- Key EBV transcription factors hijack host cell factors to reprogramme B cells.
- This is mostly directed through binding to host gene enhancers and super-enhancers.
- Binding can promote or disrupt enhancer-promoter contacts to turn genes on or off.
- EBV activation of upstream MYC enhancers may promote upstream MYC translocations.
- Enhancer hub disruption by EBV can initiate long term gene silencing.
Chromatin reorganisation in Epstein Barr virus-infected cells and its role in cancer development

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**Abstract**

The oncogenic Epstein-Barr virus (EBV) growth transforms B cells and drives lymphoma and carcinoma development. The virus encodes four key transcription factors (EBNA2, EBNA3A, EBNA3B and EBNA3C) that hijack host cell factors to bind gene control elements and reprogramme infected B cells. These viral factors predominantly target long-range enhancers to alter the expression of host cell genes that control B cell growth and survival and facilitate virus persistence. Enhancer and super-enhancer binding by these EBNAs results in large-scale reorganisation of three-dimensional enhancer-promoter architecture to drive the overexpression of oncogenes, the silencing of tumour suppressors and the modulation of transcription, cell-cycle progression, migration and adhesion.
Introduction

Epstein-Barr virus (EBV) is an oncogenic γ-herpesvirus responsible for 200,000 lymphoma and carcinoma cases worldwide per year. Despite its oncogenicity, EBV infection is usually harmless and >95% of the worldwide population carry EBV as a life-long asymptomatic infection. B cell infection by EBV results in growth transformation and the establishment of a persistent latent infection in memory B cells [1]. An effective cytotoxic T lymphocyte response controls the number of EBV-infected cells and protects from tumour outgrowth [2].

Initial B cell growth transformation by EBV in vivo and EBV infection in vitro results in the expression of all latent genes including six nuclear proteins (Epstein-Barr virus nuclear antigens; EBNAs, 1, 2, 3A, 3B, 3C and leader protein), three latent membrane proteins (LMP1, 2A and 2B), numerous viral microRNAs and two highly-expressed non-coding RNAs. Five latent proteins (EBNA1, 2, 3A, 3C and LMP1) are critical for the transformation of B cells [3-6]. EBNA2, 3A and 3C play key roles in the B-cell transcriptional reprogramming which is crucial for the outgrowth and survival of EBV-immortalised cells. Although not required for B cell transformation, EBNA3B cooperates in the regulation of large numbers of cellular genes [7]. This review focuses on recent advances in our understanding of how these four viral transcription factors (TFs) deregulate host cell genes through the hijacking of long-range regulatory elements and the reconfiguration of enhancer-promoter architecture to induce growth transforming cancer-promoting events.

EBV TFs associate with host cell factors to reprogramme the B cell transcriptome

EBNA2, EBNA3A, EBNA3B and EBNA3C alter the expression of thousands of B cell genes involved in key cellular processes such as lymphocyte activation, signalling, apoptosis,
transcription, cell cycle, cell adhesion and migration, including many proto-oncogenes and tumour suppressors. These EBV TFs do not bind DNA directly and bind gene control elements through interactions with B cell DNA-binding proteins. All four proteins bind the Notch signalling pathway TF, RBPJ (RBP-Jκ, CBF1, CSL) [8,9]. The association of EBNA2 and EBNA3 proteins with RBPJ is mutually exclusive and EBNA2 and EBNA3 proteins competitively bind to RBPJ-bound chromatin sites in the B cell genome [10-12]. The interaction between EBNA2, EBNA3A or EBNA3C and RBPJ is critical for EBV immortalised cell growth [13-16]. EBNA2 and EBNA3C also bind the ETS family member PU.1 [17,18]. More recently, EBNA3C binding to interferon regulatory factors (IRF) 4 and 8 and EBNA3A, EBNA3B and EBNA3C binding to the Runt-related transcription factor (RUNX) partner, core binding factor β (CBF β) was demonstrated [19,20].

