The Role of the B-cell Specific Transcription Factor, Pax5, in EBV Latency and Lytic Reactivation

By

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ABSTRACT

The Role of the B-cell Specific Transcription Factor, Pax5, in EBV Latency and Lytic Reactivation

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Epstein-Barr Virus (EBV) is associated with several B-cell and epithelial cell cancers, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma (NPC), and gastric carcinoma. However, to be transmitted from host to host, and from cell to cell, the virus periodically reactivates from latency and converts to the lytic form of replication. The latent-to-lytic switch of EBV in host cells is controlled at the level of the BZLF1 and BRLF1 viral immediate-early promoters and their associated gene products, Z and R, which are transcription factors. However, cellular factors which regulate the latent-to-lytic switch remain incompletely characterized.

Here I investigate the role of the B-cell specific transcription factor and master regulator, Pax5, in promoting EBV latency. Pax5 is known to play an essential role in allowing EBV to establish long-term latent infection in B cells, since it binds to and activates an essential EBV latency promoter, Wp, that drives expression of the viral EBNA2 and EBNA-LP proteins during

primary B-cell infection. In **Chapter II**, I demonstrate that Pax5 attenuates the function of the major lytic switch protein, Z, by inhibiting Z transcriptional function and DNA binding activity, and thereby prevents lytic reactivation. Thus, I have uncovered a new mechanism by which Pax5 helps to promote EBV latency in B cells, by blocking Z function. In addition, in **Chapter III**, I demonstrate that Pax5 binds directly to the terminal repeat region of the EBV genome and may positively regulate expression of the major EBV oncogene, LMP1. In conclusion, these results reveal novel mechanisms by which the Pax5 cellular transcription factor promotes B-cell specific viral latency.

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DEDICATION

To the memory of my grandfather, who lost the battle to cancer and inspired my work.

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LIST OF ABBREVIATIONS

EBV	Epstein-Barr Virus
IM	infectious mononucleosis
BL	Burkitt lymphoma
BCR	B-cell receptor
bZIP	basic leucine zipper
BART	BamHI A rightward transcript
ChIP	chromatin immunoprecipitation
CMV	Cytomegalovirus
EBNA	Epstein-Barr Nuclear Antigen
EMSA	electrophoretic mobility shift assay
GFP	green fluorescent protein
gp	glycoprotein
GP110	EBV glycoprotein 110
GRU	green Raji units
GST	glutathione S-transferase
HDAC	histone deacetylase
HL	Hodgkin lymphoma
TR	Terminal Repeat
HSV	Herpes Simplex Virus
oriLyt	Lytic origin of replication
IE	immediate-early
CBP	CREB-binding protein
IFN	interferon
Ig	immunoglobulin

IP	immunoprecipitation
KSHV	Kaposi's Sarcoma Herpesvirus
LCL	lymphoblastoid cell line
LPD	lymphoproliferative disease
LMP	Latent Membrane Protein
miRNA	micro RNA
NK	natural killer
XBP-1	X-box binding protein-1
Blimp-1	B lymphocyte-induced maturation protein-1
NPC	nasopharyngeal carcinoma
OHL	oral hairy leukoplakia
ORFs	open reading frames
RT-PCR	reverse transcription PCR
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
TPA	12-O-tetradecanoylphorbol-13-acetate
VZV	Varicella Zoster Virus
HHV	Human herpesvirus
WT	wild-type
ZRE	Z-responsive element
RRE	R-responsive element
SL	superlytic
HRS	Hodgkin/Reed-Sternberg
BSAP	B-cell-specific activator protein
EGR1	Early growth factor 1

TLR	Toll like receptor
MeC	methylated cytosine

Chapter I

General Introduction

Discovery and History of Epstein – Barr virus (EBV)

In 1957, a British surgeon named Denis Burkitt was consulted by a colleague about two children that had swellings in all four angles of the jaw while working in Kampala, Uganda (Epstein, 2005). Dr. Burkitt had no prior knowledge of or previous experience with this particular disease. Dr. Burkitt went on to establish that the tumors were lymphomas, as he traveled throughout sub-Saharan Africa in order to study the prevalence of the lymphoma among different native tribes. Much to Dr. Burkitt's surprise, he found that the lymphomas were prevalent in children in climates that were plagued by holoendemic malaria (Burkitt, 1983). Dr. Burkitt wrote and lectured about the unusual epidemiologic features of the childhood African lymphoma which raised questions about the etiology (Burkitt and O'Conor, 1961).

A pathologist in the United Kingdom, by the name of Anthony Epstein, arranged to obtain tumor biopsies from Dr. Burkitt, which led to the successful culture of the lymphoma cells. Using electron micrographs, a herpesvirus was identified in the cultured lymphoma cells (Epstein et al., 1964; Hopwood et al., 2002). Yvonne Barr and Anthony Epstein found that the Burkitt's lymphoma tumor virus was different from other known human herpesviruses, since it was noninfectious for a variety of different cultured cell lines and didn't react to antibodies to other herpesviruses. They named the herpesvirus-like particle Epstein-Barr virus (EBV), in which the name was adopted from the two researchers accredited with its discovery. EBV emerged as a human tumor gamma-herpesvirus virus, demonstrating various oncogenic properties (Epstein and Achong, 1967, 1968a, b; Henle and Henle, 1966a, b; zur Hausen et al., 1970).

Much of the immunological uniqueness of EBV was demonstrated by early U.S. pioneers, Werner and Gertrude Henle, along with their German postdoctoral researcher, Harald zur Hausen. The Henles were able to show via their seroepidemiological studies that EBV is the causative agent of infectious mononucleosis (Henle et al., 1969; Henle et al., 1968). Specifically, between 1966 and 1987, they were able to show a seroreponse to viral capsid and early antigens is induced during infectious mononucleosis (IM) (zur Hausen, 2005).

EBV-associated malignancies and diseases

EBV is transmitted from the saliva of an infected individual to a naïve individual and is commonly referred to as the "kissing disease." The age of acquisition of EBV can vary based on geographical location; however, it is estimated that over 90% of all individuals are eventually infected (Henle and Henle, 1979). EBV can initiate or promote various malignant proliferations under differing environmental conditions. Sero-epidemiology, as well as the presence of viral DNA within tumor cells, have been important in establishing the etiological role of EBV in the development of certain diseases.

The most frequent disease associated with EBV is IM, which is common among teenagers in western countries (Epstein et al., 1964; Evans et al., 1968). Patients who develop IM first develop IgM, and then IgG, EBV-specific antibodies against structural antigens (VCA*), early non-structural (EA*), and membrane antigens (MA*) (Henle et al., 1968). IM symptoms include fever, sore throat, swollen lymph glands, and fatigue, and excessive EBV-induced B-cell proliferation (Odumade et al., 2011; Odumade et al., 2012). Factors that influence the clinical

development of IM range from age of infection to host immune status to genetic predispositions. Age is one of the most significant factors as children infected by EBV prior to the age of 10 are generally much less symptomatic (Sumaya et al., 1975), whereas viral infection by EBV in adolescents and young adults often results in IM. It is thought that children under the age of 10 possibly have more effective $CD8^+$ T cells to mediate and control EBV infection (Odumade et al., 2011; Odumade et al., 2012). One suggested hypothesis is that older individuals have a larger percent of T cells in the form of memory T cells for previous infections (Odumade et al., 2011), and thus have fewer naïve T cells available to combat EBV infection.

EBV is also associated with B-cell lymphomas such as Burkitt's lymphoma (BL) in children in sub-Sahara Africa (Epstein, 2005; Epstein et al., 1964), and undifferentiated nasopharyngeal carcinoma (NPC) in Cantonese Chinese in South East Asia (Desgranges et al., 1977; Desgranges et al., 1975; Old et al., 1966; zur Hausen et al., 1970). In addition, the latent form of infection has been associated with other types of cancer such as post-transplant lymphoproliferative disease (PTLD), Hodgkin lymphoma (HL) (Weiss et al., 1989), T/NK lymphoma (Mitarnun et al., 2002), and gastric carcinoma (Burke et al., 1990). The association of EBV with endemic BL and undifferentiated NPC is almost 100% (Kieff and Rickinson, 2007a). These tumor cells also contain multiple viral genomes which seem to have been derived from one infectious event (Raab-Traub and Flynn, 1986). EBV is present in approximately 40-60% of HL cases (Niedobitek, 1999). EBV establishes a latent infection in its host, which is thought to explain why EBV infections last the lifetime of the host. Three different latency types have been categorized (Brink et al., 1997; Busson et al., 1992; Chiang et al., 1996; Deacon et al., 1993; Gilligan et al., 1990b; Gilligan et al., 1991; Grässer et al., 1994; Imai et al., 1994; Murray

et al., 1992; Niedobitek et al., 1997; Pallesen et al., 1991; Pathmanathan et al., 1995; Raab-Traub et al., 1983; Rowe et al., 1987; Tsai et al., 1996), and are further described later in this Chapter.

Herpesviruses

All herpeviruses are able to remain latent in their natural hosts and also periodically express a lytic form of infection within cells (Pellett and Roizman, 2007). Cells that harbor the latent form of infection contain viral genomes in the form of a closed circular molecule. During latency, only a small subset of viral genes are expressed and no infectious progeny are produced (Pellett and Roizman, 2007). The latent viral genome retains the capacity to replicate lytically, but the precise mechanisms which trigger the latent-to-lytic switch are not fully understood and vary by herpesvirus. The lytic form of infection results in expression of most viral genes, and is required for the production of infectious viral progeny, and often leads to the death of the infected cell (Pellett and Roizman, 2007).

Herpesviruses that infect humans include EBV, Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Human Cytomegalovirus (HCMV), Varicella-Zoster Virus (VZV), and Human Herpesviruses 6 (HHV-6), 7 (HHV-7), and 8 (HHV-8, also known as Kaposi's Sarcoma Herpesvirus or KSHV) (Pellett and Roizman, 2007). Herpesviruses can infect a wide or narrow range of host cells. In the case of EBV, infection primarily occurs in B lymphocytes and epithelial cells (Kieff and Rickinson, 2007b). Following the recovery from primary infection, EBV is found in approximately one in a million circulating memory B cells within the healthy human host (Khan et al., 1996). In contrast, latent HSV infection is detected only in neurons of

dorsal root ganglia, while latent HCMV can be found in hematopoietic stem cells (Pellett and Roizman, 2007).

Within the family *Herpesviridae*, viruses are classified into three subfamilies: alpha (α), beta (β), and gamma (γ). EBV and KSHV are members of the gamma herpesviruses. EBV is further classified in the genus *Lymphocryptovirus*. Members of this genus can transform target cells *in vitro* after recovery from primary infection. Infection of primary B cells in culture with EBV results in their immortalization and continual growth (Kieff and Rickinson, 2007b). EBV variants or subtypes, EBV-1 and EBV-2, differ by EBNA gene sequences including EBNA2, -3A, and -3C, and their ability to transform B cells *in vitro* and spontaneously enter the lytic cycle (Pellett and Roizman, 2007; Rowe et al., 1989; Sample et al., 1990; Young et al., 1987). EBV-1 is primarily found in the Western hemisphere, Southeast Asia, and Africa whereas EBV-2 is primarily found in Africa (Zimber et al., 1986).

EBV Genome Structure

The EBV B95.8 strain genome is comprised of a linear, double-stranded 172 kbp DNA, which encodes over 80 open reading frames (ORFs) (Bagni and America, 2008; Kieff and Rickinson, 2007b). The EBV genome was sequenced from BamHI digested cloned DNA fragments, and EBV ORFs were designated according to their order in a rightward or leftward direction within each of the BamHI fragments (Baer et al., 1984). The Bam A fragment is the largest and Bam Z fragment is the smallest (Sample et al., 2009). For example, the BARF1 gene product is encoded by the Bam A fragment first rightward ORF. Similarly, BZLF1 (*Bam*HI-Z-

fragment Leftward Frame 1) is the first leftward reading frame, starting in BamHI Z. A repeated 0.5 kbp DNA sequence (known as the terminal repeats) is found at both ends of the linear form of the viral genome (Arvey et al., 2012; Farrell, 2005). When EBV infects a cell, the genome then becomes a circular episome and is maintained as an extrachromosomal plasmid within the host nucleus using the host cell DNA polymerase to replicate during the latent form of infection (Pritchett et al., 1975).

Similar to other herpesviruses, EBV virions contain a DNA core which is surrounded by three components: a nucleocapsid of 162 capsomeres, an outer envelope with external glycoproteins, and finally a protein tegument which lies between the nucleocapsid and envelope (Kieff and Rickinson, 2007b). The EBV tegument is made up of many different viral and cellular proteins, which are common components to other herpesviruses. Viral tegument proteins serve important roles in reenvelopment of mature virions, as well as helping to activate transcription of the viral IE promoters in the initial stage of infection.

EBV entry into the cell

The oral cavity is the initial site of viral infection and production, and both epithelial cells and B cells are thought to contribute to infectious viral production within the oropharynx (Kieff and Rickinson, 2007b; Shannon-Lowe and Rowe, 2011). EBV entry into cells has two distinct steps. First, EBV binds to the host cell through specific receptors via envelope glycoproteins. Secondly, EBV can enter cells by either fusion at the plasma membrane or by fusion with an

endocytic membrane (Connolly et al., 2011b). However, the viral entry mechanism differs for B cells and epithelial cells.

For EBV entry into B cells, the cellular receptor CD21 and co-receptor HLA class II are utilized (Connolly et al., 2011b; Odumade et al., 2011). The viral envelope glycoprotein (gp), gp350/220 (BLLF1), interacts with cellular component receptor type 2, CR2/CD21 and helps tether EBV to the host cell (Connolly et al., 2011b; Fingeroth et al., 1984; Nemerow et al., 1987; Tanner et al., 1987). The viral glycoprotein, gp42 (BZLF2) (which also associates with EBV gH, gB, and gL complexes), binds to cellular HLA antigen class II resulting in endocytosis, where the virus is then enveloped and can fuse with the endocytic vesicle membrane to enter cells (Haan et al., 2000; Li et al., 1997; Li et al., 1995). The aggregation of CD21 and MHC class II by the EBV glycoproteins likely results in BCR-like tyrosine kinase signal transduction and NF-κB activation (Masucci et al., 1987; Nashar and Drake, 2006). When gp42 binds to MHC class II, this may also be important in blocking MHC class II antigen presentation and possibly averting $CD4^+$ T-cell activation (Spriggs et al., 1996). EBV lacking gp42 can still attach to B cells but is unable to enter (Wang and Hutt-Fletcher, 1998; Wang et al., 1998). The viral glyocoproteins gH-gL are also required for membrane fusion, whereas gB helps to catalyze membrane fusion (Connolly et al., 2011b).

The entry of EBV into epithelial cells is less understood, but does involve membrane fusion at the cell surface instead of endocytosis (Connolly et al., 2011b). EBV enters epithelial cells via BMRF2 (an EBV viral envelope glycoprotein), which interacts with α5β1 integrin to allow for attachment (Connolly et al., 2011b; Johannsen et al., 2004; Tugizov et al., 2003; Xiao et al., 2008).The glycoproteins gH/gL also bind to receptors such as integrin, and interact with

gB to help drive membrane fusion between the host plasma membrane and viral envelope. This helps to release the tegumented viral capsid into the cytoplasm (Connolly et al., 2011a).

Not only does gp42 help to facilitate viral endocytosis in B cells, but it also plays an important role in altering the tropism of the virus from epithelium to B cells. Virus that is produced from epithelial cells is rich in gp42, which provides favorable entry into B cells. This is important because virions produced in B cells lack a sufficient level of gp42 to reinfect other B cells; however these virions infect epithelial cells well. In B cells, virions that have gp42 are destroyed since they interact with endogenous HLA class II molecules. Epithelial cells however, are able to produce gp42 virions which in turn infect B cells effectively. Therefore, EBV is able to direct the spread of infection between B cells and epithelial cells and this tropism is mediated by gp42 (Borza and Hutt-Fletcher, 2002).

Immune Response to EBV

EBV persistent infection in humans is tightly controlled by T cells via cell-mediated adaptive immunity (Martorelli et al., 2012; Ning, 2011; Strowig et al., 2008). The innate immune response also plays an important role in the initial immune control of EBV. Specifically, Natural Killer (NK) cells are activated by dendritic cells, which help assist in polarization of DC primed T cell responses, which ultimately control the virus (Bickham et al., 2003; Borg et al., 2004; Ferlazzo and Munz, 2004; Morandi et al., 2012). A recent report also demonstrated that there is reciprocal activating crosstalk between dendritic cells and natural killer cells, which are important for controlling the immune defenses against viral tumors (Harizi, 2013). Anti-viral

Th1 immunity also helps prevent normal healthy hosts and EBV carriers from developing EBV-associated malignancies (Chen et al., 2009; Xiao et al., 2009). However, when a host becomes immunocompromised (such as during HIV infection or immunosuppressive drug treatment), EBV-associated malignancies may develop.

The initiation of EBV specific immune control is mediated by dendritic cells cross-presenting EBV antigens from infected B cells and epithelial cells (Bickham et al., 2003; Bickham and Munz, 2003; Strowig et al., 2008). In addition, NK cells, $CD4^+$ and $CD8^+$ T cells target EBV infected cells with the latent and lytic forms of infection. The production of type I IFNs (IFN α and β) is the earliest immune response to EBV infection. IFNα plays a crucial role in the reduction of B-cell outgrowth following EBV transformation (Garner et al., 1984; Lotz et al., 1985) via several mechanisms. Recent evidence also indicates that multiple toll like receptors (TLRs) are activated in response to EBV infection. These TLRs include TLR2, TLR3, TLR7, and TLR9 (Iwakiri et al., 2009; Odumade et al., 2011). Other cytokines that are elevated during primary EBV infection include IL6, TNFα, IL12, IL2, IL10, and TGFβ (Foss et al., 1994; Odumade et al., 2011).

The majority of antibody responses against latent and lytic antigens peak during the acute form of infection. Examples of antibody responses to virus-encoded nucleocapsid antigens and immediate-early and early lytic EBV antigens include IgM, IgA, and IgG (Kieff and Rickinson, 2007b). Most healthy carriers have anti-EBNA1, anti-gp350, and anti-VCA IgG responses during persistent EBV infection (Kieff and Rickinson, 2007b). These three are utilized to diagnose and assess whether an individual has been recently or previously infected with EBV. In some cases, any of these three responses can be absent. If all three antibody responses are

negative this indicates the absence of EBV infection, whereas the presence of either anti-gp350 or anti-VCA antibodies in the absence of anti-EBNA1 antibodies indicate acute (recent) EBV infection.

$CD8^+$ T cells are considered the main immunological effecter cells against EBV infection. During acute infection, up to 25-50% of all $CD8^+$ T cells are directed against EBV lytic antigens in certain patients who have infectious mononucleosis (Callan et al., 1998). $CD8^+$ T cells control EBV infection via cytolytic methods and anti-viral cytokine secretion. Studies have shown that EBNA3C specific $CD8^+$ T cells are able to control an outgrowth of newly EBV infected B cells via IFNγ secretion (Shi and Lutz, 2002). Also, EBNA1 specific $CD8^+$ T cells that exhibit low killing activity against LCLs produce IFNγ upon LCL recognition and can control outgrowth *in vitro* (Lee et al., 2004). $CD4^+$ T cells, which play an important role in controlling persistent infection, are also thought to direct virus specific immune responses and are very important for priming and maintenance of $CD8^+$ T cells (Bennett et al., 1998; Cardin et al., 1996; Ridge et al., 1998; Schoenberger et al., 1998; Zajac et al., 1998). Interestingly, Burkitt lymphoma cells evade $CD8^+$ T cells recognition due to limited EBV protein expression (only EBNA1 is expressed) and downregulation of the MHC class I antigen processing machinery (Henriquez et al., 1999; Khanna et al., 1997; Rowe et al., 1995) . To date, EBNA1 specific $CD4^+$ T cells appear to be the only T cell reactivity that are able to target Burkitt lymphoma cells (Paludan et al., 2002; Voo et al., 2002). In the case of HIV-infected patients that have a decreased number of $CD4^+$ T cells, there is a heightened risk for the development of EBV-induced lymphomas (Gasser et al., 2007).

EBV Latent Infection

An interesting and common characteristic of all herpesviruses is that that they establish latent infection. Latent infection persists in the host for life, usually without major pathological consequences (Thorley-Lawson, 2005). How herpesviruses establish and maintain latency is still not fully elucidated. However, the control of persistent latent infection is perhaps best understood in the case of EBV.

EBV can enter three different distinct latency programs, that are linked to the differentiation state of infected B cells (Thorley-Lawson, 2001). These three distinct latency programs are: Latency I/0, Latency II, and Latency III, and are based on the number of viral latent genes expressed as shown in **Fig. I-1**. In type I latency, where only one viral protein (EBNA1) is expressed (as well as EBERs and viral microRNAs), the viral latency Q promoter is used to drive EBNA1 expression (Nonkwelo et al., 1996; Schaefer et al., 1995; Tsai et al., 1995). EBNA1, which is the essential latent protein expressed in all latency types, is important since it is required for replication and extrachromosomal maintenance of the viral genome and EBV genome transfer to daughter cells by tethering viral genomes to mitotic chromosomes (Adams, 1987; Frappier and O'Donnell, 1991; Middleton and Sugden, 1992; Yates and Guan, 1991; Yates et al., 1985). During latent infection, EBV is replicated once per cell cycle using the host cell replication machinery, via a viral replication origin known as oriP. Most BL tumors harbor EBV infection with type I latency (Rowe et al., 1987).

