

Shair, K. H. and N. Raab-Traub (2012). Transcriptome changes induced by Epstein-Barr virus LMP1 and LMP2A in transgenic lymphocytes and lymphoma. *MBio* **3**(5).

Shaku, F., G. Matsuda, R. Furuya, C. Kamagata, M. Igarashi, M. Tanaka, M. Kanamori, Y. Nishiyama, N. Yamamoto and Y. Kawaguchi (2005). Development of a monoclonal antibody against Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) that can detect EBNA-LP expressed in P3HR1 cells. *Microbiol Immunol* **49**(5): 477-483.

Shannon-Lowe, C., E. Adland, A. I. Bell, H. J. Delecluse, A. B. Rickinson and M. Rowe (2009). Features distinguishing Epstein-Barr virus infections of epithelial cells and B cells: viral genome expression, genome maintenance, and genome amplification. *J Virol* **83**(15): 7749-7760.

Shannon-Lowe, C. and M. Rowe (2011). Epstein-Barr virus infection of polarized epithelial cells via the basolateral surface by memory B cell-mediated transfer infection. *PLoS Pathog* **7**(5): e1001338.

Shariat, S. F., M. W. Kattan, E. Traxel, B. Andrews, K. Zhu, T. M. Wheeler and K. M. Slawin (2004). Association of pre- and postoperative plasma levels of transforming growth factor beta(1) and interleukin 6 and its soluble receptor with prostate cancer progression. *Clin Cancer Res* **10**(6): 1992-1999.

Shi, W., C. Sun, B. He, W. Xiong, X. Shi, D. Yao and X. Cao (2004). GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* **164**(2): 291-300.

Shi, Y. and J. Massague (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**(6): 685-700.

Shibata, D. and L. M. Weiss (1992). Epstein-Barr virus-associated gastric adenocarcinoma. *Am J Pathol* **140**(4): 769-774.

Shimazu, T., K. Degenhardt, E. K. A. Nur, J. Zhang, T. Yoshida, Y. Zhang, R. Mathew, E. White and M. Inouye (2007). NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition. *Genes Dev* **21**(8): 929-941.

Shimizu, N., A. Tanabe-Tochikura, Y. Kuroiwa and K. Takada (1994). Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. *J Virol* **68**(9): 6069-6073.

Simon, J. A. and R. E. Kingston (2013). Occupying chromatin: Polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. *Mol Cell* **49**(5): 808-824.

Sing, G. K., J. R. Keller, L. R. Ellingsworth and F. W. Ruscetti (1988). Transforming growth factor beta selectively inhibits normal and leukemic human bone marrow cell growth in vitro. *Blood* **72**(5): 1504-1511.

Skaletsky, S. R. a. H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* M. S. Krawetz S. Totowa, NJ, Humana Press**:** 365-386.

Skalska, L., R. E. White, M. Franz, M. Ruhmann and M. J. Allday (2010). Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP. *PLoS Pathog* **6**(6): e1000951.

Skalska, L., R. E. White, G. A. Parker, A. J. Sinclair, K. Paschos and M. J. Allday (2013). Induction of p16(INK4a) is the major barrier to proliferation when Epstein-Barr virus (EBV) transforms primary B cells into lymphoblastoid cell lines. *PLoS Pathog* **9**(2): e1003187.

Smeland, E. B., H. K. Blomhoff, H. Holte, E. Ruud, K. Beiske, S. Funderud, T. Godal and R. Ohlsson (1987). Transforming growth factor type beta (TGF beta) inhibits G1 to S transition, but not activation of human B lymphocytes. *Exp Cell Res* **171**(1): 213-222.

Smith, A. L., T. P. Robin and H. L. Ford (2012). Molecular pathways: targeting the TGF-beta pathway for cancer therapy. *Clin Cancer Res* **18**(17): 4514-4521.

Smith, O. (2012). Denis Parsons Burkitt CMG, MD, DSc, FRS, FRCS, FTCD (1911-93) Irish by birth, Trinity by the grace of God. *Br J Haematol* **156**(6): 770-776.

