INVESTIGATING THE MECHANISM OF CELLULAR GENE ACTIVATION AND REPRESSION BY THE EBV TRANSCRIPTION FACTOR EBNA 2

By

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Submitted in total fulfilment of the requirements of the degree of

Master of philosophy

Department of Biochemistry

School of Life sciences

University of Sussex

January 2017

Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

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OPEOLUWA OJENIYI MASTER OF PHILOSOPHY BIOCHEMISTRY

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SUMMARY

Epstein-Barr virus (EBV) is a widespread human tropic B cell virus that is linked to several malignancies. EBV modulates the transcriptome of B lymphocytes to drive immortalisation and viral persistence. EBV nuclear antigens (EBNA) 2,3A, 3B and 3C are transcriptional regulators of both viral and cellular genes and are the primary drivers of the immortalisation and the continued proliferation of infected B-cells. EBNA 2 activates all EBV gene promoters and cellular growth control genes while EBNA3A, 3B and 3C activates or represses transcription. EBNA2 and 3 proteins do not bind directly to DNA. They bind through cellular DNA-binding proteins like RBP-Jκ and PU.1. The focus of this research was to investigate how EBNA 2 promotes immortalisation through the epigenetic reprogramming of cellular genes and how EBNA 3A, 3B and 3C antagonise or cooperate with EBNA 2 in gene regulation. Previous ChIP-seq results in our lab identified significant binding sites for EBNA 2 and EBNA 3s. I targeted three important novel shared EBNA 2 and EBNA 3s binding sites; the integrin *ITGAL,* cell cycle kinase *WEE1* and transcription repressor *CTBP2* genes. I investigated if these shared sites are functional as EBNA 2 response elements in reporter assay by transiently transfecting the endogenous promoter and any associated long range enhancer region of genes and performing luciferase assays. EBNA 2 activates the *ITGAL* promoter and EBNA 3s inhibits the activation while *WEE1* and *CTBP2* does not respond in reporter assay. I also performed site-directed mutagenesis to determine which cellular transcription factor was important for the activation of EBNA 2 at the *ITGAL* promoter. RBP-Jk site mutation disrupted the EBNA 2 activation. Another research focus was EBNA 2 association with gene activation and repression. BCR components *CD79A* and *CD79B* are involved in signal transduction and the regulation of B-cell growth and survival and transcription factor EBF1 plays an important role in B cell differentiation. I investigated the association of EBNA 2 with these repressed gene targets and if EBF1 plays a role in the mechanism of repression using reporter assay. *CD79A* and *CD79B* activates EBNA 2 and EBF1 does not significantly repress the activation in luciferase reporter assay. EBNA 2 have been mapped binding to enhancers at a new target gene interferon response factor 4 *IRF4* and microarray data implicates EBNA 2 in its activation. When *IRF4* expression is reduced in EBV transformed cells, cell proliferation rate is decreased and apoptosis enhanced so this activation may be important for B-cell transformation by EBV. I carried out reporter assays to determine if the site is EBNA 2 responsive and whether it interacts with the *IRF4* promoter and enhancers. EBNA 2 slightly activates the promoter and enhancers.

DEDICATION

This work is dedicated to my father LATE MR. VINCENT OLATUNJI OGBE, who departed this world the day this program started, I love you and I miss you. Also, to my father-in-law LATE MR. JOHN OJENIYI for always making me smile.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank my supervisor Professor Michelle West for her endless support and patience throughout the duration of this course. I would also like to thank the entire West and Sinclair lab members, in particular Andrea, David, Sarika, Hilda, Michele and Lina for never getting tired of supporting me with my experiments.

A BIG THANK YOU to my amazing husband Adeola for seeing this course through financially, putting up with moods and for being my rock and no. 1 fan. I would also like to thank my children Philip, Hadassah and Isaac for loving me all the same.

Also, many thanks to the entire Ogbe and Ojeniyi families for their prayers and support especially my sisters and Mum.

Most importantly, thank you to God for his joy has been my strength.

PUBLICATION

McClellan, M. J., C. D. Wood, O. Ojeniyi, T. J. Cooper, A. Kanhere, A. Arvey, H. M. Webb, R. D. Palermo, M. L. Harth-Hertle, B. Kempkes, R. G. Jenner and M. J. West (2013). "Modulation of enhancer looping and differential gene targeting by Epstein-Barr virus transcription factors directs cellular reprogramming." PLoS Pathog **9**(9): e1003636.

ABBREVIATIONS

ABBREVIATIONS	
AIDS	Acquired Immunodeficiency syndrome
BL	Burkitt's Lymphoma
Brd4	Bromodomain containing protein 4
ChIP	Chromatin Immunoprecipitation
Cp	C promoter
CTD	C-terminal domain
DBD	DNA binding domain
EBER	EBV encoded RNA
EBNA	Epstein-Barr Nuclear Antigen
FACT	Facilitates chromatin transcription
GTF	General transcription factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIV	Human Immunodeficiency Virus
HL	Hodgkins Lymphoma
HMT	Histone methyltransferase
IM	Infectious Mononucleosis
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
NELF	Negative elongation factor
NHL	Non-Hodgkins Lymphoma
OriP	Origin of replication
PCR	Polymerase chain reaction
Pol II	RNA Polymerase II
pTEFb	Positive Transcriptional Elongation Factor
PTLD	Post transplant lymphoproliferative disease
Qp	Q promoter
RBP-Jk	recombining binding protein J kappa
TBP	TATA-box binding protein
TF	Transcription factor
TR	Terminal Repeat
TAF	TBP associated factor
TAD	Transactivation domain
Wp	W promoter

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1. INTRODUCTION

1.1. Regulation of transcription

Transcriptional regulation is the means by which a cell controls the conversion of DNA to RNA by RNA polymerase to manage gene activity. This control allows the cells to regulate the activity of a single gene's activity by altering the amount of RNA copies being made in the cell in response to intra and extracellular signals. Eukaryotes have three RNA polymerases; RNA polymerase (Pol I), Pol II, and Pol III. Each polymerase has specific target genes and activities, and is regulated by independent mechanisms (Ranallo *et al.*, 1999, Thomas and Chiang, 2006).

1.1.1. Assembly of RNA polymerase II initiation complexes

Pol II carries out the transcription of all protein coding genes and regulation of Pol II transcription is essential for all cellular processes including cell growth, differentiation and survival. Pol II has 12 subunits Rpb 1 – 12. To initiate transcription, Pol II requires additional transcription factors known as General transcription factors (GTFs). These include Transcriptional Factor for Pol II A, B, D, E, F, H (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH). Pol II gene promoters can contain a recognition sequence called the TATA box, typically located 25bp upstream of the Transcription start site (TSS) (Matsui *et al.*, 1980, Kim *et al.*, 1993). Binding of the GTF TFIID through its TATA box binding protein (TBP) subunit is the first event in the formation of the transcription initiation complex (Figure 1).

The majority of eukaryotic promoters do not contain a TATA box but can still recruit TBP to the pre-initiation complex via another element called the initiator element that overlaps the TSS (Latchman, 2008). TFIID also contains TBP associated factors (TAFs) which are required for transcription regulation (Mizzen *et al.*, 1996). TFIIA interacts with TBP and helps in the binding of TBP to the TATA box thereby stabilizing TFIID, but TFIIA can often be unnecessary for efficient transcription initiation (Tang *et al.*, 1996). TFIIB then binds to create a binding surface for Pol II and help in the recruitment of other transcription factors and aid in the determination of the transcription start site (Ha *et al.*, 1991). TFIIF binds to Pol II when it is not in contact with any other factor and

Pol II and TFIIF are recruited together. TFIIF aids accurate initiation by stabilizing Pol II when in contact with TBP and TFIIB stopping it from contacting DNA outside of the promoter. TFIIF also recruits TFIIE and TFIIH to the complex (Kim *et al.*, 1997, Lee and Young, 2000) (Figure 1). TFIIE binds and recruits TFIIH and stimulates the DNA-dependent ATPase activities of TFIIH and the Carboxyl terminal domain (CTD) kinase of Rpb1 of Pol II. The CTD acts as a platform for interaction of many transcription and processing factors. TFIIE and TFIIH are thought to be required by RNA polymerase for promoter clearance (Peterson *et al.*, 1991, Maxon and Tjian, 1994). TFIIE is also required for DNA melting at the promoter. TFIIH functions as a catalyst of ATP-dependent DNA start site unwinding and also the phosphorylation of the CTD of the Rbp1 subunit of Pol II through its CDK7/cyclin H subunits. Once Pol II accesses the template strand, it starts the transcription of mostly abortive transcripts until a conformational change results in the release of Pol II from the promoter and transcription elongation begins (Lee and Young, 2000) (Figure 1).

The phosphorylation of the CTD of Rpb1 in Pol II plays an important role in the regulation of efficient transcription and RNA processing (Horikoshi *et al.*, 1992, Egloff and Murphy, 2008). The CTD in humans contains 52 heptapeptide sequence repeats (YSPTSPS). The CTD 'code' describes the regulation of Pol II by transient modifications of the CTD, of the second serine residue in the repeat (serine 2) during elongation and serine 5 phosphorylation at initiation. The CTD is phosphorylated by specific cyclin-dependent kinases (CDKs) (reviewed in (Egloff and Murphy, 2008). TFIIH subunits CDK7/cyclin H phosphorylate the CTD on the serine 5 residues during initiation. Two other CDKs, CDK8/cyclin C and CDK9/cyclin T comprises the positive elongation factor (pTEFb) and phosphorylate the CTD during elongation. The CDK8/cyclin C are part of the mediator complex and phosphorylate the CTD on serine 2 or 5 during initiation (Lu *et al.*, 1992, Hengartner *et al.*, 1998, Komarnitsky *et al.*, 2000).

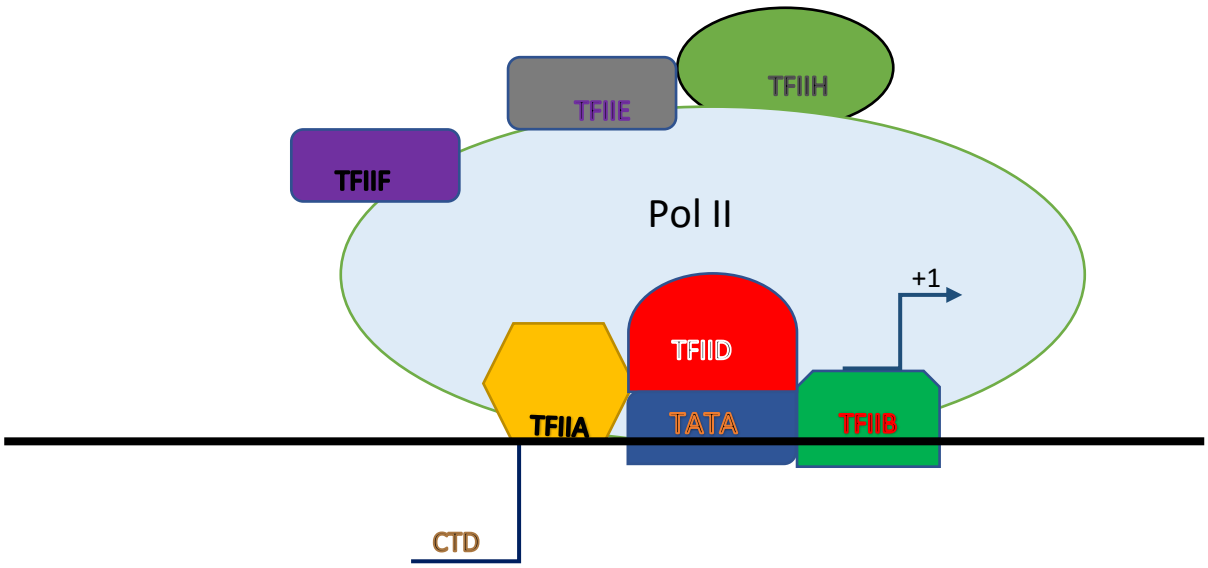

Figure 1. RNA Polymerase II initiation complexes.

Assembly of Pol II initiation complexes and highlighting the major GTF; Transcription factor for Pol II A, B, C, D, E, F, and H and the Carboxyl terminal domain (CTD) of Rpb1 projecting from the assembly.

1.1.2. Promoter clearance and elongation

Pol II is recruited to promoters with a hypophosphorylated CTD. Upon hyperphosphorylation at serine 5 residues, promoter escape is facilitated and elongation progresses (Cutting *et al.*, 1991, Yamamoto *et al.*, 2001). The phosphor-serine 5 CTD motif recruits capping enzymes immediately after promoter clearance to prevent the RNA from degrading and the activity of the capping enzyme is stimulated *in vitro* by serine 5 phosphorylation (Wen and Shatkin, 1999, Rodriguez *et al.*, 2000). The regulator of transcription 1(Rtr1), a CTD phosphatase is bound and activated as the elongation progresses and the serine 5 phosphorylation mark is removed gradually (Mosley *et al.*, 2009).

As the serine 5 phosphorylation gradually decreases serine 2 increases towards the 3' end of genes marking the Pol II complex elongation process (Saunders *et al.*, 2006). The phosphorylation of serine 2 by pTEFb is required for productive elongation (Marshall and Price, 1995). Two factors, DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) are associated with promoter proximal paused transcriptional complexes. pTEFb phosphorylates both the Spt5 subunit of DSIF and NELF relieving promoter proximal pausing of Pol II (Wada *et al.*, 1998, Fujinaga *et al.*, 2004).

In a cell, DNA is compacted in chromatin made up of basic units called nucleosomes. Each nucleosome contains an octameric core of histone proteins comprising two H3 - H4 dimers surrounded by two H2A-H2B dimers and the N-terminal histone tails protruding out from the nucleosome. DNA is wrapped twice around each histone octomer. Interestingly, it appears that NELF-induced promoter-proximal pausing is important for efficient transcription of many genes and may serve as a transcriptional checkpoint by obstructing nucleosome assembly around the promoter as the nucleosomes will impede the progress of transcription by Pol II (Gilchrist *et al.*, 2008).

Following the initiation and promoter clearance, the elongating Pol II can be affected by histones in the DNA. To aid the Pol II transcriptional elongation process the elongation factors Facilitates Chromatin Transcription (FACT) and Spt6 acts as histone chaperones by removing histones. FACT, a heterodimer that consists of Spt 16 and SSRP1 protein is recruited to elongation complexes through its association with Pol II, ATPase chromatin remodeller CHD1, and DSIF. It also mediates the removal or replacement of H3 when Anti-silencing function protein1 (Asf1) binds the Pol II associated factor1 (Paf-1) elongation complex and move along with Pol II is another representation of histone being modified to allow for the progress of transcription by Pol II (Orphanides *et al.*, 1998, Kelley *et al.*, 1999, Orphanides *et al.*, 1999, Belotserkovskaya *et al.*, 2003, Mason and Struhl, 2003).

1.1.3. Transcription factors

A transcription factor (TF) is a protein that binds to DNA and is involved in its conversion to RNA. It contains domains that help it bind to specific DNA sequences to initiate and regulate gene transcription (Karin, 1990, Latchman, 1997). TF can bind either promoter or enhancer region of DNA to regulate the gene activation or repression by promoting or obstructing RNA polymerase recruitment. In eukaryotes, an important class of TFs called general transcription factors (GTFs) forms part of the transcription initiation complex that interact with RNA polymerase to activate gene transcription. This allows genes to be expressed in specific manners and in different cell types during development. TFs either regulate the gene expression directly by attaching to specific DNA sequence through its DNA binding domain (DBDs) or with other regulatory sequences such as enhancers, these enhancers can be thousands of base pairs upstream or downstream from the gene being transcribed chromatin and requires looping to contact the activation domain as they lack DBDs (Roeder, 1996, Nikolov and Burley, 1997, Lee and Young, 2000).

TFs bind DNA directly but can bind through interactions with other DNA binding TFs. DBDs of TFs recognise a specific sequence in DNA called the response element. The DBD is sequence specific but these sequences can be degenerative, so TFs have consensus motifs created by identifying all known binding sites and determining the extent to which nucleotides are conserved (Claessens and Gewirth, 2004). The DNA binding function is either structural or regulatory. DBDs involved in DNA structure have biological roles in DNA replication, repair and storage. DBDs interact with nucleotides in a DNA sequence specific manner and the recognition type is tailored to the protein's function (Lefstin and Yamamoto, 1998). DBD may also interact with DNA in a non-sequence specific manner if there is molecular recognition between TF and DNA, the binding site sequence must be closely related to the consensus sequence.

This means transcription binding can occur randomly highlighting the difficultly in predicting where a TF will bind in a cell. To achieve more recognition specificity, TF can also bind two or more adjacent sequence of DNA by using more than one DBD

(Hahn, 2004, Wang, 2005). Some types of DBDs include Zinc finger, leucine zipper, helix-loop-helix and homeodomains. Class I, Class II, Class IV HDACs have Zn-dependent metalohydrolase activity and promote condensation of chromatin and gene repression, and are recruited by transcriptional repressors to specific genes (Kao *et al.*, 2000, Li *et al.*, 2000).

TF can stimulate transcription in many ways including increasing PIC formation through direct interactions with components of the transcriptional machinery and can affect the rate of initiation, elongation and reinitiation (Orphanides *et al.*, 1996, Lee and Young, 2000). TFs (activators) have a Transactivation domain (TAD) which act as a scaffolding domain for transcriptional coregulators. Through these they recruit chromatin modifiers to facilitate transcription by altering local chromatin structure and recent work suggests that specific transcription factor binding to DNA allows for accurate prediction of histone modifications present at that site (Benveniste *et al.*, 2014). Activators can have their activity further controlled by their interaction with co-activators e.g. TAFs and mediators. The amino acid sequence of co-activators does not exhibit many predicted functional domains and are interchangeable, they interact with DNA bound activators to determine the effect of the TFs on the DNA. Co-activators function as a bridge between DNA and TFs and they modifying chromatin landscape and altering the composition of the core transcriptional machinery (Bjorklund and Gustafsson, 2005, Lonard and O'Malley, 2005, Copland *et al.*, 2009).

TFs can also contain a signal sensing domain (SSD) that may determine whether a TF is activated or deactivated during transcription to up/down-regulate gene expression. The SSD use several mechanisms such as ligand binding, for example, nuclear receptors that senses extracellular signals, bind DNA and regulate gene expression when a ligand is present. Depending on which coregulatory protein they recruit; co-activators (which contain histone acetyltransferase HATs) or co-repressors (which contain histone deacetylases HDACs), they either promote or repress gene transcription. Other mechanisms include protein phosphorylation e.g STAT proteins and interactions with other transcription factors or coregulatory proteins (Bohmann, 1990, Weigel and Moore, 2007). In eukaryotes, combinatorial regulation of gene

expression where DBD and SSD residing on different TFs that associate within a transcription complex can occur, this type of regulation is often complicated with each specific combination resulting in different gene expression outcome (Remenyi *et al.*, 2004, Reece *et al.*, 2011) (Figure 2). This process can be complex with more factors involved and how and when they bind may also determining the effect on transcription.

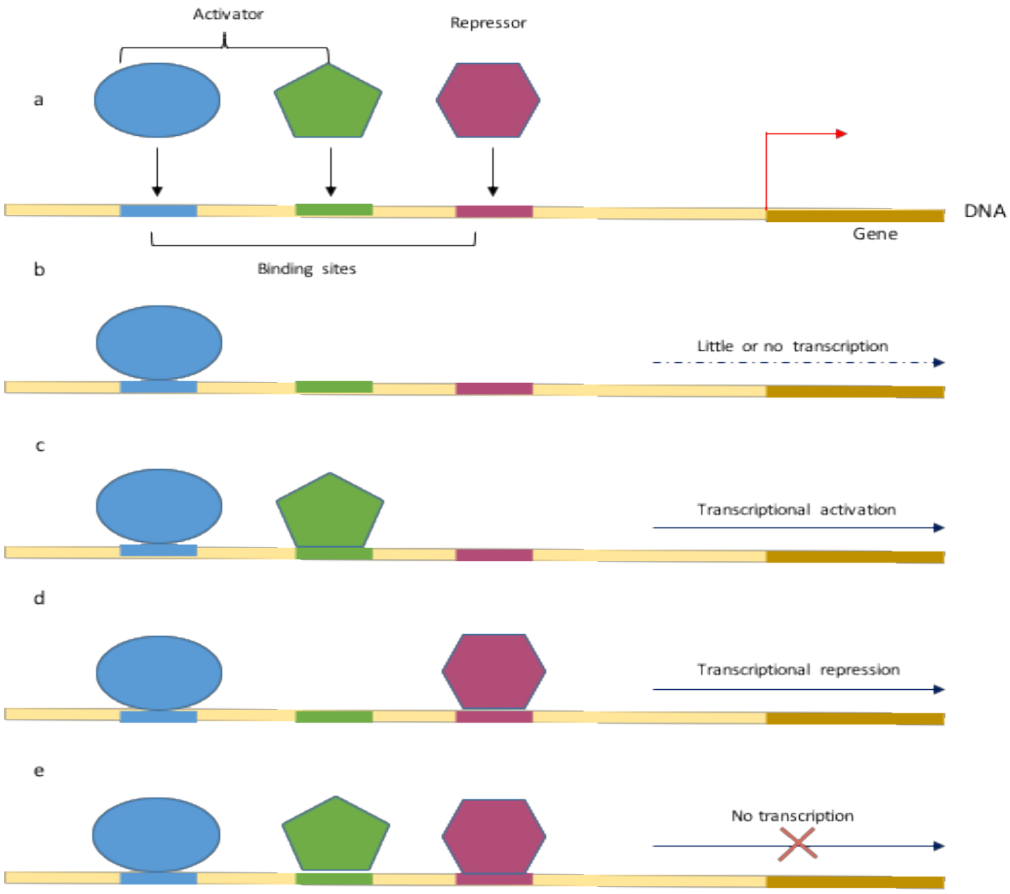

Figure 2. Schematic representation of how combinatorial regulation may occur with different outcome for each transcription factor combination. (a) Shows transcription factors (activators and repressors) and their binding sites in a gene promoter. (b) Shows little or no transcription with only one activator present. (c) Shows gene activation when two activators are present and gene repression when one activator and one repressor is present (d) while (e) shows no transcription when the all three transcription factors are bound the gene expression is blocked.

1.1.4. Epigenetics and histone modification

The nucleosome has ~147bp of DNA which completes nearly two full turns around the nucleosome with each nucleosome separated by 10-60 bp linker piece of DNA that is commonly bound by histone H1 on nucleosome near the entry of DNA (reviewed in (Peterson and Laniel, 2004). Modifications to both the histone tails and DNA regulate chromatin structure, accessibility to the gene and transcriptional machinery (Figure 3).

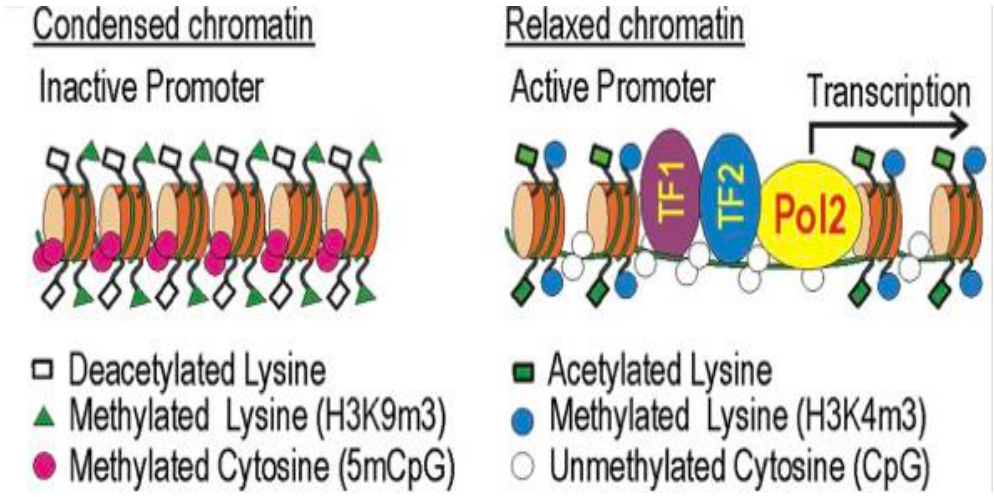

Figure 3. Diagram showing Epigenetic marks that regulate transcription. Chromosomal DNA is packaged around a histone octomer to form nucleosomes. Nucleosome spacing in the open structure that is accessible to nuclear factors is maintained, in part, by post-translational modification of histone tails, including lysine acetylation and specific lysine methylation of specific residues. CpG dinucleotides are unequally distributed throughout chromosomal DNA, and may be concentrated in regions called CpG islands that can overlap gene promoters. Methylation of cytosines in CpG dinucleotides is overall associated with inactive, condensed states of the chromosome. Inactive chromatin is also maintained by specific histone lysine modifications (Glant *et al.*, 2014).

Epigenetic modification is a heritable stable change in gene expression that does not change the DNA sequence itself; this allows multiple cell types to differ despite sharing a DNA sequence. Chromatin can be remodelled in several interconnected ways: covalent histone modifications, exchanging the core histones with variants, disrupting the nucleosome and DNA modification (Kurdistani and Grunstein, 2003). One key epigenetic modification involves DNA methylation which commonly occurs at CpG motifs, termed 'CpG islands', which are regularly found 5' to the coding sequences of constitutively expressed housekeeping genes, and are found at approximately 50% of promoters. DNA methylation of CpG islands promoters usually causes gene silencing by directly preventing transcription factor binding and recruiting methyl-binding domain proteins and histone deacetylases (HDACs) (Taby and Issa, 2010). It has been proposed that DNA methylation induced stable gene silencing may provide a memory function to progeny cells in remembering their identity (Riggs, 1990).

Histone methyltransferases can mono (me), di (me2) or tri (me3) methylate histones on lysine residues and mono or di methylate histones (HKMTs) on arginine residues (HRMTs). Histone methylation can be either a positive (methylation sites associated with transcriptionally permissive chromatin called euchromatin) or negative (methylation sites fostering heterochromatin formation) influence on transcriptional regulation. Histone modification acts as binding sites and leaves a mark that effector proteins (coactivators or corepressors) read to allow specific transcriptional events (Armstrong, 2007, Volkel and Angrand, 2007).

Post-transcriptional modification of the histone proteins, especially the histone tail, cause changes in gene expressions by either making the chromatin more or less accessible or directly recruiting other cellular factors that activate or inhibit transcription (Loizou *et al.*, 2006). Histone acetylation at the ε-amino group of lysine residues in H3 and H4 tails is usually associated with promoting transcription and is also involved in loosening DNA histone contacts, during DNA replication and histone deposition, DNA repair and recruitment of proteins with acetyl binding domains.

Histone acetyltransferases (HATs) are the enzymes that catalyse lysine residues in both histone and non-histone proteins. Histone lysine methylation primarily occurs at the 5' ends and gene promoters but can sometimes be detected throughout the gene. In the case of acetylysines, it is recognised by the bromodomain proteins (Kurdistani and Grunstein, 2003, Kim *et al.*, 2006, Wang *et al.*, 2008).

Methylation of specific lysine (K) residues H3K4me2/3, H3K36me3 and H3K79me2/3 is associated with gene activation, Set1, Set2 and Dot1 HKMTs are recruited directly by phosphorylated form of Pol II to the polymerase during elongation, for example Set1 associates with Ser5 phosphorylated form of Pol II and methylates H3K4 (Nechaev and Adelman, 2008). Effector proteins (coactivators) play an important role in maintaining the chromatin transcriptional state by disrupting chromatin structure to allow Pol II promoter access, they include histone acetyltransferases HATs, ATP-dependent remodelling complexes and HRMTs. For example, HATs targets H3 and H4 histone tails and acetylate lysine residues, chromatin remodellers NURF/ISWI (ATPase) are involved in nucleosome repositioning by limiting access to the DNA during remodelling at target promoters (Mizuguchi *et al.*, 1997, Gangaraju and Bartholomew, 2007, Li *et al.*, 2007) and HRMTs targets H3 and H4 to methylate arginine residues (Kouzarides, 2007, Li *et al.*, 2007). Activators may also recruit ATPases to remodel compacted chromatin before acetylation by HATs, for example, part of the SAGA HAT complex and chromodomain of Chd1 (ATPase) are recruited to the promoter proximal regions through Ser5 phosphorylated Pol II and bind by recognising the transcription activation mark at H3K4 methylation site (Vermeulen *et al.*, 2007, Volkel and Angrand, 2007, Nechaev and Adelman, 2008).

H3K9me3, H3K27me3 or H4K20me3 methylation states however, are associated with gene silencing or repression, for example, histone methyltransferase SUV39H1 and SUV39H2 methylate K9 on H3 and form a complex with heterochromatin protein 1 (HP1) that is involved in repression of transcription at euchromatic sites (Lachner *et al.*, 2001, Cheutin *et al.*, 2003). The association between HP1 and DNA methyltransferases such as DMNT1 facilitates chromatin compaction and catalyses

the monoubiquitylation of histone H2A at K119 through ubiquitin ligases ring finger protein1 RING1A and RING 1B (Wang *et al.*, 2004, Smallwood *et al.*, 2007).

DNMT1 maintains cellular levels of CpG methylation and during replication functions in a complex that recognises hemi methylated DNA to add methyl groups to non-methylated daughter strands (Leonhardt *et al.*, 1992, Cirio *et al.*, 2008). The polycomb protein (Pc, CBX in mammals) binds to H3K27 through its chromodomain to regulate the repression of some genes to maintain pluripotency (Lennartsson and Ekwall, 2009). When catalysed by the EZH2, a Polycomb repressive complex (PRC2) subunit, H3K27 is tri-methylated and interacts with PRC1 complexes to form a platform for the recruitment of DNA methyltransferases DNMT1, 3A and 3B highlighting cross-talk between DNA methylation and histone modification (Vire *et al.*, 2006, Bannister and Kouzarides, 2011). The deacetylation of lysine residues on histone tails by histone deacetylases (HDACs) also promotes the closed chromatin state reducing promoter access leading to gene repression (Bannister and Kouzarides, 2011, Xhemalce B *et al.*, 2011).

The HAT families, CREB binding protein (CBP), p300, MYST, and GNAT deposition of acetyl groups are mostly site specific, for example, GNAT members PCAF and GCN5 acetylate H3 at lysine residues 9, 14 and 18 differentially during biological processes (Kouzarides, 2007, Berndsen and Denu, 2008), CREB binding protein (CBP) and p300 can acetylate targets both *in vitro* and *in vivo* including H2AK5, H2BK12, H2BK15, H3K14, H3K18, H4K5 and H4K8 (Sterner and Berger, 2000, Kouzarides, 2007). Disruption to normal acetylation activity of CBP/p300 family members is associated with an autosomal dominant syndrome called the Rubenstein-Taybi syndrome,(Petrij *et al.*, 1995, Zimmermann *et al.*, 2007), highlighting the essential role these cofactors play in the regulation of proper gene expression combinations important in development and differentiation (Handy *et al.*, 2011).

