The EBNA3 Family: Two Oncoproteins and a Tumour Suppressor that Are Central to the Biology of EBV in B Cells

Martin J. Allday, Quentin Bazot and Robert E. White

Abstract Epstein-Barr virus nuclear antigens EBNA3A, EBNA3B and EBNA3C are a family of three large latency-associated proteins expressed in B cells induced to proliferate by the virus. Together with the other nuclear antigens (EBNA-LP, EBNA2 and EBNA1), they are expressed from a polycistronic transcription unit that is probably unique to B cells. However, compared with the other EBNAs, hitherto the EBNA3 proteins were relatively neglected and their roles in EBV biology rather poorly understood. In recent years, powerful new technologies have been used to show that these proteins are central to the latency of EBV in B cells, playing major roles in reprogramming the expression of host genes affecting cell proliferation, survival, differentiation and immune surveillance. This indicates that the EBNA3s are critical in EBV persistence in the B cell system and in modulating B cell lymphomagenesis. EBNA3A and EBNA3C are necessary for the efficient proliferation of EBV-infected B cells because they target important tumour suppressor pathways—so operationally they are considered oncoproteins. In contrast, it is emerging that EBNA3B restrains the oncogenic capacity of EBV, so it can be considered a tumour suppressor—to our knowledge the first to be described in a tumour virus. Here, we provide a general overview of the EBNA3 genes and proteins. In particular, we describe recent research that has highlighted the complexity of their functional interactions with each other, with specific sites on the human genome and with the molecular machinery that controls transcription and epigenetic states of diverse host genes.

M.J. Allday (✉) · Q. Bazot · R.E. White
Molecular Virology, Division of Infectious Diseases, Department of Medicine,
Imperial College London, Norfolk Place, London W2 1PG, UK
e-mail: m.allday@imperial.ac.uk

C. Münz (ed.), *Epstein Barr Virus Volume 2*, Current Topics in Microbiology
and Immunology 391, DOI 10.1007/978-3-319-22834-1_3

Contents

Abbreviations

4HT	4-Hydroxytamoxifen
ABC	Activated B cell
BAC	Bacterial artificial chromosome
BARTs	BamH1-A rightward transcripts
BL	Burkitt's lymphoma
CaHV3	Callitrichine herpesvirus 3
CBF	C promoter binding factor
CCC	Chromatin conformation capture
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation coupled to high-throughput DNA sequencing

CtBP	C-terminal binding protein
CTL	Cytotoxic T lymphocyte
DDR	DNA damage response
DLBCL	Diffuse large B cell lymphoma
EBER	EBV-encoded RNA
ER	Oestrogen receptor
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
GST	Glutathione-S-transferase
HDAC	Histone deacetylase
HL	Hodgkin's lymphoma
IM	Infectious mononucleosis
IRES	Internal ribosome entry site
KO	Knockout
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LZ	Leucine zipper
LMP	Latent membrane protein
MBC	Memory B cell
miRNA	MicroRNA
MIZ1	Myc-interacting zinc-finger protein 1
NLS	Nuclear localisation signal
NOD	Non-obese diabetic
Notch-IC	Intracellular Notch transcription factor
NSG	NOD-scid IL2 γ-null
OIS	Oncogene-induced senescence
ORF	Open reading frame
OSR	Oncogenic stress response
paHV1	Papiine herpesvirus 1
PcG	Polycomb group protein
Pol II	RNA polymerase II
PRC	Polycomb repressive complex
PTLD	Post-transplant lymphoproliferative disease
QPCR	Quantitative polymerase chain reaction
RB	Retinoblastoma protein
RBP	Recombining binding protein
REFs	Rat embryo fibroblasts
RhLCV	Rhesus lymphocryptovirus
SCID	Severe combined immunodeficiency
Su(H)	Suppressor of Hairless
TSS	Transcription start site
WT	Wild type

1 The Biology of EBV in B Cells

EBV has co-evolved with its hominid and prehominid hosts for millions of years and is now a uniquely successful intracellular parasite that persists asymptomatically after childhood infection in >90 % of the human population. Despite this benign relationship with most humans, EBV is classified as a tumour virus because of its firmly established association with several cancers of B, NK/T and epithelial cell origin, its ability to induce B cell tumours in non-human primates and its capacity to growth transform primary human B cells in culture. This capacity of EBV to 'transform' resting B cells into continuously proliferating lymphoblastoid cell lines (LCLs) was discovered about 3 years after the virus was identified in samples of Burkitt's lymphoma (BL) 50 years ago (reviewed in Crawford 2001; Epstein 2001; Thorley-Lawson and Allday 2008; Young and Rickinson 2004). This remarkably efficient process has subsequently been used countless times to establish 'normal' human B cell lines for genetic, epigenetic and immunological studies (see, for example, ENCODE, the 1000 genomes project and the HapMap project). It has also been the focus of intensive research because the process produces limitless amounts of material for study and because of the general belief that the molecular mechanisms involved in the transformation of explanted B cells might explain aspects of EBV-associated cancer in humans. Today, after several decades of study, the general strategies utilised by EBV to drive mature B cells from quiescence into the cell cycle and maintain cell proliferation and survival (while preventing the activation of lytic virus replication) are largely understood—however, many of the biochemical details are poorly characterised.

When EBV induces the activation of resting B cells, it exploits the cellular transcription and translation machineries to express nine viral latency-associated proteins. There are six nuclear proteins (Epstein-Barr nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and LP) and three membrane proteins (latent membrane proteins (LMPs) 1, 2A and 2B). In addition, two types of untranslated RNAs are expressed (EBV-encoded RNAs (EBERs) and the microRNAs (miRs) derived from the BamHI-A rightward transcripts (BARTs) and the BHRF1 transcript). The BART and BHRF1 RNAs can be processed into many miR species that probably target multiple host and viral mRNAs (comprehensively reviewed in the chapter authored by Skalsky and Cullen). This pattern of viral gene expression in B cells is known as latency III (sometimes referred to as the growth or proliferation programme) and is necessary to drive quiescent B cells into the cell cycle and sustain proliferation, while maintaining the EBV genome as extra-chromosomal 172 kb episomes (reviewed in Cai et al. 2006; Young and Rickinson 2004). The proliferating cells resemble, at least superficially, antigen-activated B cells (B blasts). As a consequence, it was a widely held view that when EBV transforms normal B cells into LCLs, the small number of viral latency-associated gene products exploits the physiological process of activation normally achieved by the interaction of a B cell with its specific antigen together with cognate T cell help. While in principle, this

might be correct, it has gradually become apparent that LCLs are trapped at a B blast-like stage of differentiation, but they have a distinctive EBV-specific phenotype. In addition, the process of EBV-induced proliferation is 'sensed' by B cells as abnormal and so activates various innate responses that cells have evolved to prevent non-physiological DNA replication and proliferation and which have to be circumvented in order to establish latent infection. Several of the latency-associated viral factors, including EBNA3C—a major focus of this review—have evolved functions that overcome cellular checkpoints and pathways that trigger anti-proliferative or suicidal responses.

During lifelong persistence in healthy seropositive individuals, extra-chromosomal EBV episomes are largely found in non-dividing ($Ki67^{-ve}$), long-lived, class-switched (IgD^{-ve}), $CD27^{+ve}$ memory B cells (MBCs) in the peripheral circulation—and they do not appear to express the latency III proteins. However, in tonsils, naïve (IgD^{+ve}) B cells can be found that express the latency III programme and proliferate as LCL-like B blasts; so it seems that, in vivo as in vitro, EBV-infected naïve B cells undergo multiple divisions—at least transiently—as part of the normal viral life cycle. A compelling explanation of how some of the proliferating B blasts end up as memory cells was provided by Thorley-Lawson and colleagues and is reviewed elsewhere in this volume. Briefly, cells in the expanding B blast population are thought to enter a germinal centre in lymphoid tissue such as tonsil. In this microenvironment, these cells differentiate to become centroblasts, then centrocytes and finally resting memory B cells that enter the peripheral circulation (Babcock et al. 2000; Roughan and Thorley-Lawson 2009 and reviewed in Thorley-Lawson and Gross 2004; Thorley-Lawson et al. 2013). While the precise series of events that the EBV-positive B cells undergo to reach the memory compartment remains unknown, the consensus view is that it involves regulated shutdown of EBV latency-associated gene expression from the initial latency III state to latency II (in which EBNA2, EBNA-LP, and the EBNA3 family have been switched off by promoter switching from Cp to Qp—Fig. 1). In quiescent MBCs, Qp also becomes silenced and no EBV proteins can be detected—a state called latency 0. If MBCs periodically divide, then EBNA1 is switched back on to facilitate episome maintenance (this state is known as latency I). However, it is still unclear whether or not the differentiation of EBV-infected B blasts to memory B cells is absolutely dependent on the microenvironment of a germinal centre, although a role for T cells is indicated. T cell-derived cytokines provide signals that trigger the repression of the EBNA promoter, Cp, and reduce the expression of EBNA2 in LCLs and a mouse model (Kis et al. 2006, 2010; Nagy et al. 2012; Salamon et al. 2012)—however, it has yet to be proven that this occurs during persistence. In normal healthy individuals, the EBV-infected B blasts are targets for EBV-specific cytotoxic lymphocytes (CTLs) that can recognise and destroy these latently infected cells. Stable persistence depends, therefore, on the equilibrium established between the proliferation of B blasts—on the one hand—and immune elimination or differentiation to the resting memory compartment on the other (Babcock et al. 1999; Hawkins et al. 2013).

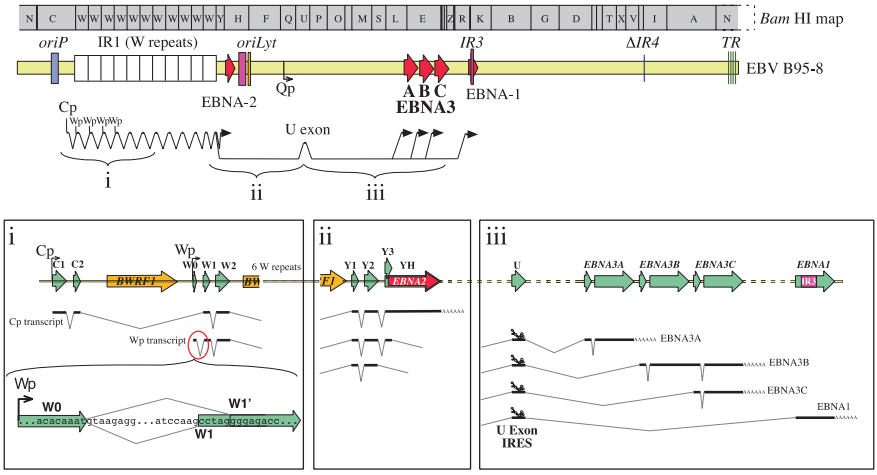

Fig. 1 The B cell transcription unit. *Top* is an overview of the EBV genome (B95-8 strain), indicating major repeats and the nuclear antigens, with the Bam HI digest map (which defines the names of exons, promoters and most EBV ORFs) above. *Below* the map is a transcript summary, indicating the overall nature of the B cell transcription unit. Numbers (i)–(iii) indicate the major splicing 'decisions' that define the alternative transcripts that can be generated from the B cell transcription unit that are defined in detail in the boxes below. No coordination between these splicing events has been reported. *i* Early after infection, transcription begins from promoter Wp (exon W0), but promoter Cp is activated by EBNA2 (Exons C1C2). Exon W0 (and exon C2—not shown) can splice to either splice acceptor W1 or W1'. Splice acceptor W1' completes a start codon across the splice junction, allowing EBNA-LP translation from an array of exons W1W2 followed by Y1Y2. Splice acceptor Exon W1 does not generate an AUG codon, enabling translation from the next AUG codon that initiates EBNA2. *ii* Within exon YH (encoding EBNA2) is a splice donor (generating exon Y3) that can splice to the internal ribosome entry site within exon U. Exon Y2 can splice to either YH/Y3 or directly to exon U. Alternatively, the transcript is polyadenylated at the end of EBNA2 as shown. *iii* The IRES within the downstream U exon allows the translation of the EBNA3s and EBNA1 as shown. Polyadenylation has been observed after EBNA3A, EBNA3C and EBNA1. Curiously, EBNA3B transcripts also contain the EBNA3C open reading frame. Not only can the splice junction from exon U to the EBNA3s define the transcribed protein, but also failure to splice the internal introns within the EBNA3s has also been reported. Switching from the Cp/Wp to using Qp (not shown) results in the loss of expression of all EBNAs except the EBNA1 transcript. Theoretically, it would be possible to also generate EBNA3 transcripts from Qp, but this has not been reported

Primary infection with EBV during adolescence can sometimes result in the relatively benign illness infectious mononucleosis (IM), but disruption of components of the immune system by co-infections, genetic/epigenetic aberrations or iatrogenic causes can result in EBV-associated B cell neoplasia including post-transplant lymphoproliferative disease (PTLD), Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL) and some diffuse large B cell lymphoma (DLBCL) (reviewed in Crawford 2001). Details of the pathogenesis of IM and these tumours are discussed elsewhere in the volume. Because, as far as we know, the EBNA3 family of proteins (along with EBNA2 and EBNA-LP) is only expressed in B cells from

the unique B cell-specific transcription unit (Fig. 1 and below), here we have only described the biology of EBV latency in B cells. The biology and pathology of EBV in epithelial cells and NK/T cells are also described elsewhere in the volume.

2 The EBNA3 Family of Genes and Proteins

The EBV latency-associated genes EBNA3A, EBNA3B and EBNA3C (historically also called EBNA3, EBNA4 and EBNA6, respectively) are a family that probably arose during primate gamma-herpesvirus evolution by a series of gene duplication events since they have a similar gene structure, are arranged in tandem in the EBV genome and share partial sequence homology. EBNA3 transcripts are alternatively spliced from very long primary transcripts generally initiated at a single latency promoter and LCLs have only a few copies of these transcripts per cell, suggesting that their expression is tightly regulated. Although it is related to one another, there is nothing to suggest that the large nuclear proteins encoded by these genes have redundant functions; moreover, they possess no obvious similarities to any known cell or viral proteins—other than their localisation to the nucleus—that provide clues to their biological roles. Genetic studies using recombinant viruses originally indicated that EBNA3A and EBNA3C are essential for efficient in vitro transformation and immortalisation of B cells, whereas EBNA3B is dispensable. However, under the appropriate conditions with feeder cells in the culture, it has more recently been possible to establish EBNA3A-negative LCLs with relative ease (Hertle et al. 2009; Skalska et al. 2010). From an increasing number of studies that have made use of bacterial artificial chromosome (BAC)-derived EBV recombinants carrying deletions, fusions or mutations in the EBNA3 locus, the molecular details underlying these roles in B cell transformation are gradually emerging and will be reviewed herein.

2.1 Genomic Organisation, the B Cell Transcription Unit and Regulation of Expression

The EBNA3 coding exons share a common structure, with a short 5′ exon, of approximately 360 nucleotides, and a longer 3′ exon of around 2.5 kb, separated by an intron of under 100 bp (Fig. 1). The coding regions of the longer exons are partially repetitive—with EBNA3B and EBNA3C containing variable length imperfect repeat regions (60 bp repeats for EBNA3B, and 45 bp repeats for EBNA3C). The C-terminal half of the EBNA3A gene comprises sequences that show evidence of historic duplication events followed by diversification, leaving regions with partial homology to each other (Baer et al. 1984; Hennessy et al. 1986). Expansions and contractions of these repetitive regions lead to EBV

isolate-specific differences in the size, hence electrophoretic mobility, of each EBNA3 protein, allowing EBV clinical isolates to be distinguished by the apparent size of their EBNAs (EBNA-type—Yao et al. 1991).

All of the EBNAs are transcribed from extended transcripts originating at a common promoter. Initially after infection, the multiple copies of the B cell-specific promoter located in the BamH1W repeats (Wp) are used, but this soon switches to a single promoter just upstream of these repeats (Cp), which remains the dominant promoter during latency type III. Cp can be epigenetically silenced in the transition to latency states type II, type I and type 0 (reviewed in Tempera and Lieberman 2014 and Lieberman in this volume). Synthesis of the various EBNA proteins from this promoter is dependent in the first instance on a complex array of alternative splicing events. For clarity, these are labelled i, ii and iii in Fig. 1. Our current understanding is that the splicing event between the promoter exon (generated by either Cp or Wp) and the W1 exon (i in Fig. 1) can select either of two splice acceptors, 5 bases apart. The most downstream of these splice acceptors (W1′) creates the start codon of EBNA-LP across the splice site, facilitating the translation of EBNA-LP. The upstream splice acceptor (W1) does not generate an AUG, and as a result, the first start codon in the transcript is that of EBNA2. Regulation of this splice is thus believed to define the balance of EBNA-LP and EBNA2 translation in the cell.

A second alternative splicing event (ii in Fig. 1) links the upstream exons to the U exon. Both the Y2 and Y3 exons splice to the U exon with similar frequencies. In so doing, the splice to the U exon necessarily excises the EBNA2 ORF. Upstream of an open reading frame, the U exon is thought to function as an internal ribosome entry site (IRES) (Isaksson et al. 2003). Thus, its splicing to downstream exons (iii in Fig. 1) allows the translation of one of the EBNA3s or EBNA1 from a second cistron of the transcript (i.e. after EBNA-LP) with the EBNA1 splice site being favoured (Arvey et al. 2012). It has further been suggested that the internal intron of each EBNA3 may not splice with perfect efficiency in LCLs, which may constitute another mechanism to modulate EBNA3 protein levels (Kienzle et al. 1999). In addition to this already complex transcriptional profile, other splice variants have been observed at lower frequency (Arvey et al. 2012), but there is no indication that these are functionally relevant to the biology of the virus.

This cascade of alternative splicing results in the ORFs located more 3′ being transcribed less frequently than EBNA2 and EBNA-LP. This probably contributes to the low levels of EBNA3 mRNAs and proteins, such that their detection in infected B cells by immunofluorescence or immunocytochemistry has proven extremely difficult. However, the use of Western blotting has shown that a constant level of EBNA3 proteins is maintained across many LCLs, suggesting that there is tight control of EBNA3 protein levels in these cells. The mechanisms that balance the expression of the EBNAs are not fully understood, but EBNA3 homoeostasis may involve modulation of splicing, IRES function and protein turnover. Our experience of engineering EBNA3 recombinants has shown that this balance of expression is easily disrupted. Specifically, we find that introducing additional sequences onto the C-terminus of EBNA3B in the virus genome disrupts

EBNA3C splicing, while N-terminal fusions of EBNA3B can either increase or decrease EBNA3B protein levels (our unpublished results). Fortunately for genetic analysis of both EBNA3A and EBNA3C, C-terminal fusions are well tolerated and have produced no unpredictable or undesirable expression patterns in LCLs. As is described in the following sections, there appears to be considerable functional and perhaps physical interaction between the EBNA3 proteins and so maintaining balanced stoichiometry is probably vitally important for LCL fitness. However, mechanisms by which RNA splicing, translation and turnover of the EBNA3s mediate this careful regulation of protein levels remain largely uncharacterised.

2.2 Sequence Variation of the EBNA3 Proteins

There are two major clades of EBV—type 1 (aka type A) and type 2 (aka type B)—defined by the sequences of their EBNA2 and EBNA3 genes. Type 1 EBV is widespread worldwide, while type 2 EBV is largely confined to sub-Saharan Africa (Young et al. 1987). EBNA2 and EBNA3 represent the major sites of genome diversity of EBV, with approximately 80 % nucleotide identity between the prototype type 1 and type 2 EBNA3B and EBNA3C gene sequences, and 90 % for EBNA3A. It is not yet clear how precisely type 2 EBNA3s are functionally equivalent to their type 1 counterparts or—as with EBNA2—they have distinct capabilities in B cell transformation. The clearest differences between type 1 and 2 EBNA3s lie in the sizes of their repetitive regions, with distinct insertions/deletions permitting PCR-based identification of type 1 versus type 2 EBNA3s (Sample et al. 1990).

The diversity of EBNA3 sequences is further complicated by recombination between distinct virus strains. Examples of EBV from both Chinese and Korean samples have been identified with a type 1 EBNA2 and type 2 EBNA3s, or type 1 EBNA2 and EBNA3A with type 2 EBNA3B and EBNA3C (Kim et al. 2006; Midgley et al. 2000, 2003). An even more complex combination was found in the DNA isolated from the blood of an Austrian post-transplant lymphoproliferative disorder (PTLD) patient, identifying a crossover incorporating almost 1 kb of type 2 sequence encoding the last 200 amino acids of EBNA3A and the first exon of EBNA3B (Gorzer et al. 2006). We also observed a recombination between distinct type 1 genotypes in the EBNA3B of a Hodgkin's lymphoma sample, although in this case, it results in a small deletion within EBNA3B (White et al. 2012). It may be that this event is unique to this tumour, but it underlines the likelihood that interstrain recombination is a driver of EBV, and EBNA3, diversity.