Virus-encoded microRNAs (miRNAs) were first identified in a human BL cell line, BL41/95 (Pfeffer et al., 2004). The expression of miRNAs differs in cell and latency type among

B cells and epithelial cells (Forte and Luftig, 2011). EBV miRNAs are located in the BamHI A rightward (BART) and BHRF1 transcripts. The BART transcripts are found in two clusters that are 5 kilobases apart. BHRF1 encodes the BHRF1 open reading frame (ORF) as well as the miRNAs. Various cellular and viral targets of these miRNAs have been identified. The miR-BART2 is antisense to the viral DNA polymerase (BALF5), and enhances BALF5 mRNA cleavage and suppression of BALF5 protein expression (Barth et al., 2008). There is also some evidence for a role of miR-BART2 in regulation of viral lytic reactivation (Barth et al., 2008; Lei et al., 2010). LMP1 and LMP2A have also been reported to be EBV miRNA targets (Lo et al., 2007; Lung et al., 2009). Interestingly, high levels of LMP1 inhibit proliferation and also lead to pro-apoptotic stress, and LMP2A is highly immunogenic. Therefore, miRNA targeting of LMP1 and LMP2A may provide EBV with a selective advantage. The expression of miRNAs (e.g. miR-BHRF1-3) in type III latency may also help in targeting interferon-inducible T-cell attracting chemokine to prevent T-cell chemoattraction (Xia et al., 2008) , and contribute to *in vitro* B-cell transformation (Feederle et al., 2011).

In all latently infected cells, the most abundant transcripts present are EBV-encoded small nonpolyadenylated RNAs, known as EBERs (Rymo, 1979). EBERs are also not translated and do not encode proteins. Downregulation of EBERs occurs in differentiated cells and during lytic viral replication (Gilligan et al., 1990a). One proposed function of EBERs is to bind to dsRNA-dependent enzymes and block their phosphorylation (activation), which contributes to viral resistance to IFN-α (Nanbo et al., 2002). Additionally, EBERs have been shown to play a role in the induction of autocrine growth factors IL-9, IGF-1, and IL-10 (Iwakiri et al., 2003; Iwakiri and Takada, 2010; Kitagawa et al., 2000; Samanta and Takada, 2010; Yang et al., 2004).

In type II latency, which is also known as the default program, EBNA1 transcription is driven by the Qp in the presence of LMP1 and LMP2a (but absence of EBNA2) (Hochberg and Thorley-Lawson, 2005). HL and undifferentiated NPC cell lines are associated with type II latency (Busson et al., 1992; Deacon et al., 1993; Gilligan et al., 1990b; Gilligan et al., 1991; Grässer et al., 1994; Murray et al., 1992; Niedobitek et al., 1997; Pallesen et al., 1991; Pathmanathan et al., 1995; Raab-Traub et al., 1983; Tsai et al., 1996).

Type III latency, also known as the "growth" program, expresses all nine of the viral latency proteins including EBNA1, EBNA2, LMP1, LMP2A, LMP2B, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP, as well as EBERs and viral microRNAs. The essential latent proteins required for transformation of B cells *in vitro* include EBNA2, EBNA3A, EBNA3C, EBNA-LP and LMP1 (Cohen et al., 1989; Humme et al., 2003; Hung et al., 2001; Kaye et al., 1993; Mannick et al., 1991; Robertson et al., 1995; Tomkinson et al., 1993). EBNA2 and EBNA-LP are the first viral proteins expressed following primary infection of B cells (Alfieri et al., 1991; Hurley and Thorley-Lawson, 1988; Rooney et al., 1989; Speck, 2005), and are initially driven from the essential viral latency promoter, Wp (Tierney et al., 2007; Tierney et al., 2000). EBNA2 is a transcription factor that does not bind directly to DNA, but instead interacts with a cellular DNA binding protein known as RBP-Jκ (Ling et al., 1994). EBNA2's interaction with RBP-Jκ activates the major EBV latent promoter, Cp, which leads to the expression of three closely related EBNA3 proteins. During type I latency, Cp mediated transcription is aborted by downstream DNA methylation which extends through the Wp (Paulson and Speck, 1999; Tierney et al., 2000). The hault of EBNA2 expression is what allows the cells to assume a germinal center phenotype and express the default program or type II latency.

During type III latency, EBNA-LP also plays an important role as a co-activator of EBNA2 and helps augment EBNA2 activation of the LMP1 promoter (Harada and Kieff, 1997; Wang et al., 1990). This is turn activates EBNA1, EBNA3A, -3B, and -3C, LMP2, and cellular genes such as *c-myc* (Cooper et al., 2003; Kempkes et al., 1995). Also, EBNA1 and EBNA3C partially regulate the LMP1 promoter during type III latency (Gahn and Sugden, 1995; Waltzer et al., 1996a). EBNA3 proteins play an important role in repressing excessive EBNA2-mediated transcriptional activation, as all EBNA3 proteins and EBNA2 compete for binding to the same region of RBP-Jκ (Waltzer et al., 1996b). EBNA3s are also known to interact with histone deacetylases (HDACs) and the C-terminal binding protein (CtBP), and can act as transcriptional repressors when bound directly to DNA (Bain et al., 1996; Hickabottom et al., 2002; Knight et al., 2003; Touitou et al., 2001). LCLs and EBV-induced post-transplant lymphoproliferative disease (PTLD) are examples of type III latency (Brink et al., 1997; Cohen et al., 2008; Pajic et al., 2001; Rea et al., 1994). BL cells are also able to convert to type III latency when grown in cell culture (Rooney et al., 1986; Rowe et al., 1987).

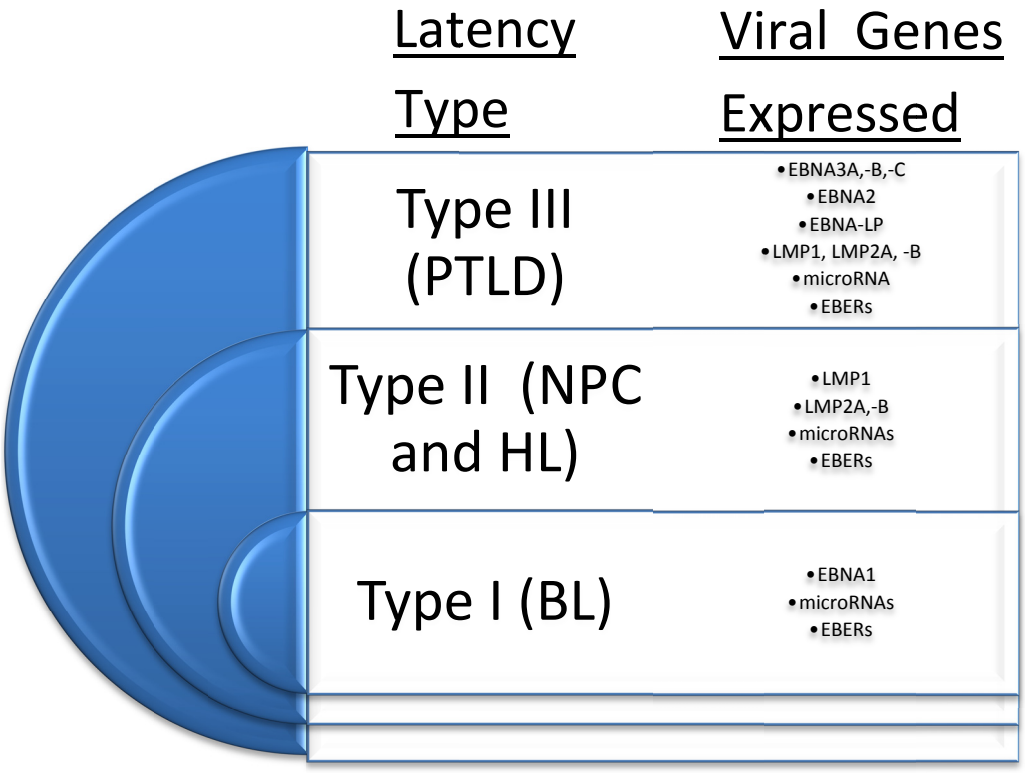

Figure I-1. Latency types I, II, and III and expression of viral proteins. The most restrictive type of latency, type I latency (commonly associated with Burkitt's lymphoma or BL) expresses only one viral protein, EBNA1, as well as EBERs and viral microRNAs. Type II latency (commonly associated with Nasopharyngeal Carcinoma or NPC, and Hodgkin's Lymphoma or HL) expresses LMP1, LMP2A and -2B in addition to EBERs and viral microRNAs. Type III Latency (commonly associated with PTLD) expresses all nine latent viral proteins (EBNA1, EBNA2, EBNA-LP, EBNA3A, -3B, -3C, LMP1, LMP2A, and -2B) as well as EBERs and viral microRNAs.

In humans, EBV establishes latency following infection of naïve B cells in the oropharynx (Thorley-Lawson and Mann, 1985). EBV can also infect memory B cells; however, naïve B cells are believed to be the primary target for infection (Thorley-Lawson, 2005). When EBV encounters and infects resting B cells, it drives that cell to become a proliferating lymphoblast (Thorley-Lawson and Mann, 1985). EBV infected, proliferating lymphoblasts resemble normal B cells that have been activated to become B blasts by antigen in a morphological (Nilsson, 1979) and phenotypical (Thorley-Lawson and Mann, 1985) manner. However, EBV-infected B cells become activated not through interaction with antigen and T cell help, but via expression of the latent proteins expressed in the growth program (type III latency) (Kempkes et al., 1995). Furthermore, the only EBV-infected cell populations within the tonsils of healthy individuals that express the growth program are naïve B cells (Babcock et al., 2000; Joseph et al., 2000).

Three events must occur for an EBV infected naïve B lymphoblast (expressing the growth program) to become a memory-like cell. First, the B lymphoblast must migrate to the follicle. Second, the latent genes which are driving proliferation must be switched off, and lastly, the cells must receive survival signals. When an infected lymphoblast migrates to the germinal center (which is regulated by chemokines), it switches to a different type of restricted latency known as type II latency. An example of a phenotypic change that a newly infected naïve B cell undergoes via the growth program is seen by induction of CCR7 (Birkenbach et al., 1993) via the essential EBV transforming protein, EBNA2 (Burgstahler et al., 1995). In order to turn off the growth program, when EBNA2 is turned off in the presence of *c-myc* (which is expressed in germinal center cells), the blasts down-regulate B blast surface markers such as CD23 and

acquire germinal center specific markers such as CD10 (Polack et al., 1996). It is thought that an infected lymphoblast acquires a germinal center phenotype once the differentiation block by EBNA2 is removed. A direct target of EBNA2 is *c-myc*, which is a regulator of cell growth and apoptosis (Kaiser et al., 1999). The assumption is that once the EBV lymphoblast arrives in the follicle, it receives signaling which turns EBNA2 and the growth program off while still allowing *c-myc* to be expressed. The signal responsible for this switch is still not clear, but may include the Notch signaling pathway (Kohlhof et al., 2009; Thorley-Lawson, 2005).

Once the viral growth program is turned off, EBV needs to provide the necessary survival signals to rescue the latently infected germinal center cell into a memory-like cell. As previously mentioned, EBV proteins expressed in cells with type II latency include EBNA1, LMP1, and LMP2. The importance of LMP1 and LMP2a expression is shown in their capability to mimic the T-cell help and BCR signals normally delivered following B-cell antigen exposure, which rescue the germinal center cell into a memory cell. LMP1 functions as a constitutively active CD40 receptor when expressed on the cellular surface, eliminating the need for CD40 ligand supplied by activated $CD4^+$ T cells (Arcipowski et al., 2011; Cahir-McFarland and Kieff, 2005; Gupta et al., 2011; Thorley-Lawson, 2001; Vrazo et al., 2012; Xie et al., 2004). LMP2A is also found in the plasma membrane and is known to mimic a partially active BCR. LMP2A is able to block excessive cellular BCR signaling and prevent the differentiation of B cells into plasma cells. It does this by sequestering kinases which are phosphorylated and activated following BCR stimulation, and in turn promotes B-cell survival during development (Longnecker, 2000). In addition, partial activation of the BCR also allows for the survival of B cells that have not undergone antigen selection (Longnecker, 2000). Therefore, both LMP1 and LMP2A provide

necessary survival signals for EBV-infected B cells, allowing for the establishment of long-term latency.

Latent Membrane Protein 1 (LMP1)

EBV Latent Infection Membrane Protein 1 (LMP1) is an essential protein required for EBV-driven B-cell immortalization (Wang et al., 1985), and is frequently expressed in latent EBV infections that are associated with B lymphocyte proliferation, as well as in EBV-positive Nasopharyngeal Carcinoma (NPC). LMP1 is uniformly expressed in type III latent EBV infection (i.e. growth program), such as what occurs during primary EBV infection of human B lymphoblasts *in vivo*, in early onset lymphoproliferative disease (LPD) in transplant recipients, or *in vitro* conversion of human B lymphocytes into lymphoblasts capable of long term proliferation (LCLs) (Cahir-McFarland and Kieff, 2005; Wang et al., 1985).

The LMP1 gene lies adjacent to the EBV genome terminal repeat and contains two approximately 80 bp introns, which encode a six transmembrane protein (Cahir-McFarland and Kieff, 2005; Liebowitz et al., 1986; Soni et al., 2007). The six transmembrane and two C-terminal cytoplasmic domains of LMP1 are essential for LMP1 induced signals (Cahir-McFarland and Kieff, 2005; Schneider et al., 2008; Soni et al., 2007; Soni et al., 2006). The six transmembrane domains cause intermolecular interaction and mediate LMP1's localization to cholesterol rich lipid rafts in the membrane, which is required for its constitutive aggregation and ligand-independent signaling (Ardila-Osorio et al., 1999; Cahir-McFarland and Kieff, 2005; Higuchi et al., 2001). The LMP1 cytoplasmic CTAR1 and 2 domains signal through tumor

necrosis factor (TNFR) associated factors or death domain proteins (Eliopoulos et al., 1996; Mosialos et al., 1995), and activate NF-κB, p38 and JNK (Dawson et al., 2003; Lavorgna and Harhaj, 2012; Soni et al., 2007). LMP1 is also significantly associated with the cytoskeleton via its C-terminal cytoplasmic domains (Higuchi et al., 2001; Liebowitz et al., 1987), and this interaction is thought to mediate stabilization of LMP1 and extend LMP1 half-life (Liebowitz et al., 1987). LMP1 manipulates host cellular processes that regulate cell proliferation, migration, and apoptosis (Cahir-McFarland and Kieff, 2005). LMP1 also protects the cell against apoptosis by upregulating expression of anti-apoptotic proteins such as Bcl-2, Mcl-1, and A20 (Gregory et al., 1991; Henderson et al., 1991; Laherty et al., 1992). LMP1 is thus able to contribute to cellular immortalization and tumorigenesis (Kilger et al., 1998; Kulwichit et al., 1998).

EBV Lytic Infection

EBV lytic infection is also an important part of the viral life cycle, since in order for EBV to persist and outlive its existing host, it must be able to transmit its genome to new hosts. This periodic lytic EBV reactivation results in production of virions for transmission between hosts. EBV is often readily detectable in the saliva of an asymptomatic host, which is suggestive of lytic replication occurring frequently throughout the lifetime of the host (Ikuta et al., 2000; Ling et al., 2003; Lucht et al., 1995; Shu et al., 1992; Walling et al., 2003).

Memory B cells primarily act as a reservoir for persistent EBV latency infection, with sporadic lytic reactivation, whereas normal differentiated oropharyngeal epithelial cells support lytic infection *in vivo* (Greenspan et al., 1985; Herrmann et al., 2002; Laichalk et al., 2002;

Walling et al., 2003; Walling et al., 2001). A study by the Thorley-Lawson group suggests that viral replication in humans is initiated upon B-cell differentiation into plasma cells (Laichalk and Thorley-Lawson, 2005). In addition, EBV lytic gene expression in oropharyngeal epithelial cells may depend upon epithelial differentiation (Greenspan et al., 1985; Young et al., 1991).

In a normal healthy host, lytic viral proteins are easy targets for T cells and are quickly eliminated. For this reason, it has been difficult to identify and isolate cells in humans undergoing lytic infection. Cell lines which efficiently support primary EBV lytic infection are currently not available, as most cell lines grown in culture contain the latent form of EBV infection. In spite of this, EBV lytic reactivation studies can be performed utilizing numerous techniques in latently infected cells that disrupt latency. For example, overexpression of the BZLF1 (Z) or BRLF1 (R) viral immediate early proteins can disrupt viral latency (Chevallier-Greco et al., 1986; Countryman et al., 1987; Countryman and Miller, 1985; Ragoczy et al., 1998; Takada et al., 1986; Zalani et al., 1996). Other methods for inducing lytic infection (in B cells) include BCR crosslinking and treatment with chemotherapy agents, HDAC inhibitors, phorbol esters and calcium ionophores (Flemington and Speck, 1990c; Israel and Kenney, 2005; Tovey et al., 1978).

The reactivation of lytic EBV infection from latently infected cells is highly regulated and initiated by activation and transcription of the immediate-early (IE) viral genes, Z and R. In latently infected memory B cells, the Z and R promoters are turned off. Therefore, the initial and most important step for viral reactivation is the activation of the IE promoters by cellular transcription factors. These two IE genes, Z and R, encode for the Z and R transcription factors.

The Z and R proteins are required for activation of the early viral genes in the context of the intact viral genome (Cox et al., 1990; Feederle et al., 2000).

Z and R promoters (Zp and Rp)

The sequence motifs in the Z promoter (Zp) have been categorized into five groups: ZI, ZII, ZIII, and ZIV, and ZV. The majority of the regulatory elements within the Zp span the region from -233 to +12, and are required for its activation by various pathways. There are 4 total ZI motifs which contain A+T-rich motifs that are negative regulators of the Zp in absence of lytic stimuli via binding of the cellular MEF2D transcription factor and its complex formation with HDAC proteins (Bryant and Farrell, 2002). These sites can also function as positive regulators in the presence of inducing stimuli such as phorbol esters, calcium ionophores, and chemotherapy agents, through MEF2D dephosphorylation (Gruffat et al., 2002b). The ZI motifs can also be bound weakly by the transcriptional activators SP1 and SP3 (Liu et al., 1997).

The Zp ZII motif, similar to a CRE site, binds to cellular transcription factors such as CREB, ATF-1, ATF-2/c-jun heterodimer, and AP1, and is required for most stimuli to successfully induce Z transcription. (Flamand and Menezes, 1996; Flemington and Speck, 1990c; Liu et al., 1998; Wang et al., 1997). Although these transcription factors are normally expressed in many cell types, they commonly require phosphorylation for activation. Numerous lytic-inducing stimuli such as BCR engagement, R expression, and chemotherapy agents can activate various stress kinases such as MAP kinases, p38, and c-jun N-terminal kinase (JNK). These kinases phosphorylate and upregulate ATF-2 and c-jun. The inhibition of JNK or p38

function by various agents also broadly inhibits the Zp through a variety of different stimuli (Adamson et al., 2000; Feng et al., 2004; Feng et al., 2002). Both the ZI and ZII motifs are required for BCR crosslinking as well as chemical inducers for the induction of Z transcription. In addition, Z is able to transactivate its own promoter via direct binding to two ZIII motifs (ZIIIA and ZIIIB), which are Z-responsive elements (ZREs), although it is still controversial whether Z can activate its own promoter in the context of the intact latent viral genome (Binné et al., 2002; Flemington et al., 1991; Le Roux et al., 1996; Yin et al., 2004; Zalani et al., 1996). The ZII and ZIIIB sites in the Zp have also been shown to be bound by cellular transcription factor, CAAT enhancer binding protein alpha (C/EBP-alpha), and synergistically activate the Zp (Wu et al., 2004). *In vitro* studies have also shown activation of the Z promoter in response to epithelial cell differentiation (Karimi et al., 1995; Li et al., 1992).