Bromodomains, a recognition domain consisting of 4 α helices and 2 hydrophobic loops contained in the CBP/p300 and PCAF and GCN5 mediate their binding to acetylated lysine residues (Haynes *et al.*, 1992). HATs deposit acetyl groups that

includes the GCN5 proteins and studies on GCN5 have revealed that it may be directed to H3K14 by surrounding residues(Rojas *et al.*, 1999), in this case a glycine and a proline, which suggests substrate specificity partly due to short preferred consensus sites (Cieniewicz *et al.*, 2014). Post translational modifications of histone tails were also shown to promote protein-protein interactions modulating transcription. TUP1, a protein involved in transcriptional silencing in yeast was shown to bind with greater affinity to unacetylated or monoacetylated histone H3 and H4 than hyperacetylated forms (Edmondson *et al.*, 1996). HATs also acetylate proteins involved in transcription like GTF TFIIE and TFIIF (Imhof *et al.*, 1997), CDK9 an elongation factor (Fu *et al.*, 2007), and p53 whose acetylation by the CBP/p300 accounts for its stability and transcriptional regulation(Lill *et al.*, 1997, Barlev *et al.*, 2001, Ito *et al.*, 2001).

The histone deacetylases (HDACs) remove the acetyl group deposited on histones by HATs through a process requiring careful regulation and balance. Deacetylation of histones contributes to the compaction of DNA, transcriptional repression and correlates with CpG methylation and the inactive state of chromatin (Marmorstein, 2001, Wade, 2001, de Ruijter *et al.*, 2003). There are 4 classes of histone deacetylase enzymes (HDACs); Class I, Class II, Class IV and Class III/SIRTUIN family with members able to deacetylate of histones and/or other protein targets (Michan and Sinclair, 2007). The HDACs have poor catalytic function without associating with other factors and are themselves subject to regulation by acetylation, phosphorylation, and sumoylation, this can affect their activity, subcellular distribution, and proteins they associate with (Codd *et al.*, 2009, Mellert and McMahon, 2009). HDACs are known to associate with repressive complexes like nucleosome remodelling deacetylating complex (NuRD), co-repressor of RE1 silencing transcription factor (Co-REST) (Ahringer, 2000, de Ruijter *et al.*, 2003, Belyaev *et al.*, 2004). HDACs have also been identified in the same complexes as HATs. Class I HDACs can interact with GCN5 and HDAC1 can interact with PCAF. Studies have also shown that GCN5 interaction with the CLR3 HDAC complex can regulate H3K14 acetylation on yeast (Yamagoe *et al.*, 2003, Johnsson *et al.*, 2009, Johnsson and Wright, 2010).

Histone modification has been intensely studied by many laboratories in recent years as we try to understand epigenetic modifications and their functions. The ENCODE project has enabled much progress as a result of different labs conducting ChIP sequencing experiments to map where different marks and TFs may localise in the human genome in different cell lines. This pool of data is publicly accessible and different users can align their own data against the resource. Some epigenetic modifications studied in the ENCODE project are listed in the Table below (Table 1).

Histone	Histone modification	Transcriptional effect/ gene or chromatin location
H3	K4me1	Gene activation/Important in development
	K4me2	Gene activation
	K4me3	Gene activation /5' end transcriptionally active gene
	K9ac	Gene activation
	K9me1	Gene silencing/ 5' end of genes/ euchromatin
	K9me2	Gene silencing/euchromatin
	K9me3	gene silencing/promoters & heterochromatin gene activation/gene coding region
	K27ac	Gene activation
	K27me1	Gene silencing/ heterochromatin
	K27me3	gene silencing/inactive X-chromosome, imprinted regions & homeotic genes
	K36me3	Gene activation (elongation)
	K79me1	Gene activation
	K79me2	Gene activation/preference for 5' end of genes
H4	K20me1	Gene silencing/Preference for 5' end of genes
	K20me2	Gene silencing/ heterochromatin
	K20me3	Gene silencing/ heterochromatin

Table 1. Table showing histone acetylation and methylation modifications, their locations, and the effects on transcription regulation (Handy *et al.*, 2011).

1.1.5. Role of enhancers in transcriptional regulation

An enhancer is short region of DNA, about 50-1500bp that can be bound by TFs to aid the transcriptional regulation of a specific gene. Enhancers are generally found scattered within the non-coding regions of the human genome and can act at long distances from their target genes. They can be located up to 1 Mbp upstream or downstream from the gene TSS, and in the forward or backward direction. The orientation may be reversed without affecting enhancer function (Blackwood and Kadonaga, 1998, Maston *et al.*, 2006a, Pennacchio *et al.*, 2013). Studies suggest that general information processing that occurs on enhancers occurs through the coordinate action of the enhanceosome, a cooperative protein complex that assembles at the enhancer and regulates target gene expression due to protein-protein interactions within the complex. An alternative mechanism for enhancer function has also been suggested. This mechanism called a flexible information display or billboard is less integrative, and postulates that multiple proteins regulate gene expression independently and the basal transcription machinery sums up their read (Arnosti and Kulkarni, 2005).

The regulation of transcription is initially coordinated by DNA elements which include the core promoter, promoter proximal elements and distal sites such as enhancers, silencers, insulators and locus control regions. These DNA elements create a module that allows cellular factor sets to bind in an ordered fashion creating a pool of unique expression patterns. When gene expression control is combined by factors bound at multiple DNA elements, it allows cells to respond rapidly to environmental or developmental stimuli (Venter *et al.*, 2001).

The core promoter is the site at which Pol II and general transcription factors bind, it identifies the transcription start site and direction of transcription(Smale and Kadonaga, 2003). The core initiation complex although able to transcribe a gene, generally only produces low levels of mRNA. DNA sequences that help recruit members of the Pol II initiation complex are present in the core promoter, however, statistical analysis of ~10,000 known promoters has shown only one eighth contain a

TATA box, and a quarter had none of the proposed core promoter DNA elements (Gershenzon and Ioshikhes, 2005). This suggests that other undescribed core promoter elements may be involved or the known sequences can be much more degenerate than initially implied. Another suggestion is that the exact sequence is secondary to the DNA secondary structure at the core promoter. Studies suggest that the way core promoter elements are composed contributes to the specific regulatory patterns of distal regulatory inputs (Morris *et al.*, 2004, Florquin *et al.*, 2005). Initiator elements (Inr) that surround the TSS can also direct accurate initiation when the TATA element is absent. The Inr is present in both TATA containing and TATA-less promoters and can direct transcription initiation itself or in association with downstream promoter elements (DPE) (Smale and Baltimore, 1989). Enhancers do not bind to the promoter region itself, they are bound through activator proteins, these activators generally bind to promoter proximal elements within a few hundred base pairs of the core promoter and this allows the enhancer to interact with GTFs and Pol II(Ptashne and Gann, 1997, Maston *et al.*, 2006b, Eichenlaub and Ettwiller, 2011). Acetylation of histone H3K27 is regarded as a marker for active enhancer sites (Creyghton *et al.*, 2010).

A single promoter can be acted upon by distinct enhancers at different times or in different tissues, allowing more unique gene expression patterns that cannot be achieved from promoter proximal elements alone (Atchison, 1988). Enhancers typically contain a cluster of transcription factor binding site TFBS, whose wide organisation and orientation to each other is important to its function as cis-regulatory elements. Studies have shown that inserting 6bp of random DNA between two TFBS in an enhancer reduces its activating ability by ~17 fold (Thanos and Maniatis, 1995). The difference between enhancers and promoter proximal elements may be the distance over which enhancers act, otherwise, they appear to function similarly and protein bound enhancers use many of the same mechanisms to stimulate transcription as promoter proximal elements and can physically contact the promoter in question via DNA looping (Vilar and Saiz, 2005).

Interestingly, it has been found that contact between enhancers and promoters on different chromosomes is possible. In fact, one enhancer was shown to interact with multiple promoters on different chromosomes, and it is likely that some promoters are contacted by multiple enhancers from different chromosomes (Spilianakis *et al.*, 2005, Lomvardas *et al.*, 2006). Genes involved in critical developmental processes contain multiple enhancers with overlapping function. Secondary or shadow enhancers may be found many kb away from the primary or first enhancer, which is often closer to the gene being regulated. On its own, each enhancer drives nearly identical gene expression patterns but in some cases, a single enhancer sometimes fails to drive the complete pattern of expression while the presence of both enhancers allows for normal gene expression to be achieved (Perry *et al.*, 2010).

In the mammalian genome, regions of putative enhancer clusters which are bound by high levels of activation-related TFs, BRD4, the mediator component Med1 and emit broad Chip-seq signals are called super-enhancers (Hnisz *et al.*, 2013, Whyte *et al.*, 2013, Pott and Lieb, 2015). Because of their proximity to genes important for controlling cell identity, they are said to play a major role in cell identity and oncogenesis. They also share typical enhancer function like looping to target genes and transcription activation, but are more sensitive to perturbation than typical enhancers. They are responsive to different signals, allowing the regulation of a single gene transcription by multiple signalling pathways (Lovén *et al.*, 2013, Hnisz *et al.*, 2015, Pott and Lieb, 2015). Notch signalling pathway is an example of pathway that regulates target genes using super-enhancers (Yashiro-Ohtani *et al.*, 2014).

1.2. Epstein-Barr virus

The Epstein-Barr virus (EBV) is a human γ-herpes virus found to asymptomatically infect the B-lymphocytes of more than 90% of the world population and establish a lifelong latent state. It was identified in 1964 by Epstein, Achong and Barr in a cell line made from a biopsy of an African patient diagnosed with Burkitt's lymphoma (Young and Murray, 2003). EBV was viewed under the electron microscope as viral-like particles in a series of Burkitt's lymphoma cell lines (LCL) (Epstein *et al.*, 1964). EBV is a member of the herpesviridae family. The herpesviruses are divided into α, β and γ

sub families. Due to its B cell tropism and its ability to replicate in lymphoblastoid and epithelial cell lines in vitro, EBV is classified as a γ-herpesvirus (Roizman *et al.*, 1981). The genome of EBV exists as a 172kb double stranded linear DNA molecule encased in a protein envelope(Hutt-Fletcher, 2007).

Infection usually occurs during the early years of childhood when the maternal antibodies recede, through contact with saliva, and is asymptomatic. If primary infection is delayed until adolescence infectious mononucleosis (IM) (glandular fever) can occur. Glandular fever symptoms vary due to differences in the immune system response, but can include fever, sweating, sore throat and severe fatigue. In addition to transmission through saliva EBV can be transmitted through blood transfusion into a seronegative person (though rarely reported) and also through sexual transmission as EBV has been discovered in high levels in male and female genital secretions (Henke *et al.*, 1973, Macsween and Crawford, 2003). Primary EBV infection in adults results in T lymphocyte proliferation and the release of cytokines, while in childhood i.e. the asymptomatic infection, T lymphocytes do not proliferate (Williams *et al.*, 2004).

1.2.1. EBV infection

EBV infects B cells and epithelial cells with different outcomes. Lytic infection is observed in epithelial cells of the nasopharynx *in vivo* and latent infection is observed in resting B-lymphocytes (Callan *et al.*, 1996, Young and Rickinson, 2004). EBV is thought to enter the body through the mouth and replicates in the oropharynx thereby releasing infectious viral particles in the oral cavity (Macsween and Crawford, 2003). The B-lymphocyte is infected by binding of the glycoprotein gp350 to CD21 (CR2 receptor) on the B-cell surface in addition to the binding of gp42 to the human leucocyte antigen class II molecule (Young and Rickinson, 2004). Once internalised, the terminal repeats at either end of the viral genome fuse to form a circular episome which can then be transcribed by the cellular transcription machinery to encode the series of EBV proteins needed to infect and immortalise cells. EBV also replicates spontaneously in latently infected B-cells in a small quantity as a result of viral reactivation. After infection of B cells, EBV enters lytic replication when sporadically

reactivated from latency, taking the cells through the cycle and producing the components of its viral progeny. The cell is arrested in the G0/G1 phase inhibiting growth and amplifying its own genome 100 – 1000 before lysis occurs. The lytic phase allows the virus to be distributed, important in establishment of the host to host transmission (Macsween and Crawford, 2003, Burns and Crawford, 2004, Tsurumi *et al.*, 2005).

EBV can immortalise resting B cells *in vitro* to generate latently infected and permanently proliferating Lymphoblastoid cell lines (LCLs) (Henle *et al.*, 1967). EBV encodes almost 90 genes, only 11 are expressed in EBV immortalised LCLs and of the 11 genes, 9 encode the latent proteins: EBV nuclear antigens 1, 2, 3A, 3B, 3C, LP and Latent membrane proteins 1, 2A and 2B (Young and Rickinson, 2004). The remaining 2 genes encode the RNAs EBER1 and EBER2 which remain untranslated and non-polyadenylated and the latency III BamH1 A rightward transcript (BART transcripts and miRNAs) which is associated with B cell growth and proliferation (Figure 3) (Murray and Young, 2001, Young and Rickinson, 2004).

Infected B cells migrate to the follicles to undergo the germinal centre (GC) reaction where they proliferate and differentiate into memory B cells(Klein and Dalla-Favera, 2008). During the GC reaction, the latency III growth programme is down regulated to a latency II transcriptional program expressing EBNA 1, LMP1, LMP2A and LMP2B only (Thorley-Lawson, 2001). The proteins expressed in latency II are thought to drive aberrant GC B cell survival stopping infected cells from undergoing apoptosis (Babcock *et al.*, 2000, Roughan and Thorley-Lawson, 2009, Spender and Inman, 2011). EBV therefore can provide the signals needed for survival in these cells (Caldwell *et al.*, 1998, Caldwell *et al.*, 2000, Bechtel *et al.*, 2005). After the GC reaction, EBV expressing either no latent genes or EBNA1 (latency 1) establishes a lifelong persistence in memory B cells(Babcock *et al.*, 1998).

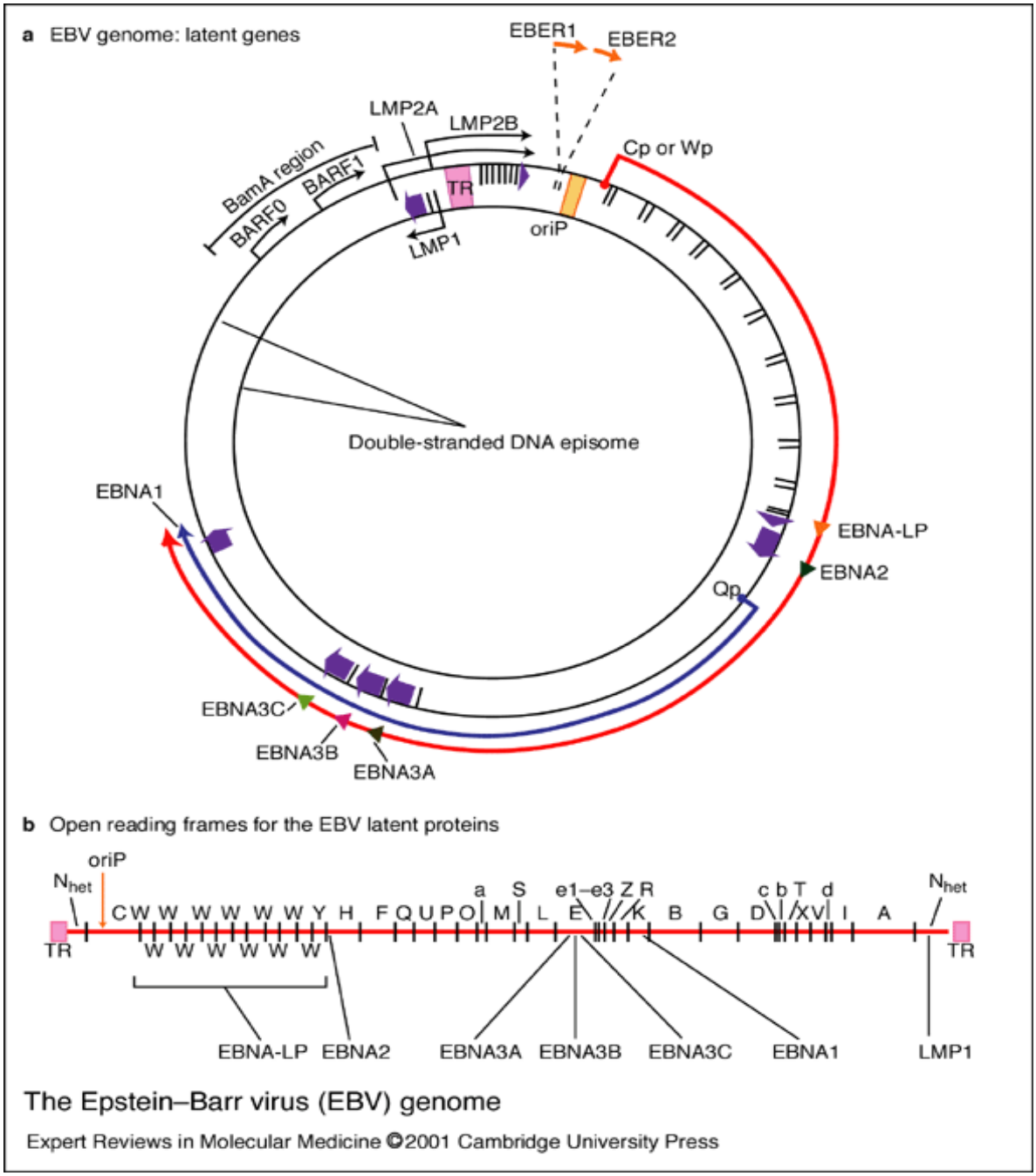

Figure 4. Diagram showing the location of latency genes within the viral genome. Origin of replication (OriP) is shown in orange and the primary message produced from Wp or Cp is shown in red. This is then alternatively spliced to produce the latent gene transcripts shown with purple arrows. The EBNA1 promoter transcript encoding EBNA1 is shown in blue. The terminal repeats where circularisation takes place are shown in pink. Location and details of the LMP promoters and locations of the EBV encoded RNAs are also displayed (Murray and Young, 2001, Young and Murray, 2003).

EBNA 1, 2, 3A, 3C and LMP1 have been shown to play a critical role in the immortalisation process; when EBNA 2, 3C and LMP1 are individually expressed in human cells they can induce LCL-like phenotypic changes (Wang *et al.*, 1990a). The role of EBV latent genes was later confirmed in the *in vitro* transformation of B cells by the generation of recombinant forms of EBV that lack individual latent genes (Knipe *et al.*, 2001). In primary infection, EBV replicates in epithelial cells and establishes Latency III, II, and I infections in B-lymphocytes, the infected cell migrates to follicles and undergo germinal centre reaction, it is during this reaction that latency III is downregulated to latency II, after the GC reaction EBV established lifelong in memory B cells by expressing latency I or 0 (Babcock *et al.*, 1998, Thorley-Lawson, 2001). Studies examining EBV latent gene expression in virus-associated tumours and cell lines have also revealed EBV latency I and II to be found in BL biopsies (Knipe *et al.*, 2001, Tao *et al.*, 2006). Of all the latent proteins, EBNA1 is the only protein consistently expressed throughout the different EBV protein expression patterns observed in tumours probably because of its role as a DNA binding protein binding the origin of plasmid replication (oriP) to induce viral replication and maintain of the episomal EBV genome (Rickinson and Kieff, 1996).

1.2.2. EBV associated diseases

In addition to Burkitt's lymphoma, EBV has been implicated in the development of tumours such as Hodgkins's disease, T-cell lymphoma, undifferentiated nasopharyngeal carcinoma, AIDS associated immunoblastic lymphoma and transplant associated immunoblastic lymphoma.

1.2.2.1. BL

Burkitt's lymphoma (BL) is classified into 3 forms; endemic, sporadic and HIV/AIDS-related and EBV was implicated in endemic BL pathogenesis after being discovered in its cell lines. The association of EBV with sporadic and HIV/AIDS related tumours is less widespread with less than 40% incidence (Burkitt, 1958, Epstein *et al.*, 1964, Magrath, 1990). EBV-positive BL tumours usually occur in young children and present as tumours around the eyes, jaws and abdomen; whereas EBV-negative tumours are seen in the abdomen of all age groups (Thorley-Lawson and Allday, 2008).

Holoendemic malaria has been shown to coincide in areas with endemic BL and BL incidence decreases in areas with malaria eradication programmes (Burkitt, 1983, van den Bosch, 2004). Therefore, malaria has been classed as an endemic BL co-factor and has been shown to cause EBV reactivation and suppression of T-cell mediated responses (Ho *et al.*, 1986, Ho *et al.*, 1988b, Donati *et al.*, 2004).

The unifying feature of BL is a translocation of the oncogene *MYC* to an immunoglobulin locus; this translocation is dependent on activation-induced cytidine deaminase (AID) which is highly expressed in the GC (Filipovich *et al.*, 1992, Dorsett *et al.*, 2007, Pasqualucci *et al.*, 2008, Allday, 2009b). The resulting hyper activation of MYC signalling as a result of its translocation to the immunoglobulin locus leads to apoptosis, perhaps the role of EBV is to counteract the high apoptosis rate either through EBERs or lingering epigenetic modifications of genes induced by other EBV factors (Allday, 2009a).

1.2.2.2. HL

Hodgkins lymphomas (HL) account for 30% of all lymphoid malignancies and, depending on the subtype of the disease, is up to 95% associated with EBV (Harris *et al.*, 1999). There are two different types; classical HL which is characterised by the presence of Reed-Sternberg cells, often infected with EBV and expressing high levels of latent transcripts EBNA 1, LMP1, LMP 2A/B, BARTS RNAs and EBERs and the nodular lymphocyte predominant HL which only has 1% of the tumour mass accounting for malignant cells in the microenvironment surrounding it (Weiss *et al.*, 1987, Weiss *et al.*, 1989, Farrell and Jarrett, 2011). In classical HL, the expression of LMP1 and LMP2A mimics CD40 and BCR signalling respectively. This may provide the aberrant survival signals that HL need to survive and contribute to the loss of B cell identity that is characteristic of classical HL (Dukers *et al.*, 2004, Mancao *et al.*, 2005, Dutton *et al.*, 2007, Kapatai and Murray, 2007, Mancao and Hammerschmidt, 2007).

1.2.2.3. NPC

Nasopharyngeal carcinoma (NPC) is very prevalent in Southeast Asia and Southern China (Chang *et al.*, 2009). It is an epithelial cell tumour and is 100% associated with EBV depending on the location and subtype. It was discovered in early pre-invasive lesions indicating the virus in the initiation of tumours (Wolf *et al.*, 1975, Raab-Traub *et al.*, 1987). NPCs express a high level of latency II transcripts (LMPs, EBNA 1 and EBERs) (Brooks *et al.*, 1992). EBNA 1 and LMP 1 have been shown to upregulate chemokine production and recruit T cells augmenting the survival of undifferentiated nasopharyngeal carcinoma (UNPC) cells, a type of NPC (Lai *et al.,* Agathanggelou *et al.*, 1995, Lai *et al.*, 2010). UNPC is characterised by the presence of a large lymphocyte infiltrate with a lower percentage UNPC cells, LMP1, LMP 2A, EBNA 1 and the ERERs are highly expressed in UNPC and EBV is present in a latency II state (Brooks *et al.*, 1992).

Various T cell lymphomas such as the one occurring in the nasal cavity has also been shown to associate with EBV with both EBNA 1 and EBER 1 transcripts detected in 90% of the lymphoma. It is prevalent in Asia and China and are characterised by an absence of T cell antigens and infection may occur during T cell activation and eradication of EBV infected cells (Brink *et al.*, 2000).

1.2.2.4. IM

Infectious mononucleosis (IM) occurs when infection is delayed until adolescence, and is characterised by up to 1% EBV-positive cells in the B cell pool (Henke *et al.*, 1973, Klein *et al.*, 1976). It can become fatal if EBV-negative transformed cells become dominant, as seen in immune-supressed patients (Falk *et al.*, 1990). In infected B cells, all EBV latency types expression can be detected and in response to delayed infection, a large hyper-activated T cell response that targets both latent and lytic proteins is generated, this contributes to the pathogenesis of the disease (Callan *et al.*, 1996, FIELDS *et al.*, 2001, Precopio *et al.*, 2003). A study of the disease pathogenesis demonstrated the activation of improper CD8+ T cells could contributed to the pathogenesis (Clute *et al.*, 2005).

1.2.2.5. PTLD

Post-transplant lymphoproliferative disease (PTLD), a disease occurring in up to 10% of transplant patients, can arise as a result of EBV infection in 50% of cases in patients who have undergone artificial knock down of the immune system to prevent graft vs host disease (Ho *et al.*, 1988a, Brink *et al.*, 1997). These cells express a latency III pattern of gene expression, the products of which are generally considered to be the primary effector in tumour development. The disease could be fatal in 50% of cases with children who acquire EBV lymphoma post-transplant having the highest mortality rate (Brink *et al.*, 1997, Nalesnik, 1998, Collins *et al.*, 2001).

1.2.3. EBV latent gene promoters

Once infection takes place, the first latent promoter to become active is the W promoter (Wp), located within the tandem IR1 repeat regions (Woisetschlaeger *et al.*, 1990) (Figure 3). The EBV genome has a variable number of IR1 repeats between viral isolates. *Ex vivo* studies showed a mean of 5 to 8 IR1 repeats present in IM patients, in accordance with the optimal number of repeats required for transformation (Tierney *et al.*, 2011). Each IR1 repeat contains a Wp, regulated by B cell transcription factors such as PAX 5 (Tierney *et al.*, 2007). Transcription from Wp results in the synthesis of detectable EBNA-LP and EBNA 2 protein between 8 and 12 hours (Allday *et al.*, 1989, Alfieri *et al.*, 1991, Tierney *et al.*, 2007). Around 48 hrs post infection, the main latent C promoter (Cp) is activated by EBNA 2 (Sung *et al.*, 1991). Following Cp activation, Wp is subsequently methylated and transcriptional activity is reduced (Tierney *et al.*, 2000b).

The C promoter (Cp) is only active in B cells and drives the transcription of an approximate 120kb pre-mRNA that is differentially spliced to generate messages encoding all the other EBNAs required for immortalisation, including EBNA 1 (Bodescot *et al.*, 1987). EBNA 2 is the main regulator of Cp, this was demonstrated when primary infection using an EBNA 2-deleted virus mainly used Wp and did not switch back to Cp (Woisetschlaeger *et al.*, 1991). Cp requires cellular transcription factors for its activity. When EBNA 2-dependant Cp is activated, RBP-Jk and Activating Transcription Factor 2 (ATF2) binding site are noted to be required (Ling *et al.*, 1993,

Fuentes-Panana and Ling, 1998). Transcriptional activators NF-Y, SP1, SP3 and C/EBP also interact with Cp upstream elements in Rael cells, furthermore, NF-Y and Sp1 were shown to be required for Cp activation in the presence of EBNA 2, which may be important for the Wp to Cp switch (Nilsson *et al.*, 2001, Borestrom *et al.*, 2003).

The binding of a chromatin binding factor known as CCCTC-binding factor (CTCF) between OriP and Cp may contribute to the maintenance of specific latency. Using the chromosome conformational capture technique, it was revealed that CTCF binding upstream of Cp and Wp together with both cellular and viral factors influenced the different tertiary chromatin structures associated with latency I and III cells. CTCF may therefore restrict access of transcriptional machinery to various latent promoters by looping DNA (Chau *et al.*, 2006, Tempera *et al.*, 2010, Tempera *et al.*, 2011). Cp can also be subjected to epigenetic regulation since in latency I or II cells where Cp is not active, Cp is methylated on CpG dinucleotide sequence stopping transcription factor binding and promoter activity (Tierney *et al.*, 2000a, Bakos *et al.*, 2007).

LMP 1 is expressed from the EBNA 2 dependent bi-directional LMP1 promoter (LMP1p) (Wang *et al.*, 1990b). The LMP 1 gene is totally contained within the LMP 2A gene locus on the opposite DNA strand which means transcription occurs in the reverse orientation (Figure 4). Furthermore two RBPJk sites control the bi-directional transcription of LMP 1 and the truncated form of LMP 2A, LMP 2B (Meitinger *et al.*, 1994). Also, required for EBNA 2 dependent transcription are key regulatory regions mapped within the LMP1 regulatory sequence (LRS) including a PU.1 binding site, cAMP response element (CRE) and AP-2 consensus site (Laux *et al.*, 1994, Johannsen *et al.*, 1995a, Sjoblom *et al.*, 1998, Jansson *et al.*, 2007). They all play a role in EBNA 2 dependent LMP 1p, LMP 1p can also be co-activated by EBNA 3C through a PU.1 binding motif (Zhao and Sample, 2000, Lin *et al.*, 2002).

In the absence of EBNA 2 in latency II, LMP 1p can be activated by a heterodimer complex of ATF-1 and CREB-1 at CRE (Sjoblom *et al.*, 1998). Additionally, an upstream E-box motif can also regulate LMP 1p activity (Sjoblom-Hallen *et al.*, 1999). Once EBNA 2 and the latency III growth programme have been switched off, only EBNA 1 and the

LMP genes are expressed. However, EBNA 1 is expressed from an alternate latent promoter Qp (Schaefer *et al.*, 1995). In healthy adults infected with EBV, memory B cells harbouring the virus are usually in latency 0 (no viral protein expressed) (Babcock *et al.*, 1998)

1.2.4. EBV latent genes

1.2.4.1. EBNA-LP

The EBNA leader protein (LP) is essential for efficient B-cell immortalisation (Mannick *et al.*, 1991, Allan *et al.*, 1992). During early infection, different isoforms of EBNA-LP can be detected because the entire EBNA-LP coding sequence is contained within the W repeat region (IR1) (Speck *et al.*, 1986, Wang *et al.*, 1987b). Therefore, the size of EBNA-LP transcripts depends on the number of W repeats contained and from which Wp transcription is initiated from (Sample *et al.*, 1986, Finke *et al.*, 1987). The Cyclin D2 gene was the first demonstration of EBNA-LP functioning as a transcriptional co-activator with EBNA 2 (Sinclair *et al.*, 1994).

In addition, EBNA-LP has been shown to co-activate all EBNA 2-dependent viral genes (Harada and Kieff, 1997, Nitsche *et al.*, 1997) and the cellular gene *hes1* (Portal *et al.*, 2011). Furthermore, the phosphorylation of the EBNA-LP Serine 35 residue is critical for the EBNA 2 dependent co-activation of LMP1 (McCann *et al.*, 2001). Although the precise mechanism of EBNA-LP co-activation is unknown, EBNA-LP has been shown to bind co-repressor complexes such as Histone deacetylase 4 (HDAC 4) and NCoR resulting in the association between EBNA 2 and RBPJk being enhanced at promoters (Portal *et al.*, 2006, Portal *et al.*, 2011).