Snapper, C. M., F. D. Finkelman and W. E. Paul (1988). Regulation of IgG1 and IgE production by interleukin 4. *Immunol Rev* **102**: 51-75.

Sorrentino, A., N. Thakur, S. Grimsby, A. Marcusson, V. von Bulow, N. Schuster, S. Zhang, C. H. Heldin and M. Landstrom (2008). The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. *Nat Cell Biol* **10**(10): 1199-1207.

Speck, P., K. A. Kline, P. Cheresh and R. Longnecker (1999). Epstein-Barr virus lacking latent membrane protein 2 immortalizes B cells with efficiency indistinguishable from that of wild-type virus. *J Gen Virol* **80 ( Pt 8)**: 2193-2203.

Spender, L. C., M. J. Carter, D. I. O'Brien, L. J. Clark, J. Yu, E. M. Michalak, L. Happo, M. S. Cragg and G. J. Inman (2013). Transforming growth factor-beta directly induces p53-up-regulated modulator of apoptosis (PUMA) during the rapid induction of apoptosis in myc-driven B-cell lymphomas. *J Biol Chem* **288**(7): 5198-5209.

Spender, L. C., G. H. Cornish, A. Sullivan and P. J. Farrell (2002). Expression of transcription factor AML-2 (RUNX3, CBF(alpha)-3) is induced by Epstein-Barr virus EBNA-2 and correlates with the B-cell activation phenotype. *J Virol* **76**(10): 4919-4927.

Spender, L. C. and G. J. Inman (2009a). Targeting the BCL-2 family in malignancies of germinal centre origin. *Expert Opin Ther Targets* **13**(12): 1459-1472.

Spender, L. C. and G. J. Inman (2009b). TGF-beta induces growth arrest in Burkitt lymphoma cells via transcriptional repression of E2F-1. *J Biol Chem* **284**(3): 1435-1442.

Spender, L. C. and G. J. Inman (2011). Inhibition of Germinal Centre Apoptotic Programmes by Epstein-Barr Virus. *Adv Hematol* **2011**: 829525.

Spender, L. C., W. Lucchesi, G. Bodelon, A. Bilancio, C. E. Karstegl, T. Asano, O. Dittrich-Breiholz, M. Kracht, B. Vanhaesebroeck and P. J. Farrell (2006). Cell target genes of Epstein-Barr virus transcription factor EBNA-2: induction of the p55alpha regulatory subunit of PI3-kinase and its role in survival of EREB2.5 cells. *J Gen Virol* **87**(Pt 10): 2859-2867.

Spender, L. C., D. I. O'Brien, D. Simpson, D. Dutt, C. D. Gregory, M. J. Allday, L. J. Clark and G. J. Inman (2009). TGF-beta induces apoptosis in human B cells by transcriptional regulation of BIK and BCL-XL. *Cell Death Differ* **16**(4): 593-602.

Stenvers, K. L., M. L. Tursky, K. W. Harder, N. Kountouri, S. Amatayakul-Chantler, D. Grail, C. Small, R. A. Weinberg, A. M. Sizeland and H. J. Zhu (2003). Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Mol Cell Biol* **23**(12): 4371-4385.

Strasser, A. (2005). The role of BH3-only proteins in the immune system. *Nat Rev Immunol* **5**(3): 189-200.

Strowig, T., F. Brilot, F. Arrey, G. Bougras, D. Thomas, W. A. Muller and C. Munz (2008). Tonsilar NK cells restrict B cell transformation by the Epstein-Barr virus via IFN-gamma. *PLoS Pathog* **4**(2): e27.

Subramanian, C., S. Hasan, M. Rowe, M. Hottiger, R. Orre and E. S. Robertson (2002). Epstein-Barr virus nuclear antigen 3C and prothymosin alpha interact with the p300 transcriptional coactivator at the CH1 and CH3/HAT domains and cooperate in regulation of transcription and histone acetylation. *J Virol* **76**(10): 4699-4708.

Sugai, M., H. Gonda, T. Kusunoki, T. Katakai, Y. Yokota and A. Shimizu (2003). Essential role of Id2 in negative regulation of IgE class switching. *Nat Immunol* **4**(1): 25-30.