On the other hand, there have also been several other elements that negatively regulate the Zp. One example of this is ZEB1 and ZEB2, which are zinc-finger proteins that bind to the ZV motif (Kraus et al., 2001; Kraus et al., 2003). Deletion of the ZV site results in a dramatic enhancement of Zp activity after BCR cross-linking (Binné et al., 2002). Other elements which negatively regulate the Zp include YYI binding to numerous ZIV motifs, and a protein complex terms ZIIR which binds to overlapping ZII and ZID sites (Ellis et al., 2010; Kraus et al., 2003; Liu et al., 1998; Montalvo et al., 1995; Montalvo et al., 1991; Yu et al., 2007). A diagram of the Zp and *cis*-acting elements is depicted in **Figure I-2.**

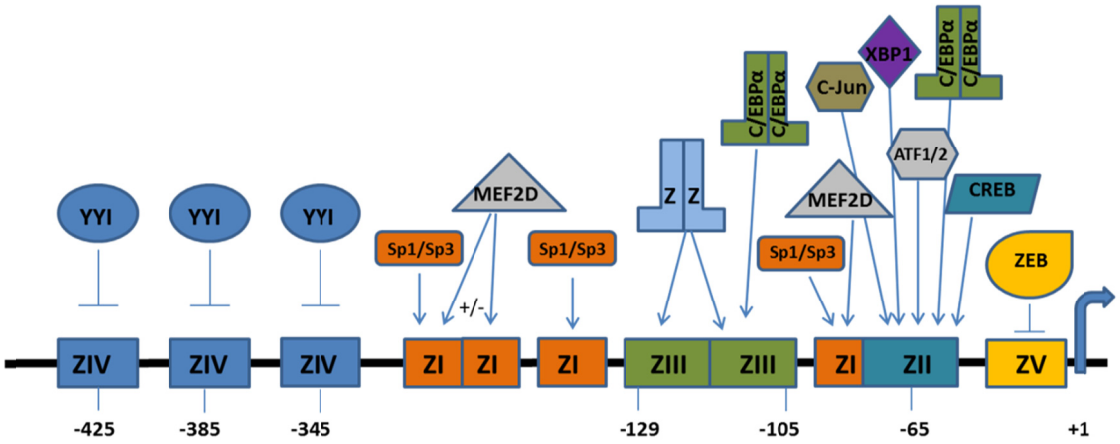

Figure I-2. Known transcription factor and *cis*-acting elements in the immediate-early Z promoter. The elements ZI, II, III, IV, and ZV are depicted and relative positions to the transcriptional start site are shown. Corresponding transcription factors which either activate (arrows) or inhibit Zp activity (block arrows) are depicted above the Z elements. The auto-activation of the Z promoter happens through Z binding sites (ZREs) that lie within the ZIII elements. Transactivation of the Z promoter by R occurs through indirect mechanisms via the ZII motif *(Figure adapted from (Israel and Kenney, 2005)).*

Our lab has shown that the cellular ataxia telangiectasia-mutated (ATM) protein (a cellular serine/threonine kinase) enhances Z promoter activity in the context of the intact EBV genome, and ATM kinase activity is required for activation of early lytic EBV proteins by many different types of stimuli (Hagemeier et al., 2012). Furthermore, p53 also contributes to the ATM effect (Hagemeier et al., 2012). Additionally, our lab has shown that a cellular transcription factor known as X-box-binding protein 1 (XBP-1), activates the both the Z and R promoters and plays an essential role in lytic viral reactivation during plasma cell differentiation. XBP-1 alone is able to activate the R promoter, whereas the combination of XBP-1 and protein kinase D (PKD) is able to activate the Z promoter (Bhende et al., 2007). The expression of XBP-1 and activated PKD was also shown to be sufficient to induce lytic gene expression in latently infected epithelial cells (Bhende et al., 2007).

Although the Rp is less well-characterized than the Zp, it contains sequence motifs that are similar to the Zp, including SP1 and SP3 binding sites. These SP1 binding sites are required for R autoregulation of its own promoter (Chang et al., 2005; Ragoczy and Miller, 2001). Since the Rp lacks an R binding site, R cannot bind to directly to its promoter to activate transcription. The mechanism involved in the autoregulation of the Rp has been shown to involve R, MCAF1, and Sp1, which form a complex and bind to Sp1 sites to activate Sp1-mediated transcription (Chang et al., 2010; Chang et al., 2005; Liu and Speck, 2003; Ragoczy and Miller, 2001). Additionally, two YYI binding sites within the Rp also function to inhibit transcription (Zalani et al., 1997). The Rp is known to be activated by EGR-1 (a transcription factor that is upregulated by BCR crosslinking), phorbol esters and chemotherapy via two EGR-1 DNA-binding sites (Feng et al., 2004; Krappmann et al., 2001; Zalani et al., 1995). The Rp also contains Z-

responsive elements (ZREs), that are responsible for Z activation of the promoter (Farrell et al., 1989).

Z and R proteins

Translation of the Z and R genes produces the proteins Z and R, respectively, which are transcription factors. Z and R transactivate each other's promoters as well as their own promoters (Adamson and Kenney, 1998; Feederle et al., 2000; Liu and Speck, 2003; Zalani et al., 1996). Expressing Z at high levels is sufficient to induce the lytic form of infection in most infected cell lines. On the other hand, some but not all latently infected cell lines reactivate in response to R overexpression (Chevallier-Greco et al., 1986; Countryman and Miller, 1985; Ragoczy et al., 1998; Rooney et al., 1989; Takada et al., 1986; Zalani et al., 1996).

The Z protein (also called Zta, ZEBRA, or EB1) is a member of the basic leucine zipper (bZIP) family of DNA binding proteins. Other leucine-zipper transactivators include the cellular transcription factors c-jun and c-fos (Farrell et al., 1989; Flemington et al., 1994; Kouzarides et al., 1991). All bZIP proteins share three regions including a basic DNA binding region, an N-terminal transactivation domain, and a C-terminal leucine zipper (Vinson et al., 2002). Z is only active and functional as a dimer, and homodimerizes via a bZIP domain at the carboxy-terminus end of the protein. Z is able to bind DNA through its basic DNA binding domain to AP-1 like sites, known as Z-responsive elements (ZREs) (Chang et al., 1990; Farrell et al., 1989; Flemington and Speck, 1990a; Lieberman and Berk, 1990, 1991). The domains of Z are illustrated in **Figure I-3**. The structure of Z is shown in **Figure I-4**.

The amino-terminal end of Z contains the transactivator domain, which is responsible for mediating Z regulation of promoter activity. When Z binds to AP-1-like sites (ZREs), the TFIIA-TFIID transcription-initiation complex is stabilized (Chi et al., 1995). Z has also been shown to recruit the CREB-binding protein (CBP) to the promoter (Adamson and Kenney, 1999; Chen et al., 2001), which upregulates transcription via its acetylation of histones and induction of chromatin remodeling (Manteuffel-Cymborowska, 1999; Zerby et al., 1999).

Interestingly, Z is the first transcription factor shown to preferentially activate certain promoters in their methylated form (Bhende et al., 2004). In addition, demethylation of viral promoters inhibits the ability of Z to activate lytic genes such as R and BRRF1 (Na) (Bhende et al., 2004; Dickerson et al., 2009; Wille et al., 2013). Upon initial viral infection in B cells, the genome is highly unmethylated (Type III latency), and Z expression does not induce lytic replication. However, the viral genome eventually becomes highly methylated by host proteins and this is a needed step for the latent-to-lytic cycle in B cells (Kalla et al., 2010). Therefore, EBV has evolved in a way to utilize host-cell methylation to its advantage, by first being able to establish latency and also being able to reactivate lytic replication efficiently from a highly methylated genome.

In addition to Z's effects on viral gene activation, Z can activate transcription of certain cellular genes, including TGF-β, IL-10, MMP1 and 9, tyrosine kinase TKT, and c-fos (Cayrol and Flemington, 1995; Flemington and Speck, 1990b; Lu et al., 2000; Lu et al., 2003; Mahot et al., 2003; Yoshizaki et al., 1999). One role of these Z-activated genes may be to suppress host immunity (TGF-β and IL-10) or contribute to oncogenesis (MMP1 and 9). Other functions of Z include dispersing promyelocytic leukemia (PML) bodies, inhibiting cellular apoptosis and

regulating p53 function, and halting cellular DNA replication (Adamson and Kenney, 2001; Kudoh et al., 2005; Mauser et al., 2002a; Mauser et al., 2002b; Zhang et al., 1994). Z is also able to inhibit expression of MHC class I on cells and decrease C/EBP-mediated activation of TNF-α-R1p (Bristol et al., 2010; Keating et al., 2002; Mahot et al., 2003; Morrison et al., 2004).

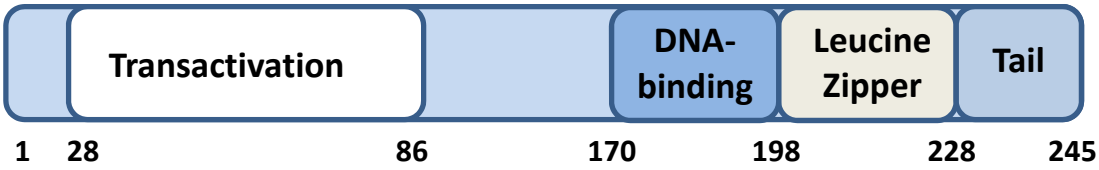

Figure I-3. Z protein schematic. Z is a 245 amino acid protein which consists of an N-terminal transactivation domain (amino acids 28-86), a C-terminal DNA-binding domain (amino acids 170-198), leucine zipper/dimerization region (amino acids 198-228), and tail (228-245). Corresponding amino acid positions are shown below the protein.

Figure I-4. Structure of Z protein/leucine zipper binding to DNA. The interaction between the C-terminal tail and the coiled coil in the structure of the Z-DNA complex. Z monomers are in green and yellow, double-stranded DNA is shown in gray. The two monomers of Z line up and grab onto DNA with their leucine zipper domains as depicted *(Figure adapted from (McDonald et al., 2009)).*

The EBV R protein is a 605 amino acid protein which contains an N-terminal dimerization/DNA-binding domain (amino acids 1-300) and a C-terminal transactivation domain (amino acids 350-605) (Gruffat et al., 1990; Manet et al., 1991). R is known to induce lytic reactivation in a subset of latently-infected cell lines. A closely related gamma herpesvirus known as KSHV, encodes a homolog to R known as ORF50 or Rta, and is a KSHV IE protein which mediates viral reactivation (Harrison and Whitehouse, 2008; Lukac et al., 1998; Sun et al., 1998; Xu et al., 2005; Zhu et al., 2004). The transactivation of a subset of lytic EBV promoters such as SM, EAD, and BHRF1 are mediated by R-binding directly to a GC-rich sequence known as an R-responsive element or RRE (Chen et al., 2005; Gruffat et al., 1992; Gruffat et al., 1990; Gruffat and Sergeant, 1994; Quinlivan et al., 1993). When R directly binds to RREs, it often leads to strong promoter activation since R is able to function as an enhancer factor (Cox et al., 1990; Gruffat et al., 1990; Kenney et al., 1989). As mentioned previously, R uses indirect mechanisms to activate both the Z and R promoters.

Our lab has recently shown that although R is impaired in its ability to activate methylated viral lytic promoters, lytic viral gene expression and replication can be induced by R, but not Z, expression in an EBV-positive telomerase-immortalized epithelial cell line (NOKs-Akata) where the viral genome remains largely unmethylated (Wille et al., 2013). This suggests that the unmethylated form of the EBV genome can undergo viral reactivation and replication, but is dependent on R. Therefore, R is important for viral reactivation in certain EBV-positive epithelial cells where the EBV genome is largely unmethylated, in which Z alone is not sufficient to induce viral reactivation.

Role of Z in Viral Replication

During EBV reactivation, the viral IE genes (Z and R) are first transcribed, followed by activation of the early genes and late genes. The IE, early, and late viral genes have been defined by either inhibiting protein synthesis or viral replication. The transcription of IE genes occurs even in the presence of protein synthesis inhibitors (Biggin et al., 1987; Flemington et al., 1991; Takada and Ono, 1989). For example, one recent report showed that Z is expressed in the presence of a protein synthesis inhibitor known as anisomycin (Wen et al., 2007). The transcription of early genes occurs prior to lytic replication but does not occur in the presence of protein synthesis inhibitors.

The expression of both Z and R leads to the activation of viral early genes. Z initiates viral replication via its binding to four ZRE sites within the lytic origin of replication (oriLyt) (Schepers et al., 1996), and interacting with various components of the replication machinery. The replication machinery interacts with both the leucine zipper and transactivation domains of Z. The transactivation domain of Z associates with the helicase (BBLF4), the DNA polymerase (BALF5), and the primase subcomplex (BSLF1-BBLF2/3), whereas the leucine zipper region interacts with the viral DNA polymerase processivity factor (BMRF1 or EAD) (Gao et al., 1998; Liao et al., 2001; Zhang et al., 1996). In addition to binding to oriLyt, Z also turns on expression of early viral genes required for replication such as the DNA polymerase (BALF5), helicase (BBLF4), primase-associated factor (BBLF2/3), mRNA export factor (BMLF1 or SM), single-stranded DNA binding protein (BALF2), and viral DNA polymerase processivity factor (EAD), (Fixman et al., 1992; Gruffat et al., 2002a; Kieff and Rickinson, 2007b; Verma et al., 2009). EAD is highly expressed in lytically infected cells and is commonly used in immunoblot assays

as a lytic induction marker. The BMLF1 (SM) lytic viral protein plays a role in the lytic cycle by aiding in the export of unspliced viral RNA from the nucleus (Boyle et al., 1999; Hiriart et al., 2003; Ruvolo et al., 1998). Since the viral mRNA is intronless, it would otherwise be trapped in the nucleus without having SM present to stabilize and effectively export it. The lytic viral protein BGLF4 (PK), which is a protein kinase, is also expressed early in infection and plays an important role in the phosphorylation and function of viral proteins (including EAD) as well as cellular proteins (such as Lamin A/C) (Asai et al., 2009; Hagemeier et al., 2010; Meng et al., 2010).

Following the transcription of early genes, and subsequent viral DNA replication, late genes are transcribed, which encode proteins involved in nucleocapsid production, virion maturation, and virion release. Late genes, which require both protein synthesis and viral DNA replication in order to be expressed, include the major and minor capsid components in addition to glycoproteins (gB, gL, gH, gp42, and gp350/220) (Kieff and Rickinson, 2007b). Since virion release can result in the destruction of the host cell (Yuan et al., 2006), EBV must maintain a stable pool of latently infected cells by tightly controlling the lytic process.

The B-cell specific transcription factor, Oct-2, inhibits Z function

Both the latent and lytic forms of EBV infection are essential for the long-term success of EBV. However, specific cellular factors which can have varying outcomes on triggering or preventing the latent-to-lytic switch after infection remain poorly defined. Our lab has shown that one important B-cell specific transcription factor, Oct-2, promotes viral latency in B cells.

Specifically, Oct-2 inhibits the function of the IE protein, Z, and prevents lytic reactivation (Robinson et al., 2012). Additionally, Oct-2 expression has been reported to decrease following B-cell differentiation into plasma cells (Buettner et al., 2012), or when B cells are treated with several different lytic inducing agents (Robinson et al., 2012). This led us to investigate whether there are additional B-cell specific transcription factors that play a role in preventing viral reactivation and promoting viral latency, as discussed later in this Chapter.

The role of the B-cell specific transcription factor, Pax5, in the EBV life cycle

EBV transformation of B cells requires activation of the viral latent promoter, Wp, which is present as tandemly repeated copies in the viral genome. Wp drives expression of the essential viral transforming proteins, EBNA2 and EBNA-LP, in the initial stages of B-cell infection (Tierney et al., 2007; Tierney et al., 2000). The Wp contains two promoter-activating regions, known as UAS1 (B-cell specific) and UAS2 (lineage independent). The UAS1 region contains two binding sites for Pax5, and Pax5 is bound to Wp sequences on the EBV genome in transformed cells (Tierney et al., 2007). Furthermore, when the Pax5 sites are mutated within the UAS1 region, EBV can deliver its genome to the B-cell nucleus, but there is no detectable Wp transcription, expression of latent viral proteins, or outgrowth of transformed cells (Tierney et al., 2007). Therefore, Pax5 is intricately tied to the EBV life cycle as it is required for EBV transformation of B cells and the establishment of long-term latent infection in the memory B-cell compartment.

Pax5 also interacts with Z

There is also evidence that Pax5 interacts directly with the main lytic switch protein, Z (Adamson et al., 2005). Z has been shown to physically associate with Pax5 *in vitro* and *in vivo*, and to inhibit Pax5-mediated transcription (but not Pax5 DNA-binding). As described in this thesis, I decided to determine if Pax5 expression in B cells helps to promote EBV latency.

Loss of Pax5 expression and activation of Blimp-1 expression correlates with EBV reactivation in B cells

A mature B cell and a plasma cell differ dramatically in their transcriptional programs (Delogu et al., 2006; Shaffer et al., 2002b). During B cell development from a pro-B cell to a mature B cell, Pax5 is constitutively expressed and controls the gene expression program of B cells (Delogu et al., 2006; Horcher et al., 2001). However, once a B cell terminally differentiates into a plasma cell (which correlates with EBV lytic reactivation), Pax5 is turned off precisely at this point.

Blimp-1 is a transcription factor that orchestrates the transcription program of terminally differentiated plasma cells (Shaffer et al., 2002a). Two separate transcriptional programs controlled by Blimp-1 and Pax5 are likely maintained by mutually antagonistic interactions between Pax5 and Blimp-1. Previous studies have shown that Blimp-1 binds to and represses the Pax5 gene and promoter in plasma cells (Lin et al., 2002), whereas Pax5 keeps Blimp-1 in check in mature B cells since the gene encoding Blimp-1 (Prdm1) is only expressed following conditional Pax5 deletion (Delogu et al., 2006). Conditional deletion of Pax5 in mature B cells

not only results in an increase in IgM secretion and the expression of Blimp-1 and XBP-1 (Calame et al., 2003), but also expression of the J chain, which is a known Pax5-repressed gene required for efficient antibody secretion (Nera et al., 2006; Reimold et al., 1996; Rinkenberger et al., 1996). Furthermore, Blimp-1 expression in plasma cells not only represses Pax5 expression, but also activates the Zp (Buettner et al., 2012). Therefore, Blimp-1 (via upregulation of the Zp and possible downregulation of Pax5) may play an important role in driving plasma cell differentiation and helping to promote EBV lytic reactivation. A diagram of Pax5 expression during B-cell development and repression by Blimp-1 upon plasma cell differentiation is shown in **Figure I-5**.

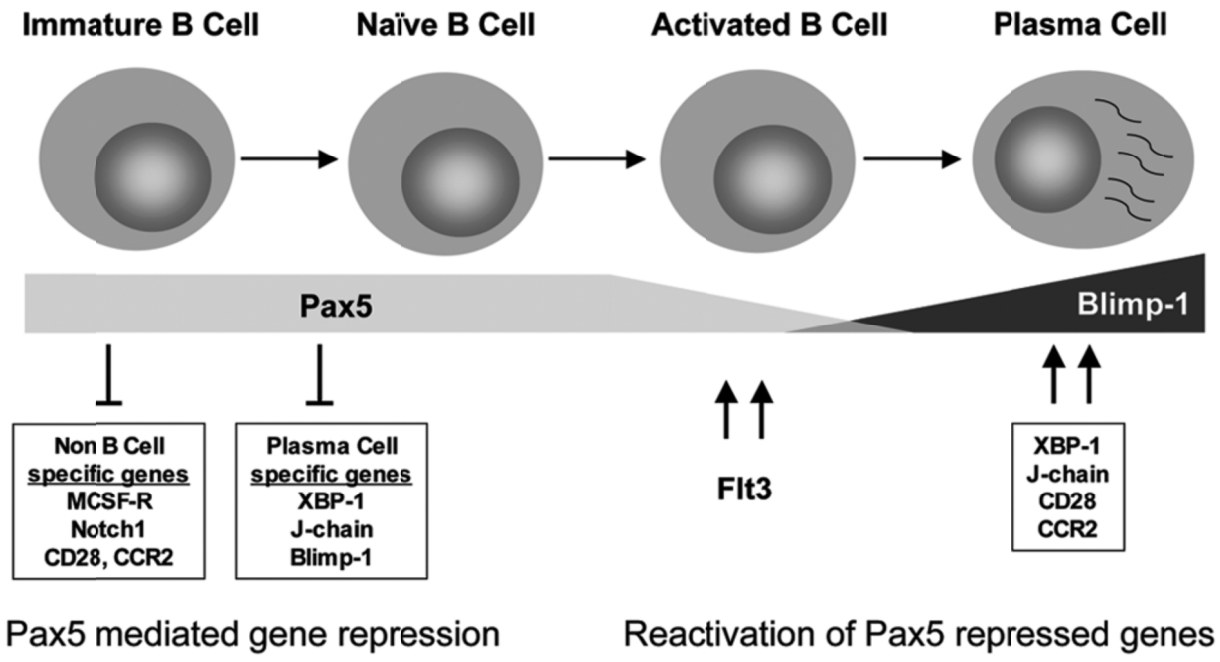

Figure I-5. Pax5 and Blimp-1 functions in late B-cell differentiation. The stages of cellular differentiation from a B-cell to an antibody-secreting plasma cell are indicated along with the levels of Pax5 (in light gray) and Blimp-1 (in black) shown below the cells. Pax5 represses non-B cells and plasma cell genes in both immature and naïve B cells (as indicated in the tables below ⊥). Upon activation of a memory B-cell with antigen stimulation, Pax5 expression and function is inhibited as Pax5-repressed genes are induced (Blimp-1, Flt3). These Pax5-repressed genes (during normal B-cell development) then become important for plasma cell functionality (*Figure adapted from (Carotta et al., 2006)).*

The interaction of antigen with the membrane-bound antibody of the B-cell receptor (BCR) is crucial in determining which clones enter the plasma cell response. One genome-wide analysis study showed that Ab-secreting cell differentiation of mouse B cells is induced by BCR activation via very rapid regulatory events emanating from the BCR (Hauser et al., 2009). In particular, IFN regulatory factor-4 activation and Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B downregulation are the main immediate early events (Hauser et al., 2009). Interestingly, Pax5 mRNA levels in B cells decrease within 30 minutes of BCR stimulation, and this decrease is mediated by loss of expression of the transcription factor, E2A, which is a known activator of the Pax5 promoter (Hauser et al., 2009). Plasma cell differentiation is regulated by calmodulin inhibition of E2A, which is required for rapid downregulation after BCR activation (Hauser et al., 2009). Therefore, there is a clear correlation between the loss of Pax5 and BCR stimulation via loss of E2A expression.

Since Pax5 downregulation is linked to both B-cell receptor (BCR) antigen stimulation (Hauser et al., 2009) and plasma cell differentiation events (Lin et al., 2002), it is an interesting candidate to negatively regulate EBV lytic reactivation.