1.2.4.2. EBNA 1

EBNA 1 is the only latent protein expressed in all latencies and all EBV-associated diseases. EBNA 1 is a multifunctional viral protein that is required for B cell immortalisation (Humme *et al.*, 2003). Through its interaction with OriP binding elements, EBNA 1 regulates viral replication, chromosome segregation and transcription (Yates *et al.*, 1984, Lupton and Levine, 1985, Rawlins *et al.*, 1985, Yates *et al.*, 1985, Sugden and Warren, 1989). OriP contains two EBNA 1 binding elements; family of repeats (FR) and dyad symmetry (DS) (Reisman *et al.*, 1985). DS is required

for viral replication (Wysokenski and Yates, 1989), while FR has been linked to mitotic segregation and transcriptional function (Reisman and Sugden, 1986).

EBNA 1 has been shown to enhance both Cp and LMP 1 transcription through OriP binding and to regulate its own expression in latency I cells through Qp (Sugden and Warren, 1989, Sample *et al.*, 1992, Gahn and Sugden, 1995). Furthermore, EBNA 1 was shown to functionally interact with the chromatin adapter protein Brd4, therefore EBNA 1-dependent transcription may be mediated through the elongation factor pTEFb, known to be a binding partner of Brd4 (Jang *et al.*, 2005, Yang *et al.*, 2005). Further interactions with cellular protein and p53 regulator USP7 elude to a potential anti-apoptotic function (Saridakis *et al.*, 2005).

1.2.4.3. EBNA 2

EBNA 2 is a transcriptional regulator of both cellular and viral genes. It initiates and maintains the growth of infected B cells during latency III. P3HR-1 an EBV strain carrying a deletion of the gene encoding EBNA2 and the last two exons of EBNA-LP gave the first indication of the important role played by EBNA2 protein in B cell immortalisation by its inability to transform B cells (Cohen *et al.*, 1989, Knipe *et al.*, 2001). Upon restoration of EBNA 2 protein into P3HR-1, functionally essential domains of the protein were identified and this confirmed the role of EBNA 2 in the B cell transformation process (Rabson *et al.*, 1982, Hammerschmidt and Sugden, 1989).

EBNA 2 transactivates Cp and drives the switch from Wp to Cp in the early stages of B cell infection. EBNA 2 does not bind DNA directly, it interacts with RBP-Jk a cellular DNA binding protein to bind upstream of and activate the latent promoters C (Sung *et al.*, 1991, Jin and Speck, 1992), LMP1, LMP 2A and LMP 2B. This makes RBP-Jk partly responsible for targeting EBNA 2 to promoters containing the RBP-Jk consensus sequence processing a common core sequence (GTGGGAAA) (Fåhraeus *et al.*, 1990, Ghosh and Kieff, 1990, Wang *et al.*, 1990b). RBP-Jk (CBF 1) is a cellular Notch-pathway adapter protein that recruits co-repressor complexes containing Ski-interacting protein (SKIP), histone deacetylases HDAC1 and HDAC2, Sin3A and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) to repress transcription by condensing DNA and blocking transcription factor access (Kao *et al.*, 1998, Zhou *et al.*, 2000a, Zhou and Hayward, 2001). EBNA 2 activates repressed

subsets of RBPJκ targeted genes by binding to and masking the RBPJκ repressive domain whilst recruiting transcriptional activators to the same sites (Figure 5) (Hsieh and Hayward, 1995).

RBP-Jk repressed promoters are also activated by Notch receptors in a similar fashion (Grossman *et al.*, 1994, Zimber-Strobl and Strobl, 2001). RBP-Jk is a downstream target in the Notch pathway. When the Notch pathway is activated by extracellular ligands bound to the Notch receptor, the intracellular domain of Notch, Notch-IC is cleaved which then interacts with RBP-Jk bound to DNA leading to transactivation by displacing the HDACs and recruiting HATs like p300. EBNA 2 can mimic the effects of intracellular (active) Notch in its association with RBP-Jk and can functionally replace the intracellular region of Notch at some targets so that the extracellular stimulation of the Notch receptor is redundant (Sakai *et al.*, 1998, Zimber-Strobl and Strobl, 2001).

Like Notch proteins, EBNA 2 can simultaneously bind RBP-Jk and SKIP to displace repressive complexes activating transcription (Sakai *et al.*, 1998, Zhou *et al.*, 2000a, Zhou *et al.*, 2000b). Isoleucine residues 307 and 308 contained in the conserved region 5 (CR5) and residues 318- 327 contained in the CR6 are crucial of EBNA 2 are crucial for EBNA 2 interaction with SKIP and RBP-Jk respectively (Figure 5) (Ling *et al.*, 1993, Yalamanchili *et al.*, 1994, Ling and Hayward, 1995, Zhou *et al.*, 2000a). It has been shown that mutation of amino acid residues 323 and 324 completely abolished RBP-Jk binding and Cp activation (Ling *et al.*, 1993). Furthermore, mutation studies on RBP-Jk showed reduced LMP1p transcriptional activation by 60% and demonstrated that removal of B cell specific transcription factor PU.1 binding site at the LMP1p eliminates EBNA 2 responsiveness as PU.1 no longer recruits EBNA 2 to this site (Johannsen *et al.*, 1995a, Sjoblom *et al.*, 1995, Sjoblom *et al.*, 1998).

In addition to viral genes, EBNA 2 has also been shown to regulate transcription of hundreds of cellular genes, most targets upregulated by mechanisms that are not yet fully understood (Thompson *et al.*, 1999, Cahir-McFarland *et al.*, 2004, Maier *et al.*, 2006). EBNA 2 is known to up-regulate CD21 (Cordier *et al.*, 1990), the cell surface receptor utilised by the EBV for internalisation (Fingeroth *et al.*, 1984). Other cellular

targets up-regulated by EBNA 2 include the B cell activation marker CD23 (Wang *et al.*, 1987a), the proto-oncogene c-MYC (Kaiser *et al.*, 1999), the B cell transcription factor RUNX3 (Spender *et al.*, 2002) and the G1 cyclin, Cyclin D2 (Sinclair *et al.*, 1994) proteins.

EBF and RUNX proteins have been implicated in targeting EBNA 2 to DNA (Zhao *et al.*, 2011). Once associated with DNA through a cellular binding partner EBNA 2 can transactivate through numerous mechanisms with its acidic transactivation domain (TAD) which has shown to be essential for transformation and transactivation (Cohen *et al.*, 1991, Zhao *et al.*, 2011). EBNA 2 TAD-GAL4 was shown to upregulate expression from plasmids containing GAL4 binding sites 125-fold compared to GAL4 only (Cohen and Kieff, 1991).

Recent studies propose a new role for EBNA 2 that rather than static binding of B cell factors to consensus binding site at the target gene promoters as in previous studies, they suggest EBNA 2 induce dynamic and combinatorial binding sites. It was suggested that EBNA 2 can drive the formation of new EBNA 2 –dependent chromosomal binding sites for RBP-Jk and EBF1 which are in close physical proximity in cellular and viral genome. It was shown in their biochemical and shRNA studies which suggests that these newly formed co-occupied sites are cooperative and highly enriched at the promoter and enhancer regulatory elements of EBV activated genes that are required for proliferation and survival of B cells. It was suggested EBNA 2 facilitates new cooperative and combinatorial interactions on DNA by reprogramming the binding patterns of transcription factors RBP-Jk and EBF1 (Lu *et al.*, 2016b).

Interestingly, gene expression microarrays have shown EBV transformed LCLs or conditional B cells expressing EBNA 2 down-regulate many genes. For example, Maier *et al* found that of 18 genes were repressed at least 2-fold by EBNA including is the B-cell receptor genes *CD79A* and *CD79B* (Thompson *et al.*, 1999, Cahir-McFarland *et al.*, 2004, Maier *et al.*, 2006). Immunoglobulin M (IgM) has also been shown to be repressed by EBNA 2, the transcriptional repression has also been shown to be

partially dependent on RBP-Jk (Jochner *et al.*, 1996, Strobl *et al.*, 2000, Maier *et al.*, 2005).

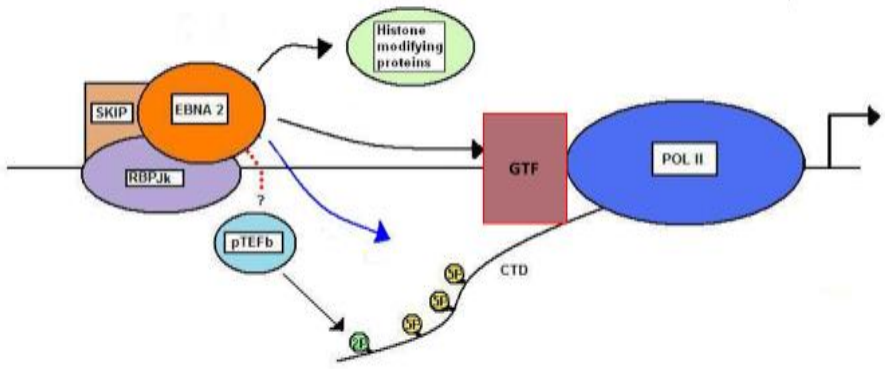

Figure 5. Schematic representation of the mechanism of EBNA 2 transcription activation. EBNA 2 is recruited upstream of promoters through interactions with cellular adapter proteins. Interactions with histone modifiers and GTFs are indicated with black arrow. The blue arrow indicates an indirect mechanism where EBNA 2 binding to promoters may facilitate serine 5 phosphorylation on the CTD.

1.2.4.3.1. Transcriptional regulation by EBNA 2

The TAD of EBNA 2 is known to interact with the histone acetyltransferases (HATs) p300, CBP and PCAF that meditate the acetylation of histone tails to activate transcription (Wang *et al.*, 2000). Phosphorylated EBNA 2 interacts with the chromatin remodelling complex hSNF5 (Wu *et al.*, 1996). The EBNA 2 TAD interacts with the basal transcription machinery components TFIIH (p62 and XPD) (Tong *et al.*, 1995a), TFIIE (p100) (Tong *et al.*, 1995b), TAF40 and TFIIB (Tong *et al.*, 1995c). Mutation of tryptophan 454 in the EBNA 2 TAD to an alanine or threonine residue blocks the ability

of EBNA 2 to interact with these proteins (Tong *et al.*, 1995a, Tong *et al.*, 1995b, Tong *et al.*, 1995c, Wang *et al.*, 2000).

Pol II serine 5 CTD phosphorylation at Cp also significantly increased on activation in the presence of EBNA 2 (Bark-Jones *et al.*, 2006). Furthermore, both Cp and LMP 1p transcription was dependent on the elongation factor pTEFb (Bark-Jones *et al.*, 2006). Therefore, EBNA 2 dependent activation of viral and cellular transcription is probably through the binding of adapter proteins, stimulating histone acetylation and Pol II Ser 5 CTD phosphorylation at viral promoters (Bark-Jones *et al.*, 2006, Day *et al.*, 2007, Fejer *et al.*, 2008). EBNA 2 recruitment to promoters appears to be regulated through the phosphorylation of its Serine 243 residue. This residue is targeted for hyper-phosphorylation by CDK1 during mitosis (Yue *et al.*, 2004, Yue *et al.*, 2006) and by the EBV encoded Serine/Threonine protein kinase PK (Yue *et al.*, 2005).

1.2.4.4. EBNA 3 family

The EBNA 3 family of proteins EBNA 3A, 3B and 3C have similar genetic organisation sharing a short 5' exon and a long 3' exon and encode hydrophilic proteins containing leucine, isoleucine or valine heptad repeats. They are tandemly located in the EBV genome and are thought to have evolved by a series of gene duplication events. The proteins share approximately 30% homology in the N terminal region (Ogiwara *et al.*, 1988, Robertson, 1997, Jiang *et al.*, 2000). They function as transcriptional regulators and deregulators of the cell cycle and are expressed following the activation of transcription from Cp by EBNA 2 (Radkov *et al.*, 1999). Each of the EBNA 3s can bind to RBP-Jk independently and prevent EBNA 2 activation in reporter assays but only EBNA 3A and 3C are essential for B cell transformation *in vitro* (Tomkinson *et al.*, 1993, Waltzer *et al.*, 1996, Zhao *et al.*, 1996, Robertson, 1997). EBNA 3B was found to be completely dispensable but has a tumour suppressive function *in vivo* (Tomkinson and Kieff, 1992, White *et al.*, 2012).

The association of the EBNA 3 proteins with RBP-Jk has been shown to disrupt the binding of EBNA 2 to Cp but removal of EBNA 3s does not increase the EBNA 2 signal in infected cells that should result from increased Cp activity (Waltzer *et al.*, 1996, Robertson, 1997, Maruo *et al.*, 2005). Like EBNA2, EBNA 3C is also able to induce CD21 expression in B-cells (Cotter and Robertson, 2000, Zhao and Sample, 2000) and

interacts with p300, prothymosin α and H1 to promote histone tail acetylation and modification of chromatin through transcriptional activation (Marshall and Sample, 1995, Cotter and Robertson, 2000).

Transcriptome analysis has shown the EBNA 3s to act synergistically, rather than individually, to regulate gene transcription (Skalska *et al.*, 2010, White *et al.*, 2010). Microarray data have identified large numbers of cellular genes involved in cell cycle, cell migration, apoptosis and B cell transcription factors differentially regulated in the EBNA 3 gene knockout (KO) BL cell lines; 210 in EBNA 3AKO, 598 in EBNA 3BKO and 839 in EBNA 3CKO of the 1201 genes 390 needed multiple EBNA 3 proteins co-regulation for gene expression to occur (White *et al.*, 2010). Pro-apoptotic Bcl-2 protein Bim regulates apoptosis through Bax activation, Bim expression is repressed by H3K27Me3 epigenetic silencing upon EBV infection and EBNA 3A and 3C were shown to downregulate Bim expression cooperatively (Anderton *et al.*, 2008, Paschos *et al.*, 2009, Wood *et al.*, 2016).

EBNA 3A and 3C have been shown to function as transcriptional repressor by associating with co-repressors such as CtBP (Hickabottom *et al.*, 2002, Paschos *et al.*, 2012). Together, they to increase repressive epigenetic maker H3K27Me3 and co-repress *CDKN2A* (which encodes $P16^{INK4a}$ and $P14^{ARF}$) and enable G_1 growth arrest to be by passed by EBV (Skalska *et al.*, 2010, Maruo *et al.*, 2011). EBNA 3C inactivation was shown to decrease phosphorylation of Retinoblastoma protein (pBb) hyperphosphorylation and accumulation of $P16^{INK4a}$ preventing the progression of cell cycle. Collectively, this data suggests the EBNA 3 family proteins uses polycomb proteins and histone modifications to co-regulate gene expressions and cell survival (White *et al.*, 2010).

In an EBV-negative BL2 cell line EBNA 3C induces low-level activation and increases the effect of EBNA2 on the LMP1 promoter. Since EBNA2 already activates the LMP1 promoter, this indicates that EBNA 3C supports EBNA2 in increasing LMP1 levels (Allday *et al.*, 1993, Allday and Farrell, 1994). EBV-positive Raji cell lines, which carry a virus deleted for EBNA 3C cultured at high density in LMP1 expression showed most

of the cells arrested at G1 with a reduction in LMP1 expression indicating that LMP1 is dependent on the cell-cycle state. LMP1 expression is influenced by EBNA 3C either by releasing the LMP1 repression or altering the state of the cell cycle because when EBNA3C was expressed in transfected Raji cells it did not reduce LMP1 expression in the growth arrested cells changes. EBNA 3C has been shown to also interact with PU.1, and co activates LMP1 though PU.1 binding sites at the promoter (Allday and Farrell, 1994, Zhao and Sample, 2000, Taby and Issa, 2010).

EBNA 3C can bind HDAC proteins (HDAC 1 and 2) in large repressive complexes associated with deacetylase activity to silence dependent transcription and its interaction with HDAC1 contribute to the transcriptional repression of Cp by RBP-Jk through this interaction (Radkov *et al.*, 1999, Knight *et al.*, 2003). As EBNA2 also acts through RBP-Jk it has been hypothesised that the two nuclear antigens (EBNA2 and EBNA3C) are further antagonistic as they compete for RBP-Jk molecules and binding sites (Lee *et al.*, 2009), however these observations were found *in vitro* and recent evidence from arrays and ChIP sequencing suggests that the two proteins act in different ways and that the effects of EBNA3C cannot be attributed to antagonism alone (McClellan *et al.*, 2012).

White *et al* performed a gene expression microarray experiment in which BL cells were infected with wild type, or 3A, 3B or 3C knock out or revertant BAC derived virus. 36 genes were shown to be regulated by all 3 EBNA 3 proteins. They revealed modest overlap between the different sets of genes. These genes were repressed by different subsets of EBNA 3 proteins. Investigation of the NOTCH2 promoters, repressed by EBNA 3A and 3C, RAS guanyl nucleotide-releasing protein 1 (RASGRP1) repressed by EBNA 3B and 3C and Thymocyte selection-associated high mobility group box protein (TOX) which is repressed by all the EBNA 3s revealed that H3K27me3 was decreased in knock out lines relative to wild type, correlating with gene repression. H3K4me3 however was unchanged creating a poised chromatin state at these genes in the absence of one or more EBNA 3 proteins. H3K9Ac increases correlated with increased TOX and RASGRP1 expression but was unchanged at NOTCH2.

This suggests that cell context is crucial for directing EBNA 3 proteins to different targets and modulating transcription. This may be due to different chromatin contexts or abundance of different TFs (17 including NOTCH2, EBF1, PU.1 and Pax5) and cofactors. Crucially work from this paper implicates EBNA 3B as an essential EBV gene in vivo as many genes regulated by it are essential in the GC reaction which is not reproduced in *in vitro* immortalisation (White *et al.*, 2010).

1.3. AIMS OF THIS PROJECT

The overall aim of this project was to study how EBNA 2 promotes immortalisation through the epigenetic reprogramming of cellular genes.

- I investigated EBNA 2 and EBNA 3 proteins coincident binding at the *ITGAL* promoter and to long range enhancers at *CTBP2* and *WEE1.* I specifically investigated regulatory elements targeted by EBNA 2 and how they antagonise or complement EBNA 3A, 3B and 3C to promote immortalisation.
- I also investigated EBNA 2 association with activated and repressed gene targets concentrating on repressed gene targets CD79A and *CD79B* and activated gene target *IRF4*.

2. MATERIALS AND METHODS

2.1. Tissue Culture

All reagents were purchased from fisher unless otherwise stated

2.1.1. Tissue culture media and supplements

100x Penicillin-Streptomycin-Glutamine (PSG) (Life Technologies)

Contains 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate and 29.2 mg/ml L-glutamine with 0.85% saline and 10 mM citrate buffer. Stored in 5 ml aliquots -20°C.

Dimethyl Sulphoxide (DMSO) (Sigma)

Dulbecco's Phosphate Buffered saline without $CaCl_2$ and $MgCl_2$ (PBS) (Life Technologies)

Fetal Bovine serum (FBS) (Gibco)

Pre-screened for endotoxins (≤5 EU/ml), haemoglobin (≤10 mg/dl) levels and heat inactivated at 56°C for 30 mins. Stored in 50ml aliquots at -20°C.

Freezing mix

80% RPMI media (supplemented with 10% FBS and PSG), 10% FBS and 10% DMSO.

RPMI 1640 media without L-glutamine (Life Technologies)

2.1.2. Maintenance of cell lines

DG75

DG75 are EBV-negative human B cell lymphoma line obtained from the pleural effusion of a 10-year-old boy with Burkitt's lymphoma. Morphologically, they can be round to polygonal and single to cluster in suspension. DG75 were cultured in RPMI 1640 media containing 10% FCS and PSG and incubated at 37°C in 5% CO_2. The cells were split 1:4 twice a week into pre-warmed media and 1:3 24hrs before transfection.

BJAB

BJAB are also EBV-negative human Burkitt's lymphoma cell line from Africa. They are obtained from peripheral blood B lymphocytes. BJABs were cultured in in RPMI 1640 media containing 10% FCS and PSG and incubated at 37°C in 5% CO_2. The cells were split 1:4 twice a week into pre-warmed media and 1:3 24hrs before transfection.

Cell lines mentioned in this study

Cell line name	Cell type	Description	Reference
3A KO BL31	BL 31	EBV negative BL cell line with 3A knockout recombinant bacmids	(White *et al.*, 2010)
3B KO BL 31	BL 31	EBV negative BL cell line with 3B knockout recombinant bacmids	(White *et al.*, 2010)
3C KO BL31	BL 31	EBV negative BL cell line with 3C knockout recombinant bacmids	(White *et al.*, 2010)
E3 KO BL31	BL 31	EBV negative BL cell line with 3 loci knockout recombinant bacmids	(White *et al.*, 2010)
Wt BAC BL31	BL31	EBV negative BL cell line with wide type bacmids	(White *et al.*, 2010)
3B KO LCL	LCL	EBV negative B-cell line with 3B knockout recombinant bacmids	(White *et al.*, 2010)

ER/EB2	BL41	EBV-negative B-cell lines fused to ER/EBNA 2	(Kempkes *et al.*, 1995)
ER/EB BJAB	LCL	EBV-negative B-cell lines fused to ER/EBNA 2	(Kempkes et al., 1995)

2.1.3. Freezing cells

200 mls of cells were pelleted by centrifugation at 13000g for 10 mins at 4°C and resuspended in 5 mls of freezing mix. Cells were aliquoted equally into 5 cryogenic vials, and frozen at -80°C in a container with isopropanol. Vials were transferred to liquid nitrogen storage after at least 24 hrs at -80°C.

2.1.4. Thawing cells

Cells were transferred from liquid nitrogen storage to a 37°C water bath. Once thawed, the 1 ml of cells were added to 10 mls pre-warmed RPMI media in a 25ml flask and incubated overnight at 37°C with 5% CO_2.

2.1.5. Haemocytometer cell counting

15 µl of cells were counted using a Neubauer haemocytometer, which is divided into 9 equally spaced squares. Cells located in the 4 corners were counted, any cells outside or on these defined regions were not counted. The average cell counts of the four squares were used to calculate the cell culture concentration using the formula:

Cells/ml = Average cell count x $1x10^4$ cells

2.1.6. Transfection by Electroporation

DG75 or BJAB cell lines were transfected by electroporation. Cells were diluted 1:3 24hrs prior to transfection, and 1 X 10^7 cells in serum-free medium were mixed with DNA and electroporated at 230V and 950 µF using the Bio-Rad Genepulser III. Transfections contained 2 µg of the luciferase reporter vector pGL3 Basic or pGL3 cloned in with either of the genes investigated *ITGAL*, *WEE1*, *CTBP2*, *IRF4*, *CD79A* and *CD79B* or *Cp1425* (EBNA2 positive control). 1 µg pRL-TK or pRL- CMV which are the transfection control. 10 µg or 20 µg pSG5-2A which expresses EBNA2, pCDNA3 EBNA3A, EBNA3B and EBNA3C expressing constructs. 2

μg of pCMVSPORT6 EBF1 expressing EBF1. DNA levels were kept constant with pSG5 or pcDNA3 vector where necessary.

2.1.7. Luciferase assay

Cells were harvested 48H after transfection, washed in 10 ml PBS and 1 ml was taken and spin down, the pelleted cells were snap frozen for western blot, then remaining 9 ml was spin down, resuspended in 1 ml and the resulting pelleted cells was lysed in 90 μl of 5x Passive lysis buffer (Promega). 10 μl aliquots of cleared lysate were assayed in duplicate in a 96-well plate with 50 μl of Luciferase assay reagent LAR (firefly) followed by 50 μl of Stop and Glo (Renilla) solutions (dual luciferase assay kit-Promega) with the sequential injector system on the Glowmax Multidetection System. The firefly reaction generates a stabilized luminescent signal and after quantification the reaction is quenched. The Renilla luciferase reaction is simultaneously initiated by adding Stop & Glo Reagent to the same well. The firefly luciferase signal was adjusted for transfection efficiency with the renilla luciferase signal from the control plasmid pRL-TK or pRL-CMV. The values for the firefly luciferase activity were corrected by dividing them by the values for the Renilla luciferase activity. For individual experiment representation, the mean of the duplicate corrected value is plotted and the standard deviation used for error bars while for more than one experiment representation, the mean of two or three experiments values are plotted and the standard deviation used for error bars.

2.2. Biochemical reagents and methods

All reagents were purchased from Fisher unless otherwise stated

2.2.1. Reagents

ECL solutions I (1 ml)

125 μM Luminol (250 mM stock in DMSO), 20 μM coumaric acid (Sigma) (90 mM stock in DMSO) and 5 mM Tris pH 8.5 in 1 ml sterile millipore water

ECL solution II (1 ml)

0.0075% Hydrogen Peroxide (H_2O_2) and 5 mM Tris pH 8.5 in 1 ml sterile millipore water.

Gel sample buffer

50 mM Tris, 4% SDS, 5% 2-Mercaptoethanol (Sigma), 10% Glycerol, 1 mM EDTA and 0.01% bromophenol blue

PBS-Tween

100 PBS tablets (Oxoid) and 10 mls Tween-20 made up in 10L dH_20.

Transfer buffer

15g Tris, 72g Glycine, 4L dH_20 and 1 ml Methanol

Stripping buffer for western blots

100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.7

NuPage Tris Acetate running buffer 20X (LIFE TECHNIOLOGIES

NuPage MOPS SDS running buffer 20X (LIFE TECHNIOLOGIES)

See Blue marker (LIFE TECHNOLOGIES)

See Blue Plus marker (LIFE TECHNOLOGIES)

2.2.2. Preparation of whole cell lysates

Cells were washed in PBS, counted and resuspended in 100 µl/1x10^6 cells of 1x GSB. Cells were sonicated on ice using the Vibra-Cell VC 750 sonicator (Sonics) for 7 pulses at 25% amplitude for 10 seconds with 10 second gaps. Samples were boiled at 95°C for 10 mins, vortexed, briefly centrifuged for 30 seconds and stored at -20°C.

2.2.3. SDS page

20 µl samples were loaded into either a pre-poured 4-12% Bis-Tris gel, 10% Bis-Tris gel or 8% Tris Acetate gel (Life Technologies) using a gel loading tip. Lysates were resolved using either 1x MOPS running buffer or 1x Tris Acetate running buffer (Life Technologies). 5 µl of SeeBlue Plus2 pre-stained standard marker (Life Technologies) was also loaded and electrophoresis carried out for 50 mins at 200 V for MOPS or 60mins at 150 V for Tris acetate.

2.2.4. Immunoblotting

After separation by SDS-PAGE, proteins were transferred onto Protran nitrocellulose membranes (Whatman) in transfer buffer at 85 V for 90 mins using a blotting cell (Bio-Rad). Membranes were incubated with ponceau stain (Sigma) for one minute to examine loading and verify a successful transfer. Membranes were cut as required to probe for multiple proteins on one blot. Membranes were washed three times for 5 mins in PBS-Tween on a shaker and blocked for 1 H using 5% milk powder (sainsburys) in PBS-Tween. Primary antibodies (Appendix A) were added to membranes in a 5% milk PBS-Tween solution and incubated overnight at 4°C with rocking. After further washing with PBS-Tween (3x10 mins), secondary antibodies (Appendix A) made up in 5% milk PBS-Tween solution conjugated to a horse-radish peroxidase (HRP) enzyme were added to membranes and incubated with rocking at room temperature for 1 H. Final washing (3x10 mins) in PBS-Tween was performed and equal volumes of ECL solutions I & II were added to membranes. Membranes were imaged on a LiCor Odyssey imaging system.

2.2.5. Stripping gels

To re-probe blots with protein band to close for the membrane to be cut, the membranes were washed with PBS-Tween 2x10mins to remove any excess ECL solutions. Membranes were heated to 50°C for 15 mins in stripping buffer and washed 4x10 mins in PBS-Tween. Membranes were then blocked in 5% milk PBS-Tween solution for 1 H and probed with a different appropriate primary antibody.

2.3. Molecular Biology

2.3.1. Buffers and Reagents

NEB enzymes: XhoI, SacI, KpnI, BglII, HindIII, NheI

Markers: Hyperladder II (BIOLINE)

Powdered agarose: low EEO agarose gel (Fischer)

5x TBE loading dye (QIAGEN)

L broth

25 g L broth powder made up in 1 litre in distilled H_2O and autoclaved

Agar

5 g agarose in 400 mls L broth solution, stirred and autoclaved

Ampicillin agar plates

10 ml of melted agar solution, 100 μg/ml ampicillin

Polymerase: Phusion high fidelity DNA polymerase (NEB)

Alkaline phosphatase: Roche

TBE:

540 g Tris, 275 g Boric acid, 46.5 g EDTA dissolved in 1 litre of distilled water and autoclaved (10x). Then 100 mls of the mix is added to 900mls of Ultra-pure H_2O to get 1X concentration.

2.3.2. pGL3 basic reporter vector – Promega

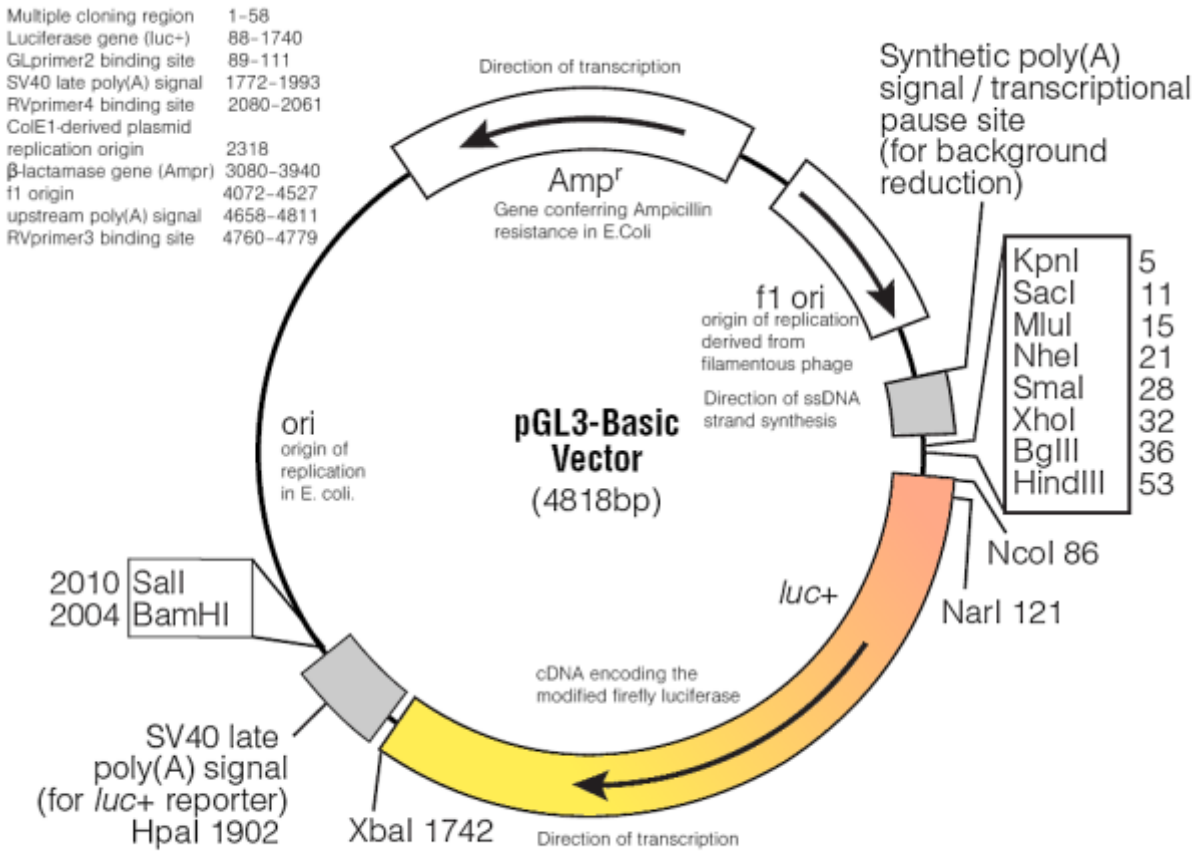

Figure 6. pGL3-Basic vector (http://www.citeulike.org/blog/Zephyrus/8307). pGL3 basic vector allows analysis of enhancer and promoter regions by luciferase assay. The multiple cloning site contains KpnI, SacI, MluI, NheI, Sma1, XhoI and BGIII sites and the vector confers ampicillin resistance to transfected cells.