Overall, it is clear that the EBNA3s are (with EBNA2) the most diverse of the EBV proteins and that, although it appears to be uncommon, linkage between EBNA3 and EBNA2 subtypes can be disrupted by recombination between diverse strains. Despite the significant amino acid sequence variation, the biological and immunological consequences of this diversity remain largely unexplored—for a more detailed consideration of EBV DNA and protein sequence variation, see the chapter authored by Paul Farrell.

2.3 The EBNA3 Counterparts in EBV-Like Primate Viruses and Their Evolution

The lymphocryptovirus (LCV) genus—to which Epstein-Barr virus belongs—appears to be restricted to primates. Evolutionary studies on LCV genes for the DNA-binding protein BALF2 and the fusion-mediating glycoprotein gB support the hypothesis that the viruses have evolved mainly through co-speciation with their hosts, with occasional horizontal transmission events, such as the apparent crossing of an ancestral LCV from macaques to hominoid apes (Orangutans; Gibbons) in Indonesia (Ehlers et al. 2003, 2010). Of the primate LCVs, only two—the Macacine herpesvirus 4 (better known as the rhesus lymphocryptovirus (RhLCV) as it was isolated from immunosuppressed rhesus macaques) and Callitrichine herpesvirus 3 (CaHV3, isolated from a common marmoset)—have been sequenced in full. *EBNA3A* and *EBNA3C* have also been cloned and sequenced from Papiine herpesvirus 1 (paHV1, host: Baboon). The *EBNA3* gene sequences of these herpesviruses are among the most divergent of all the LCV genes. The EBNA3s of paHV1 and RhLCV have only 30–50 % amino acid sequence conservation with their EBV homologues, and this is predominantly in the N-terminal half of the protein that includes the so-called homology domain (see Sect. 2.4 and Fig. 2) and—for EBNA3C—the terminal 100 amino acids (Jiang et al. 2000; Zhao et al. 2003). Initial analysis of the more distantly related CaHV3 genome—originating from a new world primate—failed to identify any *EBNA3* homologues. Only by determining the DNA sequence corresponding to the latency-associated transcripts of CaHV3 was the *EBNA3* paralogue identified, through the conserved transcript structure—an exon pair downstream of the EBNA-LP-like repeats. However, CaHV3 has only a single EBNA3 homologue, supporting the long-held supposition that the *EBNA3s* of EBV arose through gene duplication events in an ancestral virus with a single *EBNA3* gene (Rivailler et al. 2002). Functionally, the LCV EBNA3s share many properties of human EBV, such as the ability to compete with EBNA2 to suppress gene activation, and to bind RBP-JK/CBF1 (Jiang et al. 2000; Zhao et al. 2003; see Sects. 2.5 and 2.7.1). However, they are sufficiently distinct that the RhLCV EBNA3s could not support sustained transformation of human B cells when genetically substituted within the P3HR1 strain of EBV (Jiang et al. 2000).

2.4 Limited Homology but Shared Structural Features Between the EBNA3 Proteins

Immunofluorescence staining of cells ectopically expressing each of the EBNA3s revealed an exclusively nuclear distribution that at low expression levels can be speckled or punctate (particularly EBNA3C) and may spare the nucleolus (Allday et al. 1988; Hennessy et al. 1985; Krauer et al. 2004b; Petti et al. 1988, 1990;

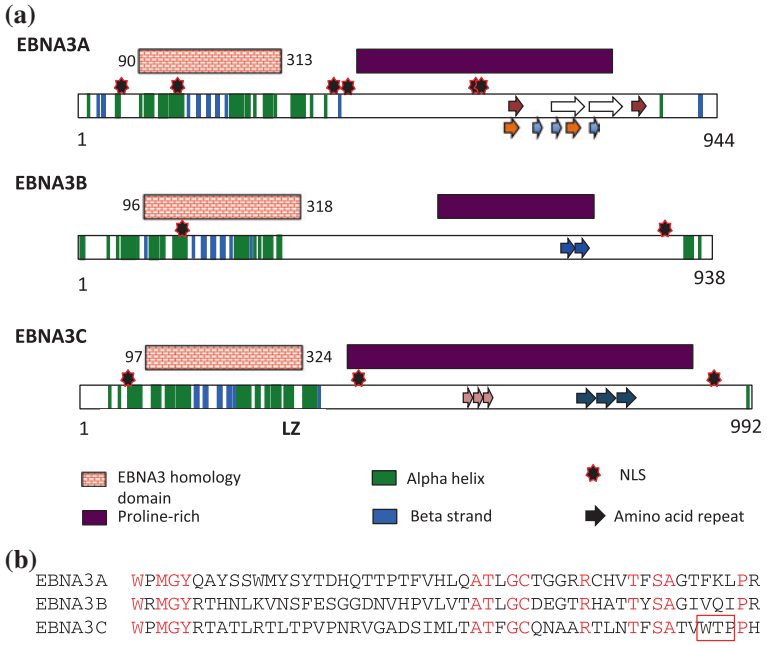

Fig. 2 The EBNA3 family of proteins. **a** Schematic representation of the EBNA3 domains. The secondary structures of the EBNA3 proteins have been predicted using the **phyre**^{2} protein fold recognition server. Domains and structural motifs are shown by the *filled green* and *blue rectangles* as indicated. The locations of nuclear localisation signals (NLS) that have been demonstrated in each protein are represented by *stars*. Similar *coloured arrows* represent similar amino acid repeats, and regions rich in *proline* are also indicated. The position of the putative leucine zipper (LZ) in EBNA3C is shown. These schematics are not drawn accurately to scale. **b** The central homology domain of the EBNA3s. Homologous amino acids are shown in *red*. The red rectangle highlights the WΦP motif of EBNA3C

Ricksten et al. 1988; Young et al. 2008 and our unpublished results). This is of no great surprise since it has been demonstrated that each of these large proteins (all are >900 aa) includes multiple nuclear localisation signals [NLS, (Fig. 2a)]: EBNA3A contains six NLSs (Buck et al. 2006; Le Roux et al. 1993), EBNA3B at least two (Burgess et al. 2006) and EBNA3C includes three (Allday et al. 1988; Krauer et al. 2004a). As well as having an exclusively nuclear distribution, the EBNA3s also associate tightly with chromatin and/or the nuclear matrix, even under high salt extraction conditions, but appear not to bind directly to DNA (Petti et al. 1990; Sample and Parker 1994 and our unpublished results). In LCLs, the proteins are remarkably stable, with a half-life of at least 24 h, and although it has been suggested that they are probably subject to ubiquitin-mediated proteosomal degradation in the production of peptides recognised by cytotoxic T cells (CTL) (Hislop et al. 2007), there is also evidence that the EBNA3s can directly bind to components of the 20S proteasome prior to degradation (Touitou et al. 2005).

Although the primary amino acid sequence of the EBNA3 proteins has diverged considerably during the course of their evolution from a common ancestor, the three proteins include an 'homology domain' near their N-terminus (Fig. 2). Within this region, EBNA3A and 3B share 28 % amino acid identity, EBNA3A and 3C share 23 %, and EBNA3B and 3C share 27 % identity (Le Roux et al. 1994). Comparative informatics analyses of the three proteins suggest that despite the very modest overall homology, there are similarities in their predicted secondary structures. Indeed, the predicted percentage similarity in secondary structure is 88 % between EBNA3A and EBNA3B, 72 % between EBNA3A and EBNAC and 72 % between EBNA3B and EBNA3C (Yenamandra et al. 2009). Predicted structural motifs are mainly alpha-helices, with few beta-strands, and are distributed primarily towards the N-terminus in the homology domain (Fig. 2a). Large regions of all three proteins have no obvious secondary structure. Other similarities between all three proteins include a proline-rich region (Fig. 2a) and repeat sequences as discussed in Sect. 2.2. A predicted structural feature that appears to be unique to EBNA3C, but for which no function has been established, is a leucine zipper (LZ (aa244-291) embedded within the homology domain (West 2006; West et al. 2004; Fig. 2a). Because of their large size and the prediction that much of each protein is disordered or flexible, structural analysis of the EBNA3s is going to be difficult. It is unlikely to be possible until functional domains can be precisely delineated, expressed and purified.

2.5 Relationship to EBNA2 and RBP-JK/CBF1—Initial Clues that the EBNA3s Are Regulators of Transcription

When EBNAs 3A, 3B and 3C were identified as nuclear proteins and mapped to the BamHI-E region of the EBV genome (Allday et al. 1988; Hennessy et al. 1985, 1986; Petti and Kieff 1988; Petti et al. 1988; Ricksten et al. 1988), the EBNA2 protein was already characterised as an essential transactivator of viral and cellular genes during B cell transformation. It was soon established that although EBNA2 does not bind DNA directly, it can be targeted to genomic response elements by binding to cellular DNA sequence-specific transcription factors including RBP-JK [also called C promoter binding factor (CBF1)]; EBNA2 then recruits multiple co-activators of transcription to these binding sites (Zimber-Strobl and Strobl 2001). Subsequently, all three EBNA3 proteins were shown to bind to the same site on RBP-JK/CBF1 as EBNA2 (Robertson et al. 1995, 1996) and that EBNA3C can probably bind PU.1/SPI1 (Jimenez-Ramirez et al. 2006; Zhao and Sample 2000). When it was shown that, in transient reporter assays, each of the EBNA3s could inhibit EBNA2-mediated activation of viral promoters (LMP2A (aka TP-1) and Cp), it was proposed that all the EBNA3s might act in a regulatory loop as functional antagonists negatively regulating all genes

activated by EBNA2 (Johannsen et al. 1996; Le Roux et al. 1994; Radkov et al. 1997; Robertson et al. 1995). For example, in the case of the EBV latency III promoter Cp, this would be a mechanism to prevent uncontrolled transactivation of the EBNA transcription unit and perhaps be involved in promoter switching during differentiation of B blasts to germinal centre B cells in vivo (Kis et al. 2010; Nagy et al. 2012; Thorley-Lawson and Allday 2008). Consistent with EBNA3s acting as repressive antagonists of EBNA2-mediated transactivation, it was discovered that when EBNA3A or EBNA3C was targeted directly to a reporter gene—by fusion to a GAL4 DNA-binding domain—both proteins exerted very robust repressor activity (Bain et al. 1996; Bourillot et al. 1998; Cludts and Farrell 1998). We have subsequently found that a Gal4-EBNA3B fusion has similar repressor activity (P. Young, PhD thesis, 2007, Imperial College London). Further support for the model came when it was shown that EBNA3A and EBNA3C interact with various repressive transcription co-factors including histone deacetylases (HDACs) HDAC-1 and HDAC-2, C-terminal binding protein (CtBP), Sin3A and NCoR (Hickabottom et al. 2002; Knight et al. 2003; Radkov et al. 1999; Touitou et al. 2001). However, the picture of EBNA3-mediated gene regulation became more complicated when it was shown in transient reporter assays that—in some circumstances—rather than exhibiting repressor activity, EBNA3C can activate transcription from viral (e.g. *LMP1* Jimenez-Ramirez et al. 2006; Zhao and Sample 2000) and cellular gene promoters (e.g. *COX-2* Kaul et al. 2006). This might be associated with its reported capacity to form complexes with transcriptional co-activators such as the histone acetyltransferase p300 (Cotter and Robertson 2000). Subsequent microarray studies have revealed the activation, as well as repression, of host genes by EBNA3A, EBNA3B and EBNA3C; so these proteins might all act as transactivators in some circumstances (see Sects. 3.2 and 4). However, in general, the molecular mechanisms of EBNA3-mediated transactivation are poorly understood.

Although there appear to be many sites across the human genome where EBNA2 and EBNA3s can apparently co-localise (see Sects. 3 and 4), it is still not known how widely EBNA2 and the EBNA3s have antagonistic roles in the regulation of host genes. Furthermore, no formal proof has been produced that the EBNA3s are involved in the negative regulation or silencing of the latency-associated EBNA promoter Cp, via RBP-JK/CBF1 response elements during viral infection and persistence. It is probable that the interaction of EBNA3s with RBP-JK/CBF1 plays an important role in the regulation of multiple genes, but until recently, no bona fide cellular targets that depend on this interaction had been identified (see Sects. 2.7.1 and 4.5). Even now, although genetic studies strongly indicate that the EBNA3A and EBNA3C binding to RBP-JK/CBF1 are important for B cell transformation (Lee et al. 2009; Maruo et al. 2005, 2009), the critical targets remain elusive and the molecular details of both protein:protein and protein:DNA interactions at regulatory loci are only now being explored (for more details, see Sects. 2.7.1 and 4).

2.6 Roles of EBNA3A and EBNA3C in Cell Cycle Regulation and as Potential Oncogenes

The initial indication that EBNA3C has activities associated with the cell cycle came from a study of its ectopic expression in Raji BL-derived cells that carry a virus with a deletion of *EBNA3C*. Complementation of the deleted gene by stable, constitutive expression from a transfected plasmid revealed that EBNA3C influences LMP1 expression only in G1 of the cell cycle (Allday and Farrell 1994). Although the basis of this phenotype was never established, it suggested that EBNA3C might have a G1-related activity. Shortly, thereafter EBNA3C was shown to 'cooperate' with oncogenic mutant Ras (Ha-Ras) in the immortalisation and transformation of primary rat embryo fibroblasts (REFs, Parker et al. 1996). In this type of assay, Ha-Ras alone induces exit from the cell cycle to a state called 'premature senescence' (Serrano et al. 1997) and other oncogenes that cooperate with Ha-Ras (e.g. adenovirus E1A; papillomavirus E7; SV40 TAg; cMyc) all subvert components of the G1 checkpoint modulated by the RB/p53 axis (reviewed in Lowe et al. 2004; Sherr 2012). This, therefore, indicated that EBNA3C might possess a similar anti-senescence activity in REFs, and perhaps also in B cells. The demonstration several years later that this activity depends on EBNA3C binding the transcriptional co-repressor CtBP (see Sect. 2.7.2) was consistent with the anti-senescence phenotype—at least in part—being linked to EBNA3C-mediated repression of transcription. The most direct and compelling evidence that EBNA3C modulates a G1 arrest checkpoint in B cells was to come from a study using an LCL established with a recombinant Akata EBV encoding a conditional EBNA3C (Maruo et al. 2006). Here, removing the inducer of EBNA3C activity from the cells resulted in the accumulation of $p16^{INK4a}$ mRNA and protein, accompanied by a substantial reduction in proliferation and considerable cell death. The molecular details and significance of these observations are discussed in detail in Sects. 4.2 and 5.

Additional evidence that EBNA3C can disrupt the regulation of the cell cycle came when it was shown that, in both rodent and human cells, constitutive overexpression of EBNA3C could induce aberrant cell division resulting in multi-nucleation, polyploidy and eventually cell death. EBNA3C also appeared to suppress the pro-metaphase arrest induced by drugs such as nocodazole that activate the mitotic spindle checkpoint (Parker et al. 2000). Together, these results suggested that unregulated expression of EBNA3C might disrupt cell cycle checkpoints occurring after DNA synthesis (S), perhaps in both G2 and mitosis. While these results raised interesting questions about the normal physiological role of EBNA3C in B cell proliferation, to date there have been no convincing genetic studies that have confirmed roles for EBNA3C in G2 or mitosis nor biochemical studies that have identified robust molecular interactions explaining these over-expression of phenotypes. A reported down-regulation of CHK2 by EBNA3C and interaction between the two proteins could have a role in facilitating the transition from G2 to mitosis (Choudhuri et al. 2007), but this has not been confirmed and it does not explain why, in LCLs, CHK2 is expressed at the same level as in mitogen-activated B cells and appears to

function normally following DNA damage (O'Nions et al. 2006). Although it has been reported that EBNA3C might repress the transcription of the mitotic regulator BUBR1 in BJAB cells, this has not been extended to LCLs nor confirmed with EBV recombinants (Gruhne et al. 2009; Skalska et al. 2013 and our unpublished data). Several reports during the past decade indicate that EBNA3C can physically associate with a variety of other factors involved in the regulation of cell cycle progression and/or the G1/S checkpoint—most of these appear to be via amino acids located at the N-terminus of EBNA3C (aa100–200). The factors include the ubiquitin ligase SCF^{SKP2}, the tumour suppressor retinoblastoma (RB), the oncoprotein MYC, MDM2, p53, cyclin A, cyclin D1, E2F1, CHK2 and Aurora kinase B (Bajaj et al. 2008; Choudhuri et al. 2007; Jha et al. 2013; Kashuba et al. 2008; Knight and Robertson 2004; Knight et al. 2004, 2005a, b; Saha et al. 2009, 2011, 2012; Yi et al. 2009). It remains to be established, by reverse genetic studies of mutants that specifically and independently disrupt these binding sites, which of these interactions are functionally important in vitro during B cell transformation or in vivo during the establishment of persistence and/or in B cell lymphomagenesis.

EBNA3A was also shown to cooperate with Ha-Ras in the transformation and immortalisation of REFs, and again, there was a remarkably good correlation between EBNA3A binding to CtBP and rescue from Ha-Ras-induced senescence (Hickabottom et al. 2002). The earliest evidence that EBNA3A might affect cell cycle regulation in B cells came from two related studies in which EBNA3A was either over-expressed (by twofold–fivefold) in an LCL from an inducible plasmid (Cooper et al. 2003) or—as with EBNA3C—using a recombinant virus conditional for EBNA3A to establish an LCL (Maruo et al. 2003). The take-home message from these experiments was that a precisely controlled level of EBNA3A was critical for optimum LCL proliferation. An excess of EBNA3A appeared to produce the down-regulation of MYC and cyclin D2 leading to a prolonged G0/G1 cell cycle arrest, but when EBNA3A was inactivated in the conditional LCL, the outcome was a gradual decline in proliferation; however, the underlying molecular mechanisms were not identified (Cooper et al. 2003; Maruo et al. 2003). Also, consistent with EBNA3A having a role in cell cycle regulation, was the recent report of an interaction between EBNA3A and MYC-interacting zinc-finger protein-1 (MIZ1) being necessary for the down-regulation of the cyclin-dependent kinase inhibitor $p15^{INK4b}$ in LCLs. Because $p15^{INK4b}$ has the potential to inhibit cell cycle progression, this could contribute to the maintenance of EBV-infected B cell proliferation. However, currently—since the role of $p15^{INK4b}$ in human B cells is unknown—the biological significance in EBV biology is still subject to speculation (Bazot et al. 2014; also see Sect. 4.2 for further discussion).

2.7 Proteins that Can Interact with the EBNA3C

EBNA3C is by far the most studied member of the EBNA3 family and has been reported to interact with many cellular proteins. In most cases, this refers to the

ability of a protein to co-immunoprecipitate with EBNA3C from EBV-infected B cells (or after co-transfection) using standard immunoprecipitation lysis buffer extraction and/or the ability to associate with EBNA3C in pull-down assays using polypeptides fused to glutathione-s-transferase (GST). To our knowledge, there is no case of a direct biochemical interaction being confirmed using proteins purified to homogeneity. Some proteins reported to associate with EBNA3C by one or both of the above criteria are listed in Table 1; however, due to constraints on the length of the review, only the interactions between EBNA3C and RBP-JK/CBF1 and CtBP will be considered in more detail. This is because (a) these two proteins associate with more than one EBNA3 (RBP-JK/CBF1 with all three; CtBP with EBNA3A and EBNA3C), (b) these are the only interactions for which essential EBNA3C amino acids have been precisely identified and—most importantly—(c) have binding mutants been analysed by reverse genetics or complementation to show a change in phenotype in EBV-infected B cells. It is perhaps no coincidence that these two proteins have also consistently been detected as 'interactors' in various EBNA3 yeast two-hybrid screens (e.g. Bazot et al. 2014; Lin et al. 2002; our unpublished results). It should be noted that EBNA3C can be co-immunoprecipitated with both EBNA3A and EBNA3B (Paschos et al. 2012; our unpublished results); however—although there is good evidence for functional crosstalk between these proteins (see Sects. 3.2 and 4)—the critical domains/residues have not yet been mapped; therefore, genetic analyses to test the significance of these potential biochemical associations have not yet been possible.