Pax5 plays multiple roles in maintaining EBV latency

There is a delicate balance between latent and lytic infection in cells, and this balance is largely controlled by cellular transcription factors. In this thesis, I demonstrate that the B-cell specific transcription factor, Pax5, regulates the latent-to-lytic switch of EBV. Pax5 is a member of the Pax gene family consisting of nine members (Pax1-9) (Bopp et al., 1986; Hill and Hanson,

1992; Hoshiyama et al., 2007; Miller et al., 2000; Noll, 1993; Underhill, 2000; Volcik et al., 2002; Zarinkamar et al., 2011). Pax genes have been shown to play an important role in human disease including cancer and genetic disorders (Robson et al., 2006). Pax5 (also known as the B-cell-specific activator protein or BSAP) is the only Pax protein that is expressed within the hematopoietic system. The conserved 'paired'-box DNA-binding domain of Pax5 consists of a bipartite DNA-binding region with N- and C-terminal subdomains (Cobaleda et al., 2007). Each subregion contains a homeodomain-like helix-turn-helix motif which binds to a distinct half-site of the Pax5 recognition sequence in the adjacent major grooves of DNA (Garvie et al., 2001). A schematic of Pax5 protein is shown in **Figure I-6,** and the crystal structure of the DNA-binding domain is shown in **Figure I-7.**

Pax5 binding to promoters can result in either the upregulation or downregulation of transcriptional activity (Revilla et al., 2012). For transcriptional activation, Pax5 utilizes both the partial homeodomain and transactivation domain. The partial homeodomain of Pax5 associates with the TATA-binding protein of the basal transcriptional machinery (Eberhard and Busslinger, 1999), and the potent C-terminal transactivation domain (Dorfler and Busslinger, 1996) regulates gene transcription through interaction with histone acetyltransferases (HAT) including the coactivator (CBP) or SAGA complex (Barlev et al., 2003). Pax5 can also be converted from a transcriptional activator to a repressor through its interaction with corepressors of the Groucho protein family (Grg4), which are part of a larger histone deacetylase (HDAC) complex (Eberhard et al., 2000).

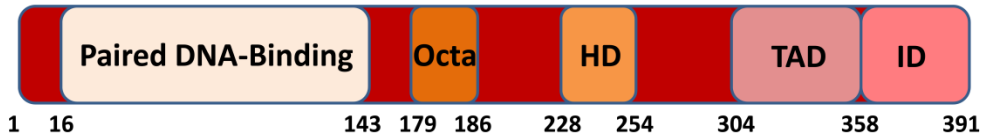

Figure I-6. Schematic of the Pax5 protein. The Pax5 protein is a 391 amino acid protein containing a Paired Box DNA-Binding domain (amino acids 16-143), an Octapeptide motif (Octa) (amino acids 176-186), Homeodomain (HD) (amino acids 228-254), Transactivation domain (TAD) (amino acids 304-358), and Inhibitory domain (ID) (amino acids 358-391).

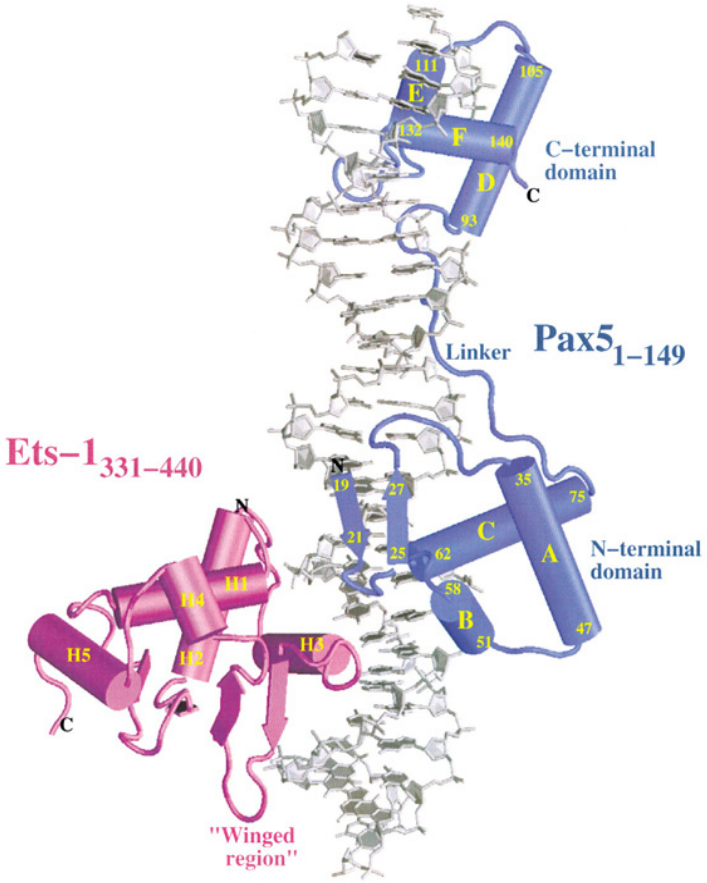

Figure I-7. Structure of the paired domain of Pax5 and the Ets domain of Ets-1 bound to DNA. Crystal structure overview of the paired domain of Pax5 (shown in blue) and Ets domain (shown in pink) of Ets-1 bound to DNA (shown in gray). Pax5 has both a C-terminal and N-terminal helix-turn-helix domain. The helices of Pax5 are labeled A to F. Residues that define the α helices and β strands of Pax5 are shown on the associated secondary elements. (*Figure adapted from (Garvie et al., 2001)*.

In **Chapter II**, I describe how Pax5 promotes EBV latency through its interaction with the viral IE protein, Z. I show that Pax5 negatively regulates Z function by inhibiting Z DNA-binding activity and transcriptional function, and demonstrate that this effect requires a protein-protein interaction between Z and Pax5. In **Chapter III**, I describe another mechanism by which Pax5 helps to promote EBV latency by positively regulating LMP1 expression during type III latency.

Together, my data show that Pax5 helps to promote EBV latency through multiple mechanisms. Pax5 not only helps establish latency during primary infection through activation of Wp-driven EBNA2 expression, but may also positively regulate LMP1 expression. In addition, Pax5 prevents Z's ability to reactivate latently infected cells and undergo lytic replication.

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Chapter II

The B-Cell Specific Transcription Factor and Master Regulator, Pax5, Promotes EBV Latency by Negatively Regulating the Viral Immediate Early Protein, BZLF1

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ABSTRACT

The latent-to-lytic switch of Epstein-Barr virus (EBV) is mediated by the immediate-early protein, BZLF1 (Z). However, the cellular factors regulating this process remain incompletely characterized. In this report, we show that a B-cell specific transcription factor, Pax5, helps to promote viral latency in B cells by blocking Z function. Although Z was previously shown to directly interact with Pax5 and inhibit its activity, the effect of Pax5 on Z function has not been investigated. Here, we demonstrate that Pax5 inhibits Z-mediated lytic viral gene expression, and release of infectious viral particles, in latently infected epithelial cell lines. Conversely, we find that shRNA-mediated knockdown of endogenous Pax5 in a Burkitt lymphoma B-cell line leads to viral reactivation. Furthermore, we show that Pax5 reduces Z activation of early lytic viral promoters in reporter gene assays, and inhibits Z binding to lytic viral promoters *in vivo*. We confirm that Pax5 and Z directly interact, and show that this interaction requires the carboxy-terminal DNA-binding/dimerization domain of Z and the amino-terminal DNA-binding domain of Pax5. A Pax5 DNA-binding mutant (V26G/P80R) that interacts with Z retains the ability to inhibit Z function, while a Pax5 mutant (Δ106-110) that is deficient for interaction with Z does not inhibit Z-mediated lytic viral reactivation. As the B-cell specific transcription factor, Oct-2, also directly interacts with Z and inhibits its function, these results suggest that EBV uses multiple redundant mechanisms to establish, and maintain, viral latency in B cells.

INTRODUCTION

Epstein-Barr virus (EBV) is an orally transmitted gamma-1 herpesvirus that is widespread in the human population (Kieff, 2007). Primary infection in humans is often asymptomatic, but can cause infectious mononucleosis if acquired during adolescence or adulthood. EBV is also associated with numerous malignancies including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, T/NK cell lymphomas, and lymphomas in immunocompromised patients (Kieff, 2007; zur Hausen et al., 1970). EBV infection persists in the host for life by establishing latency in memory B cells (Souza et al., 2005). In contrast, primary EBV infection of normal oropharyngeal epithelial cells commonly results in the lytic form of infection. EBV also periodically reactivates in B cells, particularly when B cells are stimulated by antigen and differentiate into plasma cells (Kieff, 2007; Laichalk and Thorley-Lawson, 2005).

The lytic reactivation process of EBV in host cells is controlled at the level of the BZLF1 and BRLF1 viral immediate-early promoters and their associated gene products, BZLF1 (Z) and BRLF1 (R) (Bhende et al., 2007; Feederle et al., 2000; Kieff, 2007; Vrzalikova et al., 2011; Yu et al., 2007). Z and R encode transcription factors which cooperatively activate each other's promoters as well as early lytic gene promoters involved in lytic viral replication (Adamson and Kenney, 1998; Chang et al., 2010; Countryman et al., 1987; Cox et al., 1990; Darr et al., 2001; Farrell et al., 1989; Feederle et al., 2000; Flemington and Speck, 1990; Gruffat et al., 1990; Hardwick et al., 1988; Kenney et al., 1989; Lieberman et al., 1990; Liu and Speck, 2003; Quinlivan et al., 1993; Rooney et al., 1989; Takada et al., 1986; Zalani et al., 1996). Z is a bZIP protein that binds to the consensus AP-1 motif as well as AP-1 like sites known as BZLF1-

responsive elements (ZREs). Interestingly, many of the ZREs in the EBV genome contain CpG motifs, and Z binds to, and activates, the methylated forms of most viral promoters much more efficiently than the unmethylated forms (Bergbauer et al., 2010; Bhende et al., 2004, 2005; Dickerson et al., 2009; Fernandez et al., 2009; Flower et al., 2011; Kalla et al., 2010; Wille et al., 2013). In contrast, we recently showed that R preferentially activates unmethylated lytic viral promoters (Wille et al., 2013). Since the EBV genome normally becomes highly methylated in latently infected B cells, the ability of Z to bind to and activate methylated viral promoters likely plays a key role in allowing it to overcome the normal inhibitory effects of promoter methylation on gene transcription (Boyes and Bird, 1991, 1992; Razin, 1998; Watt and Molloy, 1988).

Pax5 is a master regulator of B-cell fate that is required for B-cell identity (Cobaleda et al., 2007; Hagman and Lukin, 2005, 2006; Medvedovic et al., 2011; Zhang et al., 2006). Pax5 activates expression of many different B-cell lineage-specific genes that are necessary for B-cell development, and prevents expression of B-cell lineage 'inappropriate' genes (Carotta et al., 2006; Cobaleda et al., 2007; He et al., 2011; Pridans et al., 2008; Revilla et al., 2012; Schebesta et al., 2007; Schebesta et al., 2002; Xie et al., 2004). Pax5 also plays an essential role in allowing EBV to establish long-term latent infection in B cells, since it binds to and activates an essential EBV latency promoter (known as the W promoter or Wp) that drives the initial expression of the viral EBNA2 and EBNA-LP proteins during primary B-cell infection (Tierney et al., 2007; Tierney et al., 2000). In addition, Pax5 binds directly to the terminal repeat region of the EBV genome and may thereby regulate expression of nearby promoters that drive expression of the EBV latency proteins, LMP1 and LMP2A (Arvey et al., 2012).

Z has been shown to directly interact with Pax5 and inhibit its ability to activate promoters (Adamson, 2005; Adamson et al., 2005). However, whether Pax5 affects Z function, and whether any such effect requires a direct protein-protein interaction between Z and Pax5, is unknown. Of note, a recent study reported that depletion of Pax5 by shRNA in lymphoblastoid cell lines (LCLs) increased Z protein expression and viral genome copy number (Arvey et al., 2012), although the mechanism for this Pax5 effect was not investigated. Interestingly, the transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1), a critical inducer of plasma cell differentiation, binds to and represses the Pax5 promoter during plasma cell differentiation (Cobaleda et al., 2007; Lin et al., 2002; Shaffer et al., 2002a). Pax5 expression is also decreased following antigen stimulation of B cells (Hauser et al., 2009). Since EBV lytic reactivation can be induced by both B-cell receptor antigen stimulation (Shimizu and Takada, 1993; Takada, 1984) and plasma cell differentiation (Laichalk and Thorley-Lawson, 2005), we hypothesized that Pax5 may inhibit Z expression and/or Z function.

In this Chapter, we have examined the effect of Pax5 on lytic viral reactivation, and investigated the effect of the protein-protein interaction between Pax5 and Z on the ability of Z to bind to, and activate, lytic viral promoters. We show that Pax5 attenuates Z transcriptional function and DNA binding activity, and inhibits EBV lytic reactivation. These results suggest that the B-cell specific Pax5 protein promotes EBV latency in B cells through multiple different mechanisms, including activating the essential Wp latency promoter during primary infection of B cells, and inhibiting the function of the lytic switch protein, Z, to maintain viral latency.

MATERIALS AND METHODS

Cell lines and culture

HONE-Akata cells (a gift from Lawrence Young, University of Birmingham) and CNE-2-Akata cells (a gift from K. W. Lo at the Chinese University of Hong Kong [received via Diane Hayward]) are latently infected nasopharyngeal carcinoma (NPC) epithelial cell lines that are super-infected with an EBV (Akata strain) containing an inserted G418 resistance gene and GFP gene (Glaser et al., 1989; Lo et al., 2006). Both lines were maintained in RPMI 1640 supplemented with 10% fetal bovine seum (FBS), 1% penicillin / streptomycin (pen/ strep), and G418 (400 ug/mL). EBV-negative HONE-1 cells (a gift from Ron Glaser, The Ohio State University) were maintained in RPMI 1640 supplemented with 10% FBS and 1% pen/ strep. HeLa cells (a cervical cancer line obtained from ATCC) were maintained in DMEM supplemented with 10% FBS and 1% pen/ strep. MutuI cells (a gift from Jeff Sample and Alan Rickinson) are an EBV-positive type I Burkitt lymphoma (BL) cell line and were maintained in RPMI 1640 supplemented with 10% FBS and 1% pen/ strep. Raji cells (ATCC) are EBV-positive type III BL cell lines and were maintained in RPMI 1640 supplemented with 10% FBS and 1% pen/ strep. EBV-positive BJAB cells (a gift from Amy Ellis and Janet Mertz) and EBV-negative BJAB cells (a gift from George Klein (Menezes et al., 1975), via Joe Pagano) are BL lines (the original EBV-negative line, or an EBV-superinfected line) and were maintained in RPMI 1640 supplemented with 10% FBS and 1% pen/ strep. HEK 293 cells (obtained from the ATCC) have been previously described (Graham et al., 1977), and were used for packaging of lentiviral vectors and were maintained in DMEM supplemented with 10% FBS and 1% pen/ strep.

Plasmids, cloning, and site-directed mutagenesis

Plasmid DNA was purified using Qiagen maxi-prep columns according to the manufacturer's protocol. pSG5 was obtained from Stratagene. pSG5-Z was a gift from Diane Hayward, Johns Hopkins University, and contains the Z genomic sequence under the control of the SV40 promoter (Sarisky et al., 1996). pSG5-Pax5.S24 (which contains Pax5 cDNA cloned into pSG5 vector) was a gift from James Hagman, National Jewish Center (Fitzsimmons et al., 1996)**.** pSG5-Oct-2 was cloned as previously described (Robinson et al., 2012). The plasmids pSG5-Pax5 (V26G/P80R) and pSG5-Pax5 Δ106-110 were constructed by using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit and the following primer sequences: Pax5 V26G forward 5'-CAGCTTGGGGGGGGTTTTGTGAATGGACGG-3' and reverse 5'-CCGTC CATTCACAAAACCCCCCCCAAGCTG-3', Pax5 P80R forward 5'-GAGACAGGAAGCATC AAGCGTGGGGTAATTGGAGG-3' and reverse 5'-CCTCCAATTACCCCACGCTTGATGCT TCCTGTCTC-3'. Pax5 Δ106-110 forward 5'-AAATCGCTGAATATAAACGCCAAGCCTGG GAGATCAG-3' and reverse 5'-CTGATCTCCCAGGCTTGGCGTTTATATTCAGCGATTT-3'.

pGEX-KG was a gift from Eric Turner (University of California-San Diego) (Rhee et al., 2001). pGST-Z was constructed by inserting Z amino acids (aa) 1-245 into pGEX-KG as previously described (Robinson et al., 2012). pGST-Z 1-140, 140-180, 180-225 and 140-225 mutants were created as previously described (Robinson et al., 2012). To create pGST-Pax5, Pax5 cDNA was PCR amplified using primers forward 5'-GCCGAATTCATG GACTACAAGG ACGACGATGACAAGGATTTAGAGAAAAATTATCCGAC-3' and reverse 5'-GCCAGATC TTCAGTGACGGTCATAGGCAGTGGC-3' and cloned in-frame into BamHI-EcoRI sites of pGEX-4T (GE Healthcare). The CD19p-luciferase reporter gene construct, pGL3-CD19p-luc,

was a gift from Mikael Sigvardsson (Linkoping University, Sweden) (Gisler et al., 1999). The promoterless reporter gene construct pCpGL-basic was a gift from Micheal Rehli, Universitätsklinikum Regensburg (Klug and Rehli, 2006). Various EBV lytic promoters (BMRF1p, SMp, BRLF1p) were cloned into pCpGL-basic as previously described (Wille et al., 2013).

Glutathione S-transferase pull-down assays

GST expression vectors were propagated in DH5α *E. coli* overnight. Cultures were diluted 1:10, grown for 2 hours and induced with 0.4 mM IPTG for an additional 2 hours. GST proteins were collected by sonication followed by 1 hour incubation at 4°C with glutathione-agarose beads (Sigma-Aldrich). Beads were subject to 3 washes with GST buffer (20 mM HEPES [pH 7.7], 25 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 0.05% NP-40, protease inhibitors) and added to ^{35}S-labeled, *in vitro*-translated protein. *In vitro* translated protein was generated using the TNT T7 Quick Coupled Translation System (Promega) in accordance with the manufacturer's instructions. The reaction mixture was incubated overnight with rocking at 4°C. For one subset of GST pull-down assays, the reaction mixture of GST or GST-Z and *in vitro* translated Pax5 was DNase I treated. 10 U of DNase I was added to the reaction mixture for 15 min. EDTA was added to stop the reaction. The beads were washed 3 times in GST buffer. An equal volume of 2x SDS-sample buffer was added and proteins were eluted by heating at 95°C for 10 minutes. Proteins were subject to gel electrophoresis and ^{35}S-labeled protein was detected by autoradiography.

Transient transfections

HONE-Akata cells were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Each transfection experiment was performed at least three separate times with similar results. Cells for western blot analysis were generally transfected in 12-well dish with Z (10 ng), Pax5 WT (250ng), mutant Pax5 V26G/P80R (350ng), mutant Pax5 Δ106-110 (450ng), Oct-2 (500ng), or control expression vectors (approximately 500 ng total DNA per well).

Reporter gene assays

All pCpGL-basic reporter gene constructs were methylated using M.Sss I (NEB) according to manufacturer's protocol, and methylation was confirmed by HpaII (NEB) digestion, which cleaves the recognition sequence within the CpG motif if DNA is not methylated. HONE-1 cells were transfected with Lipofectamine 2000 Transfection Reagent (Invitrogen) in 12-well dishes with 50 ng of pCpGL-basic promoter constructs, up to 500 ng of SG5 vector control, 10 ng of Z alone, 10 ng of Z with 250 ng of Pax5, and 250 ng of Pax5 alone. Pax5 mutants V26G/P80R and Δ106-110 were also used in these studies. The cells were washed 1x with PBS and harvested in 1x Reporter Lysis Buffer (Promega) 48 hours post-transfection. The lysates were subjected to one freeze-thaw cycle and the relative luciferase units were quantified using a BD Monolight 3010 luminometer (BD Biosciences) and luciferase assay reagent (Promega). The fold change for each condition was calculated from the increase in luciferase activity relative to the activity of the promoter in the presence of the SG5 control vector (set to 1). Each condition

was performed in duplicate. Extracts were also subjected to immunoblot analysis to verify equal protein levels.

Virus Titration Assay

Virus titration assays were performed as previously described (Delecluse et al., 1998). CNE-2 Akata cells were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) in a 12-well dish with 10 ng Z, 250 ng Pax5, or vector controls. 48 hours post-transfection, the cells were washed 1x PBS and fresh RPMI was added to the cells. After 24 hours, the supernatant from the transfected cells was filtered through a 0.8-μm pore size filter. Raji cells ($2x10^5$ cells/infection) were infected with various amounts of supernatant and incubated at 37°C. Phorbol-12-myristate-13-acetate (TPA, 20 ng/ml) and sodium butyrate (3 mM final concentration) were added at 24 hours post-infection. Green fluorescent protein (GFP)-positive Raji cells were counted 48 hours after infection to determine viral titer. Each condition was performed in triplicate.