2.3.3. Plasmid Construction

Primer design

In general, the DNA sequence for the gene of interest is obtained from the genome browser and put into Primer3Plus, an online primer design tool to make a primer set which is then ordered. The primers were designed to introduce specific enzyme digestion sites needed for cloning into the vector. It region of interest was then amplified from genomic DNA extracted from an LCL using Phusion high-fidelity polymerase (NEB) on a PCR machine. The PCR product was then run on a 1% TBE agarose gel and purified (QIAGEN) as required. The DNA sequence for those difficult to amplify by PCR was synthesized (LIFE TECHNOLOGIES and EUROFINS). *ITGAL, CD79B, CTBP2, IRF4* enhancers were amplified from genomic DNA, *IRF4* promoter was amplified from plasmid containing *IRF4* promoter and *WEE1* was amplified from BAC DNA.

Enzyme digestion

3-5 µg of plasmid was digested in a 20 µl reaction with 2 µl of enzyme (NEB) and 2 µl of the appropriate buffer (NEB). The amplified PCR product was also digested with the appropriate enzyme and buffer (NEB). The samples were incubated at 37°C for 2 H. The linearized DNA fragments were separated on a 1% TBE agarose gel and then purified ad required

DNA purification

The DNA of interest is either cut out of a gel using a razor blade under UV- light or cleaned up over the column. DNA was purified using QlAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions and then eluted in 50 µl nuclease free water.

Alkaline phosphatase treatment of vector

To prevent single cut linearized vector from re-ligating, the samples were treated with alkaline phosphatase (ROCHE) to remove the 5' phosphatase group from the cut ends. In a 40 µl total volume reaction, 4 µl of alkaline phosphatase and 4 µl of 10x alkaline phosphatase buffer is added to the DNA solution. The samples were incubated at 37°C for 30 mins.

Ligation

The gene of interest is cloned into the destination vector (pGL3 Basic) at the desired site on the vector's multiple cloning site. To achieve ligation between 80-100 ng of vector was mixed with the insert in molar ratio of 1:1 and 1:3 in the presence of 2 µl of 10x T4 Ligase buffer and 1.5 µl of T4 Ligase and nuclease free water to the total volume of 20 µl. The samples were incubated overnight on ice at bench top from 4°C to room temperature, then used for transformation of DH5α.

Transformation of *E. coli* DH5α

100 µl of an *E. coli* strain called DH5α is added to the ligation products and incubated on ice for 30 mins, they were then heat shocked for 45 seconds at 42°C in a waterbath. 250 µl of L broth was added to each sample and put back on ice for 2 mins. The samples were then incubated in a shaking incubator for 30 mins at 37°C at 225 rpm. The samples were spin down and most of the supernatant removed. The pellet was then resuspended and spread on ampicillin agar plates at 37°C overnight.

2.3.4. Q-PCR

Quantitative PCR (QPCR) was performed using an Applied Biosystems step one plus real-time PCR machine. For ChIP analysis, 3 µl DNA was added to a SYBR green master mix containing 7.5 µl 2xGoTaq QPCR master mix (Promega), 150 nM forward and reverse primers (Appendix B) and sterile Millipore water to a final volume of 15 µl. Samples were heated to 95°C for 10 mins, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min and dissociation curve analysis. Input controls were serially diluted to generate a standard curve for each primer set (Appendix B). A percentage input value was obtained by measuring the crossing threshold (C_t) value in relation to each standard.

3. RESULTS

3.1. Investigating the role of coincident binding of EBNA 2 and EBNA 3A, 3B and 3C to cellular genes and regulatory elements

Previous ChIP-sequencing data from our lab using EBNA 2 and pan-specific EBNA 3 antibodies identified binding sites for EBNA 2 and EBNA 3s in the human genome (McClellan *et al.*, 2013). These binding sites were predominantly distal to transcription start sites (TSS) indicating a role in long range gene control. EBNA 2 had 75% of sites over 4kb from any TSS while EBNA 3 was more pronounced with 84% over 4kb (Figure 7.1 a, b). To identify cellular genes targeted by the binding of EBNA 2 and 3 proteins, the closest gene to each binding site was identified irrespective of distance from the gene TSS. 80% (3157) of genes targeted by EBNA 3 was also targeted by EBNA 2 suggesting a high degree of cross talk between the EBNA 2 and 3 proteins and that they likely co-regulate a subset of cellular genes (Figure 7.1 c). We also analysed whether genes were being targeted individually by EBNA 2 and EBNA 3 sites or if the EBNAs were binding to shared sites, 25% of sites were bound by both proteins (Figure 7.1 d) (McClellan *et al.*, 2013). We set out to investigate how EBNA 2 epigenetically reprogrammes cellular genes through these mapped elements, and how EBNA 3A, 3B and 3C may antagonize or cooperate with EBNA 2 in gene regulation. I focused on three EBNA 2 and EBNA 3 shared binding sites at *ITGAL*, *WEE1* and *CTBP2*.

3.1.1. EBNA 2 activates the *ITGAL* promoter and EBNA 3 proteins inhibit the activation

Integrin alpha L (*ITGAL*) forms part of the cell surface heterodimeric activation antigen LFA-1 together with the beta 2 chain (*ITGB2*) and is expressed on leukocytes. It binds members of the intercellular adhesion molecule family (CD11a/CD18, $\alpha L\beta 2$) and mediates essential adhesive interaction (Kishimoto *et al.*, 1989). *ITGAL* is located on the chromosome 16p11.2 very close to genes encoding other members of the intergrin family. LFA-1 is important in adhesive interactions between T cells, B cells, dendritic cells and macrophages. It is also important for antigen specific T cell activation, alloreactive responses, cytotoxic T cell responses and B cell help (Kishimoto *et al.*, 1989). Using deletion analysis, it was observed that the first 40 bp 5' to the transcription start site are very important for promoter function

(Cornwell *et al.*, 1993). Previous Sequence analysis also identified *SP1* and PU.1 binding motifs sites 120 bp upstream of the TSS (Cornwell *et al.*, 1993, Nueda *et al.*, 1993). Rapid and transient stimulation by cytokines presented on the cell surface can activate LFA-1 and promote firm adhesion to ICAM-1 which can subsequently cause transmigration through cells (reviewed in (Denucci *et al.*, 2009). Importantly LFA-1 has also been shown to prevent B-cell apoptosis in the GC though adhesion to antigen presenting follicular dendritic cells (Lindhout *et al.*, 1993). EBV LMP1 upregulates the expression of *ITGAL* expression and microarray studies in BL cells with recombinant knock-out EBVs have shown that *ITGAL* is repressed by EBNA 3B and EBNA 3C (Wang *et al.*, 1990a, White *et al.*, 2010).

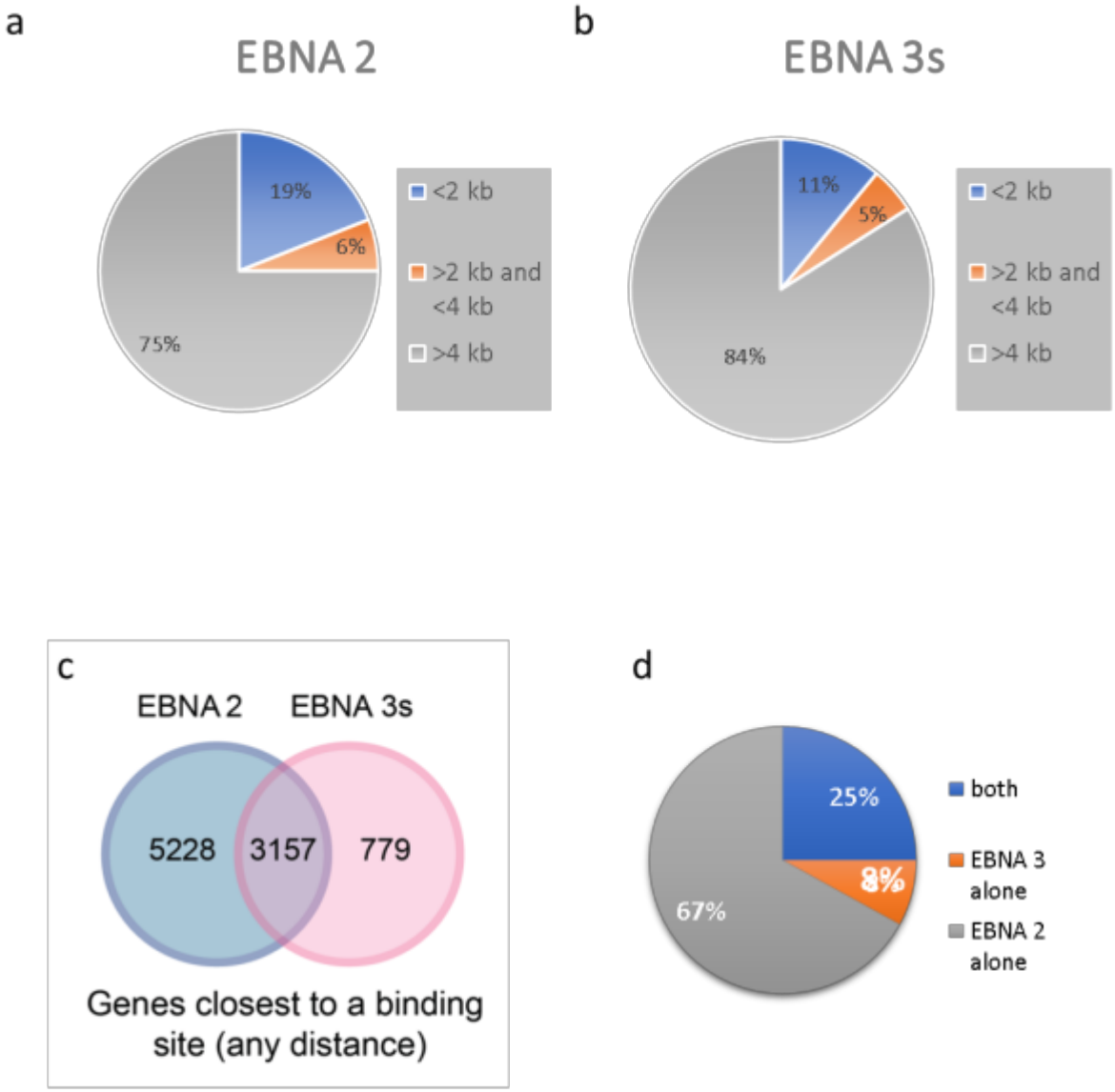

Figure 7.1 Coincident binding of EBNA 2 and 3 in the human genome. (a) Pie chart showing the distribution of all significant binding sites for EBNA 2 relative to gene TSSs. (b) Distribution of EBNA 3 family binding sites. (c) A venn diagram showing comparison of genes closest to an EBNA 2 or EBNA 3 binding site located any distance from a gene TSS. (d) Pie chart showing the proportion of sites identified for EBNA 2 and EBNA 3 family proteins that are shared or unique (McClellan et al., 2013).

Our ChIP sequencing data revealed EBNA 2 and EBNA 3 binding peaks at three distinct, coincident *ITGAL* promoter proximal regions with peak 3 being the largest for EBNA 3 proteins (Figure 7.2). This binding site was also coincident with H3K27ac in GM12878 cells from the ENCODE project highlighting that the binding sites are within gene regulatory regions that are active. To determine whether these binding sites direct EBNA 2 activation of *ITGAL*, I made *ITGAL* promoter-reporter constructs by cloning the 2 kb region encompassing all 3 binding sites into a reporter vector using primers designed to amplify all three peaks joined in the identified 2 kb region to investigate the regulation of *ITGAL* promoter by EBNA 2. The luciferase assay results demonstrating up to a 5-fold activation of the *ITGAL* promoter by EBNA 2. A similar fold activation was observed for the known EBNA 2 activated EBV C promoter. When co-expressed with the EBNA 3s, an inhibition of EBNA 2 activation was observed (Figure 7.3 a). Western blotting confirmed the expression level of EBNA 2, 3A, 3B and 3C proteins in the transfected cells (Figure 7.3 b). This result is consistent with a model where EBNA 3 proteins can compete with EBNA 2 for binding at the *ITGAL* promoter site. Although, in LCLs, additional experiments carried out in our lab, we found that EBNA 3A and EBNA 3C do not bind the *ITGAL* promoter significantly *in vivo* (McClellan *et al.*, 2013), they can compete for binding at this site with EBNA 2 in reporter assay where the expression level is high. EBNA 2 and 3 proteins competitive binding at the *ITGAL* promoter was also supported by re-ChIP analysis of Mutu III BL cell line carried out in out lab, where no simultaneous binding of EBNA 2 and EBNA 3B and 3C were detected (McClellan *et al.*, 2013).

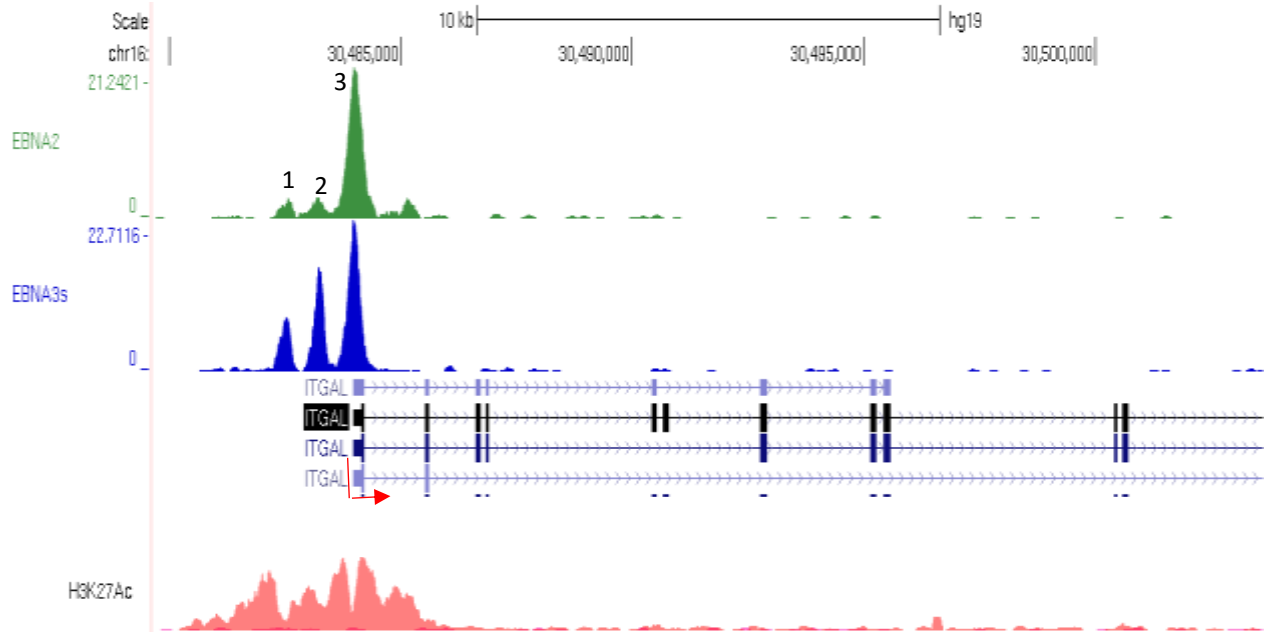

Figure 7.2 Co-incident binding sites for EBNA 2 and EBNA 3 proteins located at the *ITGAL* promoter in EBV infected cells. ChIP-sequencing data in Mutu III BL cells for proximal promoter region of *ITGAL* reveals coincident EBNA2 and EBNA3 binding. Reads per million background subtracted reads is displayed on the y axis. The four proposed isoforms of *ITGAL* and direction of transcription is indicated by the arrow are shown below along with ChIP-sequencing data for H3K27ac in from the EBV immortalised LCL GM12878. The exons are shown with the black boxes and the introns lined with arrows.

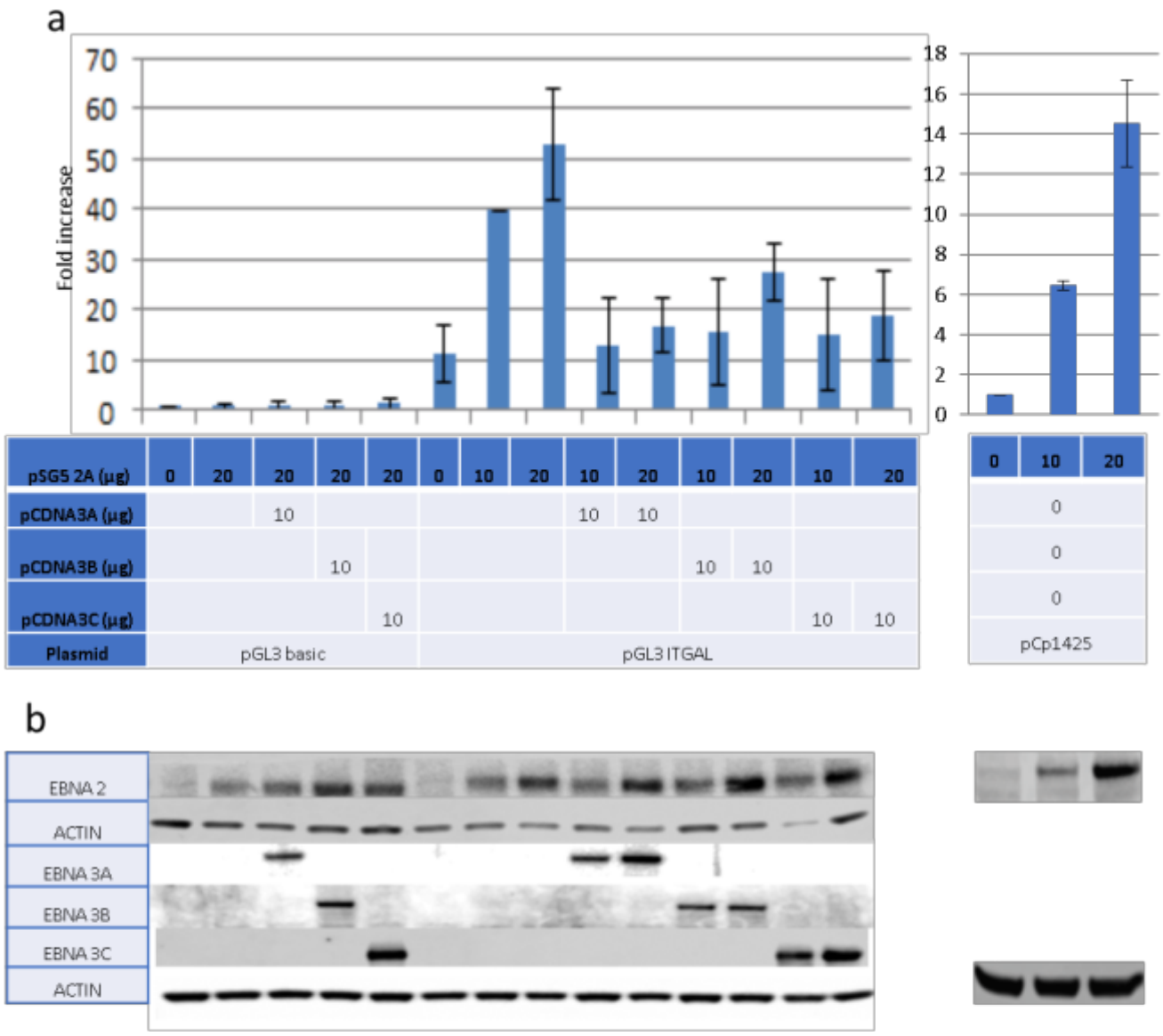

Figure 7.3 The Effect of EBNA 2 and 3 proteins on *ITGAL* expression. (a) Luciferase reporter assays carried out in DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, an *ITGAL* promoter-luciferase reporter (pGL3 *ITGAL*p) or the EBV C promoter reporter (pCp1425GL2) (right panel) in the absence or presence of 10 or 20 µg of EBNA 2, 3A, 3B or 3C expressing constructs. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-TK (1 µg). Results show the mean −/+ standard deviation of 3 independent experiments and are expressed relative to the pGL3 basic signal (left panel) or the Cp1425GL2 signal (right panel) in the absence of EBNA 2. (b) Western blot analysis of EBNA 2, 3A, 3B and 3C expression levels in transfected cells. Each set of blots was also probed for actin as a loading control.

3.1.2. Investigating the cellular transcription factors that direct EBNA 2 binding at the *ITGAL* promoter

EBNA2 and 3 proteins do not bind directly to DNA. They bind through cellular DNA-binding proteins. It has been previously reported that EBNA 2 and the EBNA 3 proteins share RBPJk as a cellular transcription factor and binding partner (Grossman *et al.*, 1994, Henkel *et al.*, 1994, Le Roux *et al.*, 1994, Waltzer *et al.*, 1994) but others studies have also implicated PU.1 in EBNA 2 and EBNA 3C gene targeting *in vivo* and EBF1 motifs have been found enriched at EBNA 2 binding sites in LCLs (Grossman *et al.*, 1994, Waltzer *et al.*, 1994, Johannsen *et al.*, 1995a, Zhao *et al.*, 2011). To understand what factors are responsible for EBNA 2 activation of the *ITGAL* promoter in reporter assays we investigated whether known binding partners of EBNA 2 may be involved by examining available ChIP-seq data. We examined ENCODE GM12878 LCL ChIP-seq data for PU.1 and EBF1 and published RBP-Jk ChIP-seq data from the EBV-immortalised LCL IB4 (Zhao *et al.*, 2011) to determine which cellular transcription factors bind at the *ITGAL* promoter. Significant $_{p}$value$<10^{-7}$ for RBP-Jk binding sites were present at all the *ITGAL* promoter peaks in IB4 cells but peak 3 was most significant, ENCODE data revealed that there were a large amount of cellular transcription factors at the *ITGAL* promoter that have not been described as EBNA 2 binding partners (Table 3). I proceeded to search for RBP-Jk motifs since this factor is already implicated as an EBNA 2 binding partner. I found two putative RBP-Jk motifs GTGAGAA (Friedmann and Kovall, 2010) at the *ITGAL* promoter peak 3 but no consensus was found at the site (Figure 7.4). I then tested whether these sites were required for EBNA 2 activation of *ITGAL* promoter.

3.1.3. RBP-Jk directs EBNA 2 activation of the *ITGAL* promoter

To determine whether RBP-Jk binding can be detected at the ITGAL promoter *in vivo*, I performed QPCR on RBP-Jk ChIP samples provided by Dr. Andrea Gunnell in our group. I used primer sets at the 3 EBNA 2 *ITGAL* promoter peaks (1, 2 & 3) and negative control primers at the PPIA gene where there is no EBNA 2 binding (McClellan *et al.*, 2013). As a positive control for RBP-Jk binding I used primers at the viral LMP1 promoter RBP-Jk site (Palermo *et al.*, 2008) and the EBNA 2 binding site at the CtBP2 gene (Figure 7.5). These data demonstrated that RBP-Jk bound at the highest level at peak 3 in the ITGAL promoter. These data are consistent with the presence of 2 consensus RBP-Jk sites at this position.

ITGALP1			ITGALP2			ITGALP3		
TF	VALUE	MOTIF	TF	VALUE	MOTIF	TF	VALUE	MOTIF
RUNX3	1000	Y	RUNX3	1000	Y	RUNX3	1000	
NFIC	1000		PAX5	719	Y	EBF1	1000	Y
POU2F2	1000	Y	EBF1	546		POLR2A	1000	
BATF	1000		POU2F2	245		SPI1	1000	Y
BHLHE40	734		FOXM1	243		BHLHE40	744	
FOXM1	654		BCL11A	230		MTA3	582	
YY1	577	Y	BATF	226		EP300	544	
EP300	542		TAF1	218		TBP	530	
ATF2	496		YY1	210		POU2F2	481	
IRF4	442		TCF3	209		PAX5	426	
POLR2A	440		RELA	177		RELA	422	
NFATC1	408		BCL3	163		FOXM1	417	
STAT5A	352		EP300	128		NFIC	406	
EBF1	343					ATF2	381	
MTA3	333					YY1	375	
RELA	332					CHD2	371	
BCL11A	329					PML	369	
CEBPB	277					SP1	368	
MEF2A	275					PBX3	365	
TBP	263					CEBPB	356	Y
MAX	234					MXI1	350	
TBL1XR1	222					TCF12	342	
PAX5	219					ELF1	309	
SPI1	219					TAF1	276	
TCF3	216					IRF4	269	Y
TCF12	196					BCL11A	269	
JUND	195					BATF	263	
BCL3	176					TBL1XR1	246	
BCLAF1	173					MAZ	244	Y
SP1	164					CHD1	235	
CHD2	149					MAX	206	
RAD21	133					TCF3	185	
						SMC3	159	
						RAD21	145	
						MEF2A	138	
						BCL3	137	
						WRNIP1	135	
						ETS1	99	Y
RBP-Jk_1							5	Y
RBP-Jk_2	7			11			68	Y

Table 3. List of transcription factor binding signals at *ITGAL* promoter peaks from ENCODE GM12878 LCL ChIP-seq data that was manually extracted from the USCS genome browser and sorted by ENCODE P - value in excel from highest maximum signal of 1000 (red) to lowest signal (blue) with (Y) indicating where motif is present. The RBP-Jk value shown was determined from the MACS peak at the *ITGAL* promoter peaks -10$\log_{10}$p-values signal.

ITGAL PEAK 3

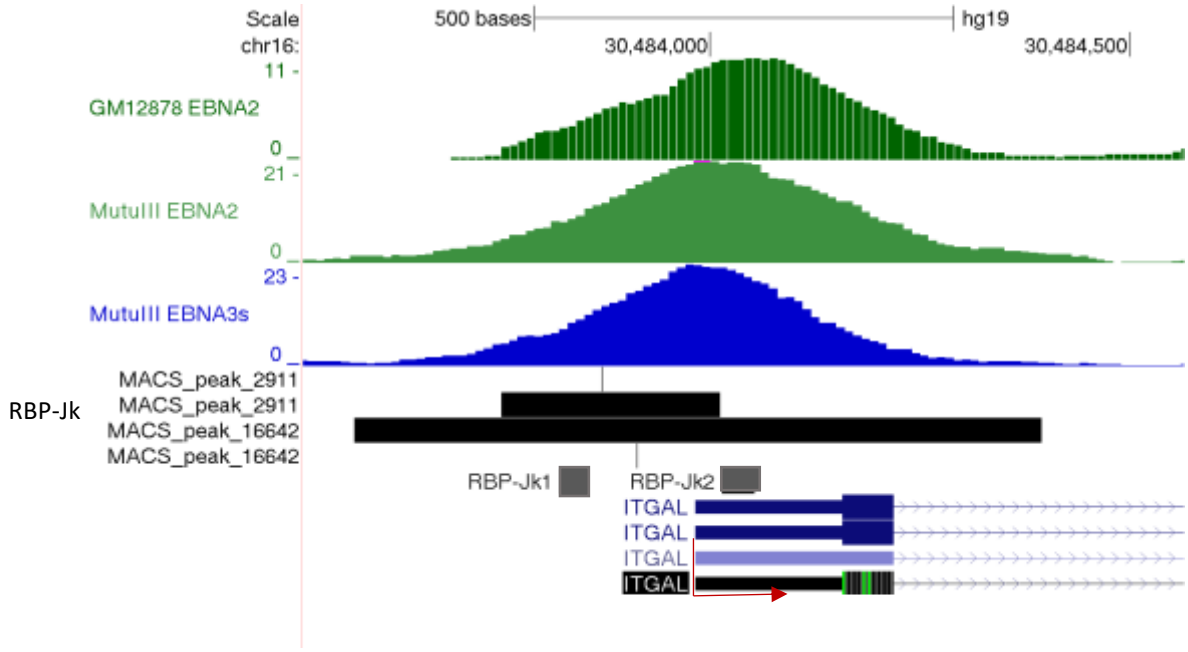

Figure 7.4. Location of RBP-Jk putative motifs at the *ITGAL* promoter site. (a) ChIP-seq data for *ITGAL* promoter and MACS peaks of RBP-Jk ChIP-seq data (black boxes) (Wang et al., 2011) showing the RBP-Jk motifs (grey boxes).

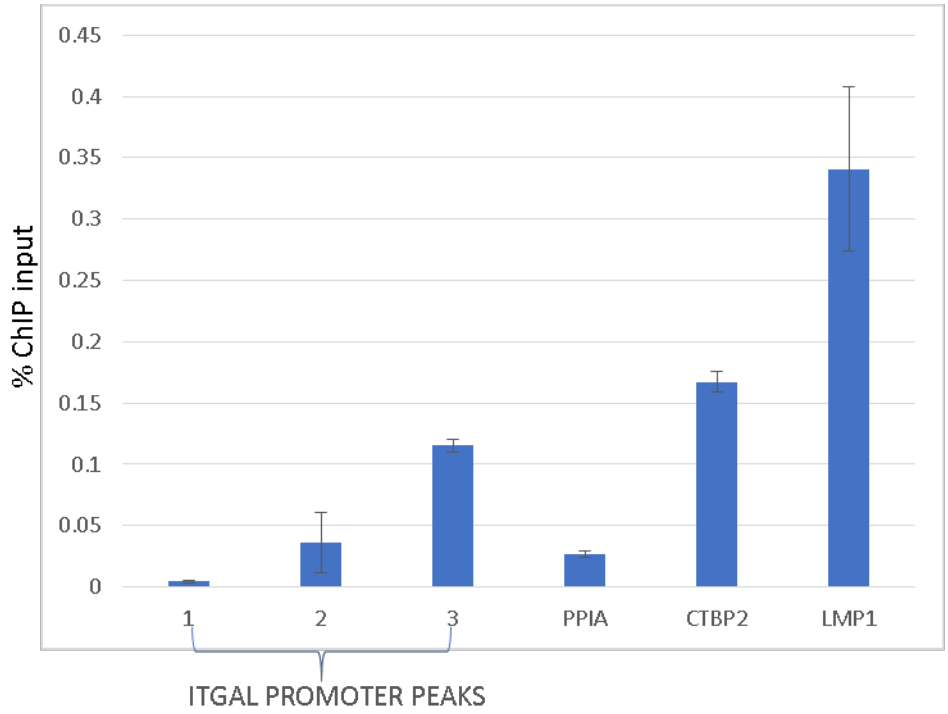

Figure 7.5. ChIP Q-PCR analysis of RBP-Jk binding carried out on Mutu III cells at EBNA 2 binding peaks 1, 2 & 3 at the ITGAL promoter site. The analysis was carried out with primer sets located at binding sites (1, 2 & 3) using against RBP-Jk ChIP samples. Binding signals at the CTBP2 and LMP1 are shown as positive controls and PPIA as a negative control. Mean percentage input signals, after subtracting the no antibody controls, are shown -/+ standard deviation for three independent ChIP experiments.