2.7.1 RBP-JK/CBF1

As indicated previously, EBNA2 and all three EBNA3 proteins can bind the conserved DNA-binding factor RBP-JK/CBF1, which is also an effector component of the Notch signalling pathway and is equivalent to *Drosophila melanogaster* Suppressor of Hairless, Su(H). In *Drosophila*, Notch pathways regulate cell fate determination, cell differentiation and developmental pattern formation. The pathways are highly conserved and are thought to play similar roles in human cells (Guruharsha et al. 2012; Zimber-Strobl and Strobl 2001). Once liberated from cellular membranes by proteolytic cleavage, the cleaved domains of Notch (Notch-IC, for intracellular) can bind to Su(H) to activate gene transcription. This process can be antagonised in *Drosophila* by competitive binding of Su(H). Hairless protein then itself recruits conserved co-repressors Groucho and dCtBP (Barolo et al. 2002; Morel et al. 2001). In EBV-transformed B cells, EBNA2 can mimic aspects of activated Notch signalling by binding—like Notch-IC—to RBP-JK/CBF1, recruiting co-activators and enhancing transcription of both viral and cellular genes that include RBP-JK/CBF1-binding sequences. In *Drosophila,* Notch signalling is antagonised by Hairless, so it has been proposed that EBNA3A, EBNA3B and EBNA3C could function in a similar manner to Hairless and repress activation of transcription mediated by EBNA2 bound to RBP-JK/CBF1 (Thorley-Lawson and Allday 2008; Zimber-Strobl and

Table 1 Factors reported to interact with EBNA3C

Interacting cellular proteins[a]	Proposed outcome	Genetic confirmation using recombinant EBV in B cells	References
RBP-JK (CBF1)	Directing EBNA3C to a subset of target genes	Yes	Robertson et al. (1996); Maruo et al. (2009); Lee et al. (2009); Calderwood et al. (2011); Harth-Hertle et al. (2013)
SPI1/PU1	Directing EBNA3C to a subset of target genes	Indirect evidence	Zhao and Sample (2000); Jiang et al. (2014); McClellan et al. (2013)
CtBP	Repression of some target genes (e.g. $p16^{INK4a}$)	Yes	Touitou et al. (2001); Skalska et al. (2010)
HDACs 1/2	Repression of target genes	Indirect evidence	Radkov et al. (1999); Knight et al. (2003); Paschos et al. (2009)
Sin3A	Repression of target genes	Indirect evidence	Knight et al. (2003); Jiang et al. (2014)
NCoR	Repression of target genes	No	Knight et al. (2003)
SUMO-1/3	Sumoylation of EBNA3C?	No	Rosendorff et al. (2004); Touitou et al. (2005)
Cyclin A	Increased cdk activity	No	Knight et al. (2004); Knight and Robertson (2004)
Cyclin D1	Cyclin D1 stabilised[b]	No	Saha et al. (2011)
SCF^{SKP2}	Recruited to RB	No	Knight et al. (2005b)
RB	RB degraded and release of E2F1	No	Knight et al. (2005a)
MYC	MYC stabilised	No	Bajaj et al. (2008)
p300	Recruited in a transcription complex	No	Cotter and Robertson (2000)
Prothymosin-α	Recruited in a transcription complex	No	Cotter and Robertson (2000)
MRS18-2	Releases E2F1 from RB	No	Kashuba et al. (2008)
NM23-H1	Recruited to nucleus	No	Subramanian et al. (2001)
MDM2	MDM2 stabilised	No	Saha et al. (2009)
ING4/5	Blocks interaction of ING4/5 with p53	No	Saha et al. (2011)
p53	Inhibits p53-mediated transcription	No	Yi et al. (2009)

(continued)

Table 1 (continued)

Interacting cellular proteins[a]	Proposed outcome	Genetic confirmation using recombinant EBV in B cells	References
CHK2	Blocks CHK2 kinase activity, might restrict DDR	Indirect evidence	Choudhuri et al. (2007) Nikitin et al. (2010)
GADD34	Counteracts unfolded protein response	No	Garrido et al. (2009)
DP103 (Gemin3)	DP103 degraded	No	Cai et al. (2011)
Aurora Kinase B	Aurora B stabilised	No	Jha et al. (2013)
E2F1	Inhibits E2F1 binding to DNA	No	Saha et al. (2012)
IRF4	IRF4 stabilised, perhaps recruits EBNA3C to DNA	Indirect evidence	Banerjee et al. (2013); Jiang et al. (2014); McClellan et al. (2013)
H2AX	Relocalisation of H2AX, might restrict DDR	Indirect evidence	Jha et al. (2014), Nikitin et al. (2010)
EBNA3A	Collaboration with EBNA3C in gene regulation	Indirect evidence	Calderwood et al. (2007); Anderton et al. (2008); Skalska et al. (2010); White et al. (2010); Paschos et al. (2012); McClellan et al. (2013)
EBNA3B	Collaboration with or antagonism of EBNA3C in gene regulation	Indirect evidence	White et al. (2010); White et al. (2012); McClellan et al. (2013); our unpublished data

[a]In some cases, this is probably direct, but for others, the interaction might involve a multi-protein complex—none of these interactions has been confirmed with purified proteins
[b]It should be noted that Cyclin D1 is not generally expressed in EBV-transformed B cells Palmero et al. (1993) and Pokrovskaja et al. (1996)

Strobl 2001). Furthermore, since EBNA3A and EBNA3C can recruit mammalian CtBP (discussed in the next section), EBV might mimic several components of the Notch effector pathway to precisely control the expression of host genes in an analogous manner to Notch in *Drosophila.* However, despite the functional similarities between Notch-IC/Hairless and EBNA2/3s, it has been shown that EBNA2 and Notch-IC are not interchangeable in regulating human genes (Kohlhof et al. 2009)—this is discussed further in the chapter authored by Kempkes and Ling.

Although there is not complete agreement on the precise details, there is an emerging consensus that the most important region of each EBNA3 in the interaction with RBP-JK/CBF1 is located in the middle of the homology domain

(central homology domain) between amino acid 170-221 for EBNA3A, 176-227 for EBNA3B and 180-231 for EBNA3C (Fig. 2; Bourillot et al. 1998; Calderwood et al. 2011; Dalbies-Tran et al. 2001; Lee et al. 2009; Maruo et al. 2005, 2009; Robertson et al. 1996). EBNA3A and EBNA3C mutants deleted for those regions no longer interact with RBP-JK/CBF1 and fail to repress EBNA2-mediated transcriptional activation of reporter genes (Maruo et al. 2005, 2009). It should be noted, however, that mutations near, but not within, the central homology region might also affect the interaction between the EBNA3s and RBP-JK/CBF1 (Maruo et al. 2005; West et al. 2004). Sequence alignment of the EBNA3 central homology regions identified three amino acid clusters conserved between the EBNA3s (Fig. 2). A refined analysis of RBP-JK/CBF1 binding to EBNA3C performed by Calderwood and colleagues revealed that residues 211–233 of EBNA3C include an amino acid sequence (WTP) that resembles the WΦP motif of Notch-IC (WFP) and EBNA2 (WWP) that interacts with RBP-JK/CBF1 (Calderwood et al. 2011). They showed using EBNA3C mutated at ATFGC and/or WΦP that both regions are involved in the interaction with RBP-JK/CBF1 (Fig. 2).

An important observation that linked EBNA3:RBP-JK/CBF1 complexes to EBV biology was the demonstration that the interaction between RBP-JK/CBF1 and EBNA3A or EBNA3C is not only necessary for the regulation of EBNA2-mediated activation in reporter assays, but also essential for maintaining LCL proliferation. The ectopic expression of wild-type EBNA3A can maintain proliferation in an LCL conditional for EBNA3A when it is cultured in non-permissive conditions, whereas an EBNA3A mutant deleted for RBP-JK/CBF1 binding cannot (Maruo et al. 2005). In comparable complementation experiments using an EBNA3C-conditional LCL, the results were similar: no interaction with RBP-JK/CBF1 and no rescue of proliferation (Lee et al. 2009; Maruo et al. 2009). So it appears that EBNA3A and EBNA3C mutants that are deficient in their capacity to repress EBNA2-mediated activation of reporters are also unable to sustain LCL proliferation. However, it is not known whether these two functions are directly linked or precisely how EBNA3A and EBNA3C (and possibly EBNA3B) antagonise gene activation by EBNA2. Two mechanisms have been proposed, but neither confirmed: either the EBNA3s might destabilise the interaction of EBNA2:RBP-JK/CBF1 complexes with DNA (Robertson et al. 1995, 1996; Waltzer et al. 1996) or alternatively the EBNA3s could replace EBNA2 on DNA-bound RBP-JK/CBF1 and recruit co-repressors (see next section and Sect. 4). It is possible that both of these mechanisms could operate, but perhaps at different targets. These issues will only be resolved when the details of molecular complexes at the regulatory elements of fully validated target genes have been determined. Remarkably, it appears that only a few (about 16 %) of the many thousands of sites across the human genome that bind EBNA3s have been shown to coincide with reported RBP-JK/CBF1 sites. However, many coincide with sites where EBNA2 can bind (Jiang et al. 2014; McClellan et al. 2013; see Sect. 4 for more detailed discussion).

2.7.2 CtBP

EBNA3A and EBNA3C, but not EBNA3B, interact with the co-repressor CtBP (Hickabottom et al. 2002; Touitou et al. 2001), a protein initially discovered as a cellular factor interacting with the C-terminal region of the adenovirus E1A oncoprotein and subsequently identified as one of a highly conserved family of co-repressors of transcription (reviewed in Chinnadurai 2007). CtBP is now a generic term used to refer to two proteins, CtBP1 and CtBP2. These closely related regulators of transcription are encoded in vertebrates by separate genes (located on human chromosomes 4p16.3 and 10q23.13, respectively). While CtBP1 and CtBP2 share significant amino acid homology overall, CtBP2 includes a unique N-terminal domain that probably contributes to its nuclear localisation (Chinnadurai 2007; Zhao et al. 2014). It remains largely unexplored to what extent these proteins are functionally redundant, but there is evidence that at some developmental stages, they might play unique roles (Chinnadurai 2007). Both appear to act primarily as transcriptional co-repressors in the process of gene silencing and both interact with factors that have the conserved, prototypical CtBP-binding Pro-Leu-Asp-Leu-Ser ('PLDLS') motif. Proteomic analysis of mammalian CtBP1 and CtBP2 complexes has shown each can interact with a similar array of transcriptional co-repressors including HDAC-1 and HDAC-2, CoREST, G9a/GLP and LSD1; they have also been implicated in polycomb group (PcG) protein-mediated repression (Sewalt et al. 1999; Shi et al. 2003, 2004; Sundqvist et al. 1998; Zhao et al. 2014). Genetic evidence indicates that CtBP2 might also activate transcription in a context-dependent manner—this could account for some of its unique properties (Chinnadurai 2007). Since both CtBP1 and CtBP2 bind NAD+ and NADH, it has been proposed that their transcriptional regulatory capacity is modulated by the ratio of nuclear NAD+:NADH and that they play a role in monitoring the redox status of a cell (Zhang et al. 2006).

Touitou and colleagues identified a perfect PLDLS motif in the C-terminal region of EBNA3C and demonstrated that this motif (aa 728-732) was essential and sufficient for EBNA3C to interact with CtBP1 (Touitou et al. 2001). Subsequently, EBNA3A was shown to interact with CtBP1 through two non-consensus motifs, ALDLS (aa 857-861) and VLDLS (aas 891-895), also located at the carboxyl terminus. In vitro studies suggested that EBNA3A binds with a higher affinity than EBNA3C—perhaps because it includes the bipartite site—but this has not been confirmed (Hickabottom et al. 2002). Because EBNA3C and EBNA3A interact with CtBP1 via PLDLS (or a variant), it has been assumed that they can interact with both CtBP proteins, but—to our knowledge—this has not been formally tested. Although both CtBPs could be present simultaneously in human B cells, most available data suggest that CtBP2 is not expressed in EBV-infected B cells and this silencing is probably related to the expression of EBNA3A (Hertle et al. 2009; McClellan et al. 2012; White et al. 2010) (see Sect. 4.5 for a more detailed discussion).

The ability of EBNA3C to bind CtBP correlates only partially with the ability of EBNA3C to repress transcription when targeted to DNA in transient reporter

assays, but correlates extremely well with EBNA3C's ability to behave as a cooperating nuclear oncoprotein when expressed in primary REFs with oncogenic Ha-ras (Touitou et al. 2001). Binding of CtBP to EBNA3A correlates well with both reporter repression and oncogenic cooperation (Hickabottom et al. 2002). Furthermore, since Marek's disease virus—a herpesvirus that induces T cell lymphoma in poultry—requires its nuclear oncoprotein MEQ to bind chicken CtBP for tumorigenesis (Brown et al. 2006), it seems likely that the interactions with EBNA3A and EBNA3C might be involved in human B cell transformation and lymphomagenesis. The importance of these EBNA3:CtBP interactions in B cell transformation became apparent when analysis of $p16^{INK4a}$ expression in LCLs established by using each of three different CtBP-binding mutant viruses ($3A^{CtBP}$; $3C^{CtBP}$ and a 3A/3C double CtBP-binding mutant called $E3^{CtBP}$) showed that CtBP is involved in the EBNA3A/3C-mediated epigenetic repression of the $p16^{INK4a}$-encoding gene locus (Skalska et al. 2010 and discussed in more detail in Sect. 4.2). Taken together, the data suggest that binding of EBNA3A and EBNA3C to CtBP augments transformation efficiency and LCL outgrowth at least in part by aiding the establishment or maintenance of repressive chromatin around the $p16^{INK4a}$ transcription start site (TSS). Both EBNA3A and EBNA3C can be readily immunoprecipitated from LCLs with CtBP (Hickabottom et al. 2002; Touitou et al. 2001); however, no EBNA3A/3C-CtBP complexes have been demonstrated on the $p16^{INK4a}$ promoter (Skalska et al. 2010). It is unclear whether this is because of technical limitations of the available reagents or whether the promoter is not actually a direct target of such complexes. Although we do not yet understand this requirement for CtBP binding, the data are consistent with reports that the C-terminus of EBNA3C—and specifically the PLDLS CtBP-binding site—is necessary to completely rescue proliferation in EBNA3C-conditional LCLs cultured without the appropriate activator (Lee et al. 2009; Maruo et al. 2009).

3 New Technologies and the Analysis of EBNA3 Function

3.1 BAC-Derived EBV Recombinants Facilitate Robust Genetic Analysis

The earliest strategy for the genetic analysis of EBV functions entailed recombination between the non-transforming P3HR1 strain, which lacks EBNA2, and fragments of B95-8 virus. This system relied on selecting recombinants that had incorporated B95-8 EBNA2 that enabled the virus to transform B cells. Inclusion of second site targeting constructs induced a remarkably high proportion (around 10–15 %) of selected virus clones that also incorporated this second change, thereby allowing the modification of other EBV genes under selection of EBNA2 inclusion. Using this strategy, it was found that incorporating a type 1 region including the EBNA3s in place of the type 2 EBNA3s of P3HR1 had no significant effect on the virus's ability to transform B cells (Tomkinson and Kieff 1992b). Parallel

attempts to generate EBNA3 knockouts—by incorporating stop codons into each EBNA3 independently within the type 1 EBNA3 cassette—found that EBNA3B knockouts could be generated with the same frequency as incorporation of the intact type 1 cassette (Tomkinson and Kieff 1992a). In contrast, recombinants containing either EBNA3A- or EBNA3C-knockout DNA grew out in <2 % of transformants, and then only where cells were co-infected with the non-transforming P3HR1 virus. In addition, these dually infected cells were prone to spontaneous reversion of the mutation through recombination with the complementing P3HR1 virus (Tomkinson et al. 1993). These data were interpreted to mean that EBNA3A and EBNA3C are essential for B cell transformation, whereas EBNA3B is dispensable.

The requirement for transformation- and replication-competent recombinant viruses, and the technical challenges of the first-generation recombination system, limited its use as a tool for the genetic analysis of EBV. However, cloning the genome of the B95-8 strain of EBV into an F factor-derived plasmid (Delecluse et al. 1998)—more commonly known as a bacterial artificial chromosome (BAC)—facilitated the generation and characterisation of EBV recombinants that lacked the ability to transform B cells (reviewed in detail in Feederle et al. 2010 and the chapter authored by H-J. Delecluse). Subsequently, the Akata strain of EBV was also cloned as a BAC, and both strains have now been used to analyse the functions of the EBNA3s. Two main modification strategies have been used to reveal functional contributions of the EBNA3s to the biology of EBV in B cells: first, introducing truncating mutations into the EBNA3s to create EBNA3 knockouts (KO) and second, creating proteins conditional for function by fusing EBNA3s to a modified oestrogen receptor (ER) in the viral genome. The resulting fusion protein is then functional only in the presence of oestrogen or its related analogue, 4-hydroxytamoxifen (4HT) that prevents the sequestration and degradation of the protein that would otherwise be mediated by the inactive ER (Maruo et al. 2006; Skalska et al. 2010). More recently, fusions of EBNA3s with epitope tags have proved to be very useful in locating sites to which individual EBNA3s are targeted on host chromatin using chromatin immunoprecipitation (ChIP) strategies (Jiang et al. 2014; Paschos et al. 2012; see below).

In the past, we and others have attempted to determine the roles of the EBNA3s in gene regulation and EBV biology by expressing single EBNA3 genes or cDNAs transiently, constitutively or from inducible vectors in B and non-B cells (for example Gruhne et al. 2009; Knight et al. 2005a; Parker et al. 2000; Yi et al. 2009; Young et al. 2008). However, while this approach provided some clues to EBNA3 functions, it was sometimes unhelpful and could potentially produce misleading data because the protein is generally over-expressed, often not in B cells and always in the absence of other EBV latency factors—that is out of its physiological context. Constructing recombinant viruses with mutations or deletions of the EBNA3 locus using EBV-BAC technology and using the resultant viruses to infect B cells has allowed the investigation of host phenotypes and transcriptomes associated with each EBNA3, in the context of latency-associated EBV gene expression in B cells. This strategy has already produced many surprising results and provided a wealth of information for future research.

3.2 Microarrays Reveal the Extent and Complexity of EBNA3-Mediated Regulation of Host Genes

A second strategic advance in the study of EBNA3 function was the determination of genome-wide gene expression patterns using microarray technology in conjunction with the knockout, conditional or mutant EBNA3 viruses. To date, around 100 or more independent B cell lines have been analysed to produce unbiased views of how the host transcriptome is modified by EBNA3 expression (Chen et al. 2006; Hertle et al. 2009; Kelly et al. 2013; McClellan et al. 2012; Skalska et al. 2010; White et al. 2010; Zhao et al. 2011). Several of these data sets (derived from BL lines and LCLs in our and the Kempkes' laboratories) describing the expression of genes that are regulated by EBV in an EBNA3-dependent manner are available in a searchable format at the Website http://www.epstein-barrvirus.org.uk. Together, these analyses have revealed the extent and complexity of EBNA3-mediated host gene control. It is now well established that the regulation of well over a 1000 host genes by EBV requires EBNA3 expression, and a considerable number of these genes appear to be regulated by combinations of EBNA3s. Confirming this original observation, many genomic loci are bound by multiple EBNA3s, and also EBNA2, but surprisingly, relatively few of these locations are also bound by RBP-JK/CBF1 (see Sect. 3.3 for further discussion).

Using the EBV-BAC system and microarrays to analyse the regulation of host genes by EBNA3s has highlighted a number of underlying principles that should be considered when interpreting the gene expression analyses undertaken to explore the function of EBV proteins. First, using the same recombinant viruses in different cell backgrounds can give very different results. For instance, only a quarter of the genes identified as regulated by EBNA3B in LCLs were also EBNA3B regulated in the Burkitt's lymphoma cell line BL31 (White et al. 2010). There are many possible reasons for such differences. They may be due to the different differentiation states of the cell or changes associated with the mutations or other transformation events that produced the BL31 cell line. Since these analyses have been carried out on cell lines that have been established with mutant viruses, selection pressures during outgrowth can also distort the transcriptome. For instance, a loss/reduction of retinoblastoma protein (RB) expression appears to assist the outgrowth of EBNA3A-KO and CtBP-binding mutant LCLs (Hertle et al. 2009; Skalska et al. 2010). Also, differences in regulation of target genes due to host genetic background have also been observed. We, and others, have found that the regulation of CXCR4 by EBNA3B occurs only in a subset of donors (Chen et al. 2006; White et al. 2010). These issues are discussed in greater depth in the relevant reports, including Hertle et al. 2009; Kelly et al. 2013; McClellan et al. 2012; Skalska et al. 2010, 2013; White et al. 2010; and Zhao et al. 2011. Nevertheless, using microarray data as an indicator of potential EBNA3 targets—that are then rigorously validated—has proven to be remarkably fruitful, and existing data sets probably still have more to offer. Examples of some host genes that reveal different features of EBNA3-mediated gene regulation and provide novel insights into viral regulation of host transcription are considered in detail in Sect. 4 and are summarised in Table 2.