EBNA2 is a transcriptional activator that interacts with histone acetyl transferases (HATs), the SWI/SNF chromatin remodeler and the basal transcription machinery, although it can also repress gene transcription through unknown mechanisms [21]. EBNA3A, EBNA3B and EBNA3C comprise a family of distantly-related genes and can repress and activate transcription individually and co-operatively [7]. EBNA3A and EBNA3C associate with the co-repressors C-terminal binding proteins 1 and 2 (CtBP) [22-24] and EBNA3C also binds the histone deacetylase co-repressor complex components HDACs 1 and 2, Sin3A and NCoR [25,26]. Gene silencing by EBNA3A and EBNA3C involves recruitment of polycomb repressor complexes (PRC1 and 2) to target gene loci and the deposition of the histone H3 trimethyl lysine 27 (H3K27me3) silencing mark by the PRC2 methyltransferase EZH2 [27-29]. EBNA3A, EBNA3B and EBNA3C can also activate cellular genes and EBNA 3C associates with the HAT p300, implicating histone acetylation in its mechanism of gene activation [7,30].
**EBV TFs predominately target long-range cellular gene regulatory elements**

EBNA2 and EBNA3 family proteins regulate viral transcription via promoter elements [31-34], but most cellular target genes do not possess EBNA-responsive promoters. Chromatin immunoprecipitation-sequencing revealed that in an EBV immortalised lymphoblastoid cell line (LCL), 86% of EBNA2 human genome binding sites were in fact outside of promoter regions (>2 kb from a TSS) [35]. Similarly in a Burkitt’s lymphoma (BL) cell line, 81% of EBNA2 and 89% of EBNA3 family binding sites were > 2kb from cell gene TSSs [36]. The majority of these EBNA binding sites coincided with histone modifications that characterise enhancer elements, indicating that EBNA2 and EBNA3 proteins were likely to reprogramme B cell transcription by predominantly targeting enhancers. A number of subsequent studies using tagged versions of EBNA3A, EBNA3B and EBNA3C confirmed these results [12,20,37,38]. Motif enrichment analysis of DNA sequences at EBNA2, 3A, 3B and 3C binding sites also identified potential new DNA targeting partners [12,20,37,38]. Of these, IRF4 and the RUNX partner CBFβ have now been confirmed as interacting proteins that are required for the targeting of specific cellular genes by EBNA3C and EBNA3B and EBNA3C, respectively [12,20].

A number of the enhancer regions bound by EBNA2 and EBNA3 proteins display characteristics of ‘super-enhancers’ [39,40]. These highly-active regulatory regions represent large clusters of individual enhancer peaks containing binding sites for multiple cell-type specific TFs and high levels of histone H3 acetylation [41]. Super-enhancers play a critical role in driving high-level expression of lineage-specific genes and specific super-enhancers are often activated in cancer cells. This makes super-enhancers an attractive target for drugs that can be used to block their activity as drivers of cancer cell growth, such as the bromodomain.
inhibitor JQ1 and inhibitors of the RNA polymerase II kinase CDK7 [42-46]. RNAs transcribed at EBV super-enhancers (eRNAs) have also been shown to be important for enhancer function, in common with eRNAs found at non-viral TF bound enhancers [47,48].

Overlap in EBNA2, EBNA3A, EBNA3B and EBNA3C binding

ChIP-sequencing analysis of EBNA2, EBNA3A, EBNA3B and EBNA3C identified extensive overlap in their binding sites. This is perhaps predictable given their shared cellular TF binding partners e.g. RBPJ. It is also consistent with the co-operative and/or antagonistic nature of gene regulation by these EBNAs [7,29,49]. The extent of binding overlap was reported for a BL cell line as 25% of all binding sites shared by EBNA2 and EBNA3 family proteins, with an estimated 80% of genes closest to EBNA2 binding sites also closest to an EBNA3 protein binding site [36]. Binding analysis of individual tagged EBNA3A, EBNA3B and EBNA3C proteins in LCLs found 21-26% binding overlap between different EBNA3 proteins with 37% of genes closest to EBNA2 binding sites also closest to a binding site for an EBNA3 protein [12]. It is therefore likely that the co-regulation of B cell genes by these EBNAs is mediated via their association with the same regulatory element or to different elements that target the same gene.