Western blot analysis

Western blot analysis was performed as previously described (Bhende et al., 2005; Swenson et al., 2001). Cell lysates were harvested in SUMO lysis buffer containing protease inhibitor cocktail (Roche) and quantified by SUMO Protein Assay (BioRad). Equivalent amounts of protein were separated in sodium dodecyl sulfate, 10% polyacrylamide gels and transferred to membranes. Membranes were blocked in PBS containing 5% milk and 0.1% Tween-20 solution and incubated with primary antibody. Immunoblots were probed with the

following antibodies: anti-Z (Santa Cruz, sc-53904; 1:250), anti-BMRF1 (Vector, 1:250), anti-β-actin (Sigma, 1:5000), anti-Pax5 (Santa Cruz, sc-13146; 1:250), and anti-Oct-2 (Santa Cruz, sc-233; 1:500). Secondary antibodies were HRP goat-anti-mouse (Fisher Scientific; 1:5000) and HRP goat-anti-rabbit (Fisher Scientific; 1:10,000).

shRNA Infection and Packaging of Lentivirus

Lentivirus vectors expressing two different shRNAs against a control sequence (Sigma Cat. #SHC002 or Addgene plasmid 1864), or two different shRNAs directed against Pax5 (Open Biosystems Cat. #RHS4533-NM_016734) were used to produce lentivirus as previously described according to the Open Biosystems (Thermo-Scientific) protocol (Robinson et al., 2012). HEK 293 cells were transfected with the lentivirus expression plasmids, and the packaging plasmids psPAX2 (Addgene plasmid 12260, a gift of Didier Trono) and VSVG (Addgene plasmid 12259, a gift of Didier Trono) using Lipofectamine 2000 reagent (Invitrogen). Supernatant was collected 3 days post-transfection and filtered through a 0.8-μm pore size filter. EBV-positive MutuI BL cells were then transduced by incubation with filtered media. After 3 days of incubation, stable cell lines were selected with 0.5 μg/ml puromycin. MutuI cells were selected for 10 days in puromycin prior to immunoblot analysis.

RT-PCR

RNA was harvested from Raji cells (treated with or without TPA/sodium butyrate) after 48 hours using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Isolated RNA was quantitated and DNase treated (RQ1 RNase-Free DNase, Promega). Reverse

transcriptase (RT)-PCR analysis was performed to determine the transcript levels of the endogenous cellular Pax5 and GAPDH genes. PCR primers used to detect Pax5 transcript were 5'-GCGTGTTTGAGAGACAGCACT-3' and 5'-AAGAATACTGAGGGTGGCTGT-3'; and GAPDH transcript 5'-TTAGCACCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCA TG-3'.

Chromatin immunoprecipitation (ChIP) assays

HONE-Akata cells were transfected in 15-cm dishes (200ng Z, 4μg Pax5 WT, 6μg mutant Pax5 V26G/P80R, 8μg mutant Pax5 Δ106-110, or control vector). Cells were cross-linked 48 hours post-transfection in fresh 1% paraformaldehyde for 10 minutes at room temperature. Cross-linking reaction was quenched using 125 mM glycine and the cells were lysed. The lysate was sonicated to yield approximately 500 bp DNA fragments. DNA-protein complexes were immunoprecipitated with the following antibodies: anti-Z (Santa Cruz, sc-53904), anti-Pax5 (Santa Cruz; sc-1975X), (Santa Cruz), and control mouse polyclonal immunoglobulin G (IgG) (Santa Cruz; 2025). Immunoprecipitated DNA-protein complexes were washed with low salt, high salt, lithium chloride and TE wash buffers. Protein-DNA cross-linking was reversed at 65°C overnight. DNA was purified using the Qiagen Gel Extraction Kit. PCR was used to determine the presence and relative amount of specific DNA fragments that were immunoprecipitated. Primers used were the following: BMRF1 promoter 5'-ATGCCCAGAAACCTGAGCAAGTAGCC-3' and 5'-CCTTGGTGGATGTGCGAGCCATAA AG-3'; SM promoter 5'-CGTGACATGGAGAAACTGGGGG-3' and 5'-CCTCTTACATCACT CACTGCACG-3'; CD19 cellular promoter 5'-GGCCTAACCTAAGGTGTGACCAC-3' and 5'-

GTGGCTGCGCAGAGGATGCTG-3'; and β-globin 5′-AGGGCTGGGCATAAAAGTCA-3′ and 5′-GCCTCACCACCAACTTCATC-3′.

EMSA

T4 Polynucleotide Kinase (NEB) and [γ-^{32}P] ATP (Perkin-Elmer) were used to label double-stranded, annealed DNA oligonucleotides for DNA-protein binding studies. The Pax5 binding probe (30,000 cpm), derived from the Pax5 binding site within the CD19 promoter, consisted of a 29-bp sequence containing the Pax5 consensus sequence (underlined) 5'-AGAATGGGGCCTGAGGCGTGACCACCGCC-3'. The protein samples used in electrophoretic mobility shift assays (EMSAs) were *in vitro* translated protein using the TNT T7 Quick Coupled Translation System (Promega). The reaction was allowed to incubate for 20 minutes at room temperature and was loaded onto a 4% polyacrylamide gel in 0.5x Tris-borate-EDTA buffer and electrophoresed at 35 mA. Gels were dried on Whatman paper under a vacuum and exposed to autoradiography film for 4 to 48 hours at -80°C.

Quantitative PCR

ChIP DNA was subject to real-time PCR in order to quantitate the level of Z binding to the SM promoter using SYBR green (Bio-Rad) according to the manufacturer's protocol. Samples were measured with an ABI Prism 7900 real-time PCR system (Applied Biosystems). SM was amplified with primers 5'-CGGTTTGCTCAAACGTGACATGGA-3' and 5'-AATGTCTGCGCCATGATAGAGGGA-3'. Input samples were diluted to 5%, 1%, and 0.2% into H_2O with 100 μg/ml sonicated salmon sperm DNA (Agilent). A standard curve was

calculated from the threshold cycle (C_T) of the input sample dilution series and used to calculate percent input bound in the tested samples. Each condition and input dilution was loaded in triplicate.

RESULTS

Pax5 inhibits Z-mediated lytic reactivation

Z and Pax5 have been reported to interact *in vitro* and *in vivo*, and this interaction has been shown to inhibit Pax5 function (Adamson et al., 2005). However, whether Pax5 affects Z function is unknown. To determine if Pax5 regulates Z transcriptional activity, we transfected a Z expression vector, in the presence or absence of a co-transfected Pax5 expression vector, into two different latently infected EBV-positive epithelial NPC cell lines (HONE-Akata and CNE-2 Akata). Immunoblot analysis was performed 48 hours following transfection to assess the level of transfected Z protein in the presence and absence of co-transfected Pax5, and its ability to induce the expression of an early lytic EBV protein, BMRF1, from the endogenous viral genome.

As shown in **Fig. II-1**, expression of Pax5 in epithelial cells (which do not normally express Pax5) greatly reduced Z-mediated activation of the early lytic EBV protein, BMRF1, in both HONE-Akata cells (**Fig. II-1A**) and CNE-2 Akata cells (Fig. 1B). The level of Pax5 expression in the transfected epithelial cells was found to be similar to that of endogenous Pax5 expression in the EBV-infected BL cell line, MutuI (**Fig. II-1C**), and therefore physiologically relevant. Since we recently reported that another B-cell specific transcription factor, Oct-2, also directly interacts with, and inhibits Z (Robinson et al., 2012), we next determined if the Pax5 inhibitory effect on Z is independent of Oct-2. We transfected Z expression vector in the presence or absence of co-transfected Pax5 or Oct-2 expression vector in HONE-Akata cells. Both Pax5 and Oct-2 inhibit Z-mediated lytic reactivation independent of each other (**Fig. II-**

1C). Therefore, Pax5's inhibitory effect on Z function is not due to an Oct-2-mediated effect or vice versa. These results suggest that Pax5 is a potent inhibitor of Z transcriptional function.

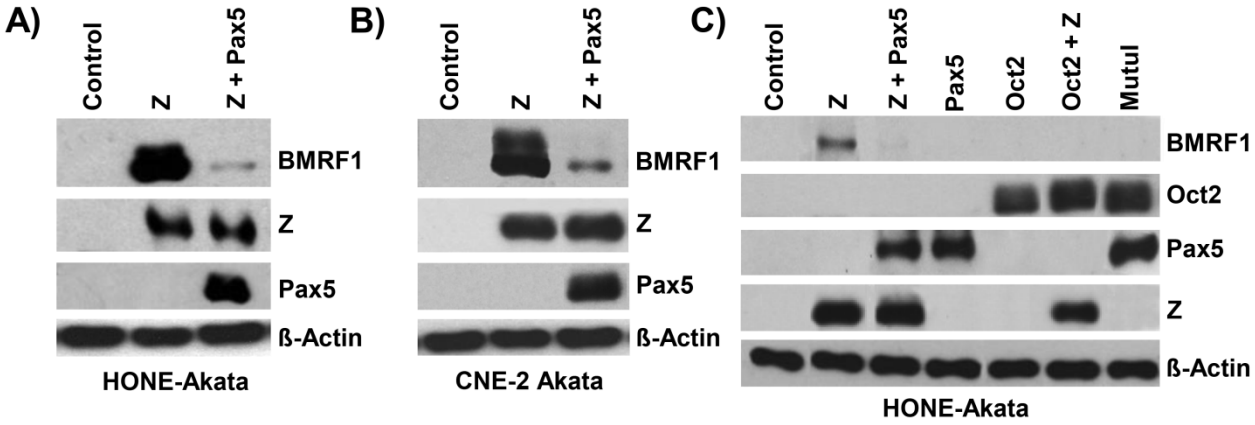

Figure II-1. Pax5 inhibits Z-mediated lytic viral reactivation. (**A**) EBV-positive HONE-Akata cells were transfected with 10 ng Z, 250 ng Pax5, or SG5 control expression vectors as indicated. Immunoblot analysis was performed two days after transfection. Transfected levels of Pax5 and Z were compared, along with the lytic viral protein, BMRF1, derived from the endogenous viral genome. β-Actin protein expression was used as the loading control. (**B**) EBV-positive CNE-2 Akata cells were transfected with 10 ng Z, 250 ng Pax5, or SG5 control expression vectors as indicated. Immunoblot analysis was performed two days after transfection. Transfected levels of Pax5 and Z were compared, along with the lytic viral protein, BMRF1, derived from the endogenous viral genome. β-actin protein expression was used as loading control. (**C**) EBV-positive HONE-Akata NPC cells were transfected with 10 ng Z, 250 ng Pax5, 500 ng Oct-2, or SG5 control expression vectors as indicated. Untransfected MutuI BL cells were also utilized to compare endogenous levels of Pax5 and Oct-2 (MutuI) to transfected levels (HONE-Akata). Immunoblot analysis was performed two days after transfection to compare the levels of Pax5, Oct-2, BMRF1 and Z. ß-Actin protein expression was used as the loading control.

Pax5 inhibits EBV lytic replication

To determine whether Pax5 also decreases viral replication induced by Z expression, CNE-2 Akata NPC cells, which are superinfected with an EBV genome containing an inserted GFP gene (Glaser et al., 1989; Lo et al., 2006), were transfected with Z in the presence or absence of Pax5, and the amount of infectious viral particles released into the supernatant was quantitated using the Green Raji cell assay (Delecluse et al., 1998). Transfected cell extracts were also examined by immunoblot analysis to confirm equal levels of Z expression. As shown in **Fig. II-2A**, transfected Z levels were similar in the presence or absence of co-transfected Pax5, although the ability of Z to induce expression of BMRF1 from the endogenous viral genome was much decreased. Furthermore, consistent with the ability of transfected Pax5 to prevent efficient expression of the essential viral replication protein, BMRF1 (the viral DNA polymerase processivity factor), the presence of Pax5 reduced the level of infectious viral particles produced by Z-transfected cells by approximately 10-fold (**Fig. II-2B**). These results further support the ability of Pax5 to act as an inhibitor of EBV lytic viral replication.

Loss of endogenous Pax5 expression in an EBV-infected Burkitt lymphoma cell line reactivates lytic EBV protein expression

Loss of endogenous Pax5 expression in a lymphoblastoid cell line (LCL) was recently reported to increase Z protein expression and EBV genome copy number (Arvey et al., 2012). However, the mechanism for this effect was not examined. Since the EBV latency protein, LMP1, has been shown to inhibit viral reactivation (Adler et al., 2002) and Pax5 binds to a region of the EBV genome near the LMP1 promoter (Arvey et al., 2012), the ability of Pax5 to

inhibit viral reactivation in the previous report could potentially be due to an effect of Pax5 on LMP1 expression. To determine if loss of endogenous Pax5 expression also reactivates lytic EBV gene expression in cells with type I latency (in which the only viral protein expressed is EBNA1), we infected MutuI cells (a BL line with type I latency) with lentiviral vectors expressing two different control shRNAs or two different shRNAs directed against Pax5, selected infected cells in puromycin for 10 days, and then performed immunoblot analysis to examine the level of Pax5, Z, Oct-2, and BMRF1 expression. As shown in **Fig. II-2C**, the expression of Pax5 was efficiently decreased in MutuI cells using either of two different shRNA constructs, and in each case was associated with an increase in lytic protein expression. Of note, the level of Oct-2 expression remained unaffected by loss of Pax5, indicating that the increase in lytic reactivation is not due to an Oct-2 effect. We also confirmed that MutuI cells do not express LMP1 (data not shown). These results indicate that Pax5 inhibits lytic EBV reactivation in cells with type I latency, independent of its effects on EBNA2 and/or LMP1 latent viral protein expression.

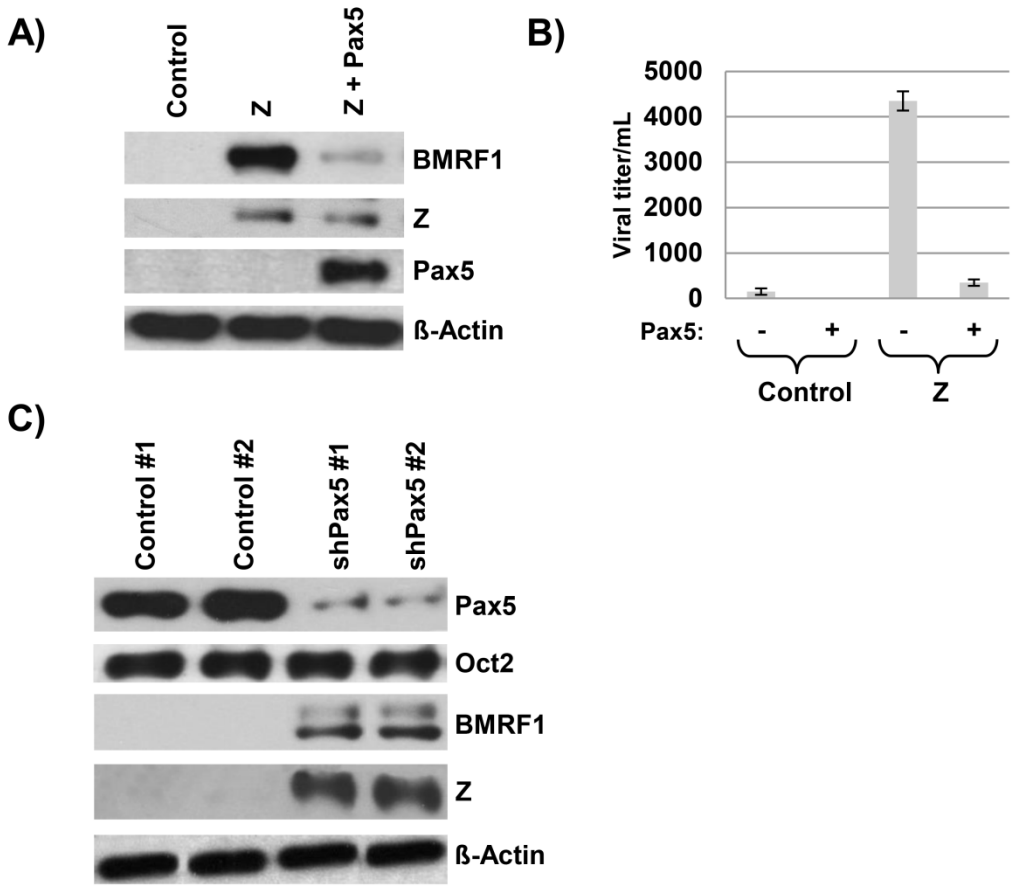

Figure II-2. Pax5 inhibits EBV replication, and loss of endogenous Pax5 increases lytic gene expression in EBV-infected B cells. (**A**) CNE-2 Akata cells were transfected with 10 ng Z, 250 ng Pax5, or control expression vectors as indicated. Immunoblot analysis was performed to compare the levels of transfected Pax5 and Z, as well as the lytic viral protein, BMRF1, from the endogenous viral genome. (**B**) The amount of infectious virus produced from conditions expressed in Fig. 2A was quantitated using the green Raji cell assay. (**C**) MutuI cells were infected with two different individual lentivirus vectors directed against Pax5, or control shRNAs. The cells were selected for 10 days using puromycin (0.5 μg/ml). Immunoblot analysis was performed to compare the levels of Pax5, Oct-2, BMRF1, Z, and β-Actin as loading control.

TPA/sodium butyrate treatment of BL cells decreases Pax5 expression

Given that two different stimuli known to induce lytic EBV reactivation in B cells (BCR stimulation and plasma cell differentiation) also have been reported to decrease Pax5 expression (Hauser et al., 2009; Lin et al., 2002), we examined whether another potent lytic inducing regimen (the combination of TPA and sodium butyrate) also reduces the level of Pax5 in B cells. Raji Burkitt lymphoma cells were treated with or without the combination of TPA/sodium butyrate for 2 days, and the level of Pax5, Z and BMRF1 proteins was examined by immunoblot analysis. Interestingly, the level of Pax5 protein was strikingly decreased in Raji cells following TPA/sodium butyrate treatment, while the level of Z and BMRF1 was increased (**Fig. II-3A**). Further studies revealed that a maximal decrease of Pax5 required both TPA and sodium butyrate, and that the TPA/sodium butyrate combination also induces a similar decrease in Pax5 expression in the EBV-negative, as well as EBV-positive, BL cell line BJAB (**Fig. II-3B**).

To determine whether the TPA/sodium butyrate combination decreases Pax5 transcript levels, we isolated RNA from Raji cells treated with or without TPA/sodium butyrate, and performed RT-PCR to examine the level of the Pax5 transcript (**Fig. II-3C**). We found that Pax5 RNA level was greatly diminished following TPA/sodium butyrate treatment, suggesting that the loss of Pax5 is likely mediated through a transcriptional mechanism. Collectively, these results suggest that the loss of Pax5 expression may aid in the ability of certain lytic inducing stimuli to reactivate EBV gene expression in latently infected B cells.

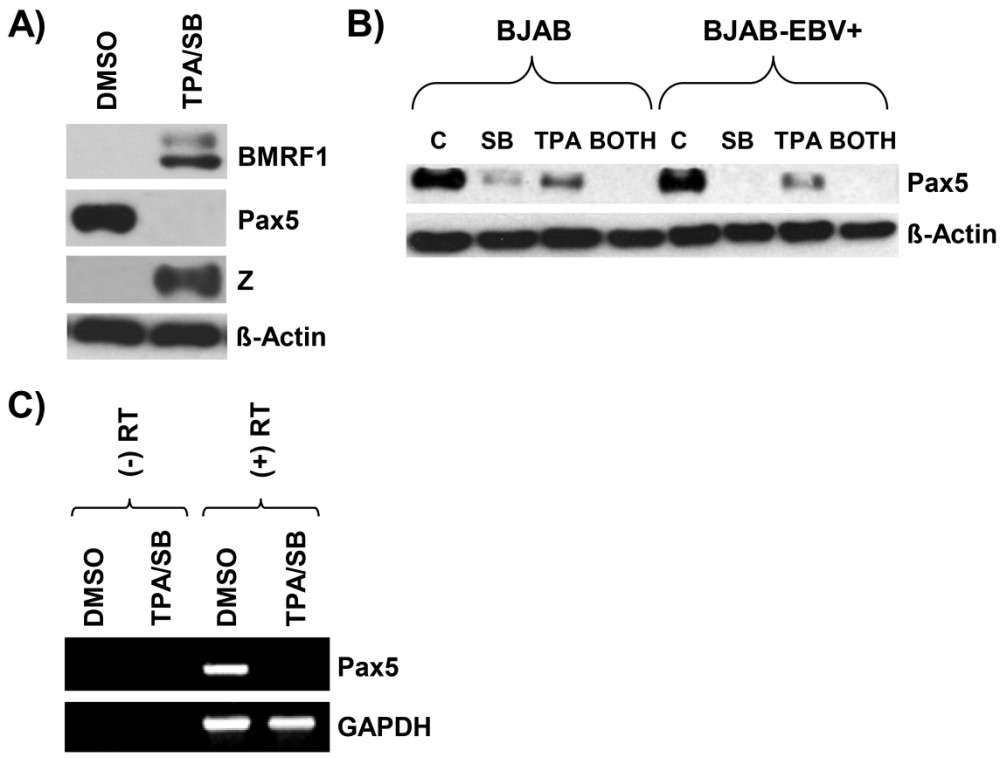

Figure II-3. Lytic reactivation stimuli decrease endogenous Pax5 expression. (**A**) Raji cells were lytically induced with a phorbol ester, TPA (20 ng/mL), and HDAC inhibitor, sodium butyrate (3 mM). Immunoblot analysis was performed two days following treatment to compare levels of endogenous Pax5, lytic viral proteins, BMRF1 and Z, and β-Actin as a loading control. (**B**) The EBV-negative BL B-cell line BJAB, and the EBV-positive superinfected BJAB line, were treated with TPA (20 ng/ml) alone, sodium butyrate (3 mM) alone, or a combination of both TPA (20 ng/ml) and sodium butyrate (3 mM). Immunoblot analysis was performed two days following treatment comparing levels of endogenous Pax5, and β-Actin. (**C**) The expression level of the Pax5 gene was examined by RT-PCR in Raji cells lytically induced with chemical inducers (20 ng/mL TPA/3 mM sodium butyrate) for 48 hours. The GAPDH gene was used as a control.