Table 2 Host genes regulated by the EBNA3 family proteins

Genes reported to be regulated by EBNA3s	EBNA3 binding to chromatin: ChIP ChIPseq	Genetic confirmation: using recombinant EBV in B cells -KO—conditional	Comments	References
Alpha-V integrin—repressed by EBNA3C	N/R N/R	− −	• Regulation involves NM23-H1, GATA1 and SPI1	Choudhuri et al. (2006)
COX-2—activated by EBNA3C	N/R N/R	− −	• Regulation involves NM23-H1, GATA1 and SPI1	Kaul et al. (2006)
BIM (BCL2L11)—repressed by EBNA3A and EBNA3C	+ + (proximal to TSS)	+ +	• Co-regulation by EBNA3A and EBNA3C • Involves PcG complexes and H3K27me3 repressive chromatin mark • Might involve inhibition of Pol II-PO_4	Anderton et al. (2008); Paschos et al. (2009, 2012); White et al. (2010); McClellan et al. (2013); Jiang et al. (2014)
TCL-1—activated by EBNA3C plus EBNA3B	N/R N/R	+ +	• Both EBNA3B and EBNA3C appear to be required for activation in LCLs • Probably involves RBP-JK/CBF1	Chen et al. (2006); Lee et al. (2009); White et al. (2010, 2012)
$p16^{INK4a}$ (CDKN2A)—repressed by EBNA3C (plus EBNA3A)	+ + (proximal to TSS)	+ +	• Co-regulation by EBNA3C and EBNA3A • Involves polycomb protein complexes and H3K27me3 repressive chromatin mark • Involves CtBP and might involve BATF/IRF4 or SPI1/IRF4 binding sites proximal to $p14^{ARF}$ TSS • Whole $p15^{INK4b}$, $p14^{ARF}$, $p16^{INK4a}$ locus regulated coordinately?	Maruo et al. (2006); Hertle et al. (2009); Skalska et al. (2010, 2013); Zhao et al. (2011); Maruo et al. (2011); Jiang et al. (2014)

(continued)

Table 2 (continued)

Genes reported to be regulated by EBNA3s	EBNA3 binding to chromatin: ChIP	ChIPseq	Genetic confirmation: using recombinant EBV in B cells -KO—conditional		Comments	References
p15^{INK4b} (*CDKN2B*)—repressed by EBNA3A	N/R	N/R	+	+	• Involves interaction between EBNA3A and the transcription factor MIZ1 • Probably co-regulated by EBNA3A and EBNA3C • Involves PcG complexes and H3K27me3 • Whole p15^{INK4b}, p14^{ARF}, p16^{INK4a} locus regulated coordinately?	Bazot et al. (2014); our unpublished results
BUBR1—repressed by EBNA3C	N/R	N/R	−	−		Gruhne et al. (2009)
p73—repressed by EBNA3C	N/R	N/R	−	−		Saha et al. (2012)
ADAM28—repressed by EBNA3A and EBNA3C	+ (distal, intergenic)	+	+	+	• EBNA3 binding between *ADAM28* and *ADAMDEC1*—locus co-regulated by chromatin looping • H3K27me3 involved	Hertle et al. (2009); McClellan et al. (2012, 2013)
ADAMDEC1—repressed by EBNA3A and EBNA3C	+ (distal, intergenic)	+	+	+	• EBNA3 binding between *ADAM28* and *ADAMDEC1*—locus co-regulated by chromatin looping • H3K27me3 involved	Hertle et al. (2009); McClellan et al. (2012, 2013); Skalska et al. (2013)
IRF8—repressed by EBNA3C	N/R	N/R	−	−	• EBNA3C reported to also bind IRF8 protein	Banerjee et al. (2013)

(continued)

Table 2 (continued)

Genes reported to be regulated by EBNA3s	EBNA3 binding to chromatin: ChIP ChIPseq	Genetic confirmation: using recombinant EBV in B cells -KO—conditional	Comments	References
CtBP2—repressed by EBNA3A	+ + (intragenic enhancer)	+ −	• EBNA3A binding on intragenic enhancer and chromatin looping involved • Co-regulation with EBNAs2, 3A and 3B that might bind the same site? • Confirmed in EBNA3AKO LCL, but not EBNA3C-conditional LCL or EBNA3BKO LCL exon microarrays	Hertle et al. (2009); Skalska et al. (2013); McClellan et al. (2013)
CXCL9/CXCL10—repressed by EBNA3A	+ + (intergenic enhancer)	+ +	• *CXCL9* and *CXCL10* locus co-regulated • EBNA3 binding on distal intergenic enhancer and RBP-JK/CBF1 involved • Leads to H3K27me3 mark across locus • Co-repression with EBNA3C • EBNA2 activates, EBNA3B potentiates? • Chromatin looping probably involved	Hertle et al. (2009); White et al. (2010, 2012); McClellan et al. (2012); Skalska et al. (2013); Harth-Hertle et al. (2013); our unpublished results
Aurora kinase B—repressed by EBNA3C	+ N/R (proximal to TSS?)	− −	• EBNA3C reported to also bind Aurora kinase B protein	Jha et al. (2013)
H2AX—repressed by EBNA3C	N/R N/R	− −	• EBNA3C reported to also bind H2AX protein	Jha et al. (2014)

N/R—Not reported

3.3 ChIP and ChIP-Seq Analyses and Mechanisms of Gene Regulation

The biological significance of interactions of nuclear proteins, such as the EBNA3s, with specific DNA fragments of defined sequence has been immensely enhanced by the advent of chromatin immunoprecipitation (ChIP) techniques. ChIP is a method by which proteins are selectively immunoprecipitated and the associated DNA sequences are determined—usually by methods involving quantitative PCR (QPCR) and/or high-throughput sequencing (ChIP-seq). This technique has been used to map the binding of transcription factors, co-factors and chromatin-modifying enzymes at particular loci and across the genome. It has also proven to be a very powerful tool for identifying the distribution of post-translationally modified histones and their spatial and functional relationship to individual genes.

Coupled with the use of EBV recombinants and transcriptome data, ChIP and ChIP-seq technologies have revolutionised the way we consider EBNA3 function. It has been possible to identify many thousands of specific genomic loci where the EBNA3s can be detected and, we assume, recruit cellular factors that exert effects on chromatin organisation. This has heralded some paradigm-shifting observations, including the EBNA3 manipulation of histone-modifying complexes to reprogram the epigenetic landscape and the modulation by EBNA3s of the three-dimensional architecture of chromatin during the regulation of gene expression (Harth-Hertle et al. 2013; Jiang et al. 2014; McClellan et al. 2012, 2013; Paschos et al. 2012; Skalska et al. 2013). Some of these studies will be considered in the next section.

4 Host Gene Regulation by the EBNA3s Involves Polycomb Proteins, Epigenetic Modifications and Chromatin Looping

Five host genes (or gene clusters) whose regulation by EBNA3s is particularly well characterised are described here in detail. Each reveals novel aspects of EBNA3 cooperation and/or mode of action in the regulation of transcription, and in some cases, how the identification of targets has provided remarkable insights into aspects of EBV biology and the virus's oncogenic potential.

4.1 BIM (BCL2L11)

The first evidence that EBNA3A and EBNA3C can cooperate to regulate specific host cell genes came using a panel of EBNA3-knockout recombinant

B95.8-derived EBVs to infect EBV-negative BL31 BL-derived cells. This revealed, among other things, that expression of both EBNA3A and EBNA3C is necessary to repress transcription of *BIM/BCL2L11* (Anderton et al. 2008).

BIM (Bcl2-interacting mediator) is a pro-apoptotic member of the BH3-only family of BCL2-like proteins and is encoded by the *BCL2L11* gene on human chromosome 2q13. BIM acts as a potent, direct initiator of apoptosis because it binds with high affinity to BCL2 and all the other pro-survival family members to inactivate them. BIM is particularly important in the immune system, acting as a major regulator of life-and-death decisions during lymphocyte development (Fischer et al. 2007; Strasser 2005). *Bim*-null mice accumulate excess lymphoid and myeloid cells, and loss of *Bim* accelerates B cell lymphomagenesis induced by an *Eμ-Myc* transgene in mice (Egle et al. 2004). The connection between deregulated *MYC* expression and the activity of BIM in human B cells was revealed by Hemann and colleagues (Hemann et al. 2005). They showed that MYC activates *BIM/BCL2L11* in EBV-negative BL, and this led to the proposal that MYC-induced apoptosis can be overridden by inactivation of any one of several MYC effectors—including p53, $p14^{ARF}$ or BIM (reviewed in Dang et al. 2005). MYC is induced and becomes constitutively expressed early after the infection of primary human B cells with EBV (Fig. 4 and Sect. 5); therefore, suppression of BIM expression by EBV is likely to be a contributory factor in B cell transformation and the development of any EBV-associated B cell lymphomas.

In cultured B cells, a reduction in BIM expression occurs very soon after infection with EBV (Anderton et al. 2008; Skalska et al. 2013). This did not involve detectable CpG methylation, but correlated with loss of histone acetylation and the deposition of the polycomb group (PcG) protein signature H3K27me3 on chromatin proximal to the transcription start site (TSS) for BIM (Paschos et al. 2009, 2012). Detailed chromatin immunoprecipitation (ChIP) analyses of the chromatin around the *BIM/BCL2L11* promoter revealed that latent EBV triggers the recruitment of polycomb repressive complex (PRC)2 core subunits and the trimethylation of histone H3 lysine 27 (H3K27me3) at this locus and represses transcription. In uninfected BL cells, PRC2 ancillary factors RbAp48 and JARID2 already associate with the chromatin proximal to the TSS, so the data suggest that at the *BIM/BCL2L11* locus, EBV infection is necessary to recruit core components SUZ12 and EZH2 (the histone methyl transferase) to establish functional PRC2. Assembly of PRC2 at the locus was absolutely dependent on both EBNA3A and EBNA3C being expressed, and, using a recombinant EBV expressing an epitope-tagged EBNA3C, ChIP showed that EBNA3C associates with chromatin near the TSS (Paschos et al. 2012). Subsequently, a broad EBNA3C-binding peak around the TSS was confirmed by ChIP-seq analyses (Jiang et al. 2014; McClellan et al. 2013; our unpublished results; Fig. 3a). It is therefore possible that EBNA3C (and/or EBNA3A) physically interacts with PRC2 and the transcription preinitiation complex, but this has not been formally demonstrated.

Since the activation mark H3K4me3 is largely unaltered at this locus irrespective of H3K27me3 or EBNA3 status, this indicates the establishment of a 'bivalent' chromatin domain. When the repressive histone modification H3K27me3 and

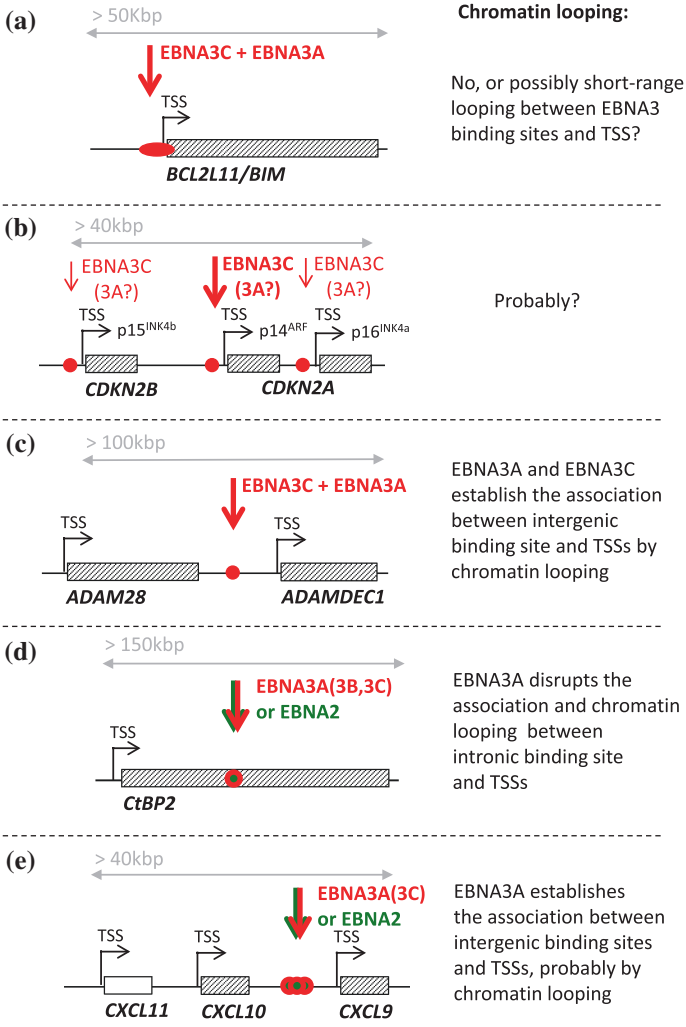

Fig. 3 Examples of EBNA3 repressed gene loci. Schematic representations of several gene loci to which EBNA3 binding has been mapped by ChIP-seq and ChIP-QPCR (not drawn accurately to scale). In each case, the result of binding is repression of transcription—for details see text. **a** Repression of *BIM/BCL2L11* results from EBNA3A/EBNA3C binding proximal to the TSS. **b** Repression of $p15^{INK4b}$ (*CDKN2B*), $p14^{ARF}$ and $p16^{INK4a}$ (*CDKN2A*) probably involves a yet to be determined pattern of chromatin looping between the three TSSs, initiated by EBNA3C binding. **c** The *ADAM28/ADAMDEC1* locus is repressed by EBNA3A/EBNA3C binding to an intergenic site between the two genes and initiating chromatin looping to the TSSs. **d** At *CtBP2*, EBNA3A binding to an intragenic site (an intronic enhancer) is thought to displace bound transactivator EBNA2 and disrupt chromatin looping between the enhancer and TSS, resulting in repression of transcription. **e** At the *CXCL9/10* locus, binding of EBNA3A to three intergenic enhancers is thought to displace the transactivator EBNA2 and initiate repression of transcription; this probably involves chromatin looping, but it has not been formally demonstrated

the permissive modification H3K4me3 occur at the same promoter, the gene is said to be in a bivalent state that can be either activated or silenced. This is associated with low-level transcription poised to increase or decrease (Bernstein et al. 2006; Stock et al. 2007). Consistent with the 'poised' nature of such domains, RNA polymerase II (Pol II) occupancy at the *BIM/BCL2L11* TSS was not diminished by EBV. However, analysis of phosphorylation of serine 5 on Pol II indicated that when EBNA3A and EBNA3C are both expressed, this phosphorylation step is inhibited and *BIM* transcripts are not initiated. It was not determined whether this involves the direct action of an EBV protein on the kinase CDK7 or is a consequence of the recruitment of PRC2 and/or PRC1 (the second PcG complex) to this particular locus. Conditional expression of EBNA3C revealed that this epigenetic repression of BIM expression was reversible, but took more than 30 days from when EBNA3C was inactivated to fully return to its active state, emphasising the stability of these chromatin modifications through rounds of cell division. Lentiviral delivery of shRNAs against polycomb complex subunits disrupted EBV repression of *BIM/BCL2L11,* thus confirming the requirement for the PcG system in maintaining the repression of BIM by EBV (Paschos et al. 2012). The mechanisms by which the PcG complexes are recruited to this locus are still unknown and the factor(s) responsible for the targeting of EBNA3C and/or EBNA3A to this specific site are also unidentified. The biological significance of *BIM/BCL2L11* repression is considered in more detail in Sect. 5.

4.2 P16^{INK4a} and the INK4a-ARF-INK4b Locus

Maruo and colleagues, using a recombinant Akata EBV encoding a conditional EBNA3C fused to a modified oestrogen receptor, revealed that functional EBNA3C is essential to repress expression of the CDK inhibitor p16^{INK4a}—but not the CIP/KIP family inhibitors p21^{WAF1} or p27^{KIP1}—in LCLs. Within the *INK4a-ARF-INK4b* locus at human chromosome 9p21, *CDKN2A* encodes two potent tumour suppressors, p16^{INK4a} and p14^{ARF} (p19^{ARF} in mice); these proteins are crucial negative regulators of cell proliferation (Fig. 3b). Although exons 2 and 3 of *CDKN2A* are shared by *INK4a* and *ARF*, the proteins result from differential splicing but have no amino acid similarity because they are encoded in alternative reading frames (reviewed in Gil and Peters 2006; Sherr 2012). Adjacent to *CDKN2A* is the gene *CDKN2B* that encodes a protein closely related to p16^{INK4a} called p15^{INK4b}. The cyclin-dependent kinase (CDK) inhibitor p16^{INK4a} acts on the cyclin D-dependent kinases (CDK4 and CDK6) abrogating their binding to D-type cyclins and so inhibiting CDK4/6-mediated phosphorylation of the retinoblastoma protein (RB). By binding CDKs and blocking RB hyperphosphorylation, increased p16^{INK4a} expression causes a G1 cell cycle arrest and senescence (Gil and Peters 2006; Sherr 2012). The CDK inhibitor p15^{INK4b} has about 85 % amino acid similarity to p16^{INK4a} and biochemically behaves in much the same way, but in mammalian hematopoietic cells—for reasons unknown—it has

differentiation-associated functions that are distinct from those of $p16^{INK4a}$ and the two CDK inhibitors are not generally interchangeable (Humeniuk et al. 2013). In contrast to these CDK inhibitors, the p14 and p19 ARF proteins regulate p53 by inactivating MDM2, thus stabilising and activating of p53. This leads to cell cycle arrest by inducing the p53-dependent CDK regulator $p21^{WAF1}$ or apoptosis by inducing p53-dependent pro-apoptotic factors such as BAX and NOXA (Sherr 2012; Vousden and Prives 2009).

Inactivating the conditional EBNA3C resulted in an accumulation of both $p16^{INK4A}$ mRNA and protein, dephosphorylation of RB and concomitant cell cycle arrest (Maruo et al. 2006, 2011; Skalska et al. 2010, 2013). The *CDKN2A* locus encoding $p16^{INK4a}$ had been identified as a target of polycomb-mediated repression in proliferating fibroblasts and epithelial cells (Gil and Peters 2006), so it was not a surprise to discover that the EBNA3C-mediated repression of *CDKN2A* in cycling B cells was accompanied by the deposition of the repressive H3K27me3 epigenetic mark across the locus—primarily around the $p16^{INK4a}$ TSS (Maruo et al. 2011; Skalska et al. 2010, 2013). Analysis of LCLs established with EBNA3A knockout and conditional viruses revealed that EBNA3A also plays a role in creating the H3K27me3 modification and the repressed state of *CDKN2A* (Maruo et al. 2011; Skalska et al. 2010). In the absence of EBNA3A, cells consistently expressed higher levels of $p16^{INK4a}$ concomitant with lower levels of H3K27me3. These data were consistent with the earlier report of a microarray analysis that showed *CDKN2A* to be a target of EBNA3A-mediated repression (Hertle et al. 2009). Furthermore, establishing LCLs with recombinant EBV encoding CtBP-binding mutants of EBNA3C and/or EBNA3A showed that their interaction with this co-repressor was also necessary for the efficient deposition of H3K27me3 and repression of $p16^{INK4a}$ expression (Skalska et al. 2010). As with *BIM/BLC2L11*, B cell lines carrying EBV encoding the conditional EBNA3C modified oestrogen receptor fusion revealed that this epigenetic repression of *CDKN2A* was reversible by adding or removing 4HT from the medium (Skalska et al. 2010). The repression of *CDKN2A* by EBNA3C is likely to be direct because ChIP analysis of epitope-tagged EBNA3C expressed in LCLs identified binding peaks localised proximal to not only the TSS of $p16^{INK4A}$ and ARF, but also the *CDKN2B* gene encoding $p15^{INK4b}$ (Skalska et al. 2013). Subsequent ChIP-seq studies using tagged EBNA3C suggested that the major EBNA3C peak is adjacent to *ARF* (Jiang et al. 2014; our unpublished results; Fig. 3b). Taken together, all the various results indicate that EBV via EBNA3C (cooperating with EBNA3A) co-ordinately regulates the whole *INK4b-ARF-INK4a* locus by binding, repressing and directing the recruitment of PRC2 to sites near the three transcriptional start sites. Although this coordinated regulation might involve interactions between EBNA3C and IRF4/BATF-containing complexes (Jiang et al. 2014), the precise mechanism of targeting to the locus has not yet been identified. The role CtBP plays is also unknown. Consistent with all these data indicating that the whole 40 kb locus is co-regulated, we have found that $p15^{INK4b}$ mRNA is co-ordinately expressed with $p16^{INK4a}$ mRNA in EBNA3C-conditional and EBNA3A-conditional LCLs (our unpublished data) and it has recently been

shown that EBNA3A can repress *CDKN2B* transcription via an interaction with MIZ1 and deposition of the H3K27me3 repressive histone modification around its TSS (Bazot et al. 2014).

Our current understanding of the *CDKN2A/CDKN2B* locus in EBV-infected B cells is far from complete. For instance, *CDKN2A/CDKN2B* regulation by the 'looping' of chromatin has been reported at the locus in various non-B cells (Kheradmand Kia et al. 2009), but this remains to be assessed after EBV infection or generally in B cells. Similarly, the roles of non-coding RNAs, such as ANRIL, that can regulate *CDKN2A* (Yap et al. 2010) require exploration in EBV-infected B cells. What determines the requirement for EBNA3A in addition to EBNA3C and what defines their relative contributions are recurring, unanswered questions. Regulation of the locus by EBNA3C in an RB-null LCL (Skalska et al. 2010) formally established that EBNA3C-mediated epigenetic control is quite independent of the degree of cell proliferation. The specific role of $p16^{INK4a}$ (rather than $p14^{ARF}$ or $p15^{INK4b}$) as a target for EBNA3C and as the major barrier to B cell transformation was further explored by making use of an 'experiment of nature' in the form of 'Leiden' B cells carrying a homozygous genomic deletion that specifically ablates production of functional $p16^{INK4a}$ (Brookes et al. 2002). A comparison of $p16^{INK4a}$-null LCLs with LCLs established from normal B cells showed that if $p16^{INK4a}$ is not functional, then active EBNA3C is unnecessary to sustain proliferation (Skalska et al. 2013). Consistent with these observations—and providing formal proof that $p16^{INK4a}$ is the main target of EBNA3C that enables the establishment of LCLs in vitro—it was possible to transform $p16^{INK4a}$-null primary B cells into stable LCLs with EBV lacking functional EBNA3C. Although EBV (via EBNA3A and EBNA3C) appears to epigenetically regulate expression of both $p15^{INK4a}$ and $p14^{ARF}$ together with 16^{INK4a}, it is unclear precisely what role $p15^{INK4b}$ and $p14^{ARF}$ play in the inhibition of B cell transformation, in proliferating LCLs, or more generally in human B cell biology.