Swaminathan, S. (2008). Noncoding RNAs produced by oncogenic human herpesviruses. *J Cell Physiol* **216**(2): 321-326.

Swaminathan, S., B. Tomkinson and E. Kieff (1991). Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. *Proc Natl Acad Sci U S A* **88**(4): 1546-1550.

Swanson-Mungerson, M., R. Bultema and R. Longnecker (2010). Epstein-Barr virus LMP2A imposes sensitivity to apoptosis. *J Gen Virol* **91**(Pt 9): 2197-2202.

Swanson-Mungerson, M. A., R. G. Caldwell, R. Bultema and R. Longnecker (2005). Epstein-Barr virus LMP2A alters in vivo and in vitro models of B-cell anergy, but not deletion, in response to autoantigen. *J Virol* **79**(12): 7355-7362.

Swerdlow, S. H., E. Campo, N. L. Harris, E. S. Jaffe, S. A. Pileri, H. Stein, J. Thiele and J. W. Vardiman (2008). *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, IARC.

Takada, K. (1984). Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. *Int J Cancer* **33**(1): 27-32.

Takada, K., K. Horinouchi, Y. Ono, T. Aya, T. Osato, M. Takahashi and S. Hayasaka (1991). An Epstein-Barr virus-producer line Akata: establishment of the cell line and analysis of viral DNA. *Virus Genes* **5**(2): 147-156.

Takahashi, Y., H. Ohta and T. Takemori (2001). Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* **14**(2): 181-192.

Takanashi, M., J. Li, M. Shirakata, S. Mori and K. Hirai (1999). Tumorigenicity of mouse BALB/c 3T3 fibroblast cells which express Epstein-Barr virus-encoded LMP1 and show normal growth phenotypes in vitro is correlated with loss of transforming growth factor-beta 1-mediated growth inhibition. *Arch Virol* **144**(2): 241-257.

Tan, L. C., N. Gudgeon, N. E. Annels, P. Hansasuta, C. A. O'Callaghan, S. Rowland-Jones, A. J. McMichael, A. B. Rickinson and M. F. Callan (1999). A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. *J Immunol* **162**(3): 1827-1835.

Tarakanova, V. L. and W. S. Wold (2003). Transforming growth factor beta1 receptor II is downregulated by E1A in adenovirus-infected cells. *J Virol* **77**(17): 9324-9336.

Thawani, J. P., A. C. Wang, K. D. Than, C. Y. Lin, F. La Marca and P. Park (2010). Bone morphogenetic proteins and cancer: review of the literature. *Neurosurgery* **66**(2): 233-246; discussion 246.

Thorley-Lawson, D. A. (2001). Epstein-Barr virus: exploiting the immune system. *Nat Rev Immunol* **1**(1): 75-82.

Thorley-Lawson, D. A. and M. J. Allday (2008). The curious case of the tumour virus: 50 years of Burkitt's lymphoma. *Nat Rev Microbiol* **6**(12): 913-924.

Thorley-Lawson, D. A. and A. Gross (2004). Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med* **350**(13): 1328-1337.

Thorley-Lawson, D. A., J. B. Hawkins, S. I. Tracy and M. Shapiro (2013). The pathogenesis of Epstein-Barr virus persistent infection. *Curr Opin Virol* **3**(3): 227-232.

Thorley-Lawson, D. A. and K. P. Mann (1985). Early events in Epstein-Barr virus infection provide a model for B cell activation. *J Exp Med* **162**(1): 45-59.

Tomkinson, B., E. Robertson and E. Kieff (1993). Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J Virol* **67**(4): 2014-2025.

Touitou, R., M. Hickabottom, G. Parker, T. Crook and M. J. Allday (2001). Physical and functional interactions between the corepressor CtBP and the Epstein-Barr virus nuclear antigen EBNA3C. *J Virol* **75**(16): 7749-7755.

Touitou, R., J. O'Nions, J. Heaney and M. J. Allday (2005). Epstein-Barr virus EBNA3 proteins bind to the C8/alpha7 subunit of the 20S proteasome and are degraded by 20S proteasomes in vitro, but are very stable in latently infected B cells. *J Gen Virol* **86**(Pt 5): 1269-1277.