EBNA2 and EBNA3 proteins regulate enhancer-promoter interactions

The effects of EBNA2, EBNA3A, EBNA3B and EBNA3C binding to long-range elements on the three-dimensional associations between cell gene enhancers and promoters has begun to be investigated using the chromosome conformation capture (3C) family of techniques.
Initial studies focused on the mechanism of activation of the MYC proto-oncogene by EBNA2, a key growth promoting event that occurs shortly after EBV infection [50]. ChIP-sequencing detected EBNA2 binding at multiple super-enhancers and enhancers in a 3 Mb region around MYC [35,36]. 3C analysis demonstrated that EBNA2 increased interactions between a -428 kb EBNA2-bound super-enhancer and the MYC promoter [35]. Further studies on the effects of EBNA2 on the wider MYC enhancer-promoter interaction landscape used 4C (circularised chromosome conformation capture) to capture all MYC promoter-interacting fragments in the absence or presence of EBNA2 [51]. 4C detected MYC promoter interactions with multiple EBNA2-bound upstream and downstream enhancers and showed that EBNA2 induced widespread changes to the three-dimensional arrangement of the MYC locus [51]. In the presence of EBNA2, MYC enhancer-promoter interactions were increased over a large upstream region encompassing multiple enhancers and super-enhancers, consistent with previous reports, but surprisingly EBNA2 reduced downstream enhancer-promoter interactions [51] (Figure 1). Upstream enhancer interactions were dependent on the presence of the SWI/SNF ATPase BRG1 which is bound and recruited by EBNA2 [51-53]. SWI/SNF is important in maintaining downstream MYC enhancer-promoter interactions in leukaemic cells and is required for the function of multiple distal lineage-specific enhancers [54,55]. Similar directional changes to MYC enhancer-promoter architecture were induced on infection of primary B cells by EBV that differed from the downstream interactions induced on short-term B cell activation by CD40 ligand and IL-4 [51]. This indicates that EBV activation of MYC involves distinct spatial reorganisation of chromatin. Multiple cell TFs are co-localised with EBNA2 at MYC enhancers including RBPJ, but which TFs are crucial for EBNA2 binding to MYC enhancers is not known. EBNA3A and EBNA3B also bind to MYC enhancers, but their effect on MYC enhancer-promoter interactions has not been studied [37,38].
Interestingly, in EBV-positive BL, the MYC-immunoglobulin translocations that characterise BL have a breakpoint located in the upstream MYC enhancer region activated by EBV [51]. EBV also upregulates the expression of the activation induced cytidine deaminase enzyme (AID), known to be an inducer of MYC translocations [56,57]. It has therefore been proposed that the specific activation of upstream MYC enhancers by EBV may increase the susceptibility of this region to translocations induced by off-target AID activity and could represent an initiating event in the genesis of BL [51].

Further studies characterised an EBNA2-bound and RBPJ-dependent RUNX3 super-enhancer 97 kb upstream of the gene TSS [40]. RUNX3 is a direct target of EBNA2, likely to play an important role in promoting growth, at least in part by its downregulation of Runx1c expression, which is growth inhibitory in B cells [58-60]. Genome-wide promoter-enhancer analysis in an EBV-immortalised cell line detected high levels of interactions between the RUNX3 super-enhancer region and RUNX3 promoters, providing evidence for the presence of RUNX3 super-enhancer-promoter interactions in EBV-infected cells [40,61] (Figure 1). The RUNX3 super-enhancer is also bound by EBNA3B and EBNA3C and gene expression analysis indicates that EBNA3 proteins have a positive effect on RUNX3 expression [7,40]. Whether these EBNA3 proteins also increase RUNX3 enhancer-promoter interactions however, has not been tested.

The influence of EBNA3A, 3B and 3C binding on enhancer-promoter interactions at sites shared by EBNA2 has also been examined at other gene loci that include a distal intronic site at CTBP2 bound by all four proteins [36]. CtBP1 and 2 are transcriptional repressors that bind EBNA3A and EBNA3C and mediate the repression of a number of key cell genes including
the cyclin-dependent kinase inhibitor (CDKI) p16\textsuperscript{INK4a} [22,23,62]. The repression of p16\textsuperscript{INK4a} by EBNA3A and EBNA3C is a critical event required for the outgrowth of EBV-infected cells [63], so regulation of CtBP2 expression by EBV TFs may influence gene repression by EBV or modulate other functions of CtBP2. Perhaps surprisingly, EBNA3A and EBNA3B repress CtBP2 transcription (EBNA3C effects were not examined), although this is consistent with the frequent silencing of CtBP2 in B cells [36]. Repression of \textit{CTBP2} by EBNA3A was shown to involve the disruption of interactions between the gene promoter and the EBNA3-bound distal element [36] (Figure 1). Since EBNA2 binds the \textit{CTBP2} site, it may direct enhancer-promoter looping in the absence of EBNA3A, but this was not directly tested. ChIP re-ChIP experiments did however show that EBNA2 and EBNA3 proteins do not bind this site simultaneously, indicative of competitive binding that may influence the extent of enhancer-promoter looping [36].