4.3 ADAM28/ADAMDEC1 Locus

ADAM28 and *ADAMDEC1* form part of a metalloproteinase gene cluster on chromosome 8p12 (McClellan et al. 2012, 2013; Fig. 3c). ADAM28 is a disintegrin/metalloproteinase expressed by lymphoid cells and in its cleaved, soluble form is thought to be a regulator of B cell adhesion and migration through endothelial cells. ADAMDEC1 (also known as Decysin-1) is also a metalloproteinase, but of unknown function. The available gene expression data derived from several microarray studies are largely consistent with both EBNA3A and EBNA3C playing a role in repressing these genes in EBV-infected B cells—probably by regulation of the whole locus (Hertle et al. 2009; McClellan et al. 2012; Skalska et al. 2013; White et al. 2010; http://www.epstein-barrvirus.org.uk). Furthermore, although in B cells infected with EBV, both EBNA3A and EBNA3C appear to be necessary for repression, when they are ectopically over-expressed, either can independently

repress the expression of ADAM28 and ADAMDEC1 in EBV-negative B cells (McClellan et al. 2012). Since ADAM28 and ADAMDEC1 probably modulate cell:cell and cell:stroma interactions, their regulation is likely to be of greatest biological significance in vivo during viral persistence or possibly EBV-associated disease.

ChIP-seq analysis and confirmatory ChIP-QPCR, using both a latency III BL-derived cell line and LCLs, showed robust EBNA3C and EBNA3A (but not EBNA3B) binding peaks at the same site in an intergenic regulatory element located between the *ADAM28* and *ADAMDEC1* genes, approximately 70 kb downstream of the *ADAM28* promoter and about 16 kb upstream of the *ADAMDEC1* promoter (McClellan et al. 2012, 2013; our unpublished results; Fig. 3c). As with *BIM/BCL2L11* and *CDKN2A*, repression of this complete locus by EBNA3A and EBNA3C is associated with the distribution of H3K27me3 around the *ADAMDEC1* transcription start site and within *ADAM28*. Chromatin conformation capture (CCC—a method that reveals contact between two defined regions on a genome Naumova et al. 2012) was used to show that chromatin looping to bring the intergenic element that binds EBNA3s into close proximity with the *ADAM28* and *ADAMDEC1* promoters requires the presence of EBNA3C (McClellan et al. 2013). This coincides with the deposition of H3K27me3 that is presumably mediated by PcG repressor complexes and sustained repression of both genes. It is not known how or why EBNA3C and EBNA3A are both involved in EBV-infected cells, nor what cell factors are responsible for targeting the EBNA3s to the intergenic binding site, nor what facilitates enhancer 'looping' to specific promoters. Also, in common with the regulation of *BIM/BCL2L11* and *CDKN2A/CDKN2B,* we do not know what triggers recruitment of PcG complexes or the repression of transcription—or which comes first. Nevertheless, although the molecular details are still awaited, these important experiments have provided some of the first examples (along with genes described below) of host gene reprogramming via viral modulation of long-range interactions requiring chromatin looping between promoters and distal regulatory elements.

4.4 CtBP2

As described in Sect. 2.7.2, 'CtBP' consists of two proteins encoded by two separate genes—*CtBP1* and *CtBP2*—and therefore in theory, any vertebrate cell could express either protein or both proteins. Microarray expression studies of EBV-infected cells indicate that in human B cells expressing the complete latency III pattern of viral proteins (e.g. BL31-B95.8 and various LCLs), CtBP2 mRNA is transcribed at very low levels or the gene is silent. The exception to this was identified by Hertle and colleagues by analysing EBNA3A-deficient LCLs. Consistently, LCLs established with EBV recombinants carrying a deletion of the whole EBNA3A open reading frame expressed relatively high levels of CtBP2 mRNA (Hertle et al. 2009). In contrast, studies of LCLs deficient in EBNA3B

or lacking functional EBNA3C did not produce this apparent derepression phenotype (Skalska et al. 2013; White et al. 2010; http://www.epstein-barrvirus.org.uk). Similar analyses of CtBP1 expression showed that in all EBV-infected cells investigated (irrespective of EBNA3 expression), the levels of CtBP1 transcription are relatively high and consistent with the level of protein detected by Western blotting.

Subsequently, McClellan and colleagues produced intriguing ChIP-seq and ChIP-QPCR data that partially explain this differential regulation of *CtBP1* and *CtBP2*. Specifically, they reported that while no EBNA3-enriched sites were seen at or anywhere near the *CtBP1* locus, EBNA3A, EBNA3B and EBNA3C could all be found at an intronic site (predicted by histone marks to be an enhancer) located in *CtBP2* (McClellan et al. 2013; Fig. 3d). Consistent with EBNA3A playing a central role in the repression of this locus, they were able to show by CCC that only in the absence of EBNA3A expression was there chromatin looping that juxtaposed the enhancer adjacent to the *CtBP2* promoter, thus facilitating transcription. That is, at this locus, EBNA3A prevents enhancer–promoter looping. Since not only EBNA3A, but also EBNA3B, EBNA3C and EBNA2 could be found binding to the intronic enhancer, a model was proposed in which the transactivator EBNA2 binding to the intragenic site would facilitate enhancer–promoter loop formation (although the role of EBNA2 was not experimentally assessed) and CtBP2 activation, only when an EBNA3 was absent. These observations provide another convincing example of EBNA3A (it was only demonstrated for EBNA3A) modulating the looping of chromatin between a distal site and a gene promoter—in this case inhibiting loop formation (McClellan et al. 2013). However, in the current model, it remains to be determined why EBNA3A acts negatively in the modulation of looping, while EBNA3B and 3C appear uninvolved despite their apparent binding to the locus. Because so little is known about the functional difference(s) between CtBP1 and CtBP2, the biological significance of this EBV-mediated pattern of expression cannot currently be assessed.

4.5 CXCL9/10 Locus

CXCL9, *CXCL10* and *CXCL11* are a family of CXC chemokine genes tandemly located on chromosome 4q21.1 (Fig. 3e). The chemokines they encode are generally interferon-inducible and upon secretion attract leucocytes to sites of infection and inflammation and probably attract anti-tumour cytotoxic T cells to cancer tissue—which correlates with improved clinical outcome (Dufour et al. 2002; Mlecnik et al. 2010). Therefore, regulation of this locus is likely to be important in vivo, in EBV persistence and disease pathogenesis (see for example Sect. 6.3). The consensus of various microarray expression studies of this gene cluster has indicated that in LCLs carrying EBV that is wild type for EBNA3s, the locus is generally repressed, but in EBNA3A-deficient LCLs, *CXCL9/10* is strikingly derepressed; in EBNA3C-deficient conditional LCLs, it is slightly derepressed;

and in EBNA3B-deficient LCLs, it is more profoundly repressed. In all the LCLs, *CXCL11* transcripts are barely detectable and not regulated by EBNA3s (Hertle et al. 2009; Skalska et al. 2013; White et al. 2010; http://www.epstein-barrvirus.org.uk). These data are largely consistent with those derived from the tumour-derived B cell line, BJAB ectopically expressing individual EBNA3 genes—that is EBNA3A and EBNA3C both repress *CXCL10*, whereas EBNA3B induces modest activation (McClellan et al. 2012).

Studying in detail the regulation of CXCL9 and CXCL10 expression by EBNA3A, Kempkes and colleagues developed a model wherein EBNA3A binds three intergenic enhancers located between *CXCL9* and *CXCL10* that can be occupied by RBP-JK/CBF1 and the transactivator EBNA2 (Harth-Hertle et al. 2013; Fig. 3e). The binding of EBNA3A to RBP-JK/CBF1 displaces EBNA2 and by impairing enhancer activity rapidly inhibits recruitment of Pol II and transcription from both the *CXCL9* and *CXCL10* promoters. Studying the kinetics of repression and chromatin modifications indicated that here silencing by EBNA3A precedes the deposition of H3K27me3, which subsequently sustains the genes in an inactive configuration; this is the steady state in LCLs unless EBNA3A is absent in which case EBNA2 binds and substantially activates the locus (Harth-Hertle et al. 2013).

Summarising what is known about the action of the EBNA3s at this gene cluster, EBNA3A clearly plays a major role in silencing intergenic enhancers between *CXCL9* and *CXCL10*. EBNA3C can probably bind at the same locations and contribute to the repression (McClellan et al. 2012), although a binding peak has not yet been reported. As with most of the target genes we have considered here, it is not clear why EBNA3A and EBNA3C are not equivalent and interchangeable. In the absence of EBNA3A or EBNA3C, this enhancer can be bound by EBNA2, resulting in transactivation. EBNA3B appears to potentiate the activation of the locus, but whether to do this it binds at the same enhancer sites or acts by a novel mechanism is currently unknown. Since regulation could not be recapitulated in RBP-JK/CBF1-negative DG75 BL cells, the binding of EBNA3A (probably EBNA3C) and EBNA2 is likely to be mediated by RBP-JK/CBF1 binding to multiple response elements within the enhancers (Harth-Hertle et al. 2013). Although it has not been proven, the data strongly suggest that EBNA3A (and EBNA3C?) simultaneously inhibits chromatin looping between distal intergenic enhancers and the promoter of two genes. When the level of EBNA3A (or EBNA3C) is reduced, EBNA2 gains access to these regulatory elements and promotes enhancer–promoter looping and transcription—but this remains to be formally tested.

4.6 Overview of Gene Regulatory Mechanisms Mediated by the EBNA3s

The gene loci with specific EBNA binding sites described above are illustrated schematically in Fig. 3. They show how EBNA3-mediated repression can be achieved by a variety of topologically different mechanisms. Furthermore, putting

these data on specific gene loci into perspective, analyses of genome-wide distributions have revealed >20,000 EBNA2 binding sites and (by using in ChIP-seq an antibody that recognises all three EBNA3 proteins) >7000 EBNA3 binding sites. Only a small proportion of all these binding sites are proximal to the TSS of genes, with the closest EBNA3 binding sites typically 10–50 kb from the nearest TSS—suggesting the modification of long-range interactions is common (Jiang et al. 2014; McClellan et al. 2013). Comparing EBNA2 and EBNA3 binding sites revealed that there was considerable overlap. At gene-proximal sites (<2 kb from TSS), about 60 % of EBNA3 binding peaks were overlapping with sites bound by EBNA2 and overall—including distal sites—about 80 % of genes closest to an EBNA3 binding peak were also closest genes to an EBNA2 binding peak. This is a strong indication that many genes targeted by EBNA3 proteins are co-regulated by EBNA2. It is interesting that (as indicated above in Sect. 2.7.1) probably only a small percentage of these overlapping sites correspond to RBP-JK/CBF1 response elements; however, many of the sites are enriched for multiple transcription factors, including PU1, SP1, EBF1, BATF, IRF4, PAX5 and p300. For detailed analyses of the available EBNA3/EBNA2 ChIP-seq data, the reader is referred to (Jiang et al. 2014; McClellan et al. 2012, 2013).

A comparison of EBNA3 binding sites with the genome-wide chromatin landscape (in LCL GM12878) available from the ENCODE database showed EBNA3s associated with active enhancers (defined as H3K4me1+ve; H3K27Ac+ve) or in many cases 'poised' enhancers (H3K4me1+ve; H3K27Ac-ve) consistent with EBNA3 functioning as both repressors and activators of transcription (McClellan et al. 2013). As we have seen above, specific repression mechanisms are starting to be understood; however, activation by EBNA3s remains largely unexplored. Adding to the global picture, Kempkes and colleagues performed a comprehensive data-mining analysis of genes regulated by EBNA3A (Harth-Hertle et al. 2013; Hertle et al. 2009). By comparing the data on EBNA3A-repressed genes with data from the ENCODE studies of genome-wide histone modifications, they made the following observations: (a) about 70 % of genes repressed by EBNA3A carried the PcG-associated signature H3K27me3; (b) 90 % of these genes were associated with PcG-mediated silencing in multiple cell types and contexts—that is, they may have an inherent capacity to undergo PcG-mediated silencing when repressed by a variety of mechanisms—and (c) about 20 % of genes repressed by EBNA3A were grouped in clusters of up to 4 genes and often H3K27me3 was enriched across the whole locus after repression by EBNA3A. It was concluded that EBNA3A-repressed genes were commonly associated with PcG modification of chromatin and that the target genes are often arranged in gene clusters (Harth-Hertle et al. 2013). As a result of these observations, the group performed the detailed study on the *CXCL9*, *CXCL10* and *CXCL11* gene cluster and its regulation by EBNA3A described above and in Fig. 3e.

These studies have highlighted the unexpected complexity of EBNA-mediated gene regulation, since *CXCL9 and CXCL10* appear to be genes co-ordinately regulated by the functional interaction of at least four EBNA proteins—EBNA2, EBNA3A, EBNA3B and EBNA3C. It is also possible that EBNA-LP might

be involved, as it can in some circumstances cooperate with EBNA2 to activate transcription (Peng et al. 2005). The study of this locus has also raised the important question of whether polycomb-mediated chromatin modifications such as H3K27me3 are a cause of the gene repression induced by EBNA3s or a consequence. At the *CXCL9/10* locus, analysis of the kinetics and sequence of events made a strong case for the latter, with displacement of EBNA2 probably being the primary cause of repression (or strictly speaking deactivation). However, not all genes repressed by EBNA3 activity are transactivated by EBNA2. For example, at *BIM/BCL2L11,* there is no indication that EBNA2 is involved and—because this has all the characteristics of a poised 'bivalent' gene, where Pol II is always present—PcG complexes may play a much more direct role in repression by blocking transcription initiation (Bernstein et al. 2006; Stock et al. 2007). Consistent with this, at *BIM/BCL2L11,* the recruitment of core PRC2 factors is unambiguously dependent on the expression of both EBNA3A and EBNA3C. Nevertheless, because repression of *BIM/BCL2L11* also involves removal of histone H3 and H4 acetylation (Paschos et al. 2009, 2012), the association of EBNA3A and EBNA3C with HDACs is also likely to be important. So, although some of the details are emerging, the overall picture of EBNA3-mediated gene regulation gets even more complicated. It will be exciting to see what general principles emerge as we discover more about the interactions between EBNA3s and components of the multi-factor complexes that regulate epigenetic modifications of chromatin and programmes of transcription.

5 EBNA3C and EBNA3A as Modifiers of Oncogenic Stress Responses and the DDR

Viruses that establish a persistent latent infection commonly stimulate cellular DNA synthesis and sometimes cell division early after infection. However, cells of many metazoans have evolved responses that normally monitor unscheduled DNA synthesis and prevent cell proliferation when, for instance, cell proto-oncogenes are 'activated' by mutation and/or deregulated expression. These defence strategies that reduce the risk of neoplasia and cancer are collectively called oncogenic stress responses (OSR). Mechanisms include the activation of tumour suppressor genes that together trigger pathways leading to cell cycle arrest (e.g. oncogene-induced senescence, OIS) or complete elimination of cells (e.g. apoptosis). Because viruses that can induce cellular DNA synthesis and cell division are capable of triggering OSR/OIS, they often co-evolve countermeasures for inactivating or bypassing OSR/OIS (Bartek et al. 2007; Braig and Schmitt 2006; Nikitin and Luftig 2012). As cell proto-oncogenes generally control signalling pathways and/or gene networks that link proliferative signals to the cell cycle machinery, when they are deregulated, this can result in unscheduled and aberrant DNA synthesis (sometimes called 'replicative stress'). Consequently, oncogene activation can produce the stalling of DNA replication forks that results in DNA double-strand

breaks. Such lesions can trigger, primarily via the ATM/CHK2-kinase signalling pathway, the stabilisation and activation of p53 and also the induction of $p16^{INK4a}$. Depending on the physiological and cellular context, this leads to DNA repair, cell death or senescence. This complex response is known as the DNA damage response (DDR). The links between DDR and OSR/OIS have been extensively reviewed (Acosta and Gil 2012; Bartek et al. 2007; Braig and Schmitt 2006; Halazonetis et al. 2008).

As detailed above, EBNA3C and EBNA3A appear to cooperate harnessing the PcG protein system for the sustained repression of two host tumour suppressor genes involved in OSR/OIS—*BCL2L11* encoding BIM and *CDKN2A* encoding $p16^{INK4a}$. An explanation for why EBV has evolved a mechanism for suppressing expression of the CDK inhibitor $p16^{INK4a}$ (and probably BIM) expression became apparent by comparing infections of normal B cells with EBNA3C-deficient EBV with infections by 'wild-type' (WT) EBV (Allday 2013; Skalska et al. 2013; Fig. 4). These experiments revealed that EBV infection led to a modest increase in $p16^{INK4a}$ transcription in the first few days after infection, when EBNA2 transactivates regulators of cell cycle progression (e.g. MYC, Cyclins D2 and E) to induce a period of hyperproliferation and concomitant activation of DDR pathways occurs (Allday et al. 1989; Nikitin et al. 2010; Shannon-Lowe et al. 2005; Sinclair et al. 1994; Spender et al. 1999; Thorley-Lawson and Strominger 1978). It is likely that entry into S phase, when uncoupled from normal signalling pathways, is interpreted as oncogenic or replicative stress and $p16^{INK4a}$ transcription is a consequence. When the infecting virus expressed functional EBNA3C (and EBNA3A), there was no further increase of $p16^{INK4a}$ transcripts from about day 7 onwards. In contrast, if functional EBNA3C was absent during infection, transcription from *CDKN2A* continued unrestrained and the level of mRNA progressively increased until most of the cells stopped proliferating and/or died. In a similar way, early after infection, BIM expression is down-regulated and within about 5 days reaches a steady state, unless EBNA3C is deleted or inactivated in the infecting EBV when the level of BIM mRNA also increases in parallel with that of $p16^{INK4a}$. This increase again continues until the cells arrest or die (Allday 2013; Skalska et al. 2013).

The EBNA3-mediated, sustained inhibition of $p16^{INK4a}$ and probably *BIM* transcription is therefore critical for EBV to bypass a host cell defence against oncogenic transformation triggered by EBNA2, acting through MYC (Nikitin et al. 2010). Thus, expression of EBNA3C (and EBNA3A) ensures expansion of the infected B cell population, LCL outgrowth and long-term latency. Strictly speaking, in this context, EBNA3C does not actually repress *CDKN2A* and *BIM/BCL2L11* transcription, but rather blocks their activation. We assume this involves the recruitment of PcG protein complexes to the loci, leading to H3K27me3 modifications on chromatin around the transcription start sites, as is seen in established LCLs; however, this has not yet been formally demonstrated in newly infected cells.

It was reported that EBNA3C might specifically reduce the DDR that is active in the first week after EBV infection of naïve B cells in vitro, when the cells are

beginning to cycle very rapidly (Nikitin et al. 2010) and during the same period that $p16^{INK4a}$ is actively transcribed (Skalska et al. 2013). It may be that this increase in $p16^{INK4a}$ is a consequence of DNA damage caused by hyperproliferation, as has been variously reported in other types of cell (reviewed in Acosta and Gil 2012; Gil and Peters 2006). Nevertheless, an increase in $p16^{INK4a}$ should activate RB, which could then lead to the repression of E2F-regulated genes, while MYC is constitutively active. This might enable cells to enter S phase with suboptimal amounts of DNA precursors and/or replication enzymes and this would lead to stalled DNA replication that is 'read' as DNA damage, triggering phosphorylation of histone H2AX—a focal marker of DNA damage. When EBNA3C is functional, the increase in $p16^{INK4a}$ is soon attenuated and the DDR will apparently subside. So it could be that the lack of EBNA3C exacerbates the DDR because of the accumulating $p16^{INK4a}$. However, during primary infection, EBNA3C might also have a direct effect on the ATM/CHK2 signalling pathway or some component(s) of the DDR complex at the site of DNA strand breaks (Nikitin et al. 2010). Although EBNA3C can apparently associate with CHK2 and/or histone H2AX (Choudhuri et al. 2007; Jha et al. 2013), the possibility that these interactions are involved in the attenuation of the DDR early after infection requires further investigation. Only a systematic genetic analysis of EBNA3C and its actions early after infection will unravel the DDR and the $p16^{INK4a}$-mediated senescence response and establish what relative contributions each make to the inhibition of B cell transformation.

Through the action of EBNA3C (and EBNA3A) and interactions with the cellular PcG protein system, EBV appears to have evolved a very effective countermeasure to OSR/OIS and this is critical in the virus life cycle to establish a latent infection and therefore initiate long-term persistence in B cells. In vitro this mechanism manifestly overcomes a major early obstacle to B cell growth transformation, making EBV one of the most potent transforming/immortalising biological agents to have been identified. This strategy of utilising the PcG system to specifically regulate key tumour suppressor genes is—to our knowledge—unique among tumour viruses.