Pax5 inhibits Z activation of early lytic EBV promoters in EBV-negative cells

Since Z and R activate each other's promoters, and both Z and R are required for efficient activation of most early lytic EBV promoters in the context of the intact viral genome, Pax5 could potentially inhibit lytic EBV reactivation by inhibiting R, rather than Z, function. To determine if Pax5 reduces Z transcriptional function independent of any R effect, we examined the effect of Pax5 on Z-mediated activation of early lytic EBV promoters in EBV-negative HONE-1 cells using reporter gene assays. Co-transfection of Pax5 with Z reduced Z-mediated activation of three different early lytic EBV promoters (SMp, BMRF1p, and BRLF1p) as shown in **Fig. II-4**. These results indicate that Pax5 inhibits Z transcriptional function and confirm that this effect is independent of other viral proteins.

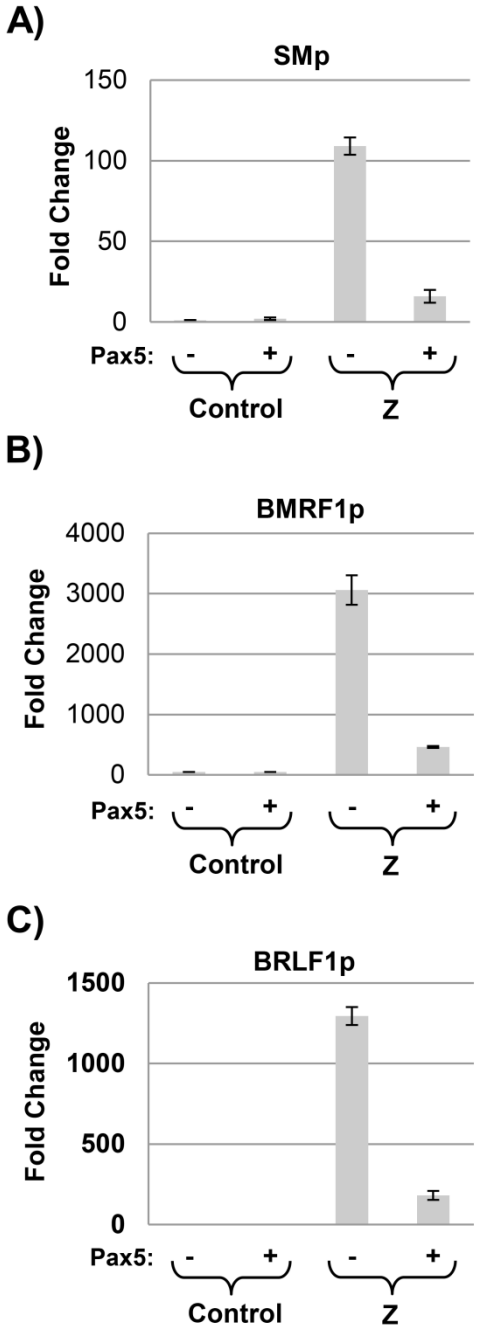

Figure II-4. Pax5 inhibits the ability of Z to transcriptionally activate multiple lytic EBV promoters. HONE-1 cells (EBV-negative) were transfected with (**A**) SMp-luciferase construct, (**B**) BMRF1p, or (**C**) BRLF1p in the presence of absence of Z (10 ng), Pax5 (250 ng), or vector control. Following transfection, cells were harvested two days later and luciferase activity was

measured. The fold change in activity under each condition is shown relative to the activity of the promoter in the presence of the SG5 control vector (set to 1). Values are given as means ± standard deviations of results from two replicates.

Pax5 inhibits Z DNA-binding to early EBV promoters *in vivo*

To determine if Pax5 inhibits the ability of Z to bind to lytic EBV promoters *in vivo,* HONE-Akata cells were transfected with Z in the presence or absence of Pax5, and Z binding to lytic EBV promoters was analyzed using the ChIP assay technique. As shown in **Fig. II-5A**, the presence of co-transfected Pax5 decreased the amount of Z binding to two different early lytic viral promoters (BMRF1 and SM). Quantitative PCR analysis of the SM promoter ChIP results confirmed that Pax5 decreases Z binding to this promoter (**Fig. II-5B**).

To determine if the direct interaction between Pax5 and Z allows Pax5 to be complexed to lytic EBV promoters in a Z-dependent manner, we also performed ChIP assays using an anti-Pax-5 antibody. Pax5 did not bind to the BMRF1 and SM EBV promoters in the presence or absence of Z (**Fig. II-5A**). These results are consistent with a model whereby Z and Pax5 form a complex that cannot bind to ZREs.

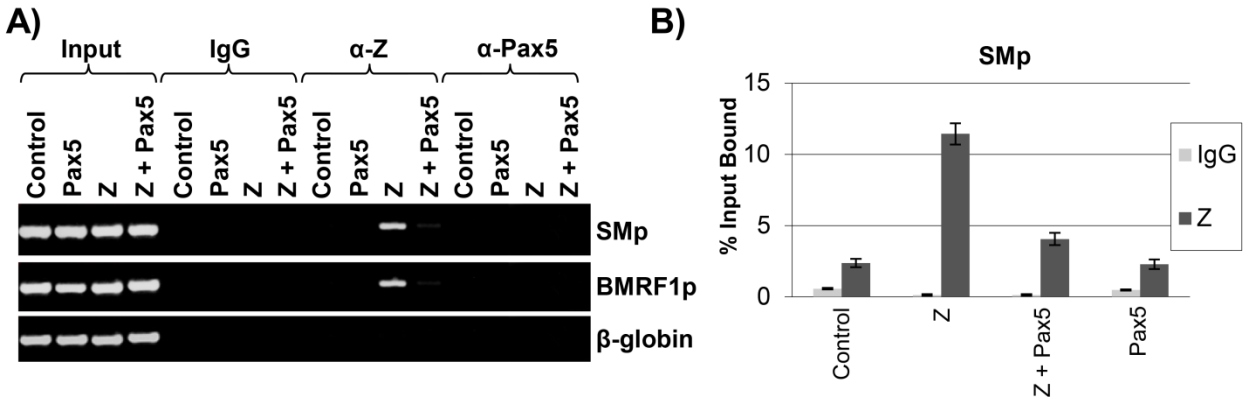

Figure II-5. Pax5 inhibits Z DNA-binding *in vivo*. (**A**) HONE-Akata cells were transfected with Z, Pax5, or vector control. Following transfection, ChIP assays were performed two days later. Cross-linked DNA-protein complexes were immunoprecipitated using anti-Z, anti-Pax5 or control mouse IgG antibodies as indicated. Antibody bound DNA sequences were subject to PCR amplification utilizing primers spanning the EBV BMRF1 and SM promoters, or β-globin (negative control). (**B**) qPCR was performed to quantitate the amount of SM promoter DNA pulled down by the anti-Z antibody in the ChIP assay.

Pax5 DNA-binding activity is not required for the Pax5 inhibitory effect on Z function

To determine whether Pax5 DNA-binding activity is important for the inhibition of lytic EBV reactivation, we constructed a Pax5 mutant altered at residues V26 and P80, which have previously been suggested to be important for Pax5 DNA-binding activity (Kurahashi et al., 2011; Mullighan et al., 2007). The Pax5 (V26G/P80R) mutant was shown to be unable to bind DNA *in vitro* (**Fig. II-6A**), and was defective for activating the CD19 promoter (known to be activated by Pax5) in comparison to the wild-type protein *in vivo* (**Fig. II-6B**). Equal levels of Pax5 wild-type and mutant (V26G/P80R) protein were obtained as determined by immunoblot analysis (data not shown). Although we show the mutant Pax5 (V26G/P80R) to be defective for DNA binding activity, we found that the Pax5 (V26G/P80R) mutant still interacts with Z *in vitro* using the GST pull-down assay (**Fig. II-6C**).

We next compared the ability of wild-type Pax5, versus the Pax5 (V26G/P80R) DNA-binding defective mutant, to inhibit Z function when co-transfected into HONE-Akata cells. As shown in **Fig. II-7A**, the Pax5 (V26G/P80R) mutant was similar to the wild-type Pax5 protein in its ability to prevent Z-mediated lytic reactivation. Furthermore, ChIP assays performed in HONE-Akata cells showed that both wild-type Pax5 and the Pax5 (V26G/P80R) mutant inhibit Z binding to early viral lytic promoters (**Fig. II-7B**). We also confirmed that Pax5 wild-type is able to bind to the cellular CD19 promoter, and that the mutant Pax5 (V26G/P80R) is defective for DNA-binding activity to the CD19 promoter *in vivo* by ChIP (**Fig. II-7B**). Collectively, these results suggest that Pax5 DNA-binding activity is not required for the ability of Pax5 to inhibit Z-mediated lytic reactivation and Z DNA-binding.

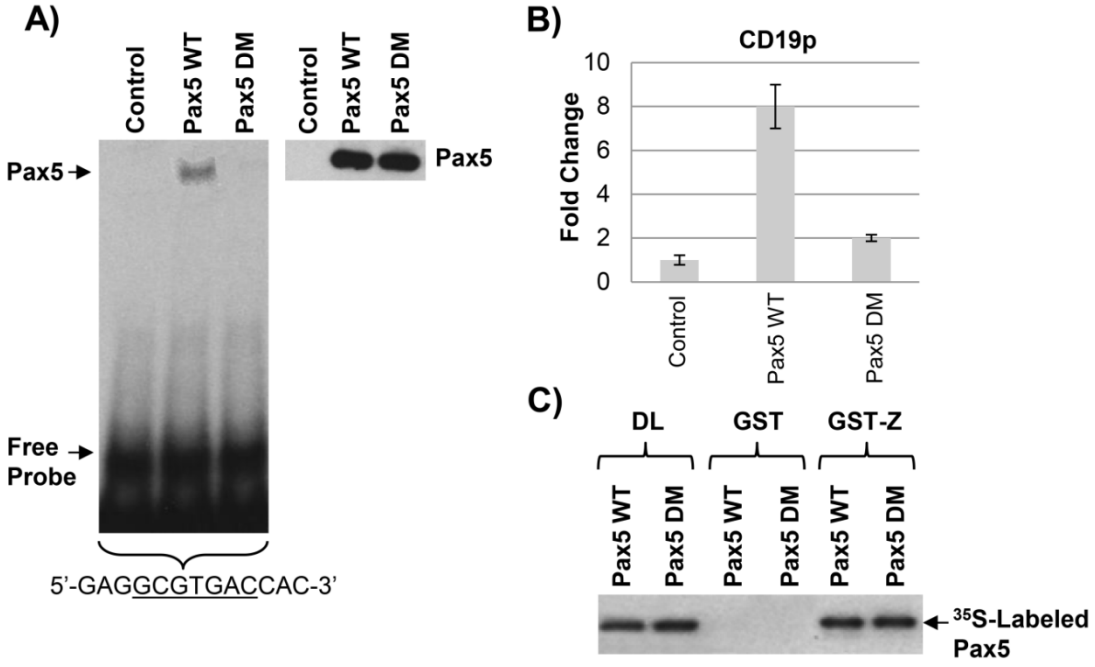

Figure II-6. A Pax5 double mutant is DNA-binding deficient and interacts with *Z in vitro*. (**A**) (Left panel) A gel-shift assay was performed using *in vitro* translated wild-type or double mutant Pax5 V26G/P80R (labeled DM) and [γ-^{32}P] ATP-labeled CD19 oligonucleotide probe containing the Pax5 consensus site. Arrows indicate protein-DNA complexes. (Right panel) Immunoblot analysis was performed to compare the levels of *in vitro* translated wild-type and double mutant Pax5 (DM) protein samples. (**B**) HONE-1 cells (EBV-negative) were transfected with a CD19p-luciferase construct and either wild-type Pax5 (250 ng), Pax5 double mutant (DM) (350 ng), or vector control. The fold change in activity under each condition is shown relative to the activity of the promoter in the presence of the SG5 control vector (set to 1). Values are given as means ± standard deviations of results from two replicates. (**C**) GST pull-down assays were performed using GST or GST-Z fusion protein incubated with ^{35}S-labeled, *in vitro*-translated wild-type Pax5 protein or the double mutant Pax5 (DM). Direct Load is labeled as DL. Ten percent of the direct load was used for autoradiography.

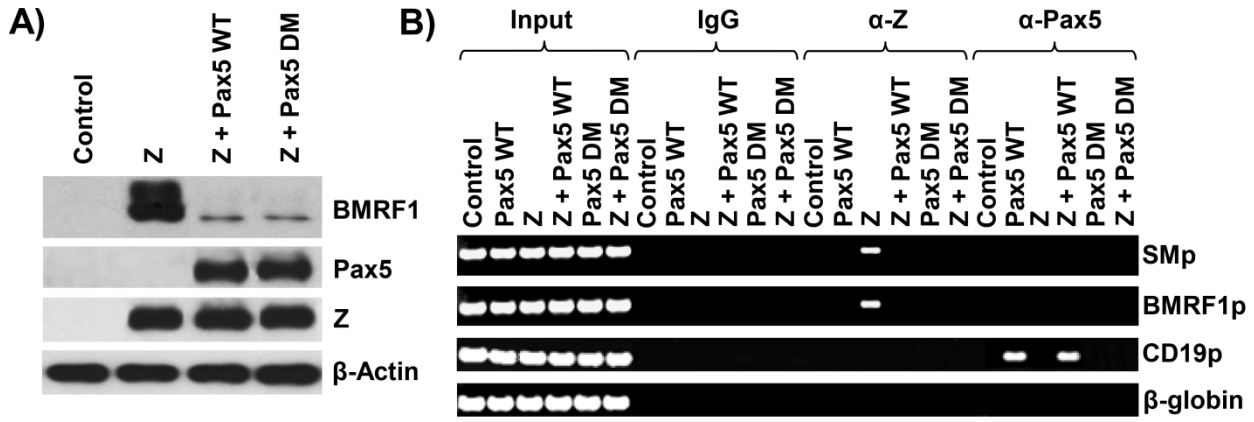

Figure II-7. The DNA-binding activity of Pax5 is not required for the Pax5 inhibitory effect on Z function. (**A**) HONE-Akata cells were transfected with 10 ng Z, 250 ng wild-type Pax5, 350 ng Pax5 double mutant (DM), or vector control. Immunoblot analysis was performed two days following transfection to compare the levels of transfected Z and Pax5, as well as the levels of BMRF1 protein derived from the endogenous viral genome. β-Actin was used as loading control. (**B**) ChIP assay was performed using HONE-Akata cells transfected with Z, wild-type Pax5, double mutant Pax5 (DM), or vector control. Cross-linked DNA-protein complexes were immunoprecipitated using anti-Z, anti-Pax5 or control mouse IgG antibodies as indicated. Antibody bound DNA sequences were subjected to PCR amplification utilizing primers spanning the EBV BMRF1 and SM promoters, cellular CD19 promoter, or β-globin (negative control).

Pax5 interacts directly with the Z DNA-binding/dimerization domain

Although Z has been shown to interact directly with Pax5 *in vivo* and *in vitro* (Adamson et al., 2005), the region of Z required for interaction with Pax5 has not been identified. To map the domain(s) of Z required for the Z/Pax5 interaction, we performed GST pull-down assays using a full-length GST-Z fusion protein, as well as various deletions of this protein constructed as shown in **Fig. II-8A**. Similar levels of the various GST-Z proteins were confirmed by Coomassie gel (data not shown). As shown in **Fig. II-8B**, we confirmed that ^{35}S-labeled, *in vitro*-translated Pax5 protein interacts with the GST-Z protein but not the GST protein alone, and that Z residues from 140 to 225 are sufficient for this interaction. However, Pax5 did not interact with Z residues 180 to 225, or Z residues 140 to 180, implying that a relatively large portion of the Z carboxy-terminus may be required for interaction with Pax5.

We also confirmed that the interaction between Z and Pax5 is not occurring through a DNA bridge by treating the Z/Pax5 protein complex with DNase. As shown in **Fig. II-8C**, ^{35}S-labeled, *in vitro*-translated Pax5 protein interacts with the GST-Z protein but not the GST protein alone in either the absence or presence of DNase treatment.

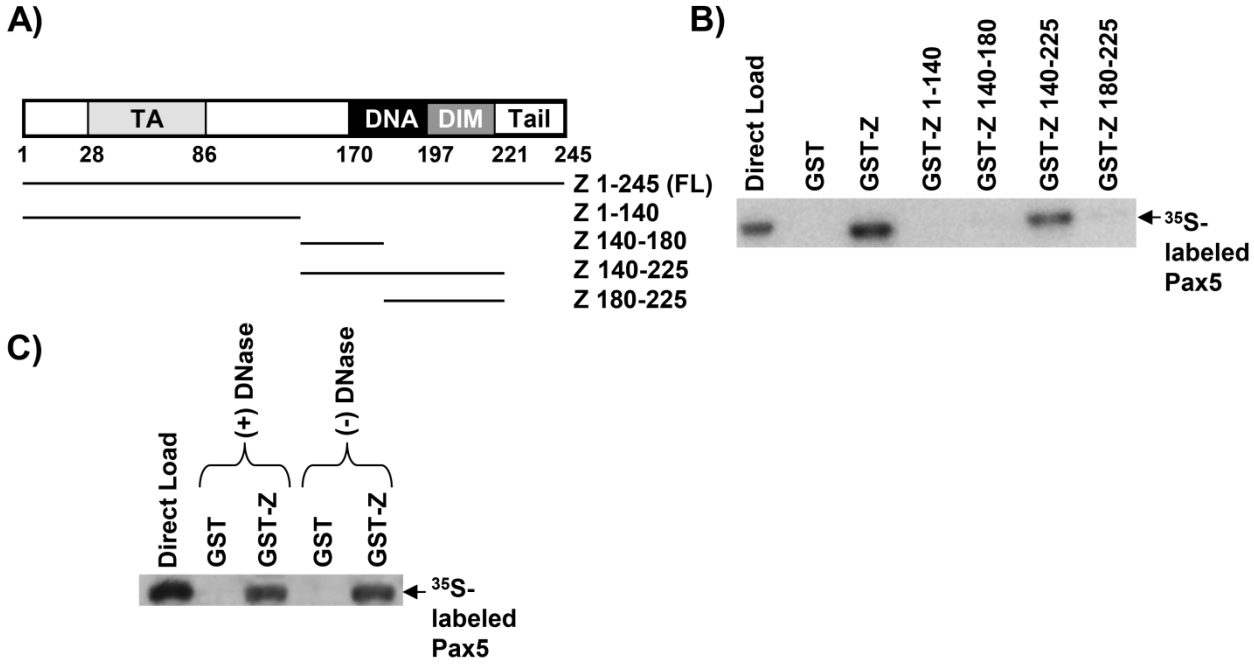

Figure II-8. Pax5 interacts with the Z DNA-binding/dimerization domain. (**A**) Schematic diagram of Z protein spanning from residues 1-245 with corresponding transcriptional activation (TA), basic DNA-binding (DNA), dimerization (DIM), and C-terminal tail (Tail) domains. Numbers represent positions of amino acids. Full-length (FL) Z was also utilized in GST pull-down assays. (**B**) GST pull-down assays were performed using GST, GST-Z full length (FL), and various GST-Z truncation mutants incubated with ^{35}S-labeled, *in vitro*- translated Pax5 protein. Ten percent of the direct load was used for autoradiography. (**C**) GST pull-down assays were performed in the presence (+) or absence (-) of DNase I treatment using GST and GST-Z full length (FL) incubated with ^{35}S-labeled, *in vitro*- translated Pax5 protein. Ten percent of the direct load was used for autoradiography.

Z interacts with the DNA-binding domain of Pax5

Since the region of Pax5 required for Z interaction has also not been previously characterized, we mapped the domain(s) of Pax5 required for interaction with Z. Our initial experiments using various Pax5 mutants indicated that the amino-terminal DNA-binding domain of Pax5 is required for interaction with GST-Z (data not shown). To further characterize the region of Pax5 required for interaction with Z, various Pax5 deletion mutants within the DNA-binding domain were constructed as shown in **Fig. II-9A**. GST pull-down assays were then performed using GST-Z and ^{35}S-labeled, *in vitro*-translated Pax5 wild-type and mutant proteins. The smallest Pax5 mutant shown to be deficient for the ability to interact with Z contained a five amino acid deletion from residues 106 to 110 (**Fig. II-9B**).

A Pax5 mutant that is deficient for interaction with Z does not inhibit Z function

To determine if the ability of Pax5 to interact directly with Z is required for its ability to inhibit Z function *in vivo,* HONE-Akata NPC cells were transfected with Z in the presence or absence of the wild-type Pax5 protein, or the Pax5 Δ106-110 mutant. As shown in **Fig. II-9C**, the Pax5 Δ106-110 mutant was deficient in its ability to inhibit Z-mediated viral reactivation in comparison to the wild-type protein. The mutant protein was confirmed to be stable and also localized in the nucleus (data not shown). In addition, we performed ChIP assays to compare the effect of wild-type Pax5 versus the Pax5 Δ106-110 mutant on Z binding to early lytic viral promoters. As shown in **Fig. II-9D**, the Pax5 Δ106-110 mutant was unable to inhibit the ability of Z to bind DNA. Together, these results strongly suggest that Pax5 must directly interact with Z to inhibit its DNA-binding activity and its ability to disrupt viral latency.