To investigate whether RBP-Jk was important in directing the binding of EBNA 2 at the *ITGAL* promoter site I performed a site-directed mutagenesis on the putative RBP-Jk sites found at peak 3. I mutated the sequences individually and also created a construct with a double mutation (Figure 7.6), this mutated the RBP-Jk sequence from GTGAGAA to GTGCCAA in the mutant, the mutation of the position 4 A to C has been shown to inhibit the binding affinity of RBP-Jk up to 3-fold at nonconsensus sites (Friedmann and Kovall, 2010). These constructs were then transiently transfected into the EBV negative DG75 B cell line and luciferase assays performed. The results showed slightly reduced EBNA 2 activation of the RBP-Jk mutant 1 promoter, in Figure 7.7a there seemed to be no effect but the basal fold is higher than the wild type basal so the activation is slightly lower compared to the wild type. There was no activation of the RBP-Jk mutant 2 promoter and the presence of the double mutation completely prevented EBNA 2 activation of the *ITGAL* promoter (Figure 7.7). This result is consistent with the known function of RBP-Jk as a cellular transcription factor for EBNA 2 binding (Grossman *et al.*, 1994, Johannsen *et al.*, 1995a). In summary, my results demonstrated that RBP-Jk is the key mediator of EBNA 2 activation of *ITGAL*.

3.1.4. EBNA 2 binds to an intragenic site at CtBP2 that does not respond in reporter assays

C-terminal binding protein (CtBP) family proteins are unique to higher eukaryotes and are essential for normal animal development. They are modulators of several essential cellular processes; they function as transcriptional co-repressors in the nucleus and in the cytosol, they play a role in membrane trafficking. The vertebrate genome encodes two different, but related, genes *CTBP1* and *CTBP2*. *CTBP1* has two isoforms while *CTBP2* has three isoforms (Boyd *et al.*, 1993, Katsanis and Fisher, 1998, Chinnadurai, 2002). Their major splice variant functions as a transcriptional corepressor while their minor splice variant displays a diverse cytosolic function (Chinnadurai, 2002). The role of *CTBP* in oncogenesis was first observed in studies with the adenovirus E1A oncogene. These studies showed that E1A mutants in the PLDLS CTBP binding motif enhanced the transformation of primary epithelial cells in rodents in cooperation with the activated Ras oncogene. This suggested that the EIA interaction restricts tumorigenesis by antagonizing the activity *CTBP* (Chinnadurai, 2009). Whilst *CTBP1*

is both nuclear and cytosolic, *CTBP2* appears to be only nuclear due to the presence of a nuclear localisation domain in its N terminus (Zhao *et al.*, 2006b).

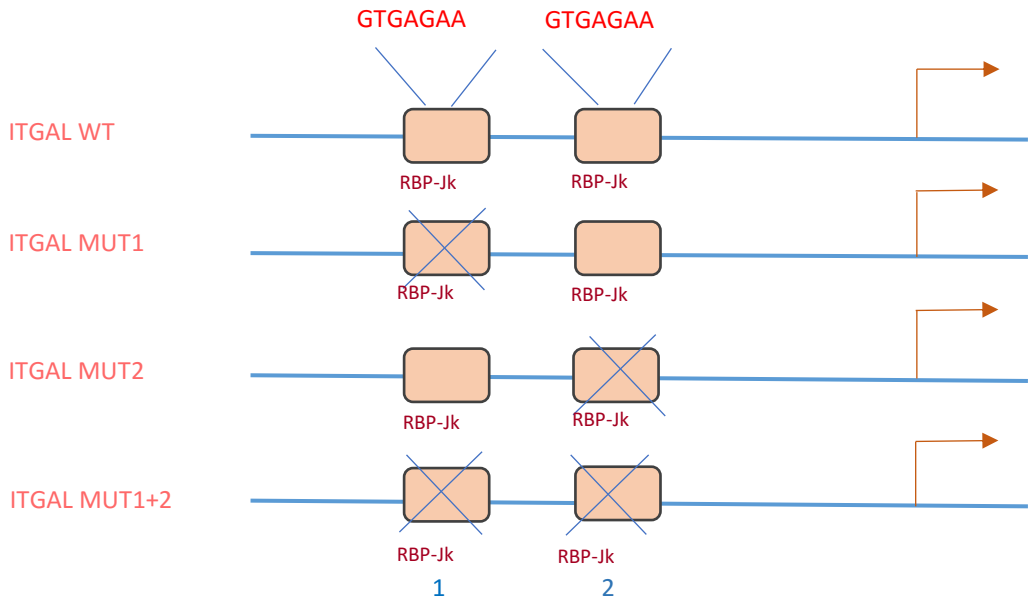

Figure 7.6 Site-directed mutagenesis of the putative RBP-Jk motifs at the *ITGAL* promoter. Diagram of the constructs made using Q5 site-directed mutagenesis kit for RBP-Jk motif site at the ITGAL promoter.

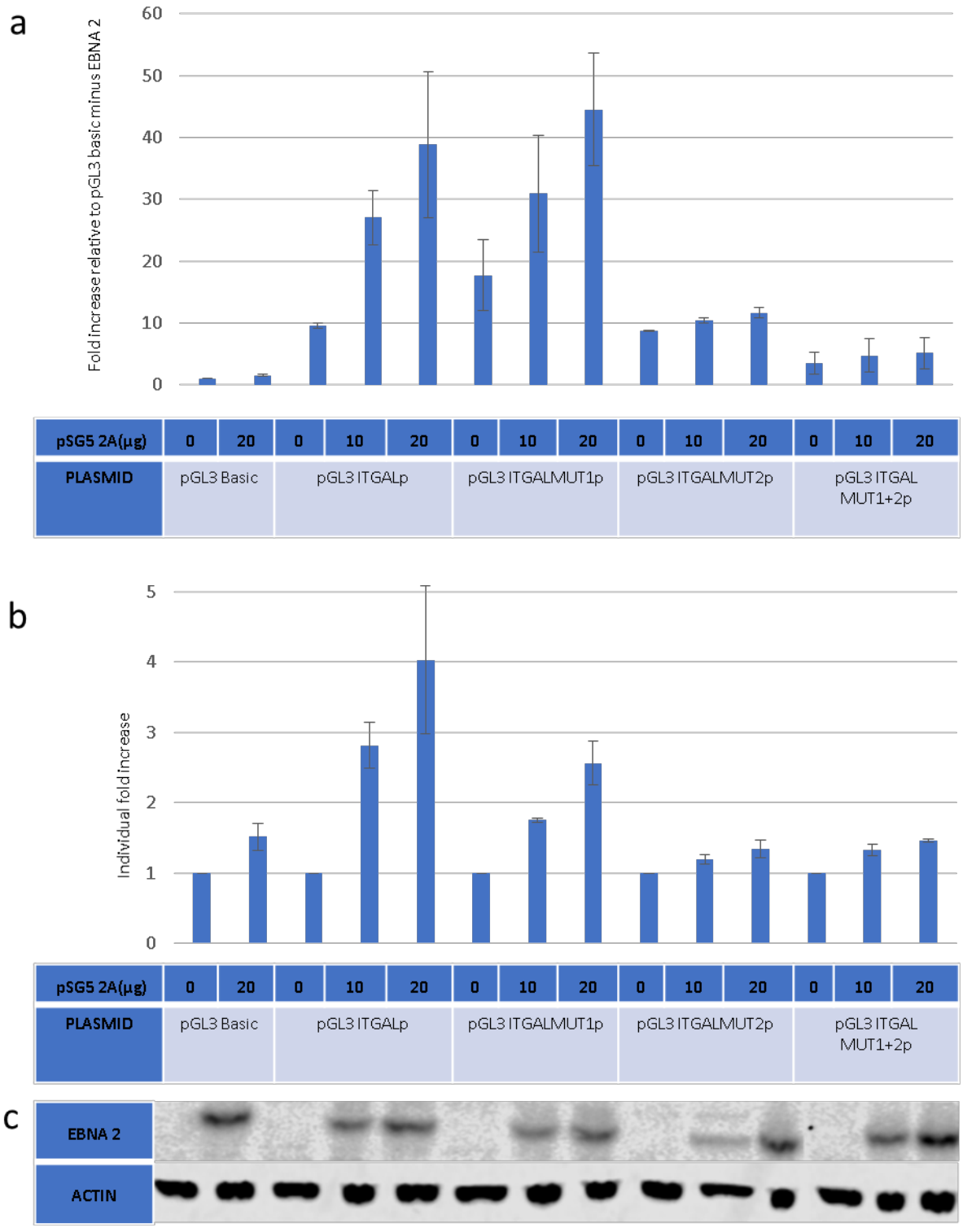

Figure 7.7. Site-directed mutation of RBP-Jk site. (a) Luciferase assay result of transiently transfected DG75 cells with 2 µg of the control vector pGL3 basic, an *ITGAL* promoter-luciferase reporter (pGL3 *ITGAL*p) or ITGAL promoter-luciferase reporter that has RBP-Jk site mutated (pGL3 *ITGALMUT1*p, pGL3 *ITGALMUT2*p, pGL3 *ITGALMUT1+2*p) in the presence or absence of EBNA2 expressing construct. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-TK (1 µg). Results show the mean −/+ standard deviation of 3 independent experiments and are expressed relative to the pGL3 basic signal. (b) Result shown in (a) expressed relative to signal for each construct in the absence of EBNA 2. (c) Western blot analysis of EBNA 2 expression levels in transfected cells. The blot was also probed for actin as a loading control.

CTBPs are known to play a key role in EBV transformation, EBNA 3A and EBNA 3C epigenetic repression of $p16^{INK4A}$ has been shown to be mediated by CtBP (Skalska *et al.*, 2010). ChIP-sequencing data from our lab found a single, large intragenic peak of EBNA 2 and EBNA 3 binding between the second and third exons of the longer isoforms of CTBP2 (Figure 7.8 a). The regulation of *CTBP2* transcription by the EBNAs had not been previously reported.

ChIP QPCR data from Michael McClellan in our group (McClellan *et al.*, 2013) confirmed that all 3 EBNA 3 proteins and EBNA 2 bind to the *CTBP2* enhancer sites in 2 different EBV-immortalised cell lines. To determine whether EBNA 2 can activate transcription via this site in reporter assays and investigate and if EBNA 2 and 3 proteins could compete for this site, I attempted to generate *CTBP2* reporter constructs containing the EBNA 2 and EBNA 3 binding peak using primers to amplify this region of the genome. Since this element could be a putative enhancer for *CTBP2* gene, I attempted to create a luciferase reporter construct containing the *CTBP2* promoter, with this region cloned upstream. However, despite multiple attempts with different primers, I was unable to amplify the *CTBP2* promoter. This may have been because of its high GC content, I therefore used a reporter construct containing the heterologous HSV TK promoter and cloned the *CTBP2* putative enhancer upstream.

When this construct and the control plasmid with the TK promoter alone were transfected into DG75 cells in the presence of EBNA 2 expressing plasmid, the TK promoter was unexpectedly activated by EBNA 2 up to 2.5-fold. EBNA 2 activated the TK promoter - *CTBP2* enhancer plasmid up to 7-fold indicating that there may be some effect of EBNA 2 on the enhancer, however, the effect between the -/+ enhancer is not significant because of the large variation between my three experiments (Figure 7.8 b, c). I repeated the experiment in EBNA 3s alone to investigate their effect on the binding site at the CTBP2 putative enhancer. There was also an unexpected repression of the TK promoter which makes it hard to make conclusions as to the effect of EBNA 3s on the *CTBP2* enhancer (Figure 7.9 a, b, c). To determine whether we could detect any effects of the EBNAs on the CtBP2 enhancer in another B cell background, I repeated the experiment using BJAB, a B cell lymphoma cell line. Similar to the previous results, the effects of EBNA 2 on the *CTBP2* peak could not be separated from the effects on the TK promoter alone (Figures 7.10). It is possible that the

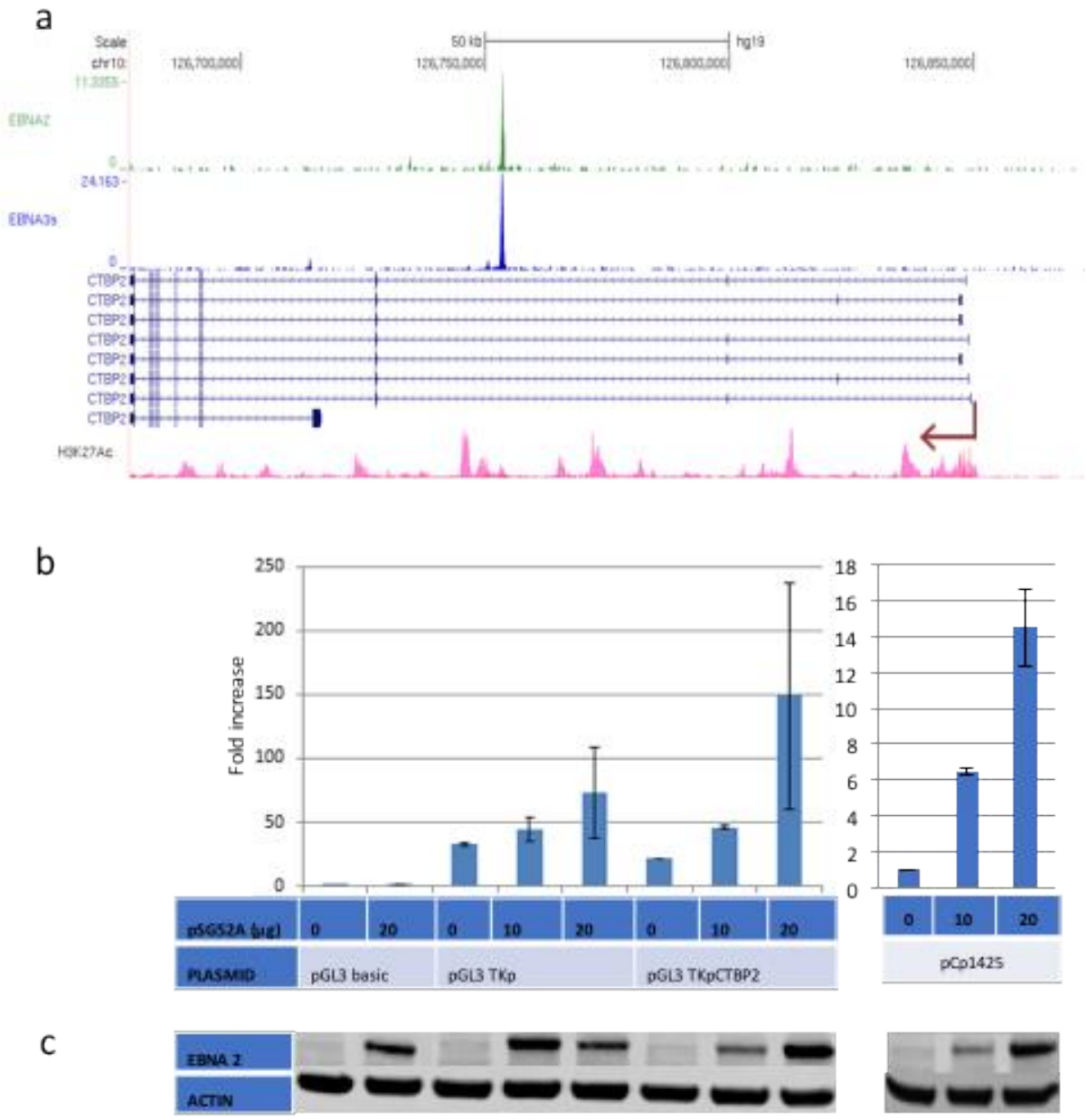

Figure 7.8 EBNA 2 bind to CTBP2 intragenic site that does not respond in reporter assay. (a) ChIP-sequencing data for intragenic site of CTBP2 showing a single significant and coincident EBNA2 and EBNA3 binding peak in the 2nd intron approximately 95 kb downstream from the TSS. (b) Luciferase assay result of DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, a TK promoter CTBP2 enhancer-luciferase reporter (pGL3 *TK*p. Pgl3 TK-CTBP2) or the EBV C promoter reporter (pCp1425GL2) (right panel) in the presence or absence of an EBNA2-expressing construct. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-CMV (1 µg). Results show the mean −/+ standard deviation of 3 independent experiments and are expressed relative to the pGL3 basic signal (left panel) or the Cp1425GL2 signal (right panel) in the absence of an EBNA2. (c) Western blot analysis of EBNA 2 expression levels in transfected cells. The blot was also probed for actin as a loading control.

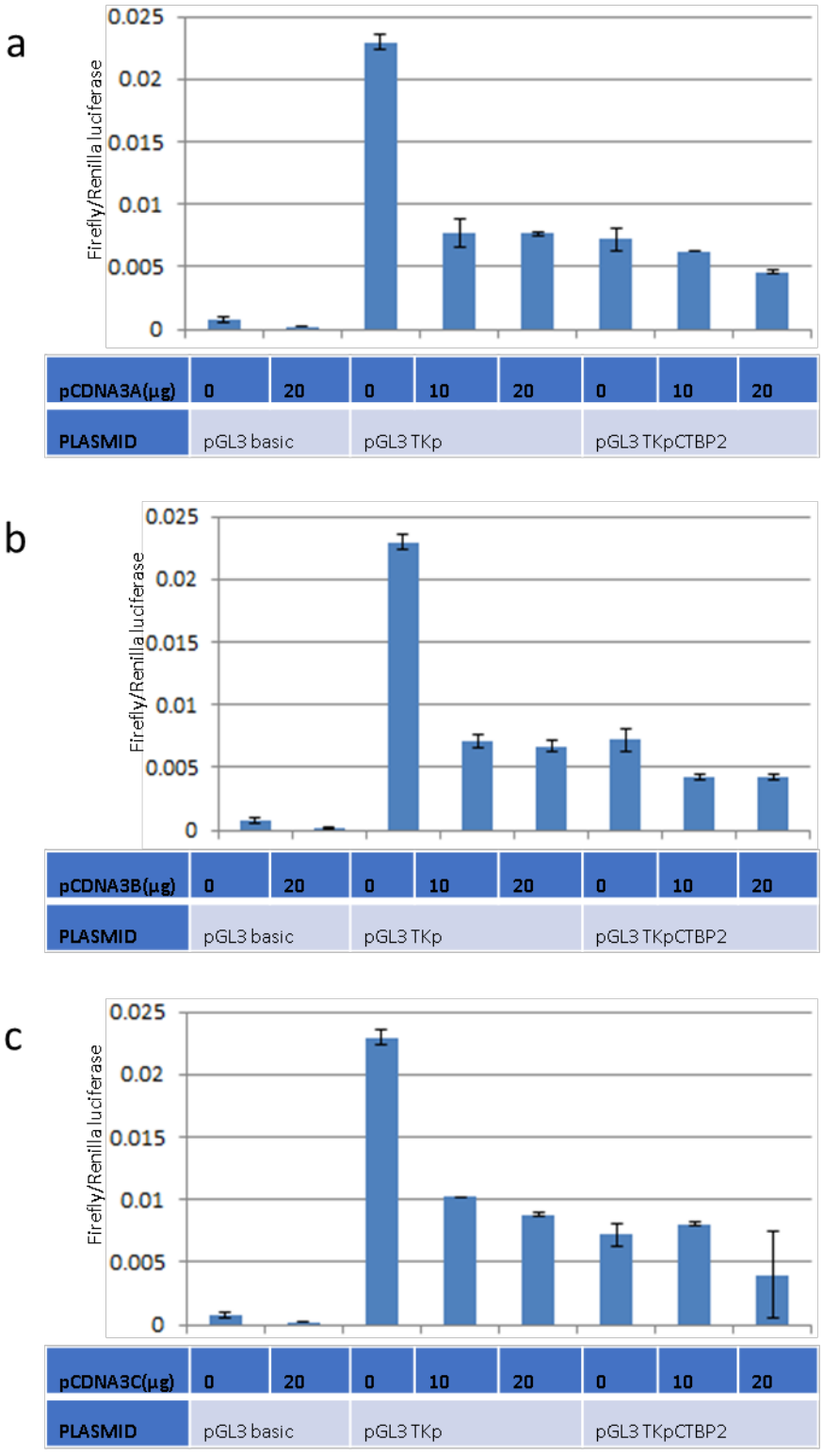

Figure 7.9 EBNA 3s bind at CTBP2 intragenic site that does not respond in reporter assay. (a) Luciferase assay result of DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, a TK promoter CTBP2 enhancer-luciferase reporter (pGL3 *TK*p, pGL3 TK-CTBP2) in the presence or absence of EBNA 3A expressing construct. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-CMV (1 µg). Result show mean Firefly reporter over Renilla control luciferase signals from one representative experiment. (b) Luciferase assay result for EBNA 3B expressing construct analysed as described in (a). (c) Luciferase assay result for EBNA 3C expressing construct analysed as described in (a).

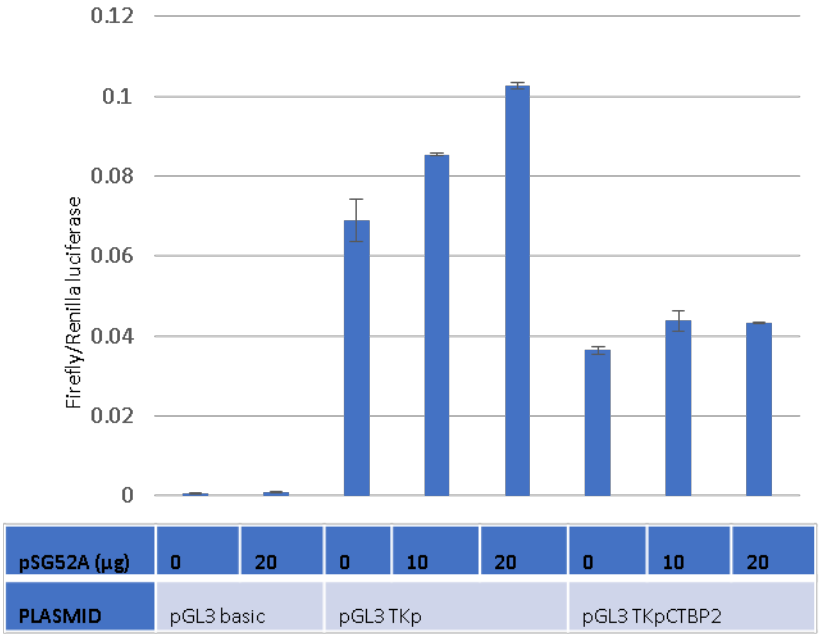

Figure 7.10 EBNA 2 bind but does not respond at CTBP2 intragenic site in reporter assay. Luciferase assay result of BJAB cells transiently transfected as described in (7.8b) in the presence or absence of EBNA 2. Result show mean Firefly reporter over Renilla control luciferase signals from one representative experiment.

CTBP2 intronic region bound by EBNA 2 is only active in the context of its own promoter or requires the correct chromatin context to function.

3.1.5. EBNA 2 binds to distal enhancers at *WEE1* that do not respond in reporter assays

Wee1 is a conserved nuclear tyrosine kinase. It was first discovered 25 years ago as a cell division cycle protein (cdc) mutant in fission yeast and is active during the S/G2 phase of the cell cycle. *WEE1* negatively regulates the activity of Cdc2/cdc28 in budding yeast and the human homologue CDK1/cyclinB by phosphorylation on Y15 throughout S phase. This prevents exit from S phase until DNA replication is complete (Russell and Nurse, 1987, Gould and Nurse, 1989, Featherstone and Russell, 1991, McGowan and Russell, 1995, Watanabe *et al.*, 1995). In the mammalian, DNA damage checkpoint G2/M, *WEE1* phosphorylates CDK1 in the CDK1/Cyclin B complex at Y15 to prevent mitotic entry until the damage is repaired (Davies *et al.*, 2011, Aarts *et al.*, 2012). EBV has previously been reported to deregulate the cell cycle at multiple points including the G2/M phase, including misregulation of CDK1 activity (Krauer *et al.*, 2004, Schlick *et al.*, 2011). *WEE1* therefore makes an attractive target for novel cell cycle regulation by EBNA 2 and 3 proteins.

ChIP-sequencing data from our lab identified *WEE1* as the closest gene to two downstream, distal sets of EBNA 2 and EBNA 3 binding sites comprising a total of five distinct peaks. These binding sites coincide with peaks of H3K27ac from ChIP sequencing data from the EBV immortalised LCL GM12878 indicating the binding sites are within gene regulatory regions that are active (McClellan *et al.*, 2013). The regulation of *WEE1* by EBNA 2 and EBNA 3s like *CTBP2* has not been previously reported.

ChIP QPCR results from our lab revealed EBNA 2 binding to each of the five peaks in two different EBV-immortalised cell lines, Mutu III and PER253 and differential binding of EBNA 3 proteins occurs at this locus, we observed low level binding of EBNA 3A binding at peak 4 and 5 in LCLs and weak EBNA 3B binding at peak 1 and 2 in Mutu III cells, however, EBNA 3C bound predominantly at peak 5 in Mutu III cells, but at both peak 4 and 5 in an LCL (McClellan *et al.*, 2013). I investigated the effect of EBNA 2 and EBNA 3s binding alone and in competition, at the *WEE1* site in reporter assays. I generated luciferase reporter constructs containing the

WEE1 promoter site alone, then added enhancer 1 peaks separately and then together with enhancer 2 peaks (Figure 7.11 a). These constructs were transiently transfected into the DG75 cells and luciferase assays performed (Figure 7.11 b), there was very little effect of EBNA 2 on transcription from constructs containing the enhancers compared to the activation at the promoter construct. Figure 7.11 c shows a western blot analysis of EBNA 2 levels in the experiments to confirm expression. To determine whether this result was cell line specific, I repeated the experiment using BJAB cell line similar to the DG75 cell line, there was no responsiveness to EBNA 2 (Figure 7.12). It is therefore possible that the *WEE1* distal sets of regions bound by EBNA 2 are not active in reporter assays because they are out of chromatin context or they may lack the transcription factors needed for activation.

3.1.6. DISSCUSION

EBNA 2, 3A, 3B and 3C transcription factors have been previously reported to play essential roles in the transcription of viral and cellular genes. EBNA 2 has been shown to be the main viral activator of the viral C promoter. EBNA 3C has also been described as a negative regulator of Cp (Sung *et al.*, 1991, Woisetschlaeger *et al.*, 1991, Jin and Speck, 1992). The activation of Cp is essential for driving gene expression of all EBNAs with EBNA 2 feeding back to up regulate Cp. Methylation of Cp on CpG dinucleotide sequences results in promoter inactivity and inability to bind transcription factors (Henkel *et al.*, 1994, Meitinger *et al.*, 1994, Tierney *et al.*, 2000b, Bakos *et al.*, 2007). EBNA 2 is also involved with the transcriptional activation of cellular promoter CD23 and viral promoters; LMP1 and LMP2 (Cordier *et al.*, 1990, Wang *et al.*, 1990a, Ling *et al.*, 1994).

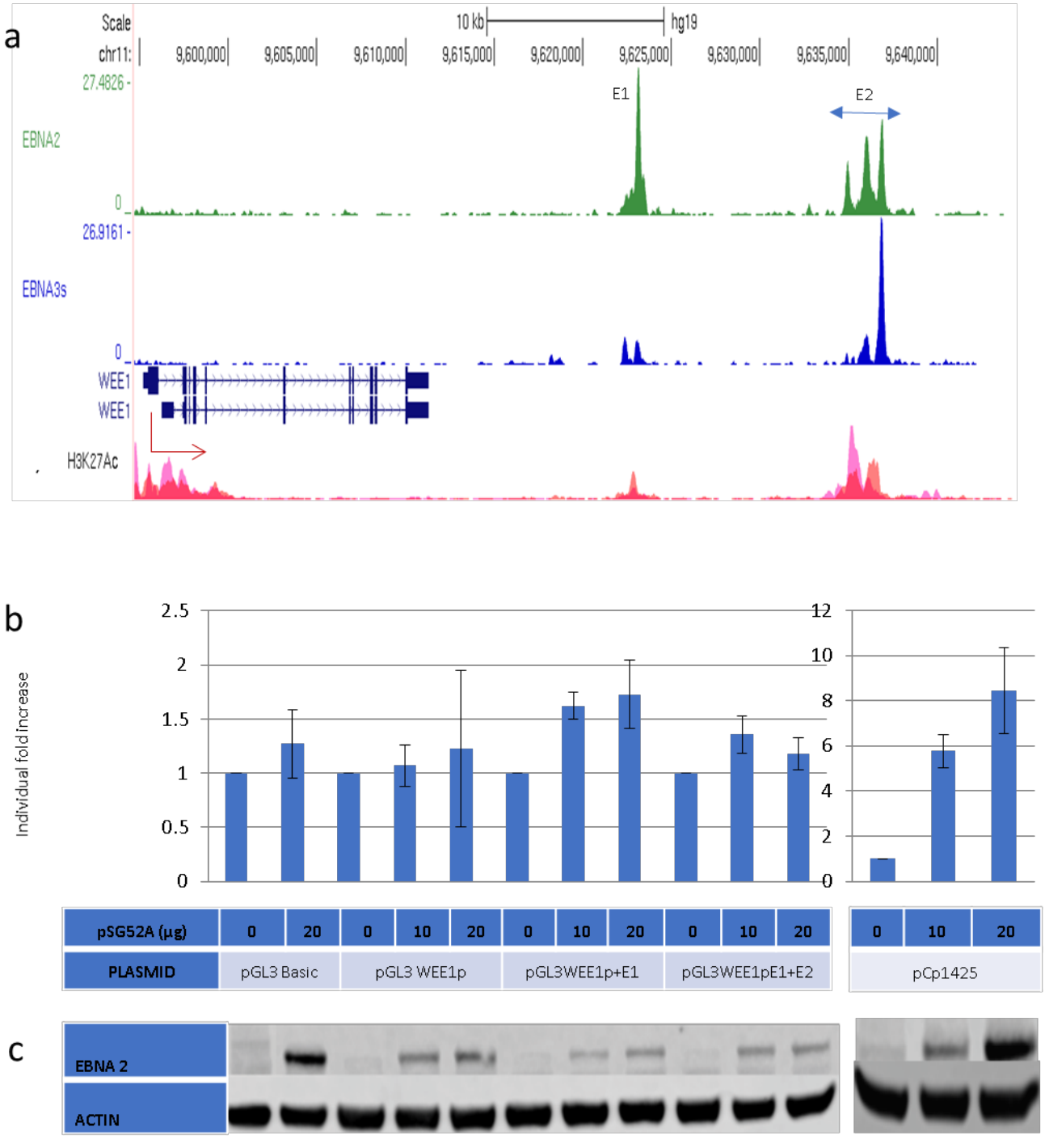

Figure 7.11 EBNA 2 and EBNA 3s bind at WEE1 distal enhancer sites that do not respond in reporter assay. (a) ChIP-sequencing data at the distal site of WEE1 showing coincident EBNA2 and EBNA3 binding sites at two clusters of putative enhancers (enhancer 1 and 2) downstream from gene. (b) Luciferase assay result of DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, a *WEE1* promoter luciferase reporter (pGL3 *WEE1*p) or WEE1 promoter-enhancer luciferase reporter (pGL3 *WEE1p-E1*, pGL3 *WEE1*p-E1+E2) or the EBV C promoter reporter (pCp1425GL2) (right panel) in the presence or absence of EBNA2 expressing construct. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-TK (1 µg). Results show the mean −/+ standard deviation of 3 independent experiments and are expressed relative to signal for each construct in the absence of EBNA 2. (c) Western blot analysis of EBNA 2 expression levels in transfected cells. The blot was also probed for actin as a loading control.