6 The EBNA3s In Vivo

6.1 EBNA3s in Asymptomatic Persistence

If in order to establish a persistent infection in vivo, EBV initially commandeers resting (naïve) B cells and drives these to proliferate as activated B blasts, we assume that—by analogy to what is seen in culture—repression of $p16^{INK4a}$ is necessary to permit the transient proliferation of the infected population (Fig. 4). Furthermore, since in vitro EBNA3C and EBNA3A expression does not seem to be required for the early rounds of rapid cell division (up to about day 10), and these proteins have clearly evolved functions that extend the proliferative life of infected

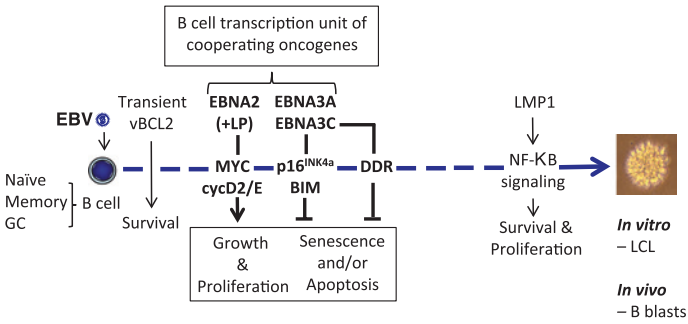

Fig. 4 Events following infection of primary resting B cells by EBV that initiate transformation into continuously proliferating LCLs. Immediately after infection, viral anti-apoptotic factors normally associated with the EBV lytic cycle (vBCL2 factors: BHRF1 and BALF1) are transiently expressed to aid the survival of newly infected B cells (Altmann and Hammerschmidt 2005). During the first 2 days post-infection (pi) with EBV, cell genes associated with growth and cell cycle are transactivated and their products (e.g. MYC, cyclin D2, cyclin E) drive cells from G0 to G1, to become enlarged, activated and start proliferating. The whole process is driven by the EBV transactivator protein EBNA2, probably assisted by the co-factor EBNA-LP (Nikitin et al. 2010; Sinclair et al. 1994; Spender et al. 1999). Cells then undergo rounds of rapid cell division (doubling time 8–10 h) that in some cells results in damage to DNA that can activate the DNA damage response (DDR, Nikitin et al. 2010). If the full complement of nine EBV latency-associated proteins is expressed, the DDR becomes attenuated (in part by EBNA3C) and cells continue to proliferate to produce LCLs that have a population doubling time of about 24 h. Early after infection, expression of BIM is down-regulated, and although the level of $p16^{INK4a}$ expression increases slightly, this soon reaches a steady state. In both cases, EBNA3C and EBNA3A cooperate to restrain the transcription of these tumour suppressor genes (Paschos et al. 2012; Skalska et al. 2013 and described in the text). EBNA3C appears more important in this partnership, and for reasons we do not understand. Although transcripts can be detected earlier, Micah Luftig and colleagues have shown that LMP1 expression and concomitant NF-κB activity are not fully evident until 3 weeks after primary B cell infection, and then, they make further contributions to cell survival and proliferation (Price et al. 2012). If EBNA3C is deleted or functionally inactivated in the infecting EBV, levels of mRNAs corresponding to $p16^{INK4a}$ and BIM progressively increase until cells arrest and/or die (Skalska et al. 2013). In vitro, the sequence of events described here is probably similar whether the B cells infected are naïve, memory or germinal centre-derived (Babcock et al. 2000; Siemer et al. 2008), but this is not known for cells in vivo. With a B cell-specific transcription unit that encodes oncogenes that collaborate in vitro to transform normal B cells into continuously proliferating LCLs, why is EBV not an acutely transforming tumour virus in vivo? We would suggest that linking expression of this transcription unit to the state of B cell differentiation, having EBNA proteins that are immunodominant in CTL recognition and having one member of EBNA3 family that encourages immune surveillance and perhaps differentiation of infected cells, all contribute to a highly evolved state of equilibrium that is mutually beneficial to virus and host (see main text for a more detailed discussion)

cells, one is tempted to speculate that prolonged B blast proliferation in vivo must be an important component of the persistence programme. The survivors from this expanding population of activated B cells might then migrate into or initiate a germinal centre, where the cells differentiate to centroblasts and centrocytes and finally emerge as MBCs after the regulated shutdown of protein-coding EBV latency genes.

Since the repression of $p16^{INK4a}$ (and BIM) expression involves polycomb-mediated covalent histone modifications, it is possible the epigenetic memory (i.e. a heritable pattern of gene expression: Campos et al. 2014) of this programme will be passed to progeny cells and carried through into the MBC population, even if the initiators, EBNA3A and EBNA3C, are no longer expressed. It is well established that polycomb-mediated gene repression is also a common precursor to promoter DNA methylation in cancer (reviewed in Cedar and Bergman 2009), so it is reasonable to hypothesise that the B cells reprogrammed in vivo by EBV will be particularly prone to aberrant DNA methylation at the *CDKN2A* and *BIM/BCL2L11* loci during tumorigenesis. This is consistent with reports describing promoter methylation of these genes in EBV-positive B cell lymphomas and derived cell lines (Klangby et al. 1998; Nagy et al. 2005; Paschos et al. 2009; Richter-Larrea et al. 2010). The role of EBNA3B in persistence and B cell lymphomagenesis is discussed in Sect. 6.3.

6.2 Immune Responses to EBNA3 Proteins

EBV infection in humans is tightly regulated by the immune system. This has been extensively reviewed elsewhere (Hislop et al. 2007) and also in several chapters of this volume. The role that the EBNA3 family proteins play in T cell-mediated immunity is very well established and is generally consistent with the notion that immune regulation of EBV-infected B blasts is critical in maintaining the virus–host balance in persistence. Indeed, the EBNA3s are the primary targets of $CD8^{+ve}$ T cells (cytotoxic T lymphocytes or CTL) in the peripheral circulation of healthy EBV carriers (Hislop et al. 2007) and have been shown to be, among all the latency-associated viral proteins, the most immunogenic. CTL responses against cells latently infected with EBV are primarily targeted against EBNA3A/3B/3C-derived epitopes that are recognised by $CD8^{+ve}$ CTL in association with specific HLA molecules (Khanna et al. 1992, 1997; Murray et al. 1992; Steven et al. 1996), but the EBNA3 proteins are also a source of epitopes for $CD4^{+ve}$ T cells, with EBNA3C-derived epitopes being more abundant than EBNA3A and 3B. A comprehensive summary of the $CD8^{+ve}$ and $CD4^{+ve}$ T cell epitopes derived from the EBNA3 proteins is shown in (Hislop et al. 2007).

The strong CTL-mediated response against the EBNA3 proteins has allowed Moss and colleagues to develop a $CD8^{+ve}$ T cell epitope-based EBV vaccine strategy—to protect against infectious mononucleosis—using EBNA3A-derived epitopes (Elliott et al. 2008). Furthermore, patients with PTLD have also been successfully treated with infusions of EBV-specific CTLs primarily directed against the EBNA3s (reviewed in Heslop et al. 2010; Nikiforow and Lacasce 2014 and the chapter in this volume authored by Gottschalk and Rooney). This focusing of CTL-mediated immune surveillance on the EBNA3s ensures that potentially oncogenic, EBV-driven, proliferating B blasts are removed before they cause morbidity or possibly mortality. It has been proposed that evolutionary pressures on the CTL epitopes in these proteins (and therefore DNA sequence variation) may be towards

their conservation rather than their inactivation, thus helping maintain a stable relationship between virus and host that favours both (Khanna et al. 1997). Given the diversity of HLA molecules in the human population, this may be one explanation of the considerable sequence variation seen in the EBNA3 genes. Conservation of some CTL epitopes across EBV subtypes and diversification of others may be driven by MHC variation in host populations to either enhance presentation or avoid it.

6.3 EBNA3B: Manipulation of Immune Surveillance and Role as Tumour Suppressor

Exon microarray analysis of the genes differentially regulated between WT (B95.8) and EBNA3B-KO LCLs identified about 200 EBNA3B-regulated genes, including many expressed at the cell surface or as secreted chemokines—for example CD28, CD305 (LAIR-1), CXCR4, CXCL10, IL10 and IL19 (White et al. 2010, 2012; http://www.epstein-barrvirus.org.uk). This led to the speculation that although these changes did not have gross effects on EBV-infected B cells in vitro, they might alter heterotypic cell:cell interactions and possibly migration or homing properties of cells in vivo. The observation that EBNA3B has not been counter-selected over millions of years of virus–host co-evolution suggested an important role for this gene in vivo during long-term EBV persistence. Furthermore, in our hands, EBNA3B-negative LCLs grow particularly robustly in vitro, and after long-term culture of LCLs, there appears to be a propensity for deletions in EBNA3B to arise. This applies to at least two widely used cord blood-derived LCLs—IB4 (Chen et al. 2005) and X50-7 (Allday and Farrell 1994)—and has been observed to arise spontaneously in adult B cell-derived LCLs established using B95.8 EBV by us and others (unpublished observations).

In order to test the hypothesis that deletion of EBNA3B would significantly alter the behaviour of EBV-infected B cells in vivo, mice engineered to be susceptible to EBV infection were used. For this purpose irradiated, newborn NOD-scid *IL2*γ^{null} (NSG) immunodeficient mice were engrafted with fetal liver-derived CD34 + haematopoietic progenitor cells (reviewed in the chapter by Christian Münz). After approximately 3 months, these mice (hu-NSG mice) were validated for successful engraftment of human immune cells and infected with similar doses of WT, EBNA3B-KO or revertant EBV-BAC-derived viruses (White et al. 2012). After a month, mice were killed and found to exhibit splenomegaly, with large tumour masses in over half of the EBNA3B-KO-infected mice, but not the WT or revertant virus-infected animals. Routine histology and immunocytochemistry confirmed that the EBNA3B-KO-infected mice had developed monomorphic, highly proliferative tumours (>90 % Ki67+ve)—described as DLBCL-like activated B cell (ABC) sub-type—that destroyed the architecture of the spleen. In contrast, spleens from the WT and revertant groups were not so enlarged and had markedly less proliferative (30–60 % Ki-67+ve), polymorphous infiltrates with significant numbers of immunoblasts and plasma cells—features similar to

human polymorphic PTLD and to the plasmacytoid lesions described previously in both hu-NSG mice (Strowig et al. 2009) and in SCID mice implanted with LCLs (Rochford et al. 1993). Furthermore, it was particularly striking that in the spleens of WT- and revertant-infected animals, there were substantial T cell infiltrates, whereas the EBNA3B-KO tumours exhibited very few infiltrating T cells. The derepression of CXCL10 by EBNA3B indicated by microarray was confirmed in ELISA to facilitate CXCL10 induction by interferon. Restoration of CXCL10 levels in culture media partially restored a migratory deficit for T cells towards EBNA3B-KO LCLs (White et al. 2012; see also Sect. 4.6).

Overall, these remarkable results indicated that failure to express EBNA3B: (a) produces aggressive B cell tumours resembling DLBCL, (b) may somehow reduce immune cell trafficking and T cell surveillance of the tumour cells, perhaps partially through loss of CXCL10 secretion, but likely also involving additional migratory signals and (c) could perhaps be inhibiting B cell/plasmacytoid differentiation. It is significant that there were at least two cases of transplant patients with PTLD that fail to express EBNA3B because of a genomic deletion in the resident EBV, one of which was an aggressive fatal PTLD (Gottschalk et al. 2001), much like those seen in the animal model. Furthermore, screening of a further eleven EBV+ve ABC-DLBCLs revealed another tumour carrying a truncated EBNA3B gene. Additional tumours carried point changes that were unique to that tumour sample (across 100 sequences)—including small internal deletions in one of 10 BL and one of 11 HLs. Without knowing either the original sequence of the virus, or having a much clearer picture of EBNA3B sequence diversity, we cannot tell whether these also represent mutations, or merely polymorphisms (White et al. 2012). It is therefore currently impossible to judge how prevalent EBNA3B mutations are in lymphoma development.

Nevertheless, these data identified EBNA3B as a bone fide tumour suppressor that could have evolved to encourage T cell interactions via chemokine secretion. T cell contact is an essential component of the germinal centre reaction and B cell differentiation, so EBNA3B may also be paving the way to allow B cell differentiation and the switch to latency II. This is supported by the observation that EBNA3B-regulated genes include several that are repressed upon entry to the germinal centre (White et al. 2010). Equally, enhanced T cell surveillance clearly helps to limit the morbidity or mortality from lymphoproliferative disease caused by the proliferating B blast-like cells induced by the latency III growth programme. Coordinating the expression of EBNA3B with the oncoproteins EBNA2, EBNA3A and EBNA3C from a single transcription unit would represent an evolutionary adaptation to minimise oncogenic risk to the host (Fig. 4).

6.4 EBNA3s in B Cell Lymphomagenesis

As described in various chapters of this volume, and briefly above, different EBV-associated tumours exhibit different viral latency gene expression profiles,

according to the type—and differentiation state—of the cell of origin of the tumour. Since the viral gene expression profile of diverse tumours in clinical samples is typically defined by the immunohistochemical detection of EBNA2 and LMP1 (and in situ hybridisation of EBERs), the expression of the EBNA3s is assumed to correlate with EBNA2 expression. By this logic, to the best of our knowledge, it is only the immunoblastic lymphomas (seen in the immunosuppressed) and a subset of DLBCLs—that express the full latency III B cell transcription unit (Fig. 1)—in which the EBNA3s are probably contributing to pathogenesis. The iatrogenic (i.e. immune-suppressive drug-induced) immunoblastic lymphomas can often be treated by removal of immunosuppression, allowing the patient's immune response to destroy the malignant cells. Where this fails, infusions of autologous or partially HLA-matched CTLs raised against EBV antigens are also able to establish an immune response that destroys the malignant cells (Heslop et al. 2010; Hislop et al. 2007). Typically, these CTLs are predominantly responsive to the EBNA3s, due to the apparent immunodominance of EBNA3-derived epitopes. The observation that latency III cells are scarce in immunocompetent individuals (Babcock et al. 1998, 1999), combined with the success of immunotherapy for latency III tumours, suggests that the EBNA3s make excellent candidates for immunotherapeutic targets, particularly once the diversity of EBNA3 CTL epitopes across viral strains and host HLA types has been fully defined (discussed in chapters by Rajiv Khanna and Gottschalk and Rooney in this volume).

The role of EBNA3s in lymphomagenesis is complicated by the apparently opposing behaviours of EBNA3A/C and EBNA3B. Where EBNA3A and EBNA3C seem to play crucial oncogenic roles in suppressing senescence and apoptosis, and perhaps overriding cell cycle checkpoints (discussed above), EBNA3B appears to have an opposing, tumour-suppressing role, as indicated by its mutation in some immunoblastic lymphomas. Mechanistically, this may be mediated by enhancing the expression of factors that promote T cell interaction, enhancing their surveillance by the immune system (see Sect. 6.3 and White et al. 2010) or some other as-yet unidentified function. Thus, inactivating mutation of EBNA3B appears to contribute to some lymphomas (White et al. 2012), whereas (at least for immunoblastic lymphomas) EBNA3C and EBNA3A seem likely to be essential.

The role of the EBNA3s in other malignancies remains much less clearly defined. The widely held supposition that the EBNA3s are absent from the latency I and latency II tumours—largely based on the assumption that EBNA3s are not present where EBNA2 is also absent—is supported by only a few early studies looking at virus gene expression in tumours or tissues. For instance, EBNA3s were not detected by immunoblotting in 24 nasopharyngeal cancers that had been passaged in nude mice (Young et al. 1988). An analysis of the latency II profile in the normal germinal centre failed to detect EBNA3 transcripts (Babcock et al. 2000), similarly the whole IgD^{-ve} fraction of tonsils was negative for EBNA3 transcripts (Joseph et al. 2000). Attempts to detect EBNA3s in HL are hard to find in the literature, with the original description of the latency II status of HL not addressing

the EBNA3s (Deacon et al. 1993). Certainly, no comprehensive survey exists to conclusively exclude the expression in HL of any of the EBNA3s.

Nevertheless, topologically, there is no reason that those latency states that use Qp should not express the EBNA3s, although Qp-driven EBNA3 has not been reported. Indeed, related transcripts (using the lytic Fp immediately upstream of Qp) have been found in latency I BL cell lines induced to enter the lytic cycle (Touitou et al. 2003). Additionally, a subset of BLs that carry a deletion of *EBNA2* express the EBNA3s from the Wp promoter (Kelly et al. 2002). Cell lines freshly established from these 'Wp-restricted' BLs exhibit a generally BL-like gene signature, but clearly represent a distinct subset, exhibiting a less germinal centre-type expression profile, that was more plasmacytoid (Kelly et al. 2013). Central players in this profile (reduced BCL6 and raised BLIMP1 and IRF4) are all regulated by EBNA3s in a BL background (White et al. 2010; http://www.epstein-barrvirus.org.uk). Notably, however, the Wp-restricted BLs are considerably more resistant to apoptosis in vitro than latency I BLs, through the effects of the BCL2 homologue BHRF1, combined with the EBNA3s and other factors acting on pro-apoptotic proteins including BIM and NOXA (Anderton et al. 2008; Kelly et al. 2009; Vereide and Sugden 2011; Watanabe et al. 2010; Yee et al. 2011). This increased robustness of the Wp-restricted BL may have implications for treatment strategies and success, but this has not yet been tested. It also remains formally possible that a Wp-restricted viral gene expression profile (or something similar) may also be found in other cancers. The expression of EBNA3s is only rarely assessed in cancers, and their low expression level (both of transcript and protein) and intimate association with chromatin make their detection potentially more challenging than for other antigens.

A few recent observations lend some credence to the possibility that EBNA3s may contribute to the onset of malignancies that do not exhibit typical latency III gene transcription. Analysis of the viral gene expression of a number of DLBCLs of the elderly identified several showing reverse transcription PCR and/or immunohistochemical evidence of tumours expressing EBNA3A but not EBNA2 (Nguyen-Van et al. 2011). Banked CTLs, whose specificity was predominantly against the EBNA3s, were able to successfully treat transplant-associated EBV lymphomas including one BL and five out of six HLs that were negative for EBNA2. Unfortunately, the authors were unable to assess EBNA3 expression in these lymphomas (McAulay et al. 2009). Furthermore, while potentially oncogenic EBNA3B truncations were identified in DLBCLs (that can adopt latency III), small internal deletions were also seen in EBNA3B genes isolated from an HL and a BL, which are not conventionally thought to express EBNA3B (White et al. 2012).

As indicated previously, the epigenetic nature of gene regulation mediated by the EBNA3s may continue after they are (presumably) no longer expressed. For instance, *BIM/BCL2L11*, repressed by EBNA3A and EBNA3C, is usually methylated in latency I EBV-positive BL, but often mutated or deleted in EBV-negative BL (Paschos et al. 2009; Richter-Larrea et al. 2010). Similar phenomena have been observed for $p16^{INK4a}$ (Klangby et al. 1998; Nagy et al. 2005). In the absence

of validated reagents for their detection, the expression of EBNA3s in lymphomas remains inconclusive, but even the absence of the proteins themselves does not preclude essential roles, through epigenetic imprinting, in the development of a variety of EBV-associated tumours.

7 Summary and Outlook

Eukaryotic gene regulation is remarkably complicated, involving very large multiprotein complexes with enzymatic and structural functions, covalent modifications to histones and DNA, changes in the 3D organisation of chromatin and even the distribution of genes within nuclear space (Bickmore 2013; Bickmore and van Steensel 2013). Added to this enormous complexity is the problem of transferring expression patterns to progeny cells after DNA replication and mitosis—that is, creating some kind of epigenetic memory of gene expression programmes (Campos et al. 2014). Our current understanding of how the EBNA3 proteins fit into this broad picture of gene regulation has barely scratched the surface—but the results so far are interesting and thought provoking. Already, it appears that these viral factors manipulate multiple facets of chromatin organisation and function, but it is obvious that many issues remain unresolved. For example:

1. What is the explanation for the variety of EBNA3-regulated host genes? Is it merely a reflection of the regulatory elements that EBNA3-targeting factors bind? Or is it a more coordinated manipulation of a physiological B cell transcription programme(s) determined by differentiation-specific combinations and permutations from a group of host transcription factors (that probably includes among others RBP-JK/CBF1, PU1, SPI1, RUNX3, ATF2, BATF and IRF4)?
2. What are the features of EBNA3A, EBNA3B and EBNA3C that are responsible for their unique activities? This is particularly important for EBNA3A and EBNA3C that often cooperate, but do not appear to be interchangeable.
3. How do EBNA3s interact with the PcG protein system—is it by direct protein:protein contacts or by less direct means involving chromatin modifications or may be non-coding RNAs?
4. Are all the EBNA3 binding sites identified across the genome functionally significant (i.e. does localisation of a particular EBNA3 as revealed by ChIP-seq always result in a change in gene expression that is physiologically relevant)?
5. Where multiple EBNAs apparently bind to the same genomic locus, what determines which is functionally dominant or are they always mutually exclusive on a single site? What are the molecular interactions responsible for specific patterns of chromatin looping?
6. Do the EBNA3s bind to and regulate chromatinised EBV episomes? Although there are models suggesting this, so far there is little direct evidence.
7. Do the EBNA3s have functions distinct from their ability to regulate gene expression? Of particular importance is their suggested role(s) in modulation of the DDR.