Additional studies of shared binding sites examined a cluster of enhancer sites up to 44 kb downstream of the TSS of the gene encoding the cell-cycle kinase \textit{wee1} [36]. \textit{Wee1} phosphorylates and inactivates the mitotic kinase CDK1 to prevent unscheduled or inappropriate entry into mitosis. EBV deregulation of the cell-cycle through its effects on multiple CDKIs and other cell-cycle regulators plays a critical role in EBV transformation, so \textit{Wee1} regulation may also contribute to cell-cycle disruption in infected cells [64]. Specific binding of EBNA3C to \textit{WEE1} enhancers repressed \textit{WEE1} expression consistent with the promotion of the transition from G2 into mitosis by EBV [36,65,66]. Surprisingly, repression by EBNA3C did not disrupt enhancer-promoter interactions, but promoted their formation [36] (Figure 1). Presumably this looping brings in co-repressors that associate with EBNA3C to the gene TSS, although this has not been investigated. EBNA2 also bound \textit{WEE1} enhancers and loss of EBNA2 binding at these \textit{WEE1} enhancers further reduced \textit{WEE1} expression [36].
the absence of EBNA2 it is possible that more EBNA3C is able to bind and increase the level
of gene repression. Re-ChIP again confirmed mutually exclusive EBNA2 and EBNA3C
binding at *WEE1* [36].

Competition between EBNA3 proteins and EBNA2 binding was also observed at a 6 kb
intergenic enhancer in the *CXCL9, CXCL10* and *CXCL11* chemokine gene cluster [11].
EBNA3A binding to this site is associated with RBPJ-dependent repression of *CXCL9* and
*CXCL10* transcription, PRC recruitment and H3K27me3 deposition across the locus [11].
Downregulation of these chemokines is likely to involve modulation of looping between this
eenhancer and the gene promoters, but this was not tested. Interestingly, *CXCL10* can also be
repressed by EBNA3C along with the adjacent *CXCL11* (if not already silenced). EBNA3C
binding at the *CXCL9/10* enhancer was not detected, so the mechanism of this repression
remains unclear [29]. Downregulation of *CXCL9, 10* and *11* may constitute an immune
avoidance mechanism for EBV-infected B cells *in vivo* since these chemokines function as key
attractors of T and NK cells. EBNA2 binding to the *CXCL9/10* enhancer reduced EBNA3A
binding and repression of *CXCL9 and CXCL10* consistent with the competitive binding and
opposing gene regulation observed at *WEE1* [11,36].

Analysis of enhancer-promoter interactions in the repression of genes targeted by EBNA3
proteins alone has thus far been examined at two gene loci; the a disintegrin and
metalloprotease (ADAM) gene cluster containing *ADAMDEC1* and *ADAM28* and the
*BCL2L11* gene locus encoding the pro-apoptotic protein Bim (Figure 1). *ADAM28* and
*ADAMDEC1* regulate B cell adhesion and migration, so their repression may provide a survival
advantage to EBV-infected B cells *in vivo*. *BCL2L11* repression by EBNA3A and EBNA3C is
known to involve PRC recruitment, increased levels of H3K27me3 and lead to long-lasting epigenetic silencing through DNA methylation at BCL2L11 promoter CpG islands [27,67].

BCL2L11 repression is a common event in lymphoma development, often providing protection from MYC-driven apoptosis in lymphomas where MYC is overexpressed e.g. BL [68]. EBV activation of MYC and repression of BCL2L11 therefore represent lymphoma driving events.