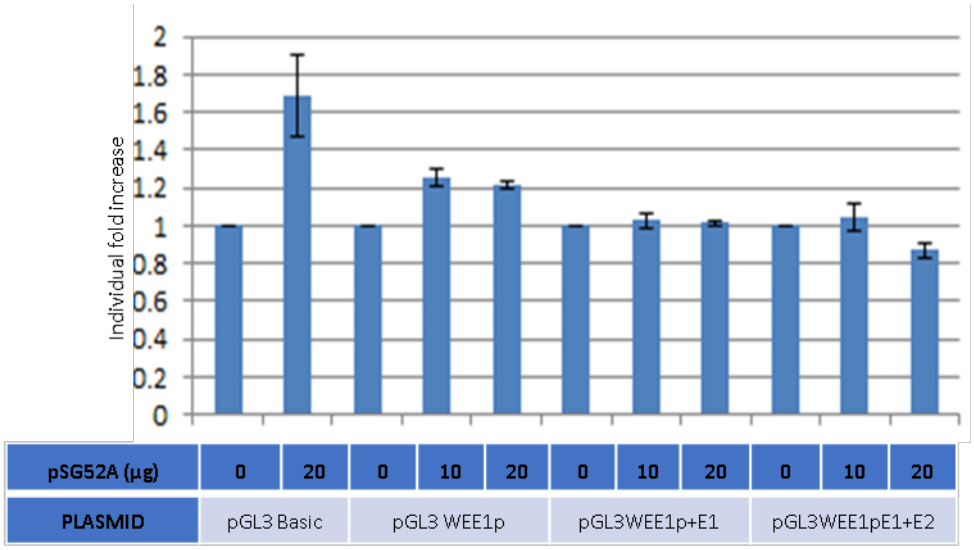

Figure 7.12 EBNA 2 effect at WEE1 distal enhancer site in reporter assay is not cell type specific. Luciferase assay result of BJAB cells transiently transfected as described in (figure 7.11b). Result shows one representative experiment and is expressed relative to signal for each construct in the absence of EBNA 2.

ITGAL encodes CD11a with (CD18)β2 intergrins to form LFA1, a heterodimer that binds ICAM1-3 and mediates homotypic and heterotypic adhesion. EBV upregulates LFA1, LFA3, ICAM1 and other activation molecules. It was previously shown that LMP1 expression in EBV negative B cells is sufficient to induce LFA1 expression on the cell surface indicating that LMP1 is involved in the activation of *ITGAL* gene expression (Wang *et al.*, 1988, Wang *et al.*, 1990a). Since LMP1 cannot be expressed in the absence of EBNA 2, is it possible that the effects of EBNA 2 observed at the *ITGAL* promoter is through directly targeting LMP1?

ChIP QPCR data from our lab shows that EBNA 2 and EBNA 3 proteins bind at the *ITGAL* promoter in infected cells (McClellan *et al.*, 2013). Since the effects of LMP1 and EBNA 2 on *ITGAL* expression cannot be separated using cells which lack functional EBNA 2, I generated *ITGAL* promoter-reporter constructs and performed luciferase reporter assays and my result have now shown that EBNA 2 up-regulates the expression of the *ITGAL* promoter. Microarray data from Dr. Rob White studys shows that EBNA 3B and 3C may play a role in the repression *ITGAL* expression in BL31 cells, EBNA 3B was also shown to play a role in the repression of *ITGAL* expression in LCLs but no EBNA 3A role was detected (Figure 7.13) (Hertle *et al.*, 2009, White *et al.*, 2010). Our lab confirmed these studies in our ChIP QPCR analysis where binding of EBNA 3B was detected in LCLs but no significant EBNA 3A or EBNA 3C binding. Using an independent set of wild types and EBNA 3B knock-out LCLs regulation by EBNA 3B was detected supporting the hypothesis that these binding sites are directly contributing to the regulation of *ITGAL* (McClellan *et al.*, 2013). My assay result showed that all EBNA 3s can compete with EBNA 2 for binding sites to repress *ITGAL* transcription when expressed in high levels. To understand if EBNA 2 and 3 proteins bind simultaneously to coincident sites or if they bind separately by recruiting their own complex, our lab performed re-ChIP analysis in Mutu III BL cell line and no simultaneous binding was observed, consistent with my data where it appears the EBNA 3s bind competitively to repress EBNA 2 activation in reporter assays (McClellan *et al.*, 2013).

Although in LCLs, EBNA 3A and EBNA 3C do not bind the *ITGAL* promoter site significantly *in vivo*, all the EBNA 3 proteins are capable of exerting their transcriptional control when overexpressed to repress EBNA 2 activation at the *ITGAL* promoter perhaps by competitive binding in reporter assays. This suggests that in vivo the differential binding of the EBNA 3s at

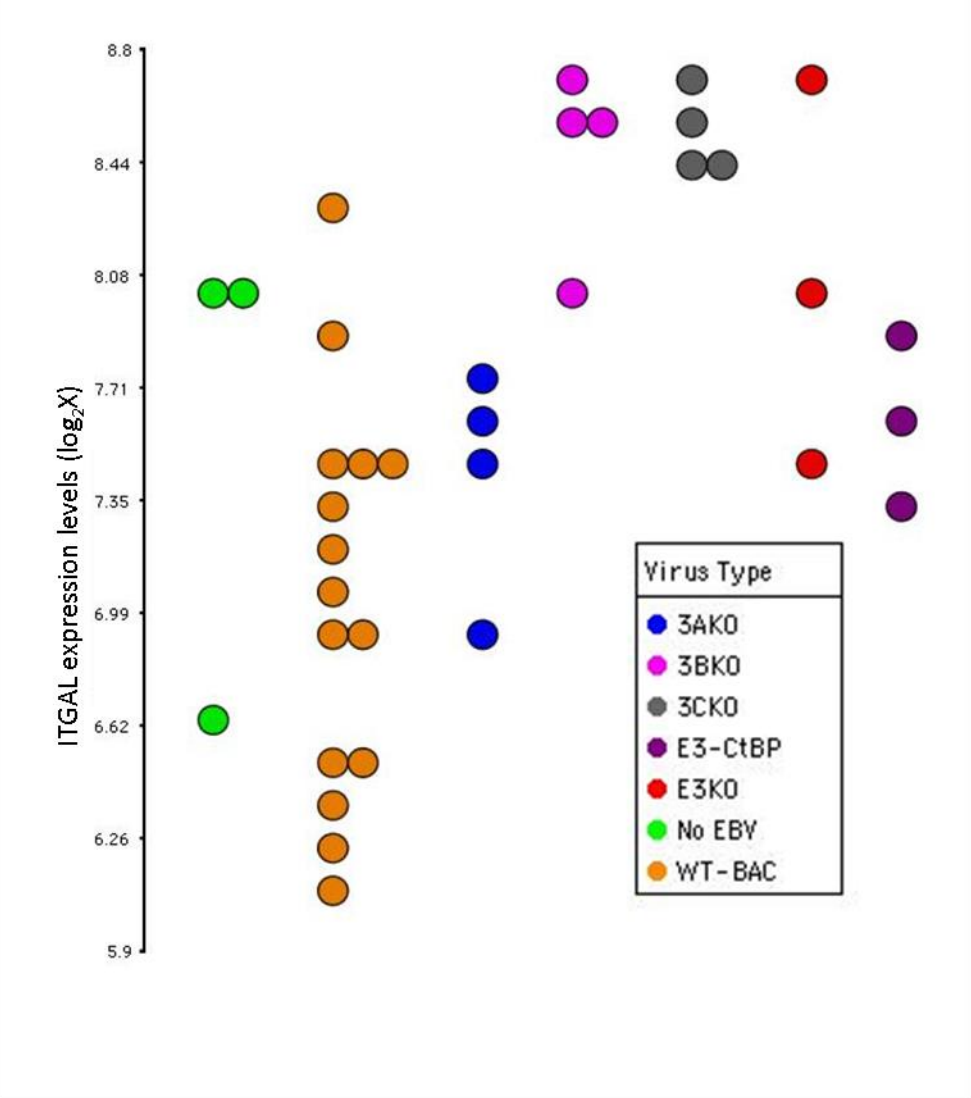

Figure 7.13 *ITGAL* array data dot plot. Screenshot from www.epstein-barrvirus.org.uk compiled by Dr Rob White. Plot shows raw data for the White *et al* array showing levels of *ITGAL* cDNA in multiple PCR and mRNA batches. Y axis is log2 values with values less than 3 indicating no expression and values over 8 indicate robust expression.

the *ITGAL* promoter is regulated by the chromatin context and the binding of cellular transcription factors. Previous B cell line analysis interestingly reported no change in *ITGAL* expression when stably expressing EBNA 2 and EBNA 3C (Wang *et al.*, 1987a, Wang *et al.*, 1990a) and microarray studies in newly EBV-infected hyperproliferating B cells where *ITGAL* expression is upregulated in the early stages of proliferation and then reduced in the resulting LCLs (Nikitin *et al.*, 2010). This suggests that EBNAs effects at the *ITGAL* promoter is context specific and may depend on the expression of DNA targeting factors and combined action of the EBNA proteins in the infected cell (McClellan *et al.*, 2013).

The inhibition of EBNA 2 activation when the second RBP-Jk site in the *ITGAL* promoter peak 3 was mutated and the Q-PCR result showing activation of EBNA 2 indicates that RBP-Jk is an important cellular transcription factor that is needed to direct EBNA 2 binding to the *ITGAL* promoter. This is consistent with EBNA 2 and the EBNA 3 proteins sharing RBP-Jk as a cellular transcription factor and binding partner (Grossman *et al.*, 1994, Henkel *et al.*, 1994, Le Roux *et al.*, 1994, Waltzer *et al.*, 1994).

CTBP2 and *WEE1* are both novel cellular gene targets identified by our lab and chosen for their proximity to EBNA 2 and 3s binding sites that may regulate their expression. CtBP protein binding by EBNA 3A and 3C is required for $p16^{INK4a}$ repression in infected cells which is essential in EBV transformation (Chinnadurai, 2009). Some studies have also implicated CtBP2 as a transcriptional activator (Paliwal *et al.*, 2012). There is no documented role for EBNA 2 and 3s in the regulation of *CTBP2*, but reanalysis of Hertle *et al* microarray data from LCLs infected with wild type and EBNA 3A knock out EBV by Dr. Rob White (Figure 7.14) (www.epstein-barrvirus.org.uk) showed upregulation of *CTBP2* expression in knock out EBNA 3A LCLs (Hertle *et al.*, 2009, Skalska *et al.*, 2010), implicating EBNA 3A in the repression of *CTBP2* transcription. As in the presence of EBNA 3A, the enhancer-promoter chromatin loops formation that correlates with increased *CTBP2* transcription is blocked (McClellan *et al.*, 2013). Our analysis of *CTBP2* mRNA expression in EBNA 3B knockout LCLs and wild type infected LCLs expressing *CTBP2* showed that *CTBP2* transcription increased when there is no EBNA 3B expression (McClellan *et al.*, 2013). Our lab mapped intragenic enhancer sites of *CTBP2* where the EBNA2 and EBNA 3s coincident bind, using ChIP-QPCR we confirmed that EBNA 2 bound *in vivo* to the *CTBP2* intragenic site and EBNA 3s interacted individually with

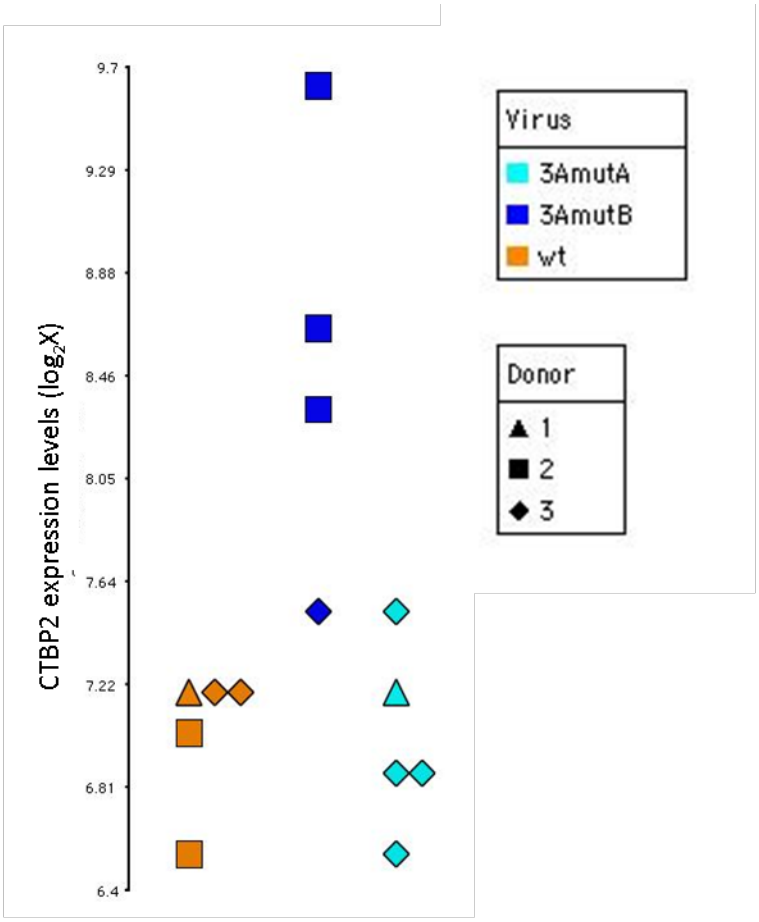

Figure 7.14 *CTBP2* array data dot plot. Screenshot from www.epstein-barrvirus.org.uk complied by Dr Rob White. Plot shows raw data for the Hertle *et al* array showing levels of *CTBP2* cDNA from individual donors within the three cell lines studied. Y axis is log 2 values with values less than 3 indicating no expression and values over 8 indicates robust expression.

the *CTBP2* site in Mutu III and LCLs. These data show all the EBNA 3s binding at the *CTBP2* site and correlates with EBNA 3A and 3B repressing *CTBP2* transcription. To investigate the effects of EBNA 2 in *CTBP2* gene expression, our lab analysed *CTBP2* mRNA levels in LCLs expressing a conditionally active estrogen receptor-EBNA 2 fusion protein (ER/EB 2.5) (Kempkes *et al.*, 1995) and although EBNA 2 bound at *CTBP2* enhancer site, it did not seem to be transcriptionally functional at the site and our lab also did not observe any effect in the *CTBP2* mRNA levels when EBNA 2 lost in the presence of the EBNA 3s (McClellan *et al.*, 2013). My reporter assay result showed no significant effect of the EBNAs on the *CTBP2* enhancer as the TK promoter was unexpectedly responsive to EBNA 2 activation and EBNA 3 repression. This could be because the binding is cell-type specific or because there is no significant effect out of chromatin context. It could also be that the transcription factors needed to recruit EBNAs to the *CTBP2* putative enhancer site is not expressed in the cell line used. It could also be an indication that it needed its own promoter to function which I tried to generate but was unsuccessful in amplifying it using different primer sets. Promoter specific enhancer function was shown in the regulation of E74-like factor 5 (Elf5) in the trophectoderm where the expression of Elf5 to the extraembryonic ectoderm and ectoplacental cone was driven by two redundant enhancers required the presence of their endogenous proximal promoter for optimal activity (Pearton *et al.*, 2011).

WEE1 encodes a cell cycle kinase that regulates the activity of mitotic kinase CDK1. EBNA 3C has been previously shown to disrupt the G2/M checkpoint through multiple potential mechanisms so regulation of *WEE1* expression may play a role in cell cycle deregulation by EBV. Microarray data showed that EBNA 3C represses *WEE1* in BL31 cells infected with a series of EBNA 3 knock out viruses while there was no significant repressive effect of EBNA 3A and 3B on *WEE1* mRNA expression (Figure 7.15) (www.epstein-barrvirus.org.uk). Our ChIP-seq data mapped binding sites for EBNA 2 and EBNA 3 protein peaks at the *WEE1* locus that may be responsible for its regulation. Our ChIP-QPCR analysis confirmed the binding of EBNA 2 in Mutu III cells and LCLs, to understand its effect on *WEE1* regulation we examined *WEE1* mRNA expression in BL31 cells infected with an EBNA 2 knock-out virus and in LCLs expressing conditionally active EBNA 2 and observed reduced *WEE1* mRNA levels suggesting a role for EBNA 2 in positive regulation of *WEE1* transcription.

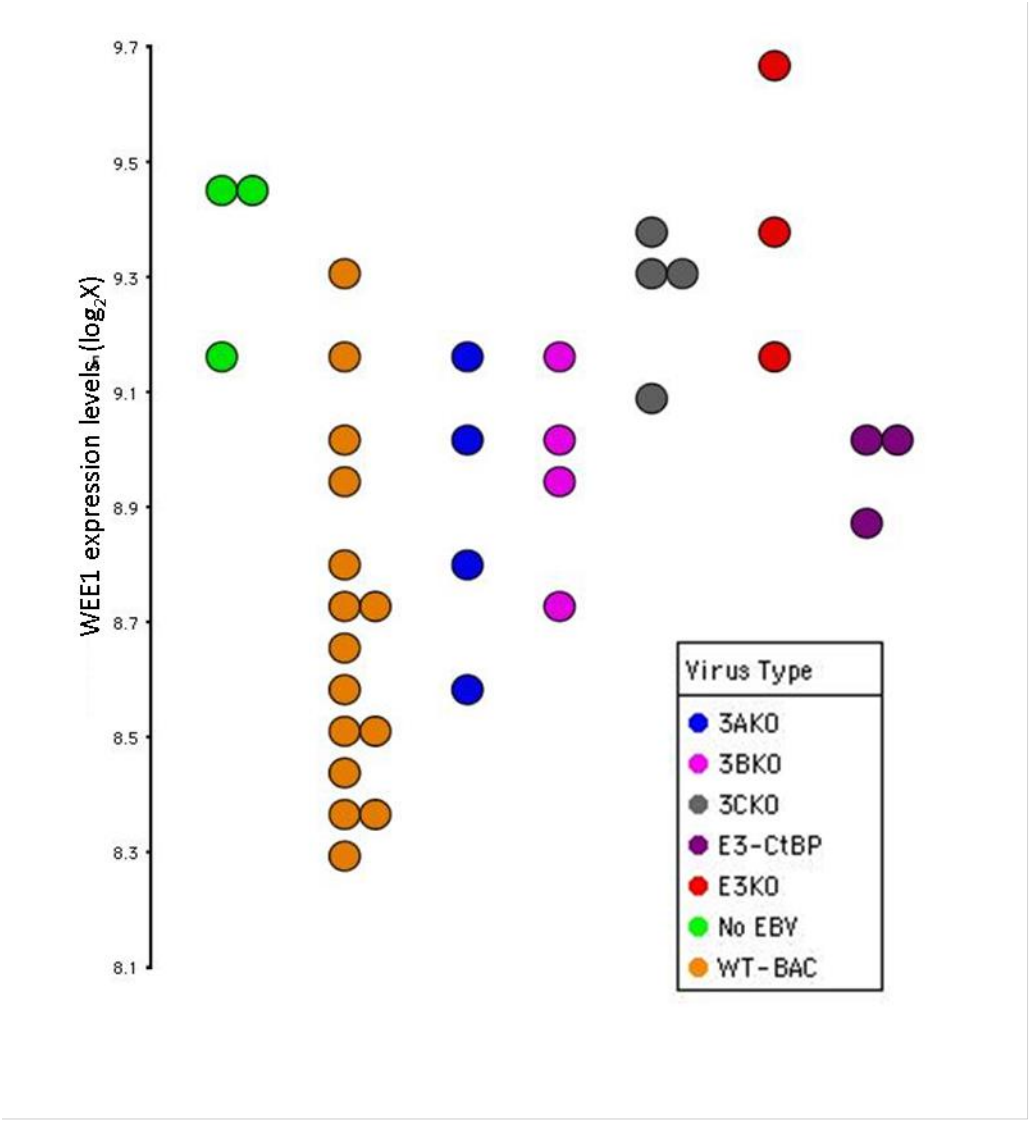

Figure 7.15 *WEE1* array data dot plot. Screenshot from www.epstein-barrvirus.org.uk compiled by Dr Rob White. Plot shows raw data for the White *et al* array showing levels of *WEE1* cDNA in multiple PCR and mRNA batches. Y axis is log2 values with values less than 3 indicating no expression and values over 8 indicate robust expression.

We also confirmed EBNA 3A and 3C binding at enhancer 2 at low level in LCLs while EBNA 3B also bind weakly at enhancer 1 in Mutu III cells. EBNA 3C binds predominantly to enhancer 2 in both Mutu III cells and LCL. We then examined *WEE1* mRNA expression in BL31 cells infected with EBNA 3C knock-out virus and observed *WEE1* transcript level increased in infected cells, we also examined LCLs expressing EBNA 3A and EBNA 3B knock-out cells and observed no significant effect on the *WEE1* mRNA levels. This data indicated EBNA 3C binding to enhancer 2 plays a dominant role in repression on *WEE1* transcription and EBNA 3A and 3B showing very low effects on the enhancer sites. This suggests that EBNA 2 antagonises the EBNA 3C repression creating a balance that determines the level of *WEE1* transcription in infected cells. It is therefore a possibility that EBNA 3C suppresses the negative effects of *WEE1* on the G2/M checkpoint during the outgrowth of EBV- immortalized cells (McClellan *et al.*, 2013).

My assay result showed the *WEE1* promoter and enhancer sites was not responsive to EBNA2 activation. This also like *CTBP2* could be because there is no significant effect out of chromatin context or that the transcription factors needed to recruit EBNAs to the *WEE1* enhancer site is not expressed in the cell line used.

In summary, EBNA 2 and 3 bind genes in cell type specific manner at a locus and their binding effects is predictive of the gene regulation, the factors at the target genes often regulate EBNA 2 and EBNA 3 coincident binding. EBNA 2 and 3 proteins compete for binding and do not bind simultaneously at a locus and differential binding of the EBNAs may be chromatin context specific. Also, luciferase reporter assays may not completely recapitulate the normal behaviour of a given promoter or enhancer.

3.2. Investigating EBNA 2 gene activation and repression

EBNA 2 is known to regulate the expression of cellular genes as well as viral genes. Microarray data from EBV BL cells expressing only EBNA 2 or EBNA 2 conditional LCLs engineered to express estrogen receptor EBNA 2 (EBNA 2-ER) fusion protein have shown regulation of hundreds of cellular genes by EBNA 2 (Thompson *et al.*, 1999, Cahir-McFarland *et al.*, 2004, Maier *et al.*, 2006). Maier *et al* in their studies of EBNA 2 regulated genes in BL41 and BJAB cells lines expressing EBNA 2-ER fusion identified over 200 genes up-regulated in BL41 and more than 100 genes up-regulated in BJAB cell lines, interestingly they also found 188 genes in BJAB and 76 genes in BL41 to be up to 2-fold repressed by EBNA 2 (Maier *et al.*, 2006). The mechanism EBNA 2 uses to regulate most gene targets are unknown, although upregulation is likely to be mediated through its association with co-activators and general TFs. The mechanism of EBNA 2 mediated repression however is unknown and has received little attention.

3.2.1. Investigating EBNA 2 association with *CD79A* and *CD79B*

CD79A, also known as Igα or mb-1 and *CD79B* also known as Igβ or B29 were identified as genes repressed by EBNA 2 expression in B cell (Maier *et al.*, 2005). *CD79A* and *CD79B* are integral membrane proteins highly conserved among species (Sims *et al.*, 2012). They are expressed in all stages of B cell development (Hermanson *et al.*, 1988, Benlagha *et al.*, 1999) and together they form a disulfide-linked heterodimer as part of the B- cell antigen receptor (BCR) complex (Hombach *et al.*, 1990). They are both essential for BCR cell surface expression and signalling that leads to B cell activation (Clark *et al.*, 1992, Grupp *et al.*, 1993). Some studies have also shown *CD79A* and *CD79B* to be critical for B cell development and maturation, especially in VDJH recombination a genetic recombination process that occurs during T and B cells early development stages where different gene segments known as variable (V), diversity (D) and joining (J) genes randomly reassemble to generate antigen receptors (Hermanson *et al.*, 1988, Papavasiliou *et al.*, 1995, Gong and Nussenzweig, 1996, Torres *et al.*, 1996). The promoters of *CD79A* and *CD79B* both contain a TATA-less promoter and share a high homology for transcription factor binding sites like EBF, OCT, SP-1, NFκB among others (Hermanson *et al.*, 1989, Travis *et al.*, 1991, Omori and Wall, 1993, Ha *et al.*,

1994). Upon EBV infection in SCID mice it has been shown that *CD79A* and *CD79B* were both significantly downregulated (Mori *et al.*, 1994). More recently microarray analysis of LCLs and EBV negative cells conditionally expressing LMP1 or EBNA 2 showed *CD79B* to be targeted for downregulation (Cahir-McFarland *et al.*, 2004, Maier *et al.*, 2006). In germinal B centre cells, it has also been shown that LMP1 downregulated *CD79A* and *CD79B* (Vockerodt *et al.*, 2008). EBNA 2 does not bind DNA directly but through binding proteins like RBP-Jk and PU.1 (Grossman *et al.*, 1994, Henkel *et al.*, 1994, Waltzer *et al.*, 1994, Johannsen *et al.*, 1995b). Previous promoter studies have shown that a RPB-Jk binding site is not present at the *CD79B* promoter but PU.1 can bind from the predominant TSS (Omori and Wall, 1993).

ChIP-seq data from our lab revealed two distinct EBNA 2 binding peaks at the *CD79A* promoter region (Figure 8.1 a) and one EBNA 2 binding peak at the *CD79B* promoter region (Figure 8.1 b). Microarray data from White *et a*l demonstrated that upon EBV infection, *CD79A* and *CD79B* expression levels are down-regulated by EBNA 3A, B and C and EBNA 3s in the BL31 series cell lines (Figure 8.2 a-b) (White *et al.*, 2010). Microarray data from Maier *et al* 2006 studies also demonstrated expression levels of *CD79A* and *CD79B* being down regulated in ER-EB and BL41 cell line (Maier *et al.*, 2006). This was supported by a gene expression analysis from Microfluidic array card carried out by Sarika Khasnis from our lab, using the EREB2.5 cell series, her data showed both genes downregulated in the presence of EBNA 2 (Figure 8.2 c-d).

To investigate the mechanism surrounding the regulation of these targets and determine whether these promoter binding sites direct the reported downregulation of *CD79A* and *CD79B* by EBNA 2, I created luciferase reporter construct containing a pGL3 basic vector and the *CD79A* promoter regions. After several failed attempts to amplify the two promoter peaks using primers I had each promoter peak region synthesised by an external supplier (Figure 8.1a). I also made constructs containing the *CD79B* promoter region (Figure 8.1b). These constructs were then transiently transfected into DG75 cells and luciferase assays performed. The result showed up to 2.6-fold activation via the peak 1 region and up to 2.75-fold activation of the construct with both *CD79A* peaks together (Figure 8.3 a), there was also an activation up to 2.6-fold at the *CD79B* peak region and a 3-fold activation at the C promoter reporter, a positive control for EBNA 2 activation (Figure 8.3 c). Western blot analysis confirmed the

expression level of EBNA 2 in these experiments (Figure 8.3 b, d). Interestingly, my reporter assay result did not support all previous data of *CD79A* and *CD79B* mRNA levels being repressed in infected cell lines by EBNA 2 as my data showed EBNA 2 activates transcription through these binding sites. Although, the activation observed at construct with both *CD79A* promoter peaks together is not much higher than that of the construct with the peak 1 region alone as the basal fold is higher.