8. Do the EBNA3s function in the same way during B cell infection in vivo as they do in vitro? For EBNA3B, there are limited data to suggest this is the case, but for EBNA3A and EBNA3C so far nothing is known. Further experiments with mice reconstituted with components of the human immune system will hopefully go some way to answer this question.

Striving for complete biochemical descriptions of EBNA3 function, coupled to precise genetic dissection of each protein will undoubtedly answer many of these questions and provide unique insights into EBV biology and disease pathogenesis, also into fundamental mechanisms of gene regulation. However, there is an important caveat that should not be overlooked—most of the studies described here have utilised the B95.8 strain of EBV or genes derived from it. This should be considered a 'laboratory-adapted' strain, chosen for its effectiveness in the ex vivo transformation of B cells, so a long-term goal must be to determine whether the EBNA network of interactions is conserved in virus strains that could be considered more representative of a 'wild-type' EBV. One should keep in mind the selection pressures that have created and 'moulded' these proteins: EBV did not evolve over millions of years to transform B cells into LCLs, nor to create B cell lymphomas. We should aim to determine what aspects of the fine virus–host equilibrium the EBNA3 proteins have evolved to maintain.

References

Acosta JC, Gil J (2012) Senescence: a new weapon for cancer therapy. Trends Cell Biol 22:211–219

Allday MJ (2013) EBV finds a polycomb-mediated, epigenetic solution to the problem of oncogenic stress responses triggered by infection. Front Genet 4:212

Allday MJ, Crawford DH, Griffin BE (1988) Prediction and demonstration of a novel Epstein-Barr virus nuclear antigen. Nucleic Acids Res 16:4353–4367

Allday MJ, Crawford DH, Griffin BE (1989) Epstein-Barr virus latent gene expression during the initiation of B cell immortalization. J Gen Virol 70(Pt 7):1755–1764

Allday MJ, Farrell PJ (1994) Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G1-arrested cells. J Virol 68:3491–3498

Altmann M, Hammerschmidt W (2005) Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. PLoS Biol 3:e404

Anderton E, Yee J, Smith P, Crook T, White RE, Allday MJ (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 27:421–433

Arvey A, Tempera I, Tsai K, Chen HS, Tikhmyanova N, Klichinsky M, Leslie C, Lieberman PM (2012) An atlas of the Epstein-Barr virus transcriptome and epigenome reveals host-virus regulatory interactions. Cell Host Microbe 12:233–245

Babcock GJ, Decker LL, Freeman RB, Thorley-Lawson DA (1999) Epstein-Barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. J Exp Med 190:567–576

Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA (1998) EBV persistence in memory B cells in vivo. Immunity 9:395–404

Babcock GJ, Hochberg D, Thorley-Lawson AD (2000) The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. Immunity 13:497–506

Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Seguin C et al (1984) DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310:207–211

Bain M, Watson RJ, Farrell PJ, Allday MJ (1996) Epstein-Barr virus nuclear antigen 3C is a powerful repressor of transcription when tethered to DNA. J Virol 70:2481–2489

Bajaj BG, Murakami M, Cai Q, Verma SC, Lan K, Robertson ES (2008) Epstein-Barr virus nuclear antigen 3C interacts with and enhances the stability of the c-Myc oncoprotein. J Virol 82:4082–4090

Banerjee S, Lu J, Cai Q, Saha A, Jha HC, Dzeng RK, Robertson ES (2013) The EBV latent antigen 3C inhibits apoptosis through targeted regulation of interferon regulatory factors 4 and 8. PLoS Pathog 9:e1003314

Barolo S, Stone T, Bang AG, Posakony JW (2002) Default repression and Notch signaling: hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to suppressor of hairless. Genes Dev 16:1964–1976

Bartek J, Bartkova J, Lukas J (2007) DNA damage signalling guards against activated oncogenes and tumour progression. Oncogene 26:7773–7779

Bazot Q, Deschamps T, Tafforeau L, Siouda M, Leblanc P, Harth-Hertle ML, Rabourdin-Combe C, Lotteau V, Kempkes B, Tommasino M, Gruffat H, Manet E (2014) Epstein-Barr virus nuclear antigen 3A protein regulates CDKN2B transcription via interaction with MIZ-1. Nucleic Acids Res 42:9700–9716

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326

Bickmore WA (2013) The spatial organization of the human genome. Annu Rev Genomics Hum Genet 14:67–84

Bickmore WA, van Steensel B (2013) Genome architecture: domain organization of interphase chromosomes. Cell 152:1270–1284

Bourillot PY, Waltzer L, Sergeant A, Manet E (1998) Transcriptional repression by the Epstein-Barr virus EBNA3A protein tethered to DNA does not require RBP-Jkappa. J Gen Virol 79(Pt 2):363–370

Braig M, Schmitt CA (2006) Oncogene-induced senescence: putting the brakes on tumor development. Cancer Res 66:2881–2884

Brookes S, Rowe J, Ruas M, Llanos S, Clark PA, Lomax M, James MC, Vatcheva R, Bates S, Vousden KH, Parry D, Gruis N, Smit N, Bergman W, Peters G (2002) INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. EMBO J 21:2936–2945

Brown AC, Baigent SJ, Smith LP, Chattoo JP, Petherbridge LJ, Hawes P, Allday MJ, Nair V (2006) Interaction of MEQ protein and C-terminal-binding protein is critical for induction of lymphomas by Marek's disease virus. Proc Natl Acad Sci USA 103:1687–1692

Buck M, Burgess A, Stirzaker R, Krauer K, Sculley T (2006) Epstein-Barr virus nuclear antigen 3A contains six nuclear-localization signals. J Gen Virol 87:2879–2884

Burgess A, Buck M, Krauer K, Sculley T (2006) Nuclear localization of the Epstein-Barr virus EBNA3B protein. J Gen Virol 87:789–793

Cai Q, Guo Y, Xiao B, Banerjee S, Saha A, Lu J, Glisovic T, Robertson ES (2011) Epstein-Barr virus nuclear antigen 3C stabilizes Gemin3 to block p53-mediated apoptosis. PLoS Pathog 7:e1002418

Cai X, Schafer A, Lu S, Bilello JP, Desrosiers RC, Edwards R, Raab-Traub N, Cullen BR (2006) Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog 2:e23

Calderwood MA, Lee S, Holthaus AM, Blacklow SC, Kieff E, Johannsen E (2011) Epstein-Barr virus nuclear protein 3C binds to the N-terminal (NTD) and beta trefoil domains (BTD) of RBP/CSL; only the NTD interaction is essential for lymphoblastoid cell growth. Virology 414:19–25

Calderwood MA, Venkatesan K, Xing L, Chase MR, Vazquez A, Holthaus AM, Ewence AE, Li N, Hirozane-Kishikawa T, Hill DE, Vidal M, Kieff, Johannsen E (2007) Epstein-Barr virus and virus human protein interaction maps. Proc Natl Acad Sci USA 104:7606–7611

Campos EI, Stafford JM, Reinberg D (2014) Epigenetic inheritance: histone bookmarks across generations. Trends Cell Biol 24:664–674

Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10:295–304

Chen A, Divisconte M, Jiang X, Quink C, Wang F (2005) Epstein-Barr virus with the latent infection nuclear antigen 3B completely deleted is still competent for B-cell growth transformation in vitro. J Virol 79:4506–4509

Chen A, Zhao B, Kieff E, Aster JC, Wang F (2006) EBNA-3B- and EBNA-3C-regulated cellular genes in Epstein-Barr virus-immortalized lymphoblastoid cell lines. J Virol 80:10139–10150

Chinnadurai G (2007) Transcriptional regulation by C-terminal binding proteins. Int J Biochem Cell Biol 39:1593–1607

Choudhuri T, Verma SC, Lan K, Murakami M, Robertson ES (2007) The ATM/ATR signaling effector Chk2 is targeted by Epstein-Barr virus nuclear antigen 3C to release the G2/M cell cycle block. J Virol 81:6718–6730

Choudhuri T, Verma SC, Lan K, Robertson ES (2006) Expression of alpha V integrin is modulated by Epstein-Barr virus nuclear antigen 3C and the metastasis suppressor Nm23-H1 through interaction with the GATA-1 and Sp1 transcription factors. Virology 351:58–72

Cludts I, Farrell PJ (1998) Multiple functions within the Epstein-Barr virus EBNA-3A protein. J Virol 72:1862–1869

Cooper A, Johannsen E, Maruo S, Cahir-McFarland E, Illanes D, Davidson D, Kieff E (2003) EBNA3A association with RBP-Jkappa down-regulates c-myc and Epstein-Barr virus-transformed lymphoblast growth. J Virol 77:999–1010

Cotter MA 2nd, Robertson ES (2000) Modulation of histone acetyltransferase activity through interaction of epstein-barr nuclear antigen 3C with prothymosin alpha. Mol Cell Biol 20:5722–5735

Crawford DH (2001) Biology and disease associations of Epstein-Barr virus. Philos Trans R Soc Lond B Biol Sci 356:461–473

Dalbies-Tran R, Stigger-Rosser E, Dotson T, Sample CE (2001) Amino acids of Epstein-Barr virus nuclear antigen 3A essential for repression of Jkappa-mediated transcription and their evolutionary conservation. J Virol 75:90–99

Dang CV, O'Donnell KA, Juopperi T (2005) The great MYC escape in tumorigenesis. Cancer Cell 8:177–178

Deacon EM, Pallesen G, Niedobitek G, Crocker J, Brooks L, Rickinson AB, Young LS (1993) Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. J Exp Med 177:339–349

Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W (1998) Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc Natl Acad Sci USA 95:8245–8250

Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD (2002) IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol 168:3195–3204

Egle A, Harris AW, Bouillet P, Cory S (2004) Bim is a suppressor of Myc-induced mouse B cell leukemia. Proc Natl Acad Sci USA 101:6164–6169

Ehlers B, Ochs A, Leendertz F, Goltz M, Boesch C, Matz-Rensing K (2003) Novel simian homologues of Epstein-Barr virus. J Virol 77:10695–10699

Ehlers B, Spiess K, Leendertz F, Peeters M, Boesch C, Gatherer D, McGeoch DJ (2010) Lymphocryptovirus phylogeny and the origins of Epstein-Barr virus. J Gen Virol 91:630–642

Elliott SL, Suhrbier A, Miles JJ, Lawrence G, Pye SJ, Le TT, Rosenstengel A, Nguyen T, Allworth A, Burrows SR, Cox J, Pye D, Moss DJ, Bharadwaj M (2008) Phase I trial of a CD8+T-cell peptide epitope-based vaccine for infectious mononucleosis. J Virol 82:1448–1457

Epstein MA (2001) Historical background. Philos Trans R Soc Lond B Biol Sci 356:413–420

Feederle R, Bartlett EJ, Delecluse HJ (2010) Epstein-Barr virus genetics: talking about the BAC generation. Herpesviridae 1:6

Fischer SF, Bouillet P, O'Donnell K, Light A, Tarlinton DM, Strasser A (2007) Proapoptotic BH3-only protein Bim is essential for developmentally programmed death of germinal center-derived memory B cells and antibody-forming cells. Blood 110:3978–3984

Garrido JL, Maruo S, Takada K, Rosendorff A (2009) EBNA3C interacts with Gadd34 and counteracts the unfolded protein response. Virol J 6:231

Gil J, Peters G (2006) Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol 7:667–677

Gorzer I, Niesters HG, Cornelissen JJ, Puchhammer-Stockl E (2006) Characterization of Epstein-Barr virus Type I variants based on linked polymorphism among EBNA3A, -3B, and -3C genes. Virus Res 118:105–114

Gottschalk S, Ng CY, Perez M, Smith CA, Sample C, Brenner MK, Heslop HE, Rooney CM (2001) An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs. Blood 97:835–843

Gruhne B, Sompallae R, Masucci MG (2009) Three Epstein-Barr virus latency proteins independently promote genomic instability by inducing DNA damage, inhibiting DNA repair and inactivating cell cycle checkpoints. Oncogene 28:3997–4008

Guruharsha KG, Kankel MW, Artavanis-Tsakonas S (2012) The Notch signalling system: recent insights into the complexity of a conserved pathway. Nat Rev Genet 13:654–666

Halazonetis TD, Gorgoulis VG, Bartek J (2008) An oncogene-induced DNA damage model for cancer development. Science 319:1352–1355

Harth-Hertle ML, Scholz BA, Erhard F, Glaser LV, Dolken L, Zimmer R, Kempkes B (2013) Inactivation of intergenic enhancers by EBNA3A initiates and maintains polycomb signatures across a chromatin domain encoding CXCL10 and CXCL9. PLoS Pathog 9:e1003638

Hawkins JB, Delgado-Eckert E, Thorley-Lawson DA, Shapiro M (2013) The cycle of EBV infection explains persistence, the sizes of the infected cell populations and which come under CTL regulation. PLoS Pathog 9:e1003685

Hemann MT, Bric A, Teruya-Feldstein J, Herbst A, Nilsson JA, Cordon-Cardo C, Cleveland JL, Tansey WP, Lowe SW (2005) Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. Nature 436:807–811

Hennessy K, Fennewald S, Kieff E (1985) A third viral nuclear protein in lymphoblasts immortalized by Epstein-Barr virus. Proc Natl Acad Sci USA 82:5944–5948

Hennessy K, Wang F, Bushman EW, Kieff E (1986) Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family. Proc Natl Acad Sci USA 83:5693–5697

Hertle ML, Popp C, Petermann S, Maier S, Kremmer E, Lang R, Mages J, Kempkes B (2009) Differential gene expression patterns of EBV infected EBNA-3A positive and negative human B lymphocytes. PLoS Pathog 5:e1000506

Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, Bollard CM, Liu H, Wu MF, Rochester RJ, Amrolia PJ, Hurwitz JL, Brenner MK, Rooney CM (2010) Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 115:925–935

Hickabottom M, Parker GA, Freemont P, Crook T, Allday MJ (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 277:47197–47204

Hislop AD, Taylor GS, Sauce D, Rickinson AB (2007) Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. Annu Rev Immunol 25:587–617

Humeniuk R, Rosu-Myles M, Fares J, Koller R, Bies J, Wolff L (2013) The role of tumor suppressor p15Ink4b in the regulation of hematopoietic progenitor cell fate. Blood Cancer J 3:e99

Isaksson A, Berggren M, Ricksten A (2003) Epstein-Barr virus U leader exon contains an internal ribosome entry site. Oncogene 22:572–581

Jha HC, Aj MP, Saha A, Banerjee S, Lu J, Robertson ES (2014) Epstein-Barr virus essential antigen EBNA3C attenuates H2AX expression. J Virol 88:3776–3788

Jha HC, Lu J, Saha A, Cai Q, Banerjee S, Prasad MA, Robertson ES (2013) EBNA3C-mediated regulation of aurora kinase B contributes to Epstein-Barr virus-induced B-cell proliferation through modulation of the activities of the retinoblastoma protein and apoptotic caspases. J Virol 87:12121–12138

Jiang H, Cho YG, Wang F (2000) Structural, functional, and genetic comparisons of Epstein-Barr virus nuclear antigen 3A, 3B, and 3C homologues encoded by the rhesus lymphocryptovirus. J Virol 74:5921–5932

Jiang S, Willox B, Zhou H, Holthaus AM, Wang A, Shi TT, Maruo S, Kharchenko PV, Johannsen EC, Kieff E, Zhao B (2014) Epstein-Barr virus nuclear antigen 3C binds to BATF/IRF4 or SPI1/IRF4 composite sites and recruits Sin3A to repress CDKN2A. Proc Natl Acad Sci USA 111:421–426

Jimenez-Ramirez C, Brooks AJ, Forshell LP, Yakimchuk K, Zhao B, Fulgham TZ, Sample CE (2006) Epstein-Barr virus EBNA-3C is targeted to and regulates expression from the bidirectional LMP-1/2B promoter. J Virol 80:11200–11208

Johannsen E, Miller CL, Grossman SR, Kieff E (1996) EBNA-2 and EBNA-3C extensively and mutually exclusively associate with RBPJkappa in Epstein-Barr virus-transformed B lymphocytes. J Virol 70:4179–4183

Joseph AM, Babcock GJ, Thorley-Lawson DA (2000) Cells expressing the Epstein-Barr virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils. J Virol 74:9964–9971

Kashuba E, Yurchenko M, Yenamandra SP, Snopok B, Isaguliants M, Szekely L, Klein G (2008) EBV-encoded EBNA-6 binds and targets MRS18-2 to the nucleus, resulting in the disruption of pRb-E2F1 complexes. Proc Natl Acad Sci USA 105:5489–5494

Kaul R, Verma SC, Murakami M, Lan K, Choudhuri T, Robertson ES (2006) Epstein-Barr virus protein can upregulate cyclo-oxygenase-2 expression through association with the suppressor of metastasis Nm23-H1. J Virol 80:1321–1331

Kelly G, Bell A, Rickinson A (2002) Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. Nat Med 8:1098–1104

Kelly GL, Long HM, Stylianou J, Thomas WA, Leese A, Bell AI, Bornkamm GW, Mautner J, Rickinson AB, Rowe M (2009) An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link. PLoS Pathog 5:e1000341

Kelly GL, Stylianou J, Rasaiyaah J, Wei W, Thomas W, Croom-Carter D, Kohler C, Spang R, Woodman C, Kellam P, Rickinson AB, Bell AI (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 87:2882–2894

Khanna R, Burrows SR, Kurilla MG, Jacob CA, Misko IS, Sculley TB, Kieff E, Moss DJ (1992) Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J Exp Med 176:169–176

Khanna R, Slade RW, Poulsen L, Moss DJ, Burrows SR, Nicholls J, Burrows JM (1997) Evolutionary dynamics of genetic variation in Epstein-Barr virus isolates of diverse geographical origins: evidence for immune pressure-independent genetic drift. J Virol 71:8340–8346

Kheradmand Kia S, Solaimani Kartalaei P, Farahbakhshian E, Pourfarzad F, von Lindern M, Verrijzer CP (2009) EZH2-dependent chromatin looping controls INK4a and INK4b, but not ARF, during human progenitor cell differentiation and cellular senescence. Epigenetics Chromatin 2:16

Kienzle N, Young DB, Liaskou D, Buck M, Greco S, Sculley TB (1999) Intron retention may regulate expression of Epstein-Barr virus nuclear antigen 3 family genes. J Virol 73:1195–1204

Kim SM, Kang SH, Lee WK (2006) Identification of two types of naturally-occurring intertypic recombinants of Epstein-Barr virus. Mol Cells 21:302–307

Kis LL, Takahara M, Nagy N, Klein G, Klein E (2006) Cytokine mediated induction of the major Epstein-Barr virus (EBV)-encoded transforming protein, LMP-1. Immunol Lett 104:83–88

Kis LL, Salamon D, Persson EK, Nagy N, Scheeren FA, Spits H, Klein G, Klein E (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 USA 107:872–877

Klangby U, Okan I, Magnusson KP, Wendland M, Lind P, Wiman KG (1998) p16/INK4a and p15/INK4b gene methylation and absence of p16/INK4a mRNA and protein expression in Burkitt's lymphoma. Blood 91:1680–1687

Knight JS, Lan K, Subramanian C, Robertson ES (2003) Epstein-Barr virus nuclear antigen 3C recruits histone deacetylase activity and associates with the corepressors mSin3A and NCoR in human B-cell lines. J Virol 77:4261–4272

Knight JS, Robertson ES (2004) Epstein-Barr virus nuclear antigen 3C regulates cyclin A/p27 complexes and enhances cyclin A-dependent kinase activity. J Virol 78:1981–1991

Knight JS, Sharma N, Kalman DE, Robertson ES (2004) A cyclin-binding motif within the amino-terminal homology domain of EBNA3C binds cyclin A and modulates cyclin A-dependent kinase activity in Epstein-Barr virus-infected cells. J Virol 78:12857–12867

Knight JS, Sharma N, Robertson ES (2005a) Epstein-Barr virus latent antigen 3C can mediate the degradation of the retinoblastoma protein through an SCF cellular ubiquitin ligase. Proc Natl Acad Sci USA 102:18562–18566

Knight JS, Sharma N, Robertson ES (2005b) SCFSkp2 complex targeted by Epstein-Barr virus essential nuclear antigen. Mol Cell Biol 25:1749–1763

Kohlhof H, Hampel F, Hoffmann R, Burtscher H, Weidle UH, Holzel M, Eick D, Zimber-Strobl U, Strobl LJ (2009) Notch1, Notch2, and Epstein-Barr virus-encoded nuclear antigen 2 signaling differentially affects proliferation and survival of Epstein-Barr virus-infected B cells. Blood 113:5506–5515