EBNA3A and EBNA3C repress ADAMDEC1 and ADAM28 by binding to an intergenic enhancer bound by RBPJ [29,36,69]. As observed at WEE1 for EBNA3C, binding of EBNA3A and EBNA3C to the ADAM enhancer mediated gene repression via looping with the gene TSSs, presumably through the formation of a repressive hub which leads to H3K27me3 deposition across the locus [36]. EBNA3A and EBNA3C silencing of BCL2L11 was originally thought to be mediated through binding to the BCL2L11 promoter [28,36], but subsequent studies identified a large chromatin hub comprising multiple enhancers bound by EBNA3A and/or EBNA3C [51]. Repression of BCL2L11 by EBNA3A and EBNA3C involves recruitment of EZH2 (PRC2) across the enhancer-promoter hub and the disruption of all enhancer-promoter interactions (Figure 1). BCL2L11 can be de-repressed and apoptosis induced by an EZH2 inhibitor [51]. Taken together, these studies demonstrate that repression of EBNA3A and EBNA3C target genes can operate through two different mechanisms, irrespective of whether EBNA2 targets the same sites; loop promotion or loop disruption (Figure 1).

Studies on the oncogenic miR-221 and miR-222 microRNA cluster provided the first evidence that EBNA3A and EBNA3C can activate transcription by promoting enhancer-promoter looping [70]. EBNA3A and EBNA3C activate expression of these microRNAs by binding to sites in and around the transcription unit of the pri-miR from which they are processed and
promoting enhancer looping with the TSS [70] (Figure 1). Importantly induction of these miRs led to reduced expression of their key target gene p57\(^{kip2}\), a key cyclin-dependent kinase inhibitor, providing another example of how EBV regulates gene expression to perturb cell-cycle regulation in infected cells.

**Conclusion**

It is clear that the targeting of long-range regulatory elements by EBV TFs in B cells induces significant changes to three-dimensional enhancer-promoter interactions to modulate the expression of multiple genes involved in a variety of B cell processes. This chromatin reorganisation is likely to be critical for the transformation of B cells and can lead to cancer-driving events that include the long-term silencing of tumour suppressor genes like \(BCL2L11\) and may include the promotion of \(MYC\) gene translocations. Studying EBV enhancers has led to the identification of new regulatory hubs that may be affected in other cancers and has identified therapeutic opportunities for super-enhancer targeting or \(EZH2\) inhibition. RBPJ plays a critical role in mediating the binding of EBV TFs to many cell gene enhancers but there is much more to learn about the roles played by other targeting co-factors and it is still unclear what determines whether a specific gene target is repressed via ‘anti-looping’ or looping.
Acknowledgements

This work was supported by Bloodwise [grant numbers 12035, 13032, 15024]; the Medical Research Council [MR/K01952X/1].

Figure caption

Figure 1. The effects of EBV transcription factors on enhancer-promoter interactions at different gene loci in B cells. The main EBV nuclear antigens (EBNAs) involved in regulation of specific genes are indicated next to the arrows. At transcribed genes the C-terminal domain of RNA polymerase II (pol II) is shown in its hyperphosphorylated state with phosphates represented as yellow spheres. EBNA 3B (3B), EBNA3A (3A) and EBNA2 (2, shown as a dimer) are shown bound to gene regulatory elements via cellular cofactors (coloured spheres). RBPJ (J) is shown at genes where it has been demonstrated to be involved in EBNA binding and gene regulation. At MYC, SWI/SNF complexes (S) are shown bound at upstream EBNA2 enhancers. An extruded loop is also formed upstream of MYC in the presence of EBNA2 as a result of interaction between two CTCF (C) bound sites. Additional coloured spheres represent various cellular co-activators or co-repressors involved in mediating enhancer-promoter interactions and gene regulation.
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Identifies the mechanism of AID induction by EBV which could contribute to the genesis of EBV associated lymphomas.


The development of a new genome-wide technique to specifically examine promoter interactions.


Demonstrates that EBNA3C actually recruits RBPJ to specific sites and does not just alter RBPJ associations with histone modifying enzymes.

The first study to describe the mechanism of gene activation by EBNA3 proteins and to identify another CDKI as an indirect target repressed by EBV.
EBV infected B cell

EBNA2

EBNA2

EBNA3A

EBNA3A

EBNA3A

EBNA3A

EBNA3C

EBNA3C

EBNA3C

EBNA3C

EBNA3C

EBNA3A

EBNA3C

MYC

RUNX3

miR-221/222

ADAMDEC1

WEE1

CTBP2

BCL2L11

Growth promotion

MYC translocation?

Growth promotion

Cell cycle progression

Reduced adhesion and migration

Cell cycle progression

Transcription regulation

Inhibition of apoptosis

Gene activation via looping

Gene repression via looping

Gene repression by loop inhibition