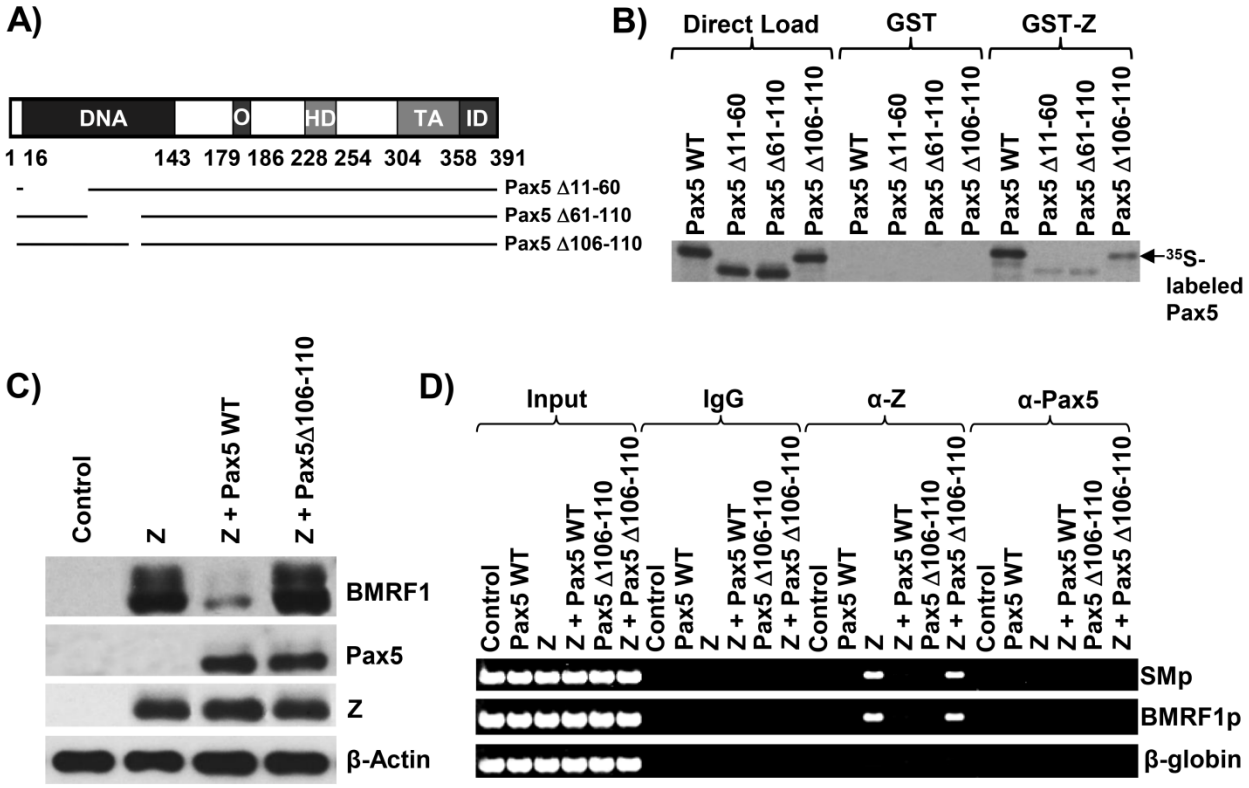

Figure II-9. Z interacts with the DNA-binding domain of Pax5, and a Pax5 mutant that is deficient for interaction with Z is unable to inhibit Z function. (**A**) Schematic of the Pax5 protein DNA-binding paired domain (DNA), octapeptide motif (O), partial homeodomain (HD), transactivation domain (TA), and inhibitory domain (ID). Numbers represent positions of amino acids. Various Pax5 truncation mutations within the DNA-binding paired domain of the in-tact full length Pax5 protein were used in subsequent GST pull-down assays. (**B**) GST pull-down assays were performed using GST or GST-Z fusion protein incubated with ^{35}S-labeled, *in vitro*-translated full-length wild-type Pax5 protein or mutant Pax5 (Δ11-60, Δ61-110, or Δ106-110) proteins. Ten percent of the direct load was used for autoradiography. (**C**) HONE-Akata cells were transfected with 10 ng Z, 250 ng wild-type Pax5, 450 ng mutant Pax5 (Δ106-110), or vector control as indicated. Immunoblot analysis was performed two days after transfection to

compare levels of transfected Pax5 and Z, as well as BMRF1 protein derived from the endogenous viral genome. β-Actin was used as loading control. (**D**) ChIP assay was performed using HONE-Akata cells transfected with Z, wild-type Pax5, mutant Pax5 (Δ106-110), or vector control. Cross-linked DNA-protein complexes were immunoprecipitated using anti-Z, anti-Pax5 or control mouse IgG antibodies as indicated. Antibody-bound DNA sequences were PCR amplified utilizing primers spanning the EBV BMRF1 and SM promoters, or β-globin (negative control).

DISCUSSION

EBV persists in the human host for life by establishing long term latency in memory B cells. The fact that EBV can infect both B cells and epithelial cells, but normally only establishes latency in B cells, suggests that B-cell specific transcription factors may promote viral latency via multiple mechanisms. Pax5, a master regulator of B-cell identity, is required for activation of the essential EBV latency promoter, Wp, which drives expression of the EBV latency proteins EBNA2/EBNA-LP during the initial stage of B-cell infection (Tierney et al., 2007). In this capacity, Pax5 is required for the establishment of persistent latent EBV infection, and transformation, in primary B cells (Tierney et al., 2007). In this report, we show that Pax5 also maintains EBV latency in B cells through a second mechanism involving its direct interaction with the Z immediate-early protein. Thus, Pax5 not only activates transcription of essential viral latency genes, but also inhibits the ability of the Z protein to induce the latent-to-lytic switch.

Our data show that Pax5 is a potent negative regulator of Z-mediated lytic viral gene expression (**Fig. II-1**), as well as Z-mediated infectious virion production (**Fig. II-2**), in EBV-positive NPC epithelial cells, which do not express endogenous Pax5. Conversely, we demonstrate that knockdown of endogenous Pax5 expression in a Burkitt lymphoma line with type I latency leads to viral reactivation (**Fig. II-2**). Pax5 knock-down was also recently reported to be associated with EBV lytic reactivation in a lymphoblastoid cell line with type III latency (Arvey et al., 2012). Together, these results confirm that Pax5 inhibits EBV reactivation when expressed at normal levels in EBV-infected B cells regardless of the type of viral latency.

Interestingly, the level of Pax5 mRNA in B cells decreases within 30 minutes of B-cell receptor (BCR) stimulation, and this effect has been shown to be mediated by reduced

expression of the E2A transcription factor, a positive regulator of the Pax5 promoter (Hauser et al., 2009). In addition, the master regulator of plasma cell differentiation, Blimp-1, binds to the Pax5 promoter and inhibits Pax5 expression in B cells (Cobaleda et al., 2007; Lin et al., 2002; Shaffer et al., 2002a). Since both BCR stimulation and plasma cell differentiation can induce EBV lytic reactivation, we hypothesized that loss of Pax5 expression at least partially contributes to lytic EBV reactivation in response to these two stimuli. Here we show that another lytic inducing regimen, (TPA and sodium butyrate), also decreases Pax5 protein and RNA levels in Raji BL cells (**Fig. II-3**), with each of these two agents contributing to the effect. Since we also found that TPA/sodium butyrate can decrease Pax5 expression in EBV-negative BJAB cells, the effect of these drugs does not seem to be mediated through virally encoded proteins, although the exact mechanism for the effect remains unknown at present.

Since the two EBV IE proteins, Z and R, synergistically activate many early lytic EBV promoters in the context of the intact viral genome (Adamson and Kenney, 1998; Chang et al., 2010; Countryman et al., 1987; Cox et al., 1990; Darr et al., 2001; Farrell et al., 1989; Feederle et al., 2000; Flemington and Speck, 1990; Gruffat et al., 1990; Hardwick et al., 1988; Kenney et al., 1989; Lieberman et al., 1990; Liu and Speck, 2003; Quinlivan et al., 1993; Rooney et al., 1989; Takada et al., 1986; Zalani et al., 1996), while the latent LMP1 viral protein (whose promoter is bound by Pax5) (Arvey et al., 2012), is known to inhibit lytic reactivation (Adler et al., 2002), we determined whether Pax5 inhibits Z-mediated activation of early lytic EBV promoters in the absence of any other EBV-encoded proteins. Using reporter gene assays, we found that Pax5 inhibits the ability of Z to turn on three different early lytic viral gene promoters (SM, BMRF1, BRLF1) in EBV-negative HONE-1 cells (**Fig. II-4**). These results confirm that Pax5 reduces Z

transcriptional function independent of any additional effects of Pax5 on other EBV viral proteins.

As Pax5 was previously reported to interact directly with Z (Adamson et al., 2005), we performed ChIP assays to ask whether the presence of Pax5 decreases Z binding to early lytic EBV promoters *in vivo*, or whether Z can tether Pax5 to lytic EBV promoters. We found that Pax5 inhibits Z binding to early lytic viral gene promoters *in vivo*, suggesting that decreased Z function in the presence of Pax5 is likely due to its decreased DNA-binding activity (**Fig. II-5, -7 and -9**). However, we did not find that Pax5 is complexed to early lytic EBV promoters in the presence or absence of co-transfected Z (**Fig. II-5**). In contrast, Z has been reported to abrogate Pax5 transcriptional activity by a mechanism that does not alter Pax5 DNA-binding (Adamson et al., 2005).

To determine whether the DNA-binding activity of Pax5 is required for its ability to inhibit Z function and/or Z DNA-binding, we constructed a DNA-binding defective Pax5 mutant (V26G/P80R) (Kurahashi et al., 2011; Mullighan et al., 2007) that still interacts with Z. We found that Pax5 DNA-binding activity is not required for its ability to inhibit Z transcriptional function or DNA-binding (**Fig. II-7**). Together, these results suggest that Pax5 inhibits Z function by forming a complex with Z that is unable to bind to ZREs in the viral genome.

To map the domain(s) of Z and Pax5 required for the direct interaction of these two proteins, we performed GST pull-down assays *in vitro* using wild-type and deleted GST-Z fusion proteins, and *in vitro* translated wild-type and mutant Pax5 proteins (**Fig. II-8 and -9**). The results of these studies suggest that Z residues spanning the basic DNA-binding domain and the adjacent bZIP dimerization domain (aa 140 to 225) are sufficient for interaction with Pax5.

However, since we were not able to identify a Z mutant which is functional (i.e. stable and transcriptionally competent) and unable to interact with Pax5, we could not determine if a Z mutant that is specifically defective in the ability to interact with Pax5 is more transcriptionally competent in B cells than the wild-type Z protein.

The Pax5 mapping studies showed that the amino-terminal DNA-binding domain of Pax5 is required for its interaction with Z (**Fig. II-9**), and we were able to identify a five amino acid deletion (residues 106 to 110) within the DNA-binding domain of Pax5 that greatly reduces its *in vitro* interaction with Z (**Fig. II-9**). Although this mutant is not competent to activate Pax5 target promoters (since we found it is defective in DNA-binding), we found that this mutant is nuclear and has reasonable stability (**Fig. II-9**). Therefore, we compared the ability of the two different DNA-binding defective Pax5 mutants, Pax5 (Δ106-110), which cannot interact with Z, and Pax5 (V26G/P80R), which does interact with Z, in regard to their ability to inhibit Z function. The results of these studies (**Fig. II-9**) showed that loss of the ability of Pax5 to interact directly with Z is correlated with loss of its ability to inhibit Z transcriptional function and Z DNA-binding activity. We conclude that a direct protein-protein interaction between Pax5 and Z is required for Pax5 inhibition of Z transcriptional function.

In many ways, these results are similar to those we recently obtained studying the interaction between another B-cell specific transcription factor, Oct-2, and Z (Robinson et al., 2012). Like Pax5, we showed that Oct-2 also interacts directly with Z and abrogates Z DNA-binding to lytic EBV promoters. Since Pax5 is required for expression of many B-cell specific promoters, a potentially confounding issue in our studies might have been that Pax5 is required for expression of Oct-2 in B cells, and/or that Pax5 expression induces Oct-2 expression in non-

B cells. However, since we found that Pax5 transfection does not induce Oct-2 expression in epithelial cells, and that loss of Pax5 expression in B cells did not reduce Oct-2 expression (**Fig. II-1 and -2**), it is unlikely that the Pax5 effect on Z is mediated indirectly through an effect on Oct-2.

These results suggest that EBV has established multiple different mechanisms to ensure that it can efficiently enter latency in B cells, and thus infect the host for life. In addition, it is increasingly clear that lytic EBV reactivation in B cells is intimately linked to the differentiation state of the cell. Lytic EBV reactivation in plasma cells may be partially due to the loss of Oct-2 expression that occurs during plasma cell differentiation (Shaffer et al., 2002b). In addition, Blimp-1 expression in plasma cells not only inhibits Pax5 expression, but also activates the Z promoter (Zp) (Buettner et al., 2012). In summary, our results show that Pax5, the master regulator of B-cell identity, promotes EBV latency in B cells not only by activating an essential viral latency promoter, but also by inhibiting Z function.

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Chapter III

The B-Cell Specific Transcription Factor, Pax5, Regulates LMP1 Expression via Binding to the EBV Terminal Repeats

This work is in progress for a possible manuscript in *PloS One*.

I would like to thank Coral Wille for creating the LMP1-TR reporter gene construct for use in reporter gene assays as shown in **Figure III-4**. I also would like to thank Dr. Eric Johannsen for analyzing ENCODE publically available ChIP-seq data indicating predicted Pax5 binding sites to the EBV genome and terminal repeat promoter as shown in **Figure III-1**.

ABSTRACT

The major oncogene of the Epstein-Barr virus (EBV), latent membrane protein 1 (LMP1) can be expressed from two different promoters, ED-L1 and L1-TR. The terminal repeat (L1-TR) promoter is active in type II latency EBV-associated malignancies such as nasopharyngeal carcinoma and Hodgkin's lymphoma. We previously reported that a B-cell specific transcription factor, Pax5, regulates EBV lytic reactivation by inhibiting the immediate early protein, BZLF1 (Z), and its function. Here we have analyzed ENCODE data from EBV LCLs to identify Pax5 DNA binding sites in the EBV genome. This analysis indicates that Pax5 binds to the EBV terminal repeats (TRs), and that Pax5 binds to the L1-TR terminal repeat promoter. We show by gel shift and ChIP assay that DNA methylation does not affect Pax5's ability to bind to the terminal repeat promoter *in vitro* and *in vivo*. However, Pax5 is inhibited in its ability to transcriptionally activate the methylated form of the terminal repeat promoter, but is able to activate the unmethylated form. Using an EBV-positive Hodgkin's Lymphoma cell line, which does not express endogenous Pax5, we found that forced Pax5 expression is not sufficient by itself to activate LMP1 expression. Additionally, we found that shRNA-mediated knockdown of endogenous Pax5 in a type III Burkitt lymphoma B-cell line led to a decrease in LMP1 expression. We conclude that Pax5 may help to drive expression of LMP1 via the ED-L1 and/or L1-TR promoter during type III latency, in which the LMP1 promoter(s) are unmethylated and EBNA2 is also expressed.

INTRODUCTION

Epstein-Barr virus (EBV) is a double-stranded DNA gamma-herpesvirus which infects over 90% of the world population. EBV is known to cause several malignancies such as infectious mononucleosis and oral hairy leukoplakia (Greenspan et al., 1985; Kieff and Rickinson, 2007a, b). In addition, EBV is commonly associated with several human malignancies such as Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), nasopharyngeal carcinoma (NPC), gastric cancer, and T/NK lymphomas (Kieff and Rickinson, 2007a, b; zur Hausen et al., 1970). EBV-positive tumor cells usually have latent infection, in which only a limited subset of viral genes are transcribed. In type I latency, the EBV nuclear protein known as EBNA1 is the only viral protein expressed. Type II latent infection is characterized by expression of EBNA1 in addition to latent membrane proteins LMP1, 2A, and 2B (Babcock and Thorley-Lawson, 2000; Brooks et al., 1992; Fahraeus et al., 1988; Rowe et al., 1992; Young et al., 1988). Cells with type III latent infection express all six EBNAs (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) and the three latent membrane proteins (LMP1, LMP2A, and LMP2B) (Babcock et al., 2000; Joseph et al., 2000; Kieff and Rickinson, 2007a; Rowe et al., 1987b; Thorley-Lawson, 2001).

LMP1 is the major EBV oncogene and promotes cell growth and transformation in multiple cell backgrounds and in transgenic mice (Cahir-McFarland and Kieff, 2005; Fernandez et al., 2009; Soni et al., 2007; Thornburg et al., 2006). LMP1 is essential for EBV-induced growth transformation of normal B cells to immortalized lymphoblastoid cell lines (LCLs) *in vitro* (Kaye et al., 1993). The expression of LMP1 in EBV-negative BL cells leads to an LCL-like phenotype (Cahir-McFarland et al., 2004; Wang et al., 1985; Wang et al., 1988). In addition,

LMP1 has the ability to transform rodent fibroblasts (Baichwal and Sugden, 1988; Fahraeus et al., 1990; Moorthy and Thorley-Lawson, 1993; Wang et al., 1985), and transgenic mice which express LMP1 from an immunoglobulin promoter have been shown to develop late B-lymphocyte tumors (Kulwichit et al., 1998).

LMP1 activates B-cell proliferation by mimicking the CD40 receptor signaling response in a ligand independent manner, and has been shown to stimulate multiple signaling pathways leading to activation of NF-κB, expression of inhibitor of differentiation (Id1 and Id3), AP-1, and STAT-1/3 (Everly et al., 2004; Gires et al., 1999; Hammarskjold and Simurda, 1992; Kaykas and Sugden, 2000; Kieser et al., 1997; Li et al., 2004). LMP1 has also been shown to have inhibitory effects on cell growth when expressed at high levels, whereas LMP1 induces proliferative phenotypes when expressed at lower levels (Coffin et al., 2003; Cuomo et al., 1992; Eliopoulos et al., 1996; Floettmann et al., 1996; Kaykas and Sugden, 2000; Lam et al., 2004). LMP1 expression varies 100-fold from cell to cell in EBV-infected B lymphocytes (Lam et al., 2004), suggesting that mechanisms which regulate LMP1 expression are crucial for understanding EBV biology.

In cells with type III latency, such as LCLs, LMP1 is primarily encoded by a 2.8-kb mRNA which initiates from the ED-L1 promoter (Chen et al., 2001; Fennewald et al., 1984; Hudson et al., 1985; Repic et al., 2010). The expression of LMP1 via the ED-L1 promoter in type III latency can be activated by viral EBNA2 protein, which interacts with cellular transcription factor RBP-Jκ bound to the ED-L1 promoter (Abbot et al., 1990; Grossman et al., 1994; Johannsen et al., 1995; Johansson et al., 2009; Ling et al., 1993; Wang et al., 1990). Alternatively, in the absence of EBNA2, the NF-κB transcription factors p50-p65 and p50-p50

can bind to and activate the ED-L1 LMP1 promoter in both B cells and epithelial cells, confirming a positive regulatory circuit between NF-κB activation and LMP1 expression (Johansson et al., 2009). Several studies have also reported additional LMP1 auto-regulatory mechanisms (Goormachtigh et al., 2006; Lee and Sugden, 2008a, b; Ning et al., 2003). Although there are several known mechanisms for activation of LMP1 via the ED-L1 promoter in type III latency, it is still unclear what cellular factors and mechanisms are responsible for LMP1 activation and regulation in type II latency in B cells.

In epithelial cells expressing type II latency (which by definition do not express EBNA2), the transcription of a 3.5-kb LMP1 mRNA initiates from the terminal repeat promoter, L1-TR (Chang et al., 1997; Gilligan et al., 1990; Sadler and Raab-Traub, 1995). The L1-TR promoter is also used to transcribe the LMP1 message in both nasopharyngeal carcinoma and Hodgkin's disease tissue (Chen et al., 2001; Repic et al., 2010). Interestingly, expression of EBNA2 is unable to activate the ED-L1 promoter in epithelial cells (Fahraeus et al., 1993), and EBNA2 transactivation of this promoter is dependent on interaction with at least two distinct sequence-specific DNA-binding proteins, RBP-Jκ and PU.1 (Johannsen et al., 1995). More recent studies also indicate that the regulation of LMP1 expression is governed by cell and latency type, cellular context of transcription factors, viral pattern of gene expression, and extracellular stimuli (Bentz et al., 2012; Fernandez et al., 2009; Gantuz et al., 2013; Johansson et al., 2009; Kung and Raab-Traub, 2010; Leonard et al., 2011; Mao et al., 2013; Noda et al., 2011; Repic et al., 2010). However, the specific cellular factors that regulate LMP1 expression have not been fully identified in type II latency cells.

The L1-TR promoter is a TATA-less promoter found in the first terminal repeat of the EBV genome, approximately 600 bp upstream of ED-L1 (Chang et al., 1997; Gilligan et al., 1990; Sadler and Raab-Traub, 1995; Speck, 2005). The termini of EBV DNA consist of a variable number of directly oriented 538-bp terminal repeats (TRs) (Zimmermann and Hammerschmidt, 1995). These EBV TRs are important for the circularization of the linear EBV genome upon entry into cells, and are essential elements for cleavage and packaging of EBV virion DNA (Hammerschmidt and Sugden, 1989; Zimmermann and Hammerschmidt, 1995). The initiation of LMP1 transcription only occurs within the first terminal repeat, and unique sequences adjacent to the first terminal repeat potentially contribute to L1-TR activity (Repic et al., 2010; Sadler and Raab-Traub, 1995). Previous studies have shown that cellular factors such as Sp1 and STAT3, in addition to X-box binding protein-1 (XBP1), contribute to L1-TR promoter activity (Chen et al., 2001; Sadler and Raab-Traub, 1995; Tsai et al., 1999). However, L1-TR promoter activation and regulation of LMP1 expression in B cells is not well understood.