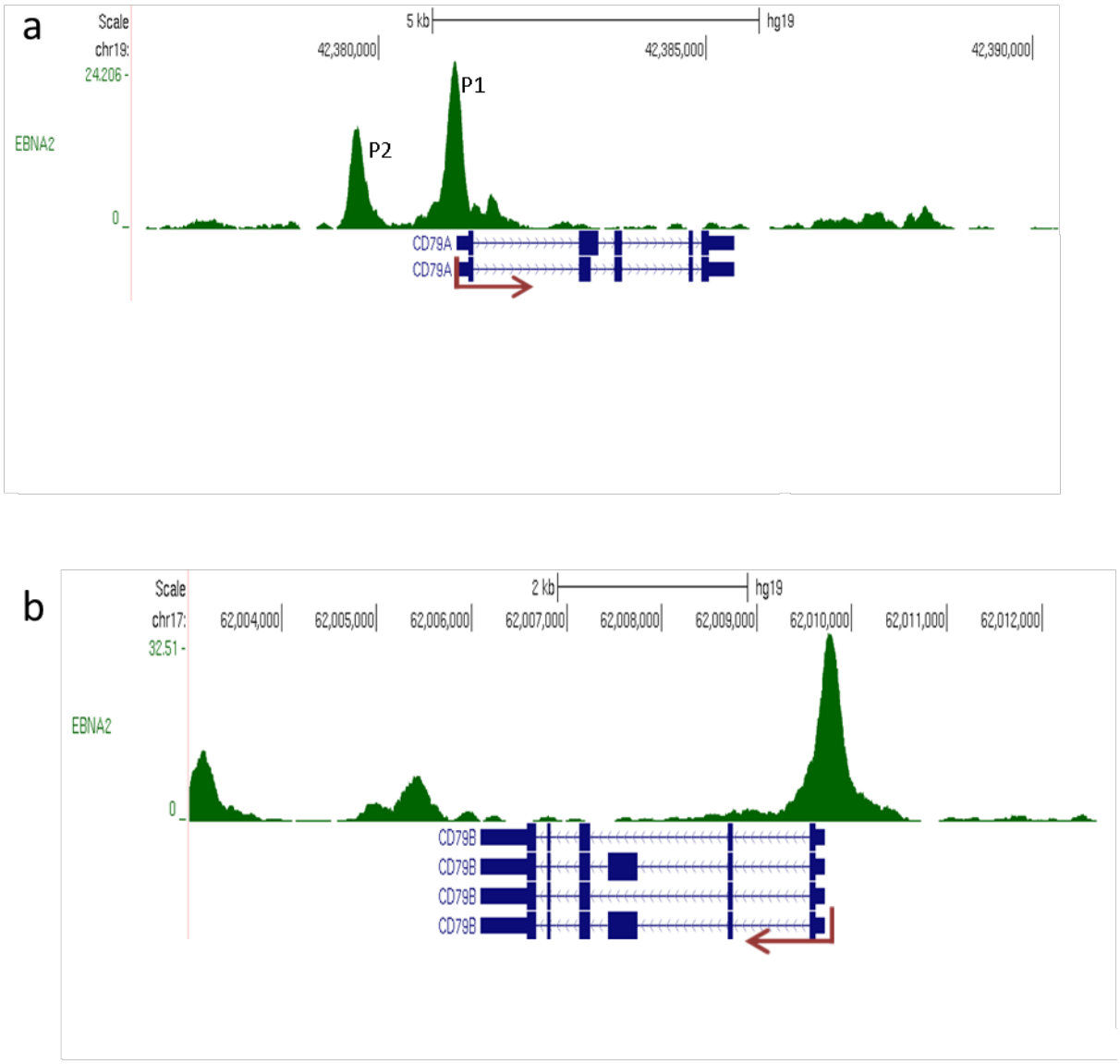

Figure 8.1 EBNA 2 binding sites located at *CD79A* and *CD79B*. (a) ChIP-sequencing data for the promoter region of *CD79A* showing two EBNA 2 binding peaks. (b) ChIP-sequencing data for the promoter region of *CD79B* showing one EBNA 2 binding peak. Read per million background subtracted read is displayed to the y axis. The arrow indicating the direction of transcription.

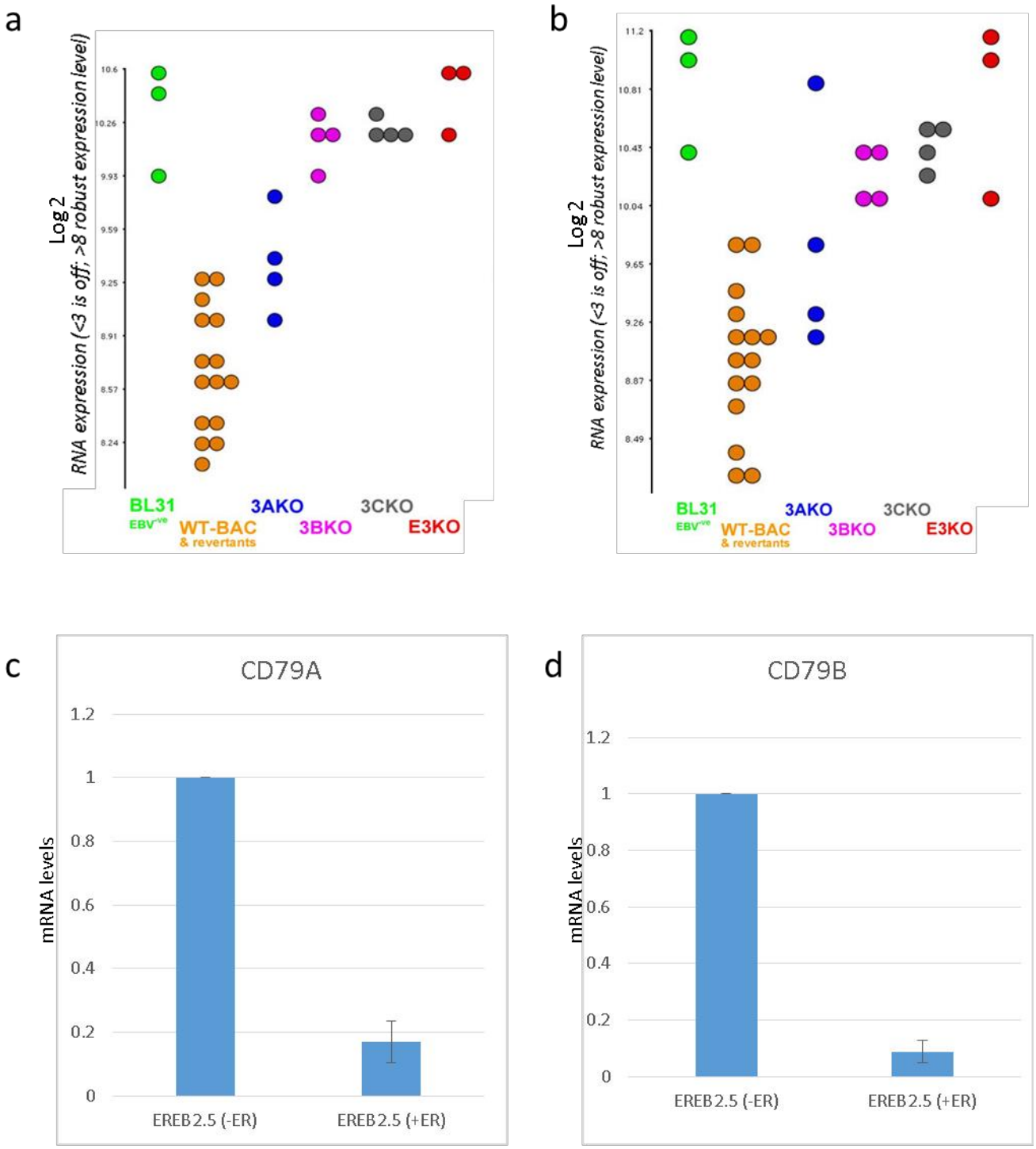

Figure 8.2 *CD79A* and *CD79B* transcriptional regulation by EBNA 2 and EBNA 3 proteins. (a) Screenshot from epstein-barrvirus.org.uk showing analysis of *CD79A* expression by microarray using the EBV-negative Burkitt's lymphoma cell line BL31 infected with a wild-type EBV virus or EBNA 3A, 3B and 3C knock-out or all EBNA 3s knock-out and revertant bacmids. (b) shows analysis as described in (a) for *CD79B*. *CD79A/B* expression levels are down-regulated by EBNA3A,B and C and combined EBNA3s in the BL31 series cell lines (White, Groves et al. 2010). (c) *CD79A* gene expression analysis from Microfluidic array card carried out by Sarika Khasnis (West lab) using the EREB2.5 cell series. (d) shows analysis as described in (c) for *CD79B*. The data suggests down regulation of *CD79A/B* in the presence of EBNA2 in the EREB 2.5 cell line.

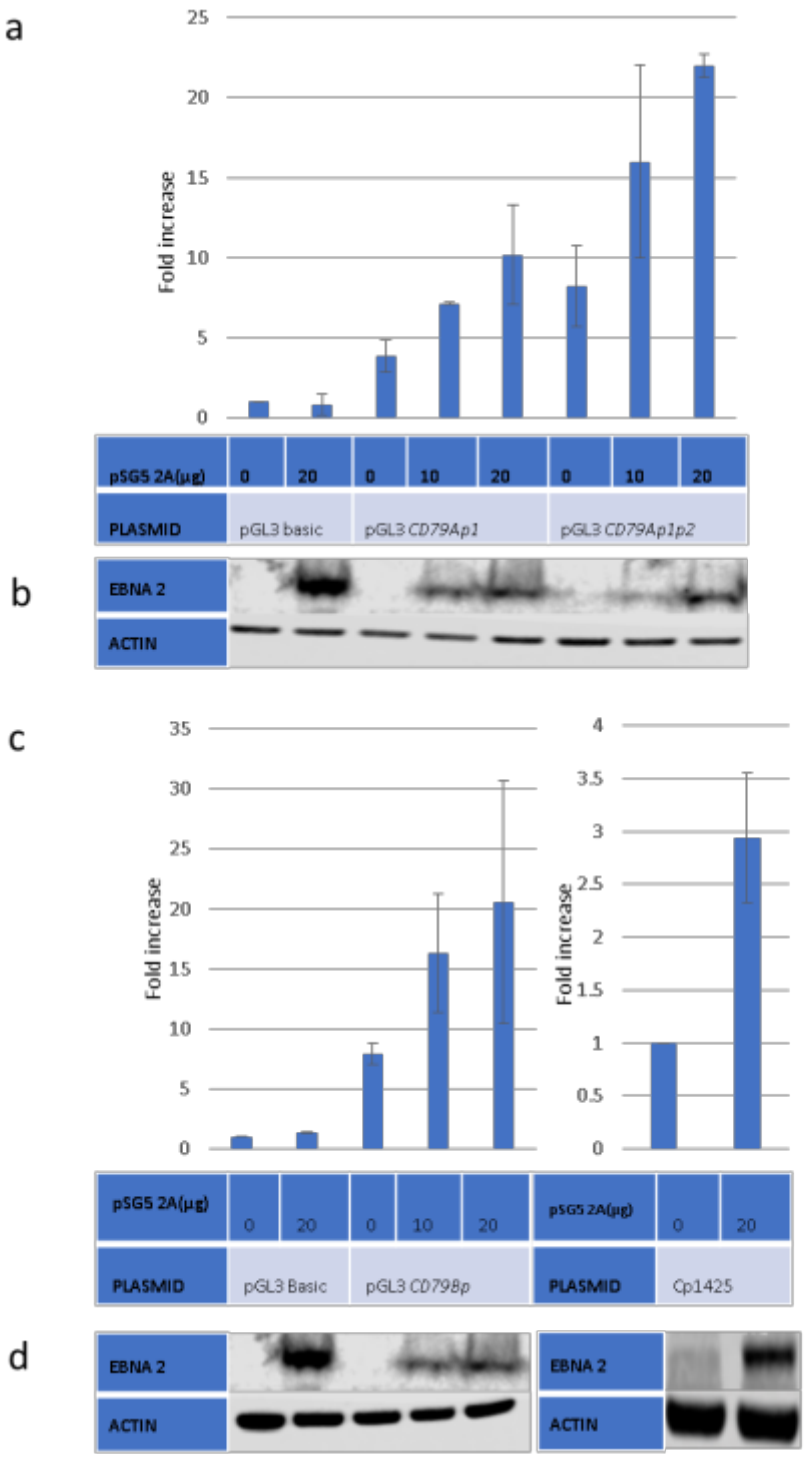

Figure 8.3 EBNA 2 activates *CD79A/B* gene in reporter assay. (a)Luciferase assay result of DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, a *CD79A* promoter P1 luciferase reporter (pGL3 *CD79A*p1) or *CD79A* promoter P1+P2 luciferase reporter (*pGL3 CD79p1+p2*) in the presence or absence of EBNA2 expressing construct showing activation. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-TK (1 µg). Results show the mean −/+ standard deviation of 2 independent experiments and are expressed relative to the pGL3 basic signal. (b) Western blot analysis of EBNA 2 expression levels in transfected cells. The blot was also probed for actin as a loading control. (c) Luciferase assay result of DG75 cells transiently transfected as described above for *CD79B* promoter (*pGL3 CD79Bp*) and EBV C promoter reporter (pCp1425GL2) (right panel)as an EBNA 2 activation control. (d) Western blot analysis of EBNA 2 and actin expression levels.

I therefore investigated whether the repressive effects of EBNA 2 may be mediated via competition with a cellular transcription factor that functions as a 'stronger' activator of these genes. I focused on Early B-cell factor 1 (EBF1), a sequence specific DNA binding protein that is expressed throughout B-cell development and induces numerous B-cell genes and maintains B-cell specific transcription programs (Dudziak *et al.*, 2003, Pongubala *et al.*, 2008, Lin *et al.*, 2010, Treiber *et al.*, 2010). EBF1 has also been implicated in epigenetic modification of *CD79A* promoter. EBF1 has been shown to activate *CD79A* and *CD79B* transcription (Hagman *et al.*, 1991, Maier *et al.*, 2004, Hagman and Lukin, 2005, Bohle *et al.*, 2013). I transiently transfected DG75 cells with the *CD79A* and *CD79B* reporter constructs in the presence and absence of EBF1 expressing plasmid. The results demonstrated activation at both *CD79A* and *CD79B* constructs by up to 13-fold and 11-fold respectively supporting previous reports (Figure 8.4 a). However, when transfected in competition with EBNA 2, EBNA 2 failed to inhibit this activation significantly. The luciferase assay result showed EBNA 2 adding a very small further increase to the activation seen in the presence of EBF1 alone at *CD79A* and a slight repression effect *CD79B* when expressed at a high concentration (Figure 8.4 c). However, my data demonstrated that EBF1 is a better activator of *CD79A* and *CD79B* than EBNA 2 and the lack of additional EBNA 2 activation of these promoters could indicate that the effects of EBNA 2 and EBF 1 activation is mutually exclusive (Figure 8.4 c). Western blot analysis showed the expression levels of EBNA 2 and EBF1 (Figure 8.4 b, d). This data also does not support the down regulation of *CD79A* and *CD79B* seen in the presence of EBNA2 or upon EBV infection in the EBNA 3s knockout cells (Maier *et al.*, 2006, White *et al.*, 2010). Perhaps other factors are involved at the binding sites in luciferase reporter assays that are not present *in vivo* or the expression of both genes in chromatin context is different.

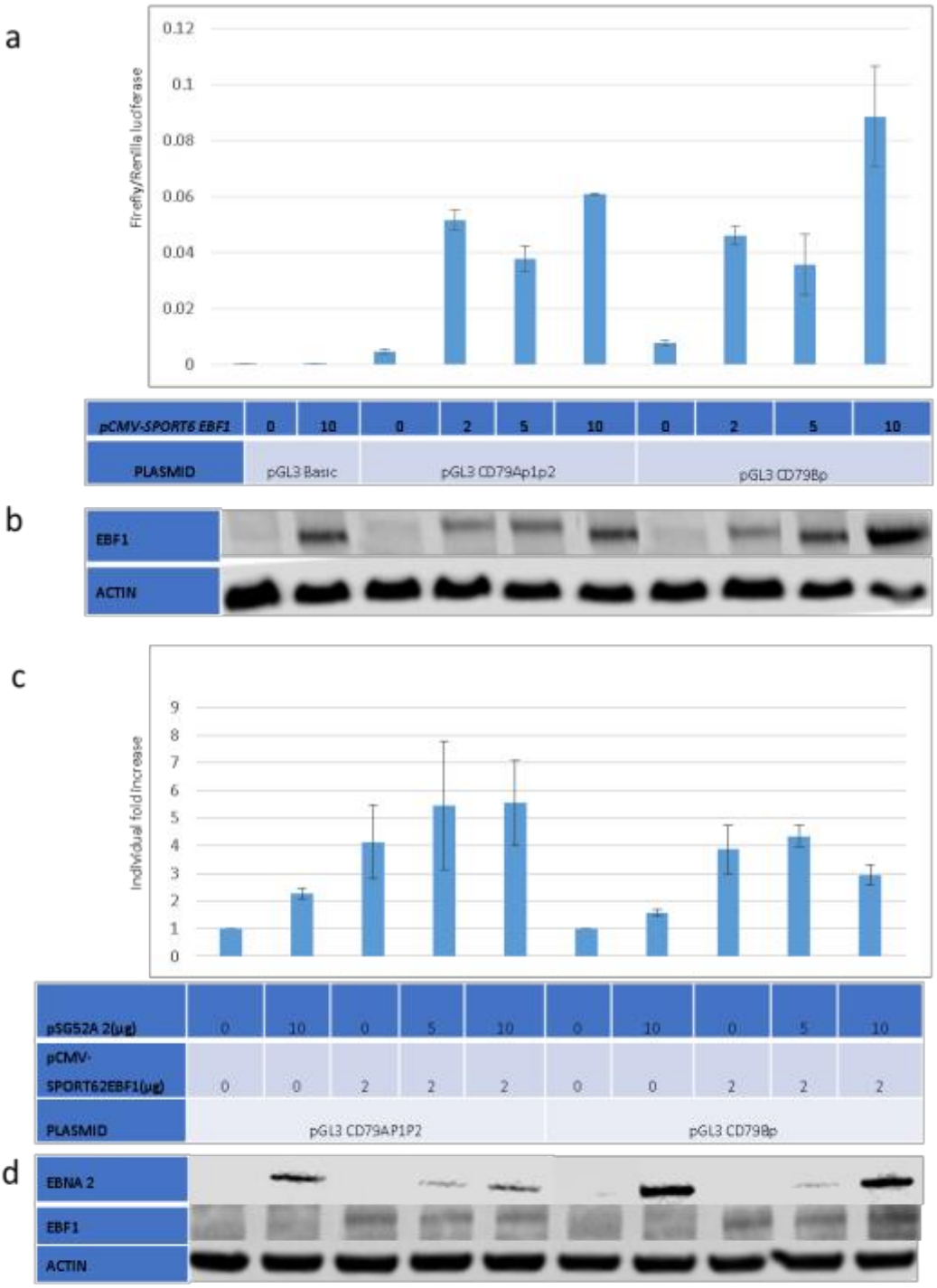

Figure 8.4 EBNA 2 and EBF1 effects on the *CD79A* and *CD79B* promoters. (a) Luciferase assay result of DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, a *CD79A* promoter peak 1 luciferase reporter (pGL3 *CD79A*p1) or *CD79A* promoter peak 1 and peak 2 luciferase reporter (*pGL3 CD79p1+p2*) in the presence or absence of EBF1 expressing construct. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-TK (1 µg). Result show mean Firefly reporter over Renilla control luciferase signals from one representative experiment. (b) Western blot analysis of EBF1 expression levels in transfected cells. The blot was also probed for actin as a loading control. (c) Competitive assay result of *CD79A* and *CD79B* with 2 µg of EBF1 and in the presence and absence of EBNA 2 transfected as described above (a). Results show the mean −/+ standard deviation of 2 independent experiments and are expressed relative to signal for each construct in the absence of EBNA 2 and EBF1. (d) Western blot analysis of EBNA 2 and EBF1 expression levels in transfected cells. The blot was also probed for actin as a loading control.

3.2.2. Investigating EBNA 2 association with *IRF4*

Interferon regulatory factor 4 (IRF4) is part of the interferon (IFN) regulatory factors (IRF) family, the family of transcription factor is made up of 9 members in mammalian cells. They are recognized with the consensus DNA of at least two GAAA repeats (Hiscott, 2007, Takaoka *et al.*, 2008). They are multi-functional and play significant roles in multiple aspects of defense systems (Zhang *et al.*, 2004, Honda and Taniguchi, 2006, Savitsky *et al.*, 2010), e. g. involvement in the regulation of tumorigenesis and cell growth, differentiation and development. They all share a conserved DNA binding domain (DBD) at their N-terminus and a variable C-terminus that defines their biological functions (Baer *et al.*, 1984, Nguyen *et al.*, 1997, Taniguchi *et al.*, 2001, Zhang and Pagano, 2001, Amon *et al.*, 2004). *IRF4* functions as an interacting partner of PU.1. It is expressed in all B-cell development stages in both mature T-cells and macrophages and play critical role in their function (Eisenbeis *et al.*, 1995, Mittrucker *et al.*, 1997). It also interacts with EBV latency programs and play a key role in mediating the EBV transformation processes of human B lymphocytes. During EBV transformation, its expression is induced in primary B lymphocytes *in vitro* and detected in primary CNS lymphomas specimens *in vivo,* suggesting *IRF4* is associated with EBV infection *in vivo.* When *IRF4* expression is reduced in EBV transformed cells, it decreases cell proliferation rate and enhances apoptosis (Xu *et al.*, 2008). Reporter assay studies shows *IRF4* to be induced by LMP1/NFkB signaling, studies in EBV LMP1 driven tumors in mice revealed *IRF4* expression in all the mice (Xu *et al.*, 2008, Hu *et al.*, 2012). EBNA 2 has been shown in several microarray studies to regulate many genes (Spender *et al.*, 2006b, Zhao *et al.*, 2006a). Lucchesi *et al* in their studies implicated EBNA 2 as a direct inducer of *IRF4* (Lucchesi *et al.*, 2008).

ChIP seq data from our lab showed two EBNA 2 binding enhancers peaks upstream of the *IRF4* promoter (Figure8.5 a). Previous microarray expression profiling analysis showed EBNA 2 to directly target *IRF4* (Spender *et al.*, 2006a). When *IRF4* expression is reduced in EBV transformed cells, cell proliferation rate is decreased and apoptosis enhanced so this activation may be important for B-cell transformation by EBV (Xu *et al.*, 2008). To investigate whether EBNA 2 activates *IRF4* enhancer sites in reporter assays, I created reporter constructs containing the promoter region of *IRF4* and designed primers to amplify the two enhancer

regions (Figure 8.5 a). Constructs were generated with the promoter region alone, with the first enhancer peak E1 and with enhancer peak E1 and E2 (pGL3 IRF4pE1+E2). I transiently transfected these constructs into DG75 cells in the presence and absence of EBNA 2. The luciferase assay results demonstrated up to 1.4-fold activation at the IRF4 promoter and up to 2.2-fold and 3.4-fold activation at both *IRF4* enhancer peaks constructs E1 and E1+E2 respectively (Figure 8.5 b). We commonly find that enhancer alone decreases basal signal (enhancers contain repressors and activators) (Gunnell *et al.*, 2016). When we see effects of EBNA 2, this often means we see a higher fold activation, so may be a mechanism to increase specificity and magnitude of activator effects. These data suggest that EBNA 2 interacts with the promoter though no binding peak is present there and the enhancer peaks are activation by EBNA 2. Western blot analysis showed this the expression level of EBNA 2 in the experiment (Figure 8.5 c). This result is shows EBNA 2 activates the *IRF4* via the enhancer peaks supporting it as a direct target gene.

3.2.3. DISCUSSION

EBNA 2 is one of the genes initially expressed upon EBV infection and plays a critical role in the growth transformation process, through the activation of a multitude of viral and cellular gene targets, it can initiate transcriptional events that result in B-cell activation, LCLs maintenance and cell cycle entry (Cohen *et al.*, 1989, Hammerschmidt and Sugden, 1989, Sinclair *et al.*, 1994, Kempkes *et al.*, 1995). The mechanism by which EBNA 2 targets cellular genes is not as widely understood as viral gene targets, studies have shown that all EBNA 2 activated viral promoters share a RBP-Jk binding site (Lai, 2002, Hayward, 2004). ChIP-seq data from our identified cellular gene targets that have been shown to be repressed or activated by EBNA 2.

Microarray data have shown B cell receptor (BCR) components: *CD79A* and *CD79B* to be downregulated by EBNA 2 in LCLs and EBV negative BL cells conditionally expressing EBNA 2 and even the EBNA 3s in BL31 cells and LCLs (Maier *et al.*, 2006, White *et al.*, 2010). The mechanism however is unclear has it has not been shown that EBNA 2 recruits co-repressive complexes or stimulate negative histone markers linked to transcriptional inhibition like EBNA

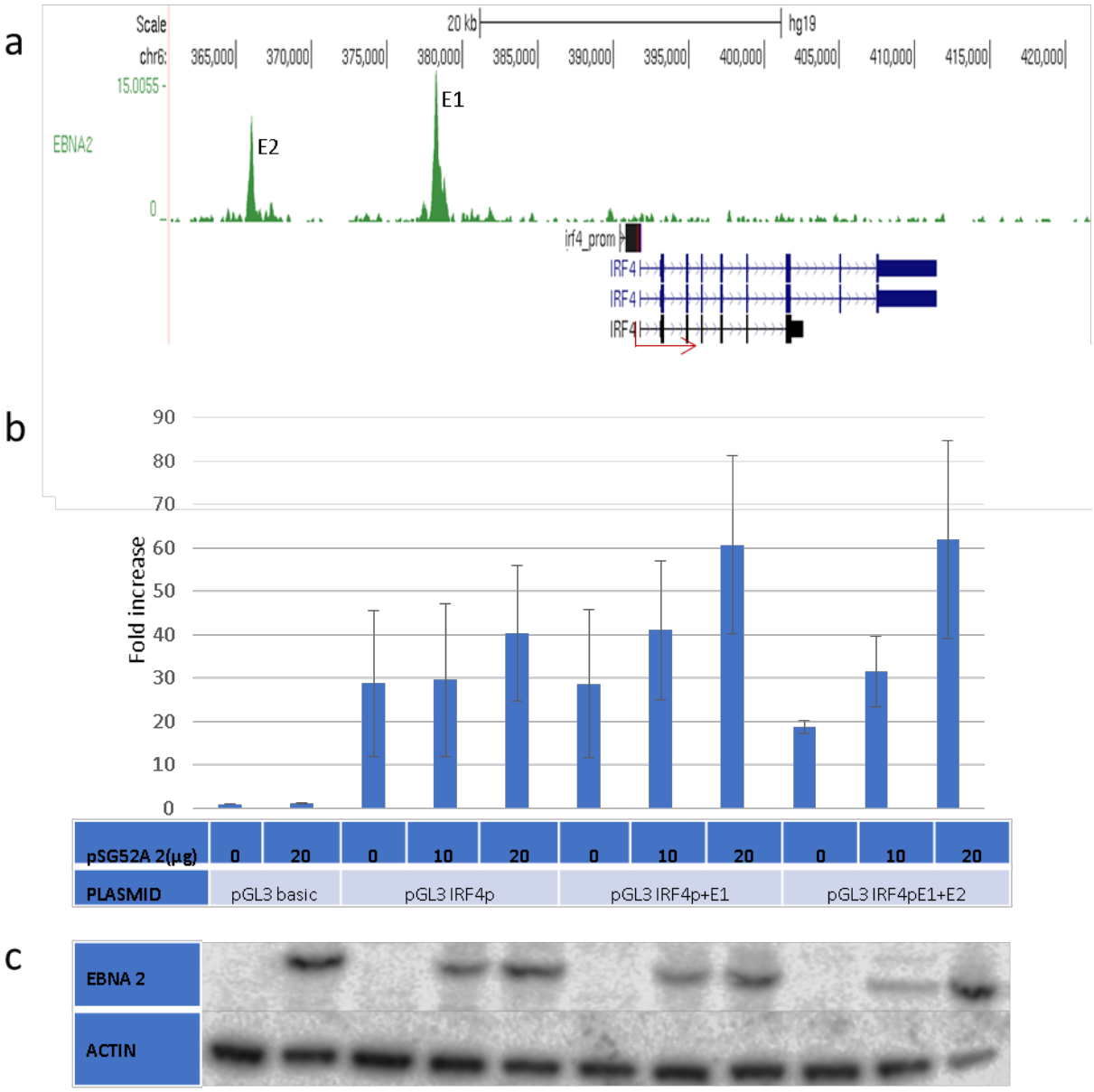

Figure 8.5. EBNA 2 association and effect on *IRF4* promoter and putative enhancers in reporter assays . (a) ChIP sequencing data for *IRF4* showing two EBNA2 enhancer binding peaks. Read per million background subtracted read is displayed to the y axis. The arrows indicate the direction of transcription. (b) Luciferase assay result of DG75 cells transiently transfected with 2 µg of the control vector pGL3 basic, a *IRF4* promoter luciferase reporter (pGL3 *IRF4*p) or *IRF4* promoter-enhancer luciferase reporter (pGL3 *IRF4p-E1*, pGL3 *IRF4*p-E1+E2) in the presence or absence of EBNA2 expressing construct showing up to a 6-fold activation. Firefly luciferase signals were normalised to Renilla luciferase signals from the co-transfected control plasmid pRL-TK (1 µg). Results show the mean –/+ standard deviation of 3 independent experiments and are expressed relative to the pGL3 basic signal. (c) Western blot analysis of EBNA 2 expression levels in transfected cells. The blot was also probed for actin as a loading control.

3C. It could be that EBNA 2 acts at the promoter directly or through upregulating repressor proteins. (Cahir-McFarland *et al.*, 2004, Vockerodt *et al.*, 2008, Faumont *et al.*, 2009).

I searched for RBP-Jk motif at *CD79A* and *CD79B* promoter sites and did not find any, it has also been reported that both *CD79A* and *CD79B* promoters do not contain a RBP-Jk binding site but they bind PU.1. *CD79B* has been shown to have two PU.1 sites with one of binding PU.1 *in vivo* (Omori and Wall, 1993, Thompson *et al.*, 1996, Xie *et al.*, 2004). I set out to investigate the mechanism of the reported repression through reporter assay. Surprisingly, my data showed *CD79A* and *CD79B* activation at the EBNA 2 binding region. *CD79A* and *CD79B* forms a dimer and non-covalently assembles together with membrane bound IgM to form the BCR signalling complex (Weiss and Littman, 1994) and EBNA 2 has been shown to repress IgM transcription by partial dependency on RBP-Jk (Jochner *et al.*, 1996, Strobl *et al.*, 2000, Maier *et al.*, 2005), as *CD79A* and *CD79B* does not contain RBP-Jk site, it could be that the transcription factor EBNA 2 needs for downregulation at the target genes is absent when out of chromatin context. Previous work from our lab by Richard Palermo investigating if EBNA 2 could bind directly and downregulate *CD79B* promoter in Mutu III cells using EBNA 2 with a differentially methylated poly-arginine and glycine region (poly-RG) in ChIP assays demonstrated enhanced binding of asymmetrically di-methylated EBNA 2 (aDMA-EBNA 2) compared to symmetrically (s) sDMA-EBNA 2.

This data suggests that EBNA 2 may bind *CD79Bp* directly and that the differential methylation of EBNA 2 determines its transcriptional regulation of genes by altering the way it interacts with adaptor proteins. Using ChIP to investigate histone modification at the *CD79B* promoter, he also demonstrated reduced H3K27ac in Mutu III cells compared to Mutu I consistent with reduced activation at *CD79B*, however, no difference could be seen in detected in the H3K27me3 status of both cells suggesting that the repression is not mediated through polycomb-dependent mechanisms.

Previous studies have shown LMP1 independently downregulates *CD79A* and *CD79B* in the germinal centre B cell (Vockerodt *et al.*, 2008), EBNA 2 may indirectly inhibit the transcription of *CD79B* promoter through upregulation of LMP1p and cell signalling suggesting the existence of a potential co-repressive mechanism.