Turley, R. S., E. C. Finger, N. Hempel, T. How, T. A. Fields and G. C. Blobe (2007). The type III transforming growth factor-beta receptor as a novel tumor suppressor gene in prostate cancer. *Cancer Res* **67**(3): 1090-1098.

Tuscano, J. M., K. M. Druey, A. Riva, J. Pena, C. B. Thompson and J. H. Kehrl (1996). Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. *Blood* **88**(4): 1359-1364.

Uchida, J., T. Yasui, Y. Takaoka-Shichijo, M. Muraoka, W. Kulwichit, N. Raab-Traub and H. Kikutani (1999). Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. *Science* **286**(5438): 300-303.

Ueda, S., S. Uchiyama, T. Azzi, C. Gysin, C. Berger, M. Bernasconi, Y. Harabuchi, A. S. Zinkernagel and D. Nadal (2014). Oropharyngeal group a streptococcal colonization disrupts latent Epstein-Barr virus infection. *J Infect Dis* **209**(2): 255-264.

Urashima, M., A. Ogata, D. Chauhan, M. Hatziyanni, M. B. Vidriales, D. A. Dedera, R. L. Schlossman and K. C. Anderson (1996). Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. *Blood* **87**(5): 1928-1938.

van Eijk, M., T. Defrance, A. Hennino and C. de Groot (2001). Death-receptor contribution to the germinal-center reaction. *Trends Immunol* **22**(12): 677-682.

van Vlasselaer, P., J. Punnonen and J. E. de Vries (1992). Transforming growth factor-beta directs IgA switching in human B cells. *J Immunol* **148**(7): 2062-2067.

Vereide, D. T., E. Seto, Y. F. Chiu, M. Hayes, T. Tagawa, A. Grundhoff, W. Hammerschmidt and B. Sugden (2013). Epstein-Barr virus maintains lymphomas via its miRNAs. *Oncogene* **Epub ahead of print**.

Victora, G. D., D. Dominguez-Sola, A. B. Holmes, S. Deroubaix, R. Dalla-Favera and M. C. Nussenzweig (2012). Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood* **120**(11): 2240-2248.

Victora, G. D. and M. C. Nussenzweig (2012). Germinal centers. *Annu Rev Immunol* **30**: 429-457.

Vire, E., C. Brenner, R. Deplus, L. Blanchon, M. Fraga, C. Didelot, L. Morey, A. Van Eynde, D. Bernard, J. M. Vanderwinden, M. Bollen, M. Esteller, L. Di Croce, Y. de Launoit and F. Fuks (2006). The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**(7078): 871-874.

Volkmann, N., F. M. Marassi, D. D. Newmeyer and D. Hanein (2014). The rheostat in the membrane: BCL-2 family proteins and apoptosis. *Cell Death Differ* **21**(2): 206-215.

Vrazo, A. C., M. Chauchard, N. Raab-Traub and R. Longnecker (2012). Epstein-Barr virus LMP2A reduces hyperactivation induced by LMP1 to restore normal B cell phenotype in transgenic mice. *PLoS Pathog* **8**(4): e1002662.

Vrzalikova, K., M. Vockerodt, S. Leonard, A. Bell, W. Wei, A. Schrader, K. L. Wright, D. Kube, M. Rowe, C. B. Woodman and P. G. Murray (2011). Down-regulation of BLIMP1alpha by the EBV oncogene, LMP-1, disrupts the plasma cell differentiation program and prevents viral replication in B cells: implications for the pathogenesis of EBV-associated B-cell lymphomas. *Blood* **117**(22): 5907-5917.

Waltzer, L., F. Logeat, C. Brou, A. Israel, A. Sergeant and E. Manet (1994). The human J kappa recombination signal sequence binding protein (RBP-J kappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements. *EMBO J* **13**(23): 5633-5638.