Krauer K, Buck M, Flanagan J, Belzer D, Sculley T (2004a) Identification of the nuclear localization signals within the Epstein-Barr virus EBNA-6 protein. J Gen Virol 85:165–172

Krauer KG, Buck M, Belzer DK, Flanagan J, Chojnowski GM, Sculley TB (2004b) The Epstein-Barr virus nuclear antigen-6 protein co-localizes with EBNA-3 and survival of motor neurons protein. Virology 318:280–294

Le Roux A, Berebbi M, Moukaddem M, Perricaudet M, Joab I (1993) Identification of a short amino acid sequence essential for efficient nuclear targeting of the Epstein-Barr virus nuclear antigen 3A. J Virol 67:1716–1720

Le Roux A, Kerdiles B, Walls D, Dedieu JF, Perricaudet M (1994) The Epstein-Barr virus determined nuclear antigens EBNA-3A, -3B, and -3C repress EBNA-2-mediated transactivation of the viral terminal protein 1 gene promoter. Virology 205:596–602

Lee S, Sakakibara S, Maruo S, Zhao B, Calderwood MA, Holthaus AM, Lai CY, Takada K, Kieff E, Johannsen E (2009) Epstein-Barr virus nuclear protein 3C domains necessary for lymphoblastoid cell growth: interaction with RBP-Jkappa regulates TCL1. J Virol 83:12368–12377

Lin J, Johannsen E, Robertson E, Kieff E (2002) Epstein-Barr virus nuclear antigen 3C putative repression domain mediates coactivation of the LMP1 promoter with EBNA-2. J Virol 76:232–242

Lowe SW, Cepero E, Evan G (2004) Intrinsic tumour suppression. Nature 432:307–315

Maruo S, Johannsen E, Illanes D, Cooper A, Kieff E (2003) Epstein-Barr virus nuclear protein EBNA3A is critical for maintaining lymphoblastoid cell line growth. J Virol 77:10437–10447

Maruo S, Johannsen E, Illanes D, Cooper A, Zhao B, Kieff E (2005) Epstein-Barr virus nuclear protein 3A domains essential for growth of lymphoblasts: transcriptional regulation through RBP-Jkappa/CBF1 is critical. J Virol 79:10171–10179

Maruo S, Wu Y, Ishikawa S, Kanda T, Iwakiri D, Takada K (2006) Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression and growth maintenance of lymphoblastoid cells. Proc Natl Acad Sci USA 103:19500–19505

Maruo S, Wu Y, Ito T, Kanda T, Kieff ED, Takada K (2009) Epstein-Barr virus nuclear protein EBNA3C residues critical for maintaining lymphoblastoid cell growth. Proc Natl Acad Sci USA 106:4419–4424

Maruo S, Zhao B, Johannsen E, Kieff E, Zou J, Takada K (2011) Epstein-Barr virus nuclear antigens 3C and 3A maintain lymphoblastoid cell growth by repressing p16INK4A and p14ARF expression. Proc Natl Acad Sci USA 108:1919–1924

McAulay KA, Haque T, Urquhart G, Bellamy C, Guiretti D, Crawford DH (2009) Epitope specificity and clonality of EBV-specific CTLs used to treat posttransplant lymphoproliferative disease. J Immunol 182:3892–3901

McClellan MJ, Khasnis S, Wood CD, Palermo RD, Schlick SN, Kanhere AS, Jenner RG, West MJ (2012) Downregulation of integrin receptor-signaling genes by Epstein-Barr virus EBNA 3C via promoter-proximal and -distal binding elements. J Virol 86:5165–5178

McClellan MJ, Wood CD, Ojeniyi O, Cooper TJ, Kanhere A, Arvey A, Webb HM, Palermo RD, Harth-Hertle ML, Kempkes B, Jenner RG, West MJ (2013) Modulation of enhancer looping and differential gene targeting by Epstein-Barr virus transcription factors directs cellular reprogramming. PLoS Pathog 9:e1003636

Midgley RS, Bell AI, McGeoch DJ, Rickinson AB (2003) Latent gene sequencing reveals familial relationships among Chinese Epstein-Barr virus strains and evidence for positive selection of A11 epitope changes. J Virol 77:11517–11530

Midgley RS, Blake NW, Yao QY, Croom-Carter D, Cheung ST, Leung SF, Chan AT, Johnson PJ, Huang D, Rickinson AB, Lee SP (2000) Novel intertypic recombinants of epstein-barr virus in the chinese population. J Virol 74:1544–1548

Mlecnik B, Tosolini M, Charoentong P, Kirilovsky A, Bindea G, Berger A, Camus M, Gillard M, Bruneval P, Fridman WH, Pages F, Trajanoski Z, Galon J (2010) Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. Gastroenterology 138:1429–1440

Morel V, Lecourtois M, Massiani O, Maier D, Preiss A, Schweisguth F (2001) Transcriptional repression by suppressor of hairless involves the binding of a hairless-dCtBP complex in Drosophila. Curr Biol 11:789–792

Murray RJ, Kurilla MG, Brooks JM, Thomas WA, Rowe M, Kieff E, Rickinson AB (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 176:157–168

Nagy E, Veress G, Szarka K, Csoma E, Beck Z (2005) Frequent methylation of p16INK4A/p14ARF promoters in tumorigenesis of Epstein-Barr virus transformed lymphoblastoid cell lines. Anticancer Res 25:2153–2160

Nagy N, Adori M, Rasul A, Heuts F, Salamon D, Ujvari D, Madapura HS, Leveau B, Klein G, Klein E (2012) Soluble factors produced by activated CD4+T cells modulate EBV latency. Proc Natl Acad Sci USA 109:1512–1517

Naumova N, Smith EM, Zhan Y, Dekker J (2012) Analysis of long-range chromatin interactions using chromosome conformation capture. Methods 58:192–203

Nguyen-Van D, Keane C, Han E, Jones K, Nourse JP, Vari F, Ross N, Crooks P, Ramuz O, Green M, Griffith L, Trappe R, Grigg A, Mollee P, Gandhi MK (2011) Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly expresses EBNA3A with conserved CD8 T-cell epitopes. Am J Blood Res 1:146–159

Nikiforow S, Lacasce AS (2014) Targeting Epstein-Barr virus-associated lymphomas. J Clin Oncol 32:830–832

Nikitin PA, Luftig MA (2012) The DNA damage response in viral-induced cellular transformation. Br J Cancer 106:429–435

Nikitin PA, Yan CM, Forte E, Bocedi A, Tourigny JP, White RE, Allday MJ, Patel A, Dave SS, Kim W, Hu K, Guo J, Tainter D, Rusyn E, Luftig MA (2010) An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. Cell Host Microbe 8:510–522

O'Nions J, Turner A, Craig R, Allday MJ (2006) Epstein-Barr virus selectively deregulates DNA damage responses in normal B cells but has no detectable effect on regulation of the tumor suppressor p53. J Virol 80:12408–12413

Palmero I, Holder A, Sinclair AJ, Dickson C, Peters G (1993) Cyclins D1 and D2 are differentially expressed in human B-lymphoid cell lines. Oncogene 8:1049–1054

Parker GA, Crook T, Bain M, Sara EA, Farrell PJ, Allday MJ (1996) Epstein-Barr virus nuclear antigen (EBNA)3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. Oncogene 13:2541–2549

Parker GA, Touitou R, Allday MJ (2000) Epstein-Barr virus EBNA3C can disrupt multiple cell cycle checkpoints and induce nuclear division divorced from cytokinesis. Oncogene 19:700–709

Paschos K, Parker GA, Watanatanasup E, White RE, Allday MJ (2012) BIM promoter directly targeted by EBNA3C in polycomb-mediated repression by EBV. Nucleic Acids Res 40:7233–7246

Paschos K, Smith P, Anderton E, Middeldorp JM, White RE, Allday MJ (2009) Epstein-barr virus latency in B cells leads to epigenetic repression and CpG methylation of the tumour suppressor gene Bim. PLoS Pathog 5:e1000492

Peng R, Moses SC, Tan J, Kremmer E, Ling PD (2005) The Epstein-Barr virus EBNA-LP protein preferentially coactivates EBNA2-mediated stimulation of latent membrane proteins expressed from the viral divergent promoter. J Virol 79:4492–4505

Petti L, Kieff E (1988) A sixth Epstein-Barr virus nuclear protein (EBNA3B) is expressed in latently infected growth-transformed lymphocytes. J Virol 62:2173–2178

Petti L, Sample C, Kieff E (1990) Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. Virology 176:563–574

Petti L, Sample J, Wang F, Kieff E (1988) A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. J Virol 62:1330–1338

Pokrovskaja K, Ehlin-Henriksson B, Bartkova J, Bartek J, Scuderi R, Szekely L, Wiman KG, Klein G (1996) Phenotype-related differences in the expression of D-type cyclins in human B cell-derived lines. Cell Growth Differ 7:1723–1732

Price AM, Tourigny JP, Forte E, Salinas RE, Dave SS, Luftig MA (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 86:11096–11106

Radkov SA, Bain M, Farrell PJ, West M, Rowe M, Allday MJ (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 71:8552–8562

Radkov SA, Touitou R, Brehm A, Rowe M, West M, Kouzarides T, Allday MJ (1999) Epstein-Barr virus nuclear antigen 3C interacts with histone deacetylase to repress transcription. J Virol 73:5688–5697

Richter-Larrea JA, Robles EF, Fresquet V, Beltran E, Rullan AJ, Agirre X, Calasanz MJ, Panizo C, Richter JA, Hernandez JM, Roman-Gomez J, Prosper F, Martinez-Climent JA (2010) Reversion of epigenetically mediated BIM silencing overcomes chemoresistance in Burkitt lymphoma. Blood 116:2531–2542

Ricksten A, Kallin B, Alexander H, Dillner J, Fahraeus R, Klein G, Lerner R, Rymo L (1988) BamHI E region of the Epstein-Barr virus genome encodes three transformation-associated nuclear proteins. Proc Natl Acad Sci USA 85:995–999

Rivailler P, Cho YG, Wang F (2002) Complete genomic sequence of an Epstein-Barr virus-related herpesvirus naturally infecting a new world primate: a defining point in the evolution of oncogenic lymphocryptoviruses. J Virol 76:12055–12068

Robertson ES, Grossman S, Johannsen E, Miller C, Lin J, Tomkinson B, Kieff E (1995) Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein J kappa. J Virol 69:3108–3116

Robertson ES, Lin J, Kieff E (1996) The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa). J Virol 70:3068–3074

Rochford R, Hobbs MV, Garnier JL, Cooper NR, Cannon MJ (1993) Plasmacytoid differentiation of Epstein-Barr virus-transformed B cells in vivo is associated with reduced expression of viral latent genes. Proc Natl Acad Sci USA 90:352–356

Rosendorff A, Illanes D, David G, Lin J, Kieff E, Johannsen E (2004) EBNA3C coactivation with EBNA2 requires a SUMO homology domain. J Virol 78:367–377

Roughan JE, Thorley-Lawson DA (2009) The intersection of Epstein-Barr virus with the germinal center. J Virol 83:3968–3976

Saha A, Bamidele A, Murakami M, Robertson ES (2011) EBNA3C attenuates the function of p53 through interaction with inhibitor of growth family proteins 4 and 5. J Virol 85:2079–2088

Saha A, Lu J, Morizur L, Upadhyay SK, Aj MP, Robertson ES (2012) E2F1 mediated apoptosis induced by the DNA damage response is blocked by EBV nuclear antigen 3C in lymphoblastoid cells. PLoS Pathog 8:e1002573

Saha A, Murakami M, Kumar P, Bajaj B, Sims K, Robertson ES (2009) Epstein-Barr virus nuclear antigen 3C augments Mdm2-mediated p53 ubiquitination and degradation by deubiquitinating Mdm2. J Virol 83:4652–4669

Salamon D, Adori M, Ujvari D, Wu L, Kis LL, Madapura HS, Nagy N, Klein G, Klein E (2012) Latency type-dependent modulation of Epstein-Barr virus-encoded latent membrane protein 1 expression by type I interferons in B cells. J Virol 86:4701–4707

Sample C, Parker B (1994) Biochemical characterization of Epstein-Barr virus nuclear antigen 3A and 3C proteins. Virology 205:534–539

Sample J, Young L, Martin B, Chatman T, Kieff E, Rickinson A (1990) Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. J Virol 64:4084–4092

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602

Sewalt RG, Gunster MJ, van der Vlag J, Satijn DP, Otte AP (1999) C-Terminal binding protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins. Mol Cell Biol 19:777–787

Shannon-Lowe C, Baldwin G, Feederle R, Bell A, Rickinson A, Delecluse HJ (2005) Epstein-Barr virus-induced B-cell transformation: quantitating events from virus binding to cell outgrowth. J Gen Virol 86:3009–3019

Sherr CJ (2012) Ink4-arf locus in cancer and aging. Wiley Interdiscip Rev Dev Biol 1:731–741

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953

Shi Y, Sawada J, Sui G, el Affar B, Whetstine JR, Lan F, Ogawa H, Luke MP, Nakatani Y, Shi Y (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature 422:735–738

Siemer D, Kurth J, Lang S, Lehnerdt G, Stanelle J, Kuppers R (2008) EBV transformation overrides gene expression patterns of B cell differentiation stages. Mol Immunol 45:3133–3141

Sinclair AJ, Palmero I, Peters G, Farrell PJ (1994) EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus. EMBO J 13:3321–3328

Skalska L, White RE, Franz M, Ruhmann M, Allday MJ (2010) Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP. PLoS Pathog 6:e1000951

Skalska L, White RE, Parker GA, Sinclair AJ, Paschos K, Allday MJ (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:e1003187

Spender LC, Cannell EJ, Hollyoake M, Wensing B, Gawn JM, Brimmell M, Packham G, Farrell PJ (1999) Control of cell cycle entry and apoptosis in B lymphocytes infected by Epstein-Barr virus. J Virol 73:4678–4688

Steven NM, Leese AM, Annels NE, Lee SP, Rickinson AB (1996) Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. J Exp Med 184:1801–1813

Stock JK, Giadrossi S, Casanova M, Brookes E, Vidal M, Koseki H, Brockdorff N, Fisher AG, Pombo A (2007) Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat Cell Biol 9:1428–1435

Strasser A (2005) The role of BH3-only proteins in the immune system. Nat Rev Immunol 5:189–200

Strowig T, Gurer C, Ploss A, Liu YF, Arrey F, Sashihara J, Koo G, Rice CM, Young JW, Chadburn A, Cohen JI, Munz C (2009) Priming of protective T cell responses against virus-induced tumors in mice with human immune system components. J Exp Med 206:1423–1434

Subramanian C, Cotter MA 2nd, Robertson ES (2001) Epstein-Barr virus nuclear protein EBNA-3C interacts with the human metastatic suppressor Nm23-H1: a molecular link to cancer metastasis. Nat Med 7:350–355

Sundqvist A, Sollerbrant K, Svensson C (1998) The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein-histone deacetylase complex. FEBS Lett 429:183–188

Tempera I, Lieberman PM (2014) Epigenetic regulation of EBV persistence and oncogenesis. Semin Cancer Biol 26:22–29

Thorley-Lawson DA, Allday MJ (2008) The curious case of the tumour virus: 50 years of Burkitt's lymphoma. Nat Rev Microbiol 6:913–924

Thorley-Lawson DA, Gross A (2004) Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med 350:1328–1337

Thorley-Lawson DA, Hawkins JB, Tracy SI, Shapiro M (2013) The pathogenesis of Epstein-Barr virus persistent infection. *Curr Opin Virol*

Thorley-Lawson DA, Strominger JL (1978) Reversible inhibition by phosphonoacetic acid of human B lymphocyte transformation by Epstein-Barr virus. Virology 86:423–431

Tomkinson B, Kieff E (1992a) Second-site homologous recombination in Epstein-Barr virus: insertion of type 1 EBNA 3 genes in place of type 2 has no effect on in vitro infection. J Virol 66:780–789

Tomkinson B, Kieff E (1992b) Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro. J Virol 66:2893–2903

Tomkinson B, Robertson E, Kieff E (1993) Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. J Virol 67:2014–2025

Touitou R, Arbach H, Cochet C, Feuillard J, Martin A, Raphael M, Joab I (2003) Heterogeneous Epstein-Barr virus latent gene expression in AIDS-associated lymphomas and in type I Burkitt's lymphoma cell lines. J Gen Virol 84:949–957

Touitou R, Hickabottom M, Parker G, Crook T, Allday MJ (2001) Physical and functional interactions between the corepressor CtBP and the Epstein-Barr virus nuclear antigen EBNA3C. J Virol 75:7749–7755

Touitou R, O'Nions J, Heaney J, Allday MJ (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 86:1269–1277

Vereide DT, Sugden B (2011) Lymphomas differ in their dependence on Epstein-Barr virus. Blood 117:1977–1985

Vousden KH, Prives C (2009) Blinded by the light: the growing complexity of p53. Cell 137:413–431

Waltzer L, Perricaudet M, Sergeant A, Manet E (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 70:5909–5915

Watanabe A, Maruo S, Ito T, Ito M, Katsumura KR, Takada K (2010) Epstein-Barr virus-encoded Bcl-2 homologue functions as a survival factor in Wp-restricted Burkitt lymphoma cell line P3HR-1. J Virol 84:2893–2901

West MJ (2006) Structure and function of the Epstein-Barr virus transcription factor, EBNA 3C. Curr Protein Pept Sci 7:123–136

West MJ, Webb HM, Sinclair AJ, Woolfson DN (2004) Biophysical and mutational analysis of the putative bZIP domain of Epstein-Barr virus EBNA 3C. J Virol 78:9431–9445

White RE, Groves IJ, Turro E, Yee J, Kremmer E, Allday MJ (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:e13979

White RE, Ramer PC, Naresh KN, Meixlsperger S, Pinaud L, Rooney C, Savoldo B, Coutinho R, Bodor C, Gribben J, Ibrahim HA, Bower M, Nourse JP, Gandhi MK, Middeldorp J, Cader FZ, Murray P, Munz C, Allday MJ (2012) EBNA3B-deficient EBV promotes B cell lymphomagenesis in humanized mice and is found in human tumors. J Clin Invest 122:1487–1502

Yao QY, Rowe M, Martin B, Young LS, Rickinson AB (1991) The Epstein-Barr virus carrier state: dominance of a single growth-transforming isolate in the blood and in the oropharynx of healthy virus carriers. J Gen Virol 72(Pt 7):1579–1590

Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, Gil J, Walsh MJ, Zhou MM (2010) Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell 38:662–674

Yee J, White RE, Anderton E, Allday MJ (2011) Latent Epstein-Barr virus can inhibit apoptosis in B cells by blocking the induction of NOXA expression. PLoS ONE 6:e28506

Yenamandra SP, Sompallae R, Klein G, Kashuba E (2009) Comparative analysis of the Epstein-Barr virus encoded nuclear proteins of EBNA-3 family. Comput Biol Med 39:1036–1042

Yi F, Saha A, Murakami M, Kumar P, Knight JS, Cai Q, Choudhuri T, Robertson ES (2009) Epstein-Barr virus nuclear antigen 3C targets p53 and modulates its transcriptional and apoptotic activities. Virology 388:236–247

Young LS, Dawson CW, Clark D, Rupani H, Busson P, Tursz T, Johnson A, Rickinson AB (1988) Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J Gen Virol 69(Pt 5):1051–1065

Young LS, Rickinson AB (2004) Epstein-Barr virus: 40 years on. Nat Rev Cancer 4:757–768

Young LS, Yao QY, Rooney CM, Sculley TB, Moss DJ, Rupani H, Laux G, Bornkamm GW, Rickinson AB (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

Young P, Anderton E, Paschos K, White R, Allday MJ (2008) Epstein-Barr virus nuclear antigen (EBNA) 3A induces the expression of and interacts with a subset of chaperones and co-chaperones. J Gen Virol 89:866–877

Zhang Q, Wang SY, Nottke AC, Rocheleau JV, Piston DW, Goodman RH (2006) Redox sensor CtBP mediates hypoxia-induced tumor cell migration. Proc Natl Acad Sci USA 103:9029–9033

Zhao B, Dalbies-Tran R, Jiang H, Ruf IK, Sample JT, Wang F, Sample CE (2003) Transcriptional regulatory properties of Epstein-Barr virus nuclear antigen 3C are conserved in simian lymphocryptoviruses. J Virol 77:5639–5648

Zhao B, Mar JC, Maruo S, Lee S, Gewurz BE, Johannsen E, Holton K, Rubio R, Takada K, Quackenbush J, Kieff E (2011) Epstein-Barr virus nuclear antigen 3C regulated genes in lymphoblastoid cell lines. Proc Natl Acad Sci USA 108:337–342

Zhao B, Sample CE (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 74:5151–5160

Zhao LJ, Subramanian T, Vijayalingam S, Chinnadurai G (2014) CtBP2 proteome: role of CtBP in E2F7-mediated repression and cell proliferation. Genes Cancer 5:31–40

Zimber-Strobl U, Strobl LJ (2001) EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes. Semin Cancer Biol 11:423–434