To investigate further if the down-regulation by EBNA 2 at the target genes may be through competition with another transcription factor that acts as a 'stronger' activator at the binding sites, I conducted a reporter assay with EBF1 expressing plasmid and my data showed it can activate transcription through the EBNA 2 binding site consistent with known studies of EBF1 able to activate *CD79A* and *CD79B* transcription (Hagman *et al.*, 1991, Maier *et al.*, 2004). However, in the competition experiment, EBNA 2 failed to inhibit this activation at the *CD79A* promoter and only a slight decrease in activation was observed at *CD79B*, inconsistent with EBNA 2 downregulating these targets in EBNA 2 activated cell lines (Maier *et al.*, 2006) which was also demonstrated in our lab. My data suggest that the repressive effects of EBNA 2 may not be easily reproduced out of cellular chromatin context and interactions with other chromatin-associated cellular factors may be involved. However, my data indicated that EBF1 is a better activator of *CD79A* and *B* than EBNA 2 and the lack of additional EBNA 2 activation of these promoters could indicate that the effects of EBNA 2 and EBF 1 activation is mutually exclusive. This suggests EBF1 prevents EBNA 2 binding perhaps via the EBF1 site or an adjacent site. Interestingly, an EBF1 site is required for EBNA 2 activation of the EBV LMP1p by EBNA 2 (Zhao *et al.*, 2011, Tzellos *et al.*, 2014).

A recent study has also put forward a model that EBNA 2 bind to sequence specific factors like EBF1 and RBP-Jk that are already bound to their correlated binding sites and displace co-repressors bound to these factors then form a stable co-activation complex at selected promoters and enhancers to stimulate the transcription of the targeted gene, they indicated that EBNA 2 could drive the formation of new chromosomal occupancies for transcriptional factors like RBP-Jk and EBF1. RBP-Jk, EBF1 and EBNA 2 commonly co-occupy this new sites that are associated with activated chromatin and transcription function. They also indicated that EBNA 2 may function as a stabilizer of multiple protein interactions that includes cooperative binding between RBP-Jk and EBF1 at some genomic locations (Lu *et al.*, 2016a).

Several studies have shown *IRF4* to be expressed in all B-cell development stages in both mature T-cells and macrophages and play critical role in their function (Eisenbeis *et al.*, 1995, Mittrucker *et al.*, 1997). *IRF4* may play a key role in EBV transformation process. It is overexpressed in EBV-transformed cells and revealed to be induced through the LMP1/NFkB signaling (Cahir-McFarland *et al.*, 2004, Xu *et al.*, 2008), it has also been shown to be stabilized

by EBNA 3C in EBV-transformed cells (Banerjee *et al.*, 2013). *IRF4* has been shown to induce B-cell integration cluster (*BIC*) which encodes miR-155 that is associated with EBV latency, BIC/ miR-155 has been shown to be induced by EBV LMP1, LMP 2A and BCR engagement among others (Jiang *et al.*, 2006, Kluiver *et al.*, 2006, Du *et al.*, 2011, Wang *et al.*, 2011). EBNA 2 has been shown to indirectly target *IRF4* through LMP1 and directly as shown in microarray analysis (Arguello *et al.*, 2003, Spender *et al.*, 2006a).

I set out to investigate how EBNA 2 regulates the activation of *IRF4* in reporter assay. Studies have shown that regulatory elements, particularly enhancers, control transcription of their target gene by physically interacting with the gene promoter via chromatin loops, and it has been reported that multiple distal enhancers can regulate a target gene simultaneously or cooperatively (Chepelev *et al.*, 2012, Marsman and Horsfield, 2012). Furthermore, cell type specific transcription factor can regulate the activity of enhancers and are involved in loop formation indirectly by recruiting co-factors or directly by driving the loop formation (Marsman and Horsfield, 2012). My data demonstrated EBNA 2 activates the IRF4 via the two enhancer peaks and is consistent with microarray expression profiling analysis showing IRF4 as a direct gene target for EBNA 2 (Spender *et al.*, 2006a). These data concludes EBNA 2 is able directly target and activate *IRF4* in or out chromatin context.

In summary, these data suggest that EBNA 2 association with activated and repressed genes may be chromatin context specific and the mechanism of regulation may depend on the its interaction with other factors present at the target gene locus.

4. DISCUSSION

The EBV EBNA 2, 3A, 3B and 3C transcription factors have been previously reported to play essential roles in the transcription of viral and cellular genes. EBNA 2 activates transcription of numerous cellular and viral genes and the EBNA 3s function as both activators and repressors of cellular gene expression. In this study, I set out to investigate the mechanism through which EBNA 2 regulates cellular gene activation and repression and the role the EBNA 3 family of proteins play in this regulation. To do this, I used luciferase reporter constructs containing target gene promoters and/or enhancers identified through ChIP-sequencing in our lab (McClellan *et al.*, 2013), and studied their response to the EBNAs in transient reporter assays. Our ChIP-sequencing results identified coincident binding sites for EBNA 2 and EBNA 3 at the *ITGAL* promoter and at putative enhancers distal to *CTBP2* and *WEE1*. We demonstrated that EBNA 2 activated the *ITGAL* promoter via the EBNA2 binding site and that this activation was inhibited when the EBNA 3s were co-expressed. We demonstrated that RBP-Jk was the key mediator of the EBNA 2 activation of *ITGAL*. The effects of the EBNA3s is therefore consistent with reports of the EBNA 3 proteins associating with RBP-Jk and inhibiting EBNA 2 activation. All EBNA 3 proteins can bind RBP-Jκ to inhibit EBNA 2 activation via RBP-Jk sites in reporter assays (Le Roux *et al.*, 1994, Robertson *et al.*, 1995, Waltzer *et al.*, 1996). It is also consistent with a model where EBNA 3 proteins can compete with EBNA 2 for binding to RBP-Jκ at the *ITGAL* promoter site.

EBNA 2 has been shown to interact with long-range enhancer sites to regulate transcription. For example, *MYC* expression was shown to be upregulated by EBNA 2 via long-range enhancer interactions and promoter looping (Zhao *et al.*, 2011, Wood *et al.*, 2016). I set out to investigate the regulation of transcription by the long-range intragenic *CTBP2* and the two distal *WEE1* EBNA 2 and EBNA 3 enhancer binding sites. Data from our lab demonstrated EBNA 2 and EBNA 3s compete for binding at the *CTBP2* locus (McClellan 2013). EBNA 2 binding and EBNA 3 cell-type specific differential binding was also demonstrated at the *WEE1* locus (McClellan 2013).

Analysis of mRNA expression in infected LCLs, implicated EBNA 3A and EBNA 3B in the repression of *CTBP2* transcription by preventing enhancer-promoter loop formation, RNA

level increased when EBNA 3A and B are knocked-out. *CTBP2* was not expressed in some cell lines so it was not possible to assess the effects of EBNA 3C on *CTBP2* expression. As a result of the problems with finding cell lines that expressed *CTBP2*, it was not possible to fully determine the effects of EBNA 2 loss on endogenous *CTBP2* expression. In fact, one of the cell-lines conditionally expressing EBNA 2 that we examined initially (ER-EB 2.5) where no effect of EBNA 2 on *CTBP2* expression was observed (McClellan *et al.*, 2013), was subsequently found to lack EBNA 2 binding at the enhancer site. (White *et al.*, 2010, McClellan *et al.*, 2013). Unfortunately, I was unable to provide any evidence of this from my reporter assay data as the TK promoter used was unexpectedly responsive to EBNA 2 activation and was repressed by expression of EBNA 3 alone. The effects of EBNA 2 when the *CTBP2* enhancer was also present, were therefore difficult to assess. Further assessment of the *CTBP2* enhancer will therefore require the generation of a construct containing either the endogenous promoter or a different heterologous promoter. The endogenous promoter sequence could be obtained by having it synthesised externally.

Analysis of mRNA expression in infected LCLs demonstrated EBNA 2 activates the *WEE1* enhancers and that repression by the EBNA 3s creates a balance that determines the level of *WEE1* transcription consistent with Rob White re-analysed microarray analysis in BL31s, the EBNA 3C repression is directed by enhancer-promoter loop formation (McClellan *et al.*, 2013). Based on previous results and our observations at *ITGAL*, it is possible that *WEE1* RNA levels are affected in cell lines by EBNA 2 and 3s. Even though my constructs contained the endogenous *WEE1* promoter, I did not observe any significant response to EBNA 2 in constructs containing the *WEE1* promoter and enhancer sites. This could be because EBNA 2 and EBNA 3 binding at these locations is cell-type specific or because there is no significant effect out of chromatin context. It could also be that the transcription factors needed to recruit EBNAs to the *CTBP2* putative and *WEE1* enhancer site are not expressed in the cell line used. It is also possible that the enhancer site we have identified is not the site through which *WEE1* transcription is regulated by the EBNAs and that binding of the EBNAs here is redundant and has no function. However, other experiments in the lab have shown that this site does loop to the promoter in EBV-infected cells and its interaction is disrupted by EBNA 3C, consistent with EBNA3C repression of *WEE1* transcription. It is possible that this enhancer works together with another enhancer to regulate WEE1 transcription.

I also studied IRF4 as a gene targeted by EBNA2 alone. I demonstrated that EBNA 2 activates *IRF4* via the enhancer peaks we identified near the locus. This is consistent with microarray expression profiling analysis that showed EBNA 2 to directly regulate *IRF4* (Spender *et al.*, 2006a). Interestingly, many transcription factors including IRF4 have also been shown to co-occupy EBNA 2 enhancer and super-enhancer sites, so EBNA2 activates the expression of a TF that is possibly involved in its targeting to DNA , thus enhancing its function in cellular gene regulation (Zhou *et al.*, 2015). *IRF4* functions as an interacting partner of PU.1 and the composite PU.1/IRF4 element is implicated in EBNA 2 EBV type-specific regulation of specific cellular genes (McClellan *et al.*, 2013, Tzellos *et al.*, 2014), so *IRF4* works together with other TFs involved in EBNA2 targeting. A search for RBP-Jk, EBF1 and PU.1 could be carried out at The IRF4 enhancer sites to identify which TFs are required for EBNA 2 activation. Site-directed mutagenesis could then be used to assess the roles of these sites to obtain more information on how EBNA 2 activates IRF4.

The mechanism of EBNA 2 mediated repression has not received as much attention as EBNA 2 mediated activation. ChIP-seq data from our lab identified EBNA 2 binding sites at the *CD79A* and *CD79B* promoters and microarray experiments indicate that these two genes are repressed by EBNA 2 (Maier *et al.*, 2006, White *et al.*, 2010). I examined if EBNA 2 affected the expression of these genes using reporter assays. To investigate the mechanism of EBNA 2-dependent downregulation of these genes, I carried out luciferase reporter assays using *CD79A* and *CD79B* promoter constructs. My data showed EBNA 2 activating the *CD79A* and *CD79B* promoters. This was surprising as these targets have been reported to be downregulated by EBNA 2 and EBNA 3 *in vivo*. *CD79A* and *CD79B* assembles together with membrane bound IgM to form the BCR signalling complex (Weiss and Littman, 1994) and EBNA 2 has been shown to repress IgM transcription by partial dependency on RBP-Jk (Jochner *et al.*, 1996, Strobl *et al.*, 2000, Maier *et al.*, 2005). I did a motif search at *CD79A* and *CD79B* and did not find any RBP-Jk sites and it has also been previously reported that they do not contain RBP-Jk sites (Omori and Wall, 1993). To further my investigation I carried out a competitive reporter assay with an EBF1 expressing plasmid to examine if EBNA 2 downregulation is through competition with a stronger activator of the target gene. My data demonstrated EBNA 2 was unable to inhibit EBF1 activation at the *CD79A* promoter or

significantly inhibit activation at *CD79B* promoter, suggesting that the repressive effects of EBNA 2 may not be easily reproduced out of cellular chromatin context and interactions with other chromatin-associated cellular factors may be involved. However, my data did indicate that EBF1 is a better activator of *CD79A* and *B* than EBNA 2 and the lack of additional EBNA 2 activation of these promoters could indicate that the effects of EBNA 2 and EBF 1 activation is mutually exclusive. This could indicate that EBF1 prevents EBNA 2 binding perhaps via the EBF1 site or an adjacent site. Interestingly, an EBF1 site is required for EBNA 2 activation of the EBV LMP1p by EBNA 2 (Zhao *et al.*, 2011, Tzellos *et al.*, 2014).

Previous studies have shown that LMP1 independently downregulates *CD79A* and *CD79B* (Vockerodt *et al.*, 2008). EBNA 2 may therefore indirectly inhibit the transcription of *CD79B* promoter through the upregulation of LMP1p and cell signalling. However, microarray analysis of *CD79B* expression in cells only expressing EBNA 2 also detected repression of these genes by EBNA 2 (Maier *et al.*, 2006). This indicates that both direct and indirect repression mechanisms may be used by EBNA 2. Studies by Richard Palermo in our lab (unpublished) confirmed that EBNA 2 binds *CD79Bp* directly by ChIP-QPCR. He examined *CD79B* expression in two BL cell lines, Mutu I and Mutu III. Mutu I cells express only EBNA 1, where Mutu III cells express all EBNAs and LMPs (Gregory *et al.*, 1991). Using ChIP-QPCR to look at specific histone modifications, he found reduced H3K27ac levels in Mutu III cells compared to Mutu I, consistent with reduced activation of *CD79B*. He detected no difference in the H3K27me3 status of the promoter in both cell lines suggesting that the repression is not mediated through polycomb-dependent mechanisms. These data support our hypothesis that EBNA 2 may compete with a 'better' activator leading to reduced *CD79B* expression. Further experiments to test this using ChIP-PCR in cells where EBNA 2 activity can be switched on and off are needed to address this further. This way, EBF1 binding to the *CD79A* and *B* promoters and the binding of other TFs involved in *CD79* gene activation Pax5 and E2A can be monitored in the presence and absence of EBNA 2 and correlated with the effects on *CD79* transcription.

In this study, we have shown that EBNA 2 activates *IRF4* via a newly-identified enhancer and that it activates *ITGAL* via an RBP-Jk binding site. At *ITGAL*, EBNA 3 proteins repress EBNA 2 activation. The lack of effect of EBNA 2 on *CTBP2* and *WEE1* regulatory elements bound by EBNA 2 *in vivo* suggests that differential binding of EBNA 2 with genes may be chromatin

context specific and regulation may depend on the its interaction with other factors present at the target gene locus. Also, luciferase reporter assays may not completely recapitulate the normal behaviour of a given promoter or enhancer, as it is an *in vitro* assay outside of the nuclear environment. Many factors such as dysregulation due to the amount of plasmid DNA in transfected cells, nucleosomal distribution and looping ability of endogenous enhancers may be lacking in the plasmids.

Ultimately, understanding the mechanism behind the regulation of cellular gene will provide insight into the development of EBV-associated diseases and could help identify therapeutic targets for new drug inventions. Further studies using alternative strategies including ChIP-QPCR and the creation of integrated reporter constructs using CRISPR gene targeting will be needed to study cellular gene regulation in more detail.

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6. APPENDICES

6.1. Appendix A Antibodies for western blotting

Protein	Antibody	Antibody species	Antibody dilution	Company/generated by	Secondary antibody	Antibody dilution	Company/generated by
Actin	Actin	Rabbit polyclonal	1:2000	Sigma	Anti Rabbit-HRP	1:1000	CELL SIGNALLING
EBNA2	PE2	Mouse monoclonal	1:300	Gift from Martin Rowe	Anti-Mouse-HRP	1:1000	CELLSIGNALLING
EBNA3A	T2.78	Mouse monoclonal	1:1000	Gift from Martin Rowe	Anti-Mouse-HRP	1:1000	Cell signalling
EBNA3B	EX ALPHA	Sheep polyclonal	1:500	Ex alpha	Anti-Sheep-HRP	1:1000	sigma
EBNA3C	E3A10	Mouse monoclonal	1:300	Gift from Martin Rowe	Anti-Mouse-HRP	1:1000	Cell signalling
EBF1	EBF1	Mouse monoclonal	1:300	Santa cruz	Anti-mouse-HRP	1:1000	Cell signalling

6.2. Appendix B Real time primers for QPCR

	Appendix B - primers		
	Real time PCR primers for QPCR		
primer set	primer	sequence 5' to 3'	notes
	ITGAL		
1	Forward MW826	TGCACCTGTGGTTTCAGCTA	peak primer B
	Reverse MW827	CGATCACAGCTCAATGCAAC	peak primer B
2	Forward MW828	ACCCAGCCTCCAATTCTTTAG	
	Reverse MW829	TTTCTCTGGACCTTGAAAGATGT	
3	Forward MW830	TGCTTACACTTCCTCCCTGAA	peak primer C
	Reverse MW831	TTTCTCACAGAGGCAACAGG	peak primer C

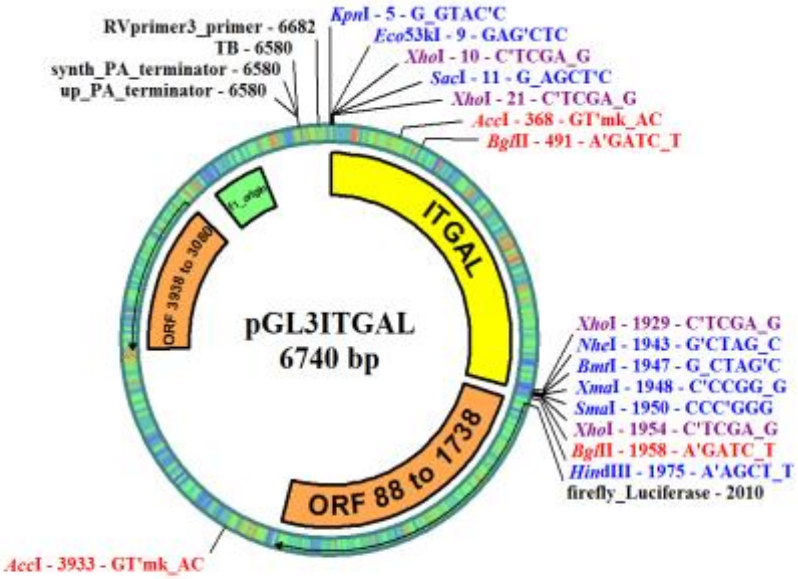
- Obtained from (McClellan *et al.*, 2013)

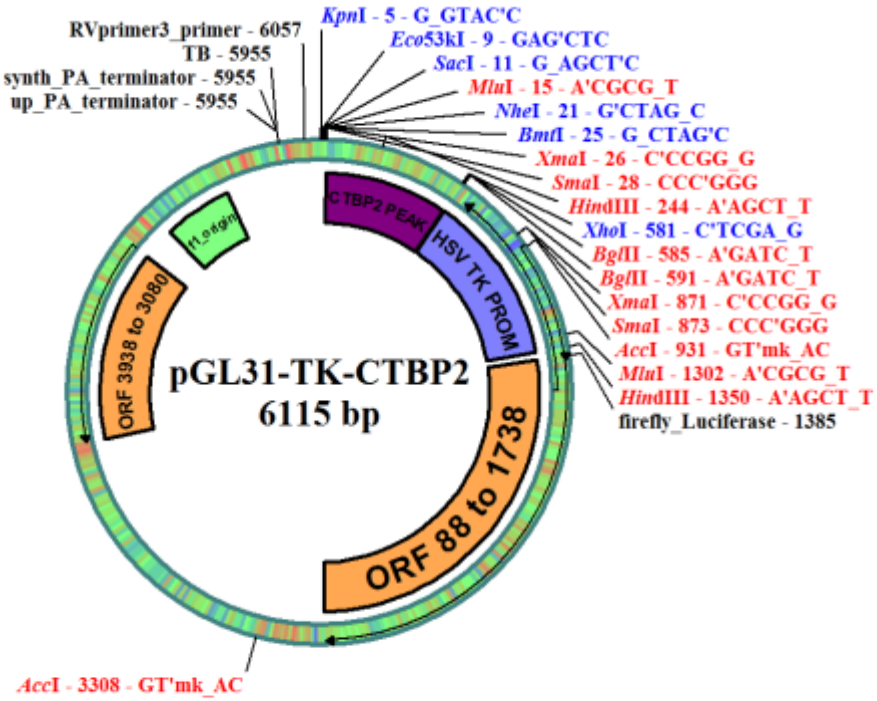
6.3. Appendix C DNA amplifying primers

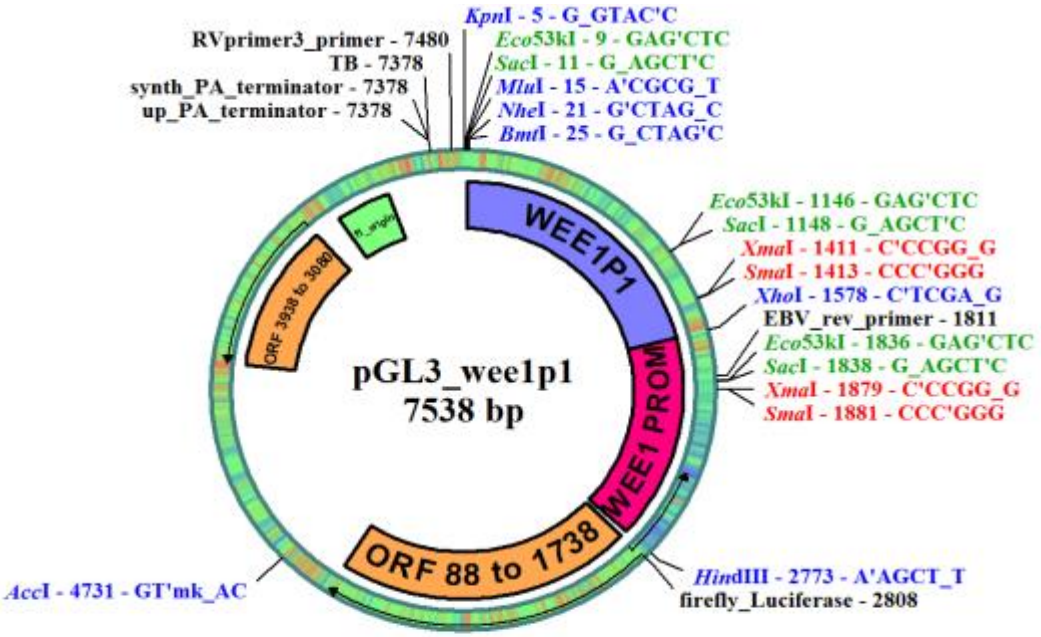
Appendix C- primers		
DNA Amplifying primers		
Sequence 5' to 3'	Gene name/region/number	PCR Product size/ sites introduced into primers
GATATCTCGAGGTTGCAGGTTGTAGTGAGCCGAG	WEE1 Promoter Fw MW842	1250bp/ Xhol
GCATAAGCTTCGAGGACAGGAGAGCGGA	WEE1 Promoter Rv MW843	HindIII
GTATGCTAGCACACAGTGTAGTGGAGGTATTAGGCAGA	WEE1 Enhancer peak 1 Fw MW844	1550bp/ Xhol
GATATCTCGAGACTCCAGCCTGGGTGACCA	WEE1 Enhancer peak 1 Rv MW845	NheI
GGATGCTAGCTGCTTGTCACTTGGTGCAGACA	WEE1 Enhancer peak 2 Fw MW900	2952bp/ NheI
GTATGCTAGCTGAGCAACAGAGTGAGACACCGT	WEE1 Enhancer peak 2 Rv MW847	SacI
GTATGAGCTCGAGAATGACTCGAGCCCGTGAG	ITGAL Promoter peaks Fw MW848	1950bp/ SacI
GCATAAGCTTTTCCAGCACTCGAGGGACC	ITGAL Promoter peaks Rv MW849	HindIII
GAATACTCGAGATTCCCGCCACGCCAGTGT	CTBP2 Enhancer peak Fw MW850	549bp/ Xhol
GTAGGTACCGCAGAGTGCCCCAGTTGC	CTBP2 Enhancer peak Rv MW851	KpnI
GATGTTAGTGccAAACCATGACAGC	ITGAL_MUT1 Fw MW1524	1950bp/ SacI
CCAACTAAGGGCTCTGTAAAG	ITGAL_MUT1 Rv MW1525	HindIII
TGCCTCTGTGccAAAGTACCACTGTAAG	ITGAL_MUT2 Fw MW1526	1950bp/ SacI
ACAGGCTGGTGACACTGG	ITGAL_MUT2 Rv MW1527	HindIII
GTCTCGAGATTACAGGCTTGAGCCACA	IRF4 Promoter	969bp/ Xhol

	Fw MW1497	
GACTCGAGCTGGACTCGGAGCTGAGG	IRF4 Promoter Rv MW1498	XhoI
GAGCTAGCATCGCTTGAGGTTGCAGTG	IRF4 Enhancer peak 1 Fw MW1076	1236bp/ XhoI
GTCTCGAGTGAAGCAGGCACTGTGATTC	IRF4 Enhancer peak 1 Rv MW1077	NheI
GAGAGCTCAGCCATCTCCATCATCTGGT	IRF4 Enhancer peak 2 Fw MW1078	996bp/ NheI
GAGCTAGCATGTGGAACGCTGGTCCT	IRF4 Enhancer peak 2 Rv MW1079	SacI
GTGCTAGCAAGAGTGAGAGACAGAGGAGGAG	CD79B Promoter Fw MW1495	942bp/ SacI
GAGAGCTCAGCTGTCTCCACCTACATCCA	CD79B Promoter Rv MW1496	NheI
Synthesized DNA by Eurofins	CD79A Promoter Peak 1	971bp/ XhoI HindIII
Synthesized DNA by Life Technologies	CD79A Promoter Peak 2	655bp/ XhoI NheI

6.4. Appendix D Examples of designed reporter constructs



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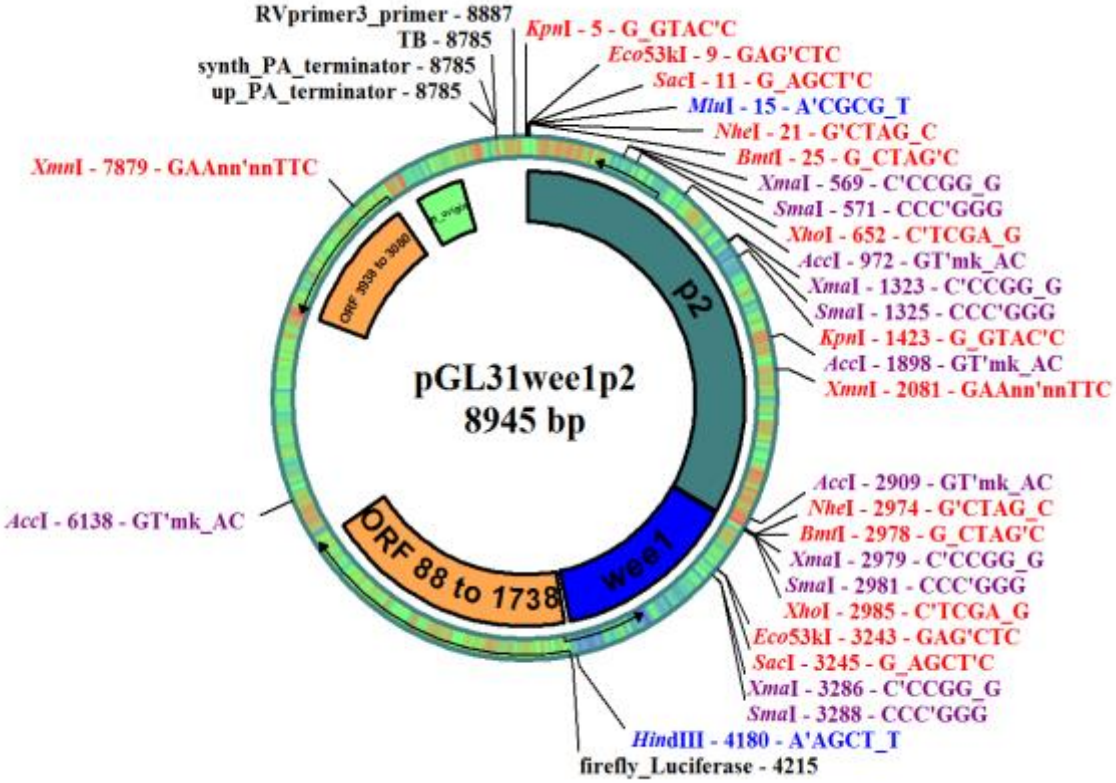
RVprimer3_primer - 7480
Eco53kI - 9 - GAG'CTC
TB - 7378
SacI - 11 - G_AGCT'C
synth_PA_terminator - 7378
MluI - 15 - A'CGCG_T
up_PA_terminator - 7378
NheI - 21 - G'CTAG_C
BmtI - 25 - G_CTAG'C
Eco53kI - 1146 - GAG'CTC
SacI - 1148 - G_AGCT'C
XmaI - 1411 - C'CCGG_G
SmaI - 1413 - CCC'GGG
XhoI - 1578 - C'TCGA_G
EBV_rev_primer - 1811
Eco53kI - 1836 - GAG'CTC
SacI - 1838 - G_AGCT'C
XmaI - 1879 - C'CCGG_G
SmaI - 1881 - CCC'GGG
WEE1P1
ORF 3938 to 3680
pGL3_wee1p1
7538 bp
WEE1 PROM
ORF 88 to 1738
AccI - 4731 - GT'mk_AC
HindIII - 2773 - A'AGCT_T
firefly_Luciferase - 2808


RVprimer3_primer - 8887
TB - 8785
synth_PA_terminator - 8785
up_PA_terminator - 8785
KpnI - 5 - G_GTAC'C
Eco53kI - 9 - GAG'CTC
SacI - 11 - G_AGCT'C
MluI - 15 - A'CGCG_T
NheI - 21 - G'CTAG_C
BmtI - 25 - G_CTAG'C
XmaI - 569 - C'CCGG_G
SmaI - 571 - CCC'GGG
XhoI - 652 - C'TCGA_G
AccI - 972 - GT'mk_AC
XmaI - 1323 - C'CCGG_G
SmaI - 1325 - CCC'GGG
KpnI - 1423 - G_GTAC'C
AccI - 1898 - GT'mk_AC
XmnI - 2081 - GAAnn'nnTTC
XmnI - 7879 - GAAnn'nnTTC
ORF 3938 to 3680
P2
pGL31wee1p2
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AccI - 2909 - GT'mk_AC
NheI - 2974 - G'CTAG_C
BmtI - 2978 - G_CTAG'C
XmaI - 2979 - C'CCGG_G
SmaI - 2981 - CCC'GGG
XhoI - 2985 - C'TCGA_G
Eco53kI - 3243 - GAG'CTC
SacI - 3245 - G_AGCT'C
XmaI - 3286 - C'CCGG_G
SmaI - 3288 - CCC'GGG
AccI - 6138 - GT'mk_AC
ORF 88 to 1738
wee1
HindIII - 4180 - A'AGCT_T
firefly_Luciferase - 4215