Waltzer, L., M. Perricaudet, A. Sergeant and E. Manet (1996). Epstein-Barr virus EBNA3A and EBNA3C proteins both repress RBP-J kappa-EBNA2-activated transcription by inhibiting the binding of RBP-J kappa to DNA. *J Virol* **70**(9): 5909-5915.

Wang, D., D. Liebowitz and E. Kieff (1985). An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43**(3 Pt 2): 831-840.

Wang, D., D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer and E. Kieff (1988). Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J Virol* **62**(11): 4173-4184.

Wang, D., J. Long, F. Dai, M. Liang, X. H. Feng and X. Lin (2008). BCL6 represses Smad signaling in transforming growth factor-beta resistance. *Cancer Res* **68**(3): 783-789.

Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson and E. Kieff (1990a). Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J Virol* **64**(5): 2309-2318.

Wang, F., S. F. Tsang, M. G. Kurilla, J. I. Cohen and E. Kieff (1990b). Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *J Virol* **64**(7): 3407-3416.

Wang, X. F., H. Y. Lin, E. Ng-Eaton, J. Downward, H. F. Lodish and R. A. Weinberg (1991). Expression cloning and characterization of the TGF-beta type III receptor. *Cell* **67**(4): 797-805.

Warner, B. J., S. W. Blain, J. Seoane and J. Massague (1999). Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G(1) arrest pathway. *Mol Cell Biol* **19**(9): 5913-5922.

Wasil, L. R., M. J. Tomaszewski, A. Hoji and D. T. Rowe (2013). The effect of epstein-barr virus latent membrane protein 2 expression on the kinetics of early B cell infection. *PLoS One* **8**(1): e54010.

Watanabe, M., N. Masuyama, M. Fukuda and E. Nishida (2000). Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal. *EMBO Rep* **1**(2): 176-182.

White, R. E., I. J. Groves, E. Turro, J. Yee, E. Kremmer and M. J. Allday (2010). Extensive co-operation between the Epstein-Barr virus EBNA3 proteins in the manipulation of host gene expression and epigenetic chromatin modification. *PLoS One* **5**(11): e13979.

White, R. E., P. C. Ramer, K. N. Naresh, S. Meixlsperger, L. Pinaud, C. Rooney, B. Savoldo, R. Coutinho, C. Bodor, J. Gribben, H. A. Ibrahim, M. Bower, J. P. Nourse, M. K. Gandhi, J. Middeldorp, F. Z. Cader, P. Murray, C. Munz and M. J. Allday (2012). EBNA3B-deficient EBV promotes B cell lymphomagenesis in humanized mice and is found in human tumors. *J Clin Invest* **122**(4): 1487-1502.

Wiater, E. and W. Vale (2003). Inhibin is an antagonist of bone morphogenetic protein signaling. *J Biol Chem* **278**(10): 7934-7941.

Widschwendter, M., H. Fiegl, D. Egle, E. Mueller-Holzner, G. Spizzo, C. Marth, D. J. Weisenberger, M. Campan, J. Young, I. Jacobs and P. W. Laird (2007). Epigenetic stem cell signature in cancer. *Nat Genet* **39**(2): 157-158.

Wildey, G. M., S. Patil and P. H. Howe (2003). Smad3 potentiates transforming growth factor beta (TGFbeta )-induced apoptosis and expression of the BH3-only protein Bim in WEHI 231 B lymphocytes. *J Biol Chem* **278**(20): 18069-18077.

Woisetschlaeger, M., X. W. Jin, C. N. Yandava, L. A. Furmanski, J. L. Strominger and S. H. Speck (1991). Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. *Proc Natl Acad Sci U S A* **88**(9): 3942-3946.

Woisetschlaeger, M., J. L. Strominger and S. H. Speck (1989). Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. *Proc Natl Acad Sci U S A* **86**(17): 6498-6502.

Wrana, J. L., L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X. F. Wang and J. Massague (1992). TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* **71**(6): 1003-1014.

Wrighton, K. H., X. Lin, P. B. Yu and X. H. Feng (2009). Transforming Growth Factor {beta} Can Stimulate Smad1 Phosphorylation Independently of Bone Morphogenic Protein Receptors. *J Biol Chem* **284**(15): 9755-9763.