Multiple latent EBV promoters, such as the Cp, Wp, and LMP1 ED-L1p, are methylated and silent in the more restrictive forms of latency and in tumors (Altiok et al., 1992; Imai et al., 1994; Jansson et al., 1992; Minarovits, 2006; Robertson et al., 1996; Rowe et al., 1992; Takacs et al., 2010). In type I Burkitt Lymphoma (BL) lines, both the LMP1 promoters are silent and ED-L1 is highly methylated (Minarovits, 2006; Takacs et al., 2010), whereas in LCLs, ED-L1 is unmethylated and both promoters are active (Li and Minarovits, 2003; Minarovits, 2006; Takacs et al., 2010). Furthermore, treatment of type I BL lines with certain demethylating agents can activate LMP1 transcription (Li and Minarovits, 2003). In NPC, the ED-L1 promoter is unmethylated and LMP1 is transcribed, but the activity of this promoter versus L1-TR in type II

latency remains to be established (Brooks et al., 1992; Busson et al., 1992; Chiang et al., 1996; Deacon et al., 1993; Falk et al., 1998; Hu et al., 1991; Minarovits, 2006; Minarovits et al., 1994; Takacs et al., 2010). It is still also not clear whether the L1-TR promoter is fully or partially methylated in EBV-positive Hodgkin's Lymphomas in patients. Therefore, the epigenetic regulation of the L1-TRp remains to be elucidated.

In our previous report, we demonstrated that Pax5 plays an important role in promoting EBV latency by inhibiting the function of the EBV immediate early protein, BZLF1 (Z) (Raver et al., 2013). In addition, Pax5 has been shown by others to activate the EBV latent W promoter (Wp), which drives expression of EBNA2 and is essential for B-cell transformation (Tierney et al., 2007). Recently, Pax5 was reported by another group, as well as our group (shown here), to bind to the viral terminal repeats directly at two sites. This study suggested that Pax5 binding to the terminal repeats may decrease LMP1 and LMP2A transcription in EBV-transformed B cells (Arvey et al., 2012). However, the mechanism by which Pax5 regulates LMP1 expression in B cells is unknown, and whether Pax5 is a negative or positive regulator of LMP1 expression depending on EBV latency type and methylation status was not investigated.

Here we have investigated the role(s) of Pax5 in regulating LMP1 expression in EBV-infected B cells with various forms of latency. We utilized an EBV-positive, Pax5- and LMP1-negative, KMH2-Akata Hodgkin's Lymphoma (HL) cell line in our present study, which has type I latency (Baumforth et al., 2005). A unique aspect of Hodgkin disease tumors, in contrast to other B-cell lymphomas, is their lack of certain B-cell specific transcription factors, including Pax5, Oct-1, Oct-2, BOB.1, and PU.1 (Loddenkemper et al., 2004; Marafioti et al., 2000; Re et al., 2001; Stein et al., 2001; Theil et al., 2001; Tzankov et al., 2003). Thus, the KMH2-Akata

line is potentially ideal for examining the role of Pax5 on the regulation of LMP1 expression in an HL cellular environment.

We found that Pax5 is able to bind to three sites within the L1-TR terminal repeat promoter, two of which were also recently reported by another group (Arvey et al., 2012), and that Pax5 binds to all three sites in either the methylated or unmethylated forms in gel shift assays. We confirmed that Pax5 binds to the L1-TR promoter in cells with either type I or type III latency *in vivo* using ChIP assays, further suggesting that methylation does not inhibit the ability of Pax5 to bind DNA at the terminal repeat sites. However, using reporter gene assays, we found that methylation of the L1-TR promoter inhibits Pax5's ability to transcriptionally activate this promoter. Additionally, we found that restoration of Pax5 expression in KMH2 EBV-positive HL cells does not activate LMP1 expression. In contrast, upon shRNA-mediated knockdown of Pax5 in a type III BL cell line, we observed a decrease in LMP1 expression, contrary to what was previously reported in LCLs (Arvey et al., 2012). Our results indicate that Pax5 may be a positive regulator of LMP1 expression during type III latency.

MATERIALS AND METHODS

Cell lines and culture

EBV-negative HONE-1 nasopharyngeal carcinoma cells (a gift from Ron Glaser, The Ohio State University) and an EBV-positive type III latency Burkitt lymphoma (BL) line, KemIII, (a gift from Jeff Sample) were maintained in RPMI 1640 supplemented with 10% FBS and 1% pen/strep. Human embryo kidney (HEK) 293 cells (obtained from the ATCC) were used for packaging of lentiviral and retroviral vectors, and were maintained in DMEM supplemented with 10% FBS and 1% pen/strep as previously described (Graham et al., 1977). EBV-negative KMH2-neo Hodgkin Lymphoma and EBV-positive KMH2-Akata Hodgkin Lymphoma cells (a gift from Paul Murray, University of Birmingham, United Kingdom) (Baumforth et al., 2005) were maintained in RPMI 1640 supplemented with 10% FBS, 1% pen/strep, and G418 1mg/ml.

Plasmids and cloning

Plasmid DNA was purified using Qiagen maxi-prep columns according to the manufacturer's protocol. pSG5 was obtained from Stratagene. pSG5-Pax5.S24 was a gift from James Hagman, National Jewish Center (Fitzsimmons et al., 1996). The plasmid pSG5-Pax5 DNA-binding Mutant (V26G/P80R) was constructed as previously described (Raver et al., 2013). The promoterless reporter gene construct pCpGL-basic was a gift from Michael Rehli, Universitatsklinikum Regensburg (Klug and Rehli, 2006). The LMP1 terminal repeat promoter, L1-TRp, was cloned into pCpGL-basic using primers forward 5'-AGCAGACGGCGGATATG GGAATTT-3' and reverse 5'-TTTGTCAGGGTTGCCTGTGTCA-3'. The retroviral vector, pBABE-puro (plasmid #1764), was obtained from Addgene. A lentiviral Pax5 vector was also

obtained from Addgene (Malin Parmar, Plasmid #35003), from which Pax5 was excised by cutting the vector with EcoRI and SalI enzymes and then inserted into pBABE via the EcoRI-Sal I sites to create the retroviral vector pBABE-Pax5. Pax5 was cloned into the BamHI and EcoRI sites of the pCDH713 lentiviral vector (SBI, Cat# CD713B-1-SBI), under the control of the murine stem cell virus (MSCV) promoter to create CDH713-Pax5.

Immunoblotting

Immunoblotting was performed as previously described (Bhende et al., 2005; Swenson et al., 2001). Cells were harvested in SUMO lysis buffer containing proteasome inhibitor cocktail (Roche) and quantitated by SUMO Protein Assay (BioRad). Equivalent amounts of protein were separated in sodium dodecyl sulfate, 10% polyacrylamide gels and transferred to membranes. Membranes were blocked in PBS containing 5% milk and 0.1% Tween-20 solution and incubated with primary antibody. The following antibodies were used: anti-LMP1 (Provided by Elliott Kieff (Wang et al., 2000), received via Micah Luftig and Eric Johannsen, clone no. S12; 1:5000); anti-RBP-Jκ (Cosmobio, SIM-2ZRBP1; 1:500) anti-PU.1 (Santa Cruz, sc-352; 1:500); anti-EBF1 (Abcam, ab108369; 1:1000); anti-EBNA2 (Leica, clone no. PE2; 1:100); anti-EBNA1 (Clone no. IH4 EBNA1; 1:50 (Grässer et al., 1994)), anti-BMRF1 (Vector, G3-E31; 1:250), anti-β-actin (Sigma, 1:5000), and anti-Pax5 (Santa Cruz, sc-13146; 1:250). Secondary antibodies were HRP goat-anti-mouse (Fisher Scientific; 1:5000), HRP goat-anti-rabbit (Fisher Scientific; 1:10,000), and HRP goat-anti-rat (Fisher Scientific; 1:5000).

Packaging of Retrovirus

The control retroviral vector, pBABE, and the retroviral vector expressing Pax5, pBABE-Pax5, were used to produce retrovirus as previously described according to the protocol from Addgene (Morgenstern and Land, 1990). HEK 293 cells were transfected with the retrovirus expression plasmids, and the packaging plasmids pUMVC (Addgene #8449) and VSV-G (Addgene #8454) using Lipofectamine 2000 reagent (Invitrogen). Supernatant was collected 3 days post-transfection and filtered through a 0.8-μm pore size filter. EBV-positive KMH2-Akata Hodgkin and EBV-negative KMH2 Hodgkin cells were then transduced by incubation with filtered media. After 3 days of incubation, stable cell lines were selected with 1.5 μg/ml puromycin. KMH2-Akata Hodgkin Lymphoma cells were selected for 14 days in puromycin prior to immunoblot analysis.

Packaging of Lentivirus

A lentiviral control vector, pCVL.EuB29.MCS.T2A-GFP was a gift from Blythe Sather and David Rawlings, Seattle Children's Hospital. A lentiviral Pax5 vector was also obtained from Addgene (Plasmid #35003). Additionally, pCDH713 lentiviral vector (SBI, Cat# CD713B-1-SBI), and CDH713-Pax5 were utilized in a separate set of experiments (stable infection with puromycin selection). The control and Pax5 lentiviral vectors were used to produce lentivirus as previously described (Morgenstern and Land, 1990; Raver et al., 2013) according to the protocol from Addgene. HEK 293 cells were transfected with the lentiviral expression plasmids, and the packaging plasmids psPAX2 (Addgene Plasmid #12260, a gift from Didier Trono) and VSVG (Addgene #12259, a gift from Didier Trono) using Lipofectamine 2000 reagent (Invitrogen).

Supernatant was collected 3 days post-transfection and filtered through a 0.8-μm pore size filter. EBV-positive KMH2-Akata Hodgkin Lymphoma and EBV-negative KMH2 Hodgkin Lymphoma cells were then transduced by incubation with filtered media. For one set of experiments (control vector and #35005 Addgene-Pax5 vector), KMH2-Akata Hodgkin Lymphoma cells were harvested for immunoblot analysis, following 5 days of incubation time. For the second set of experiments (pCDH713 and pCDH713-Pax5), after 3 days of incubation, stable cell lines were selected with 1.5 μg/ml puromycin and KMH2-Akata Hodgkin Lymphoma cells were selected for 14 days in puromycin prior to immunoblot analysis.

Chromatin immunoprecipitation (ChIP) assays

KemI and KemIII cells were plated in 10-cm dishes at $1x10^6$ cells and harvested 48 hours later. Cells were crosslinked in fresh 1% paraformaldehyde for 10 minutes at room temperature. The cross-linking reaction was quenched using 125mM glycine. Following cell lysis and DNA fragmentation by sonication, DNA-protein complexes were immunoprecipitated with the following antibodies: anti-Pax5 (Santa Cruz; sc-1975X) and control mouse polyclonal immunoglobulin G (IgG) (Santa Cruz; 2025). Immunoprecipitated DNA-protein complexes were washed with low salt, high salt, lithium chloride and TE wash buffers. Protein-DNA cross-linking was reversed at 65°C overnight. DNA was purified using the Qiagen Gel Extraction Kit. PCR was used to determine the presence and relative amount of specific DNA fragments that were immunoprecipitated. Primers used were the following: LMP1 L1-TR promoter 5'- GCACG CCGTTGGAGGGTAGAATGA-3' and 5'- CTTTGTCAGGGTTGCCTGTGTCACC-3'; cellular Blnk promoter 5'-ACACAACGCACAGTAATCAGC-3' and reverse 5'-CCTGGACTT

CCTGGGGAGCAG-3'; and β-globin 5'-AGGGCTGGGCATAAAAGTCA-3' and 5'-GCCTC ACCACCAACTTCATC-3'. Extracts were also subjected to immunoblot analysis to verify Pax5 protein levels.

EMSA

T4 Polynucleotide Kinase (Zimber et al.) and [γ-^{32}P] ATP (Perkin-Elmer) were used to label double-stranded, annealed DNA oligonucleotides for DNA-protein binding studies. The Pax5 binding probe (30,000 cpm), derived from the L1-TR promoter, consisted of three different sequences containing the Pax5 consensus sequence (underlined) with unmethylated or methylated CpG sites (asterick): Site #1 5'-TGACCCAGCCAA GC*GTGACCAAGGGGCC CGT-3'; Site #2 5'-GCGGGCAATGGAGC*GTGAC*GAAGGG CCCCAG-3'; and Site #3 5'-GGGCGCAGCCATGC*GTGACCGTGATGAGGGG-3'. Methylated and unmethylated probes were commercially obtained from IDT. The protein samples used in electrophoretic mobility shift assays (EMSAs) were *in vitro* translated protein using the TNT T7 Quick Coupled Translation System (Promega). The reaction was allowed to incubate for 20 minutes at room temperature and was loaded onto a 4% polyacrylamide gel in 0.5x Tris-borate-EDTA buffer and electrophoresed at 35 mA. Gels were dried on Whatman paper under a vacuum and exposed to autoradiography film for 4 to 48 hours at -80°C.

***In vitro* methylation of reporter gene constructs**

Reporter gene constructs were methylated *in vitro* using CpG methyltransferase M.SssI according to the manufacturer's instructions. After completion of the methylation reaction, the

DNA was cleaned by phenol chloroform extraction and salt precipitation. Successful methylation was confirmed by enzymatic digestion with two restriction enzymes that recognize the same cut site: HpaII (digestion blocked by methylation) and MspI (cuts regardless of methylation status).

Reporter gene assays

Methylated or unmethylated pCpGL-L1-TRp-luc reporter gene construct was used in reporter gene assays. HONE-1 cells were transfected with Lipofectamine 2000 Transfection Reagent (Invitrogen) in 12-well dishes with a total of 500 ng of DNA using SG5 vector control, 50 ng of pCpGL-L1-TRp-luc vector control, 250 ng of Pax5, and 350 ng of Pax5 V26G/P80R. The cells were washed 1x with PBS and harvested in 1x Reporter Lysis Buffer (Promega) 48 hours post-transfection. The lysates were subjected to one freeze-thaw cycle and relative luciferase units were quantified using a BD Monolight 3010 Luminometer (BD Biosciences) and luciferase assay reagent (Promega). The fold change for each condition was calculated from the increase in luciferase activity relative to the activity of the promoter in the presence of the SG5 control vector (set to 1). Each condition was performed in duplicate in each experiment and repeated in two biologic replicates. Extracts were also subjected to immunoblot analysis to verify equal Pax5 protein levels.

ChIP-seq analysis of EBV genome Pax5 binding sites

Fastq files from four different Pax5 ChIP-seq experiments in LCLs were downloaded from the ENCODE website (Rosenbloom et al., 2012). Sequence reads were aligned to the B95-8 genome (V01555.2) using the Mapping and Assembly with Qualities (MAQ) rapid aligner and

displayed with a local installation of the UCSC genome browser. Position-specific read depth was calculated using a python script that summed the forward and reverse reads at each location in the EBV genome or using Fseq to calculate the tag sequence density at each position.

shRNA Infection and Packaging of Lentivirus.

Lentivirus vectors expressing two different shRNAs against a control sequence (Sigma Cat. #SHC002 or Addgene plasmid 1864), or two different shRNAs directed against Pax5 (Open Biosystems Cat. #RHS-4533-NM_016734) were used to produce lentivirus as previously described according to the Open Biosystems (Thermo-Scientific) protocol (Raver et al., 2013). HEK 293 cells were transfected with the lentivirus expression plasmids, and the packaging plasmids pxPAX2 (Addgene plasmid 12260, a gift from Didier Trono) and VSVG (Addgene plasmid 12259, a gift from Didier Trono) using Lipofectamine 2000 reagent (Invitrogen). Supernatant was collected 3 days post-transfection and filtered through a 0.8 μm pore size filter. EBV-positive KemIII BL cells were then transduced by incubation with filtered media. After 3 days of incubation, stable cell lines were selected with 0.5 μg/ml puromycin. KemIII cells were selected for 10 days in puromycin prior to immunoblot analysis.

RESULTS

Mining of ENCODE data reveals that Pax5 binds to each EBV terminal repeat at three different sites

In collaboration with Dr. Eric Johannsen, we performed analysis of mined ChIP-seq data available from the public ENCODE site to identify Pax5 binding sites in the EBV genome. This analysis revealed that Pax5 binds with high occupancy to each of the viral terminal repeats in the EBV genome, as revealed by multiple binding peaks. As shown in **Fig. III-1A**, based on the Pax5 consensus sequence, and the ChIP-seq results, we predicted that there are at least three Pax5 binding sites in the first terminal repeat, which overlaps the L1-TR promoter. Two of the predicted terminal repeat binding sites were recently identified by another group (Arvey et al., 2012); however, the third site was not identified. **Fig. III-B** shows Pax5 binding peaks mapping to the terminal repeats within the EBV genome. In **Fig. III-1C**, the three Pax5 binding sites within the first TR and their sequences (with consensus sites indicated) are depicted. The relative positions of the three binding sites relative to the promoter start site are shown in **Fig. III-1A**.

Interestingly, the loss of Pax5 expression in LCLs was previously reported to increase LMP1 and LMP2 expression and decrease EBNA1 and EBNA2 (Arvey et al., 2012). However, whether Pax5 is a negative or positive regulator of LMP1 expression via the terminal repeat promoter has not been determined.

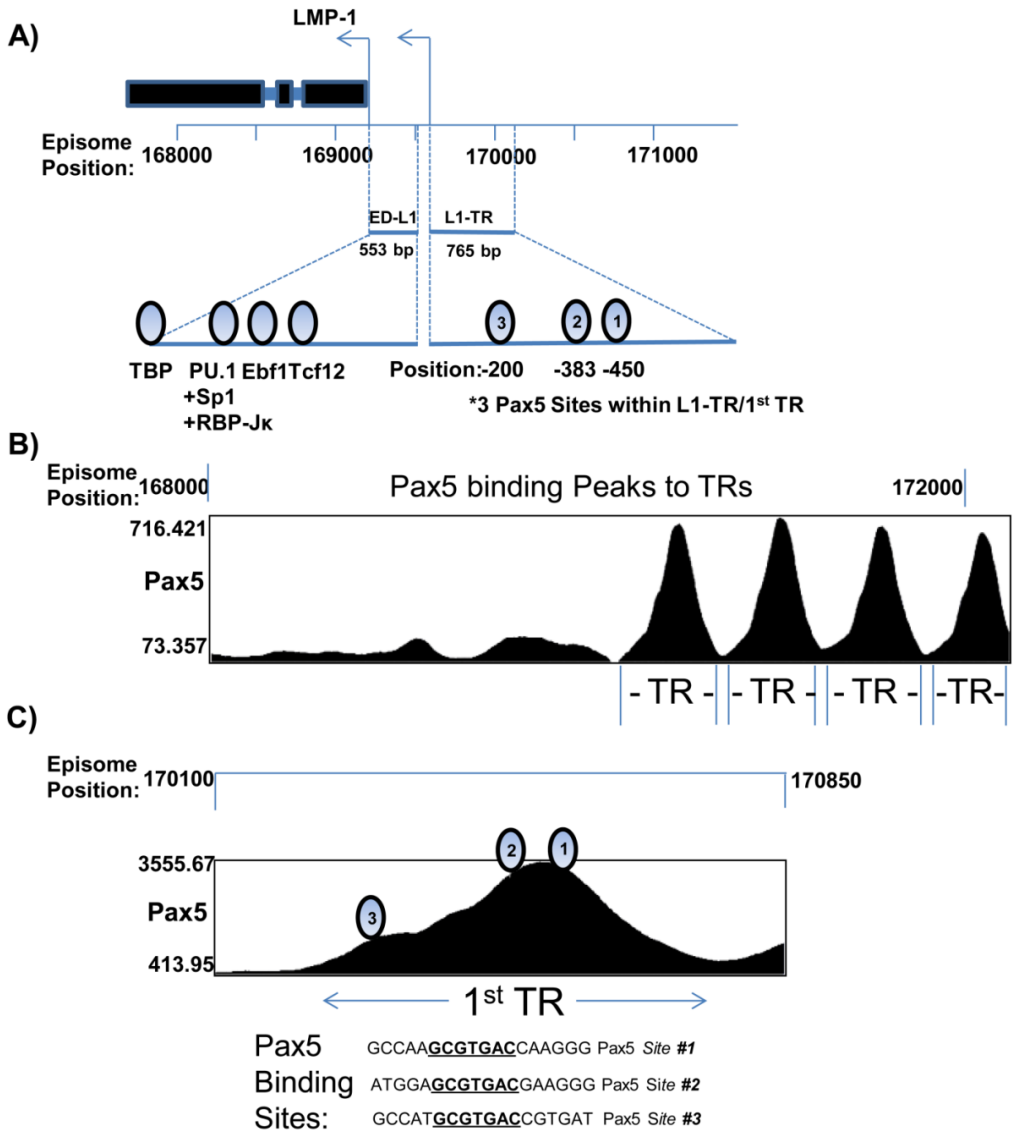

Figure III-1. Pax5 binds the viral terminal repeats at three sites. (**A**) Pax5 binds the viral terminal repeat promoter (L1-TR) at three sites, as supported by ChIP-seq. EBF1, PU.1, SP1, RBP-Jκ, and TCF12 sites in the ED-L1 promoter are also shown. (**B**) Pax5 binding peaks to the EBV terminal repeats (TRs). (**C**) A zoomed in schematic of the first viral terminal