Wurstle, M. L., M. A. Laussmann and M. Rehm (2012). The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and activity on the apoptosome. *Exp Cell Res* **318**(11): 1213-1220.

Yagi, K., D. Goto, T. Hamamoto, S. Takenoshita, M. Kato and K. Miyazono (1999). Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3. *J Biol Chem* **274**(2): 703-709.

Yajima, M., K. Imadome, A. Nakagawa, S. Watanabe, K. Terashima, H. Nakamura, M. Ito, N. Shimizu, M. Honda, N. Yamamoto and S. Fujiwara (2008). A new humanized mouse model of Epstein-Barr virus infection that reproduces persistent infection, lymphoproliferative disorder, and cell-mediated and humoral immune responses. *J Infect Dis* **198**(5): 673-682.

Yamamoto, N., T. Takizawa, Y. Iwanaga, N. Shimizu and N. Yamamoto (2000). Malignant transformation of B lymphoma cell line BJAB by Epstein-Barr virus-encoded small RNAs. *FEBS Lett* **484**(2): 153-158.

Yamamura, Y., X. Hua, S. Bergelson and H. F. Lodish (2000). Critical role of Smads and AP-1 complex in transforming growth factor-beta -dependent apoptosis. *J Biol Chem* **275**(46): 36295-36302.

Yamashita, M., K. Fatyol, C. Jin, X. Wang, Z. Liu and Y. E. Zhang (2008a). TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. *Mol Cell* **31**(6): 918-924.

Yamashita, S., S. Takahashi, N. McDonell, N. Watanabe, T. Niwa, K. Hosoya, Y. Tsujino, T. Shirai and T. Ushijima (2008b). Methylation silencing of transforming growth factor-beta receptor type II in rat prostate cancers. *Cancer Res* **68**(7): 2112-2121.

Yanagisawa, K., H. Osada, A. Masuda, M. Kondo, T. Saito, Y. Yatabe, K. Takagi, T. Takahashi and T. Takahashi (1998). Induction of apoptosis by Smad3 and down-regulation of Smad3 expression in response to TGF-beta in human normal lung epithelial cells. *Oncogene* **17**(13): 1743-1747.

Yang, X., Z. He, B. Xin and L. Cao (2000). LMP1 of Epstein-Barr virus suppresses cellular senescence associated with the inhibition of p16INK4a expression. *Oncogene* **19**(16): 2002-2013.

Yang, Y. A., B. Tang, G. Robinson, L. Hennighausen, S. G. Brodie, C. X. Deng and L. M. Wakefield (2002). Smad3 in the mammary epithelium has a nonredundant role in the induction of apoptosis, but not in the regulation of proliferation or differentiation by transforming growth factor-beta. *Cell Growth Differ* **13**(3): 123-130.

Yang, Z. Z., D. M. Grote, S. C. Ziesmer, B. Xiu, N. R. Yates, F. J. Secreto, L. S. Hodge, T. E. Witzig, A. J. Novak and S. M. Ansell (2013). Soluble and membrane-bound TGF-beta-mediated regulation of intratumoral T cell differentiation and function in B-cell non-Hodgkin lymphoma. *PLoS One* **8**(3): e59456.

Yee, J., R. E. White, E. Anderton and M. J. Allday (2011). Latent Epstein-Barr virus can inhibit apoptosis in B cells by blocking the induction of NOXA expression. *PLoS One* **6**(12): e28506.

Yenamandra, S. P., R. Sompallae, G. Klein and E. Kashuba (2009). Comparative analysis of the Epstein-Barr virus encoded nuclear proteins of EBNA-3 family. *Comput Biol Med* **39**(11): 1036-1042.

Yin, Q., X. Wang, C. Fewell, J. Cameron, H. Zhu, M. Baddoo, Z. Lin and E. K. Flemington (2010). MicroRNA miR-155 Inhibits Bone Morphogenetic Protein (BMP) Signaling and BMP-Mediated Epstein-Barr Virus Reactivation. *J Virol* **84**(13): 6318-6327.

Young, L. S. and A. B. Rickinson (2004). Epstein-Barr virus: 40 years on. *Nat Rev Cancer* **4**(10): 757-768.

Young, L. S., Q. Y. Yao, C. M. Rooney, T. B. Sculley, D. J. Moss, H. Rupani, G. Laux, G. W. Bornkamm and A. B. Rickinson (1987). New type B isolates of Epstein-Barr virus from Burkitt's lymphoma and from normal individuals in endemic areas. *J Gen Virol* **68 ( Pt 11)**: 2853-2862.

Zawel, L., J. L. Dai, P. Buckhaults, S. Zhou, K. W. Kinzler, B. Vogelstein and S. E. Kern (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* **1**(4): 611-617.

Zhang, H. T., X. F. Chen, M. H. Wang, J. C. Wang, Q. Y. Qi, R. M. Zhang, W. Q. Xu, Q. Y. Fei, F. Wang, Q. Q. Cheng, F. Chen, C. S. Zhu, S. H. Tao and Z. Luo (2004). Defective expression of transforming growth factor beta receptor type II is associated with CpG methylated promoter in primary non-small cell lung cancer. *Clin Cancer Res* **10**(7): 2359-2367.

Zhang, X., L. Ding and A. J. Sandford (2005). Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. *BMC Mol Biol* **6**: 4.

Zhang, Y., C. Chang, D. J. Gehling, A. Hemmati-Brivanlou and R. Derynck (2001). Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. *Proc Natl Acad Sci U S A* **98**(3): 974-979.

Zhao, B., J. C. Mar, S. Maruo, S. Lee, B. E. Gewurz, E. Johannsen, K. Holton, R. Rubio, K. Takada, J. Quackenbush and E. Kieff (2011). Epstein-Barr virus nuclear antigen 3C regulated genes in lymphoblastoid cell lines. *Proc Natl Acad Sci U S A* **108**(1): 337-342.

Zhao, B., S. Maruo, A. Cooper, R. C. M, E. Johannsen, E. Kieff and E. Cahir-McFarland (2006). RNAs induced by Epstein-Barr virus nuclear antigen 2 in lymphoblastoid cell lines. *Proc Natl Acad Sci U S A* **103**(6): 1900-1905.

Zhao, B. and C. E. Sample (2000). Epstein-barr virus nuclear antigen 3C activates the latent membrane protein 1 promoter in the presence of Epstein-Barr virus nuclear antigen 2 through sequences encompassing an spi-1/Spi-B binding site. *J Virol* **74**(11): 5151-5160.

Zhao, H., H. Shiina, K. L. Greene, L. C. Li, Y. Tanaka, H. Kishi, M. Igawa, C. J. Kane, P. Carroll and R. Dahiya (2005). CpG methylation at promoter site -140 inactivates TGFbeta2 receptor gene in prostate cancer. *Cancer* **104**(1): 44-52.

Zhao, S., K. Venkatasubbarao, S. Li and J. W. Freeman (2003). Requirement of a specific Sp1 site for histone deacetylase-mediated repression of transforming growth factor beta Type II receptor expression in human pancreatic cancer cells. *Cancer Res* **63**(10): 2624-2630.

Zhu, H., P. Kavsak, S. Abdollah, J. L. Wrana and G. H. Thomsen (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400**(6745): 687-693.

Zimber-Strobl, U., B. Kempkes, G. Marschall, R. Zeidler, C. Van Kooten, J. Banchereau, G. W. Bornkamm and W. Hammerschmidt (1996). Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. *EMBO J* **15**(24): 7070-7078.

Zimber-Strobl, U. and L. J. Strobl (2001). EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes. *Semin Cancer Biol* **11**(6): 423-434.

Zimber-Strobl, U., L. J. Strobl, C. Meitinger, R. Hinrichs, T. Sakai, T. Furukawa, T. Honjo and G. W. Bornkamm (1994). Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless. *EMBO J* **13**(20): 4973-4982.

zur Hausen, H., F. J. O'Neill, U. K. Freese and E. Hecker (1978). Persisting oncogenic herpesvirus induced by the tumour promotor TPA. *Nature* **272**(5651): 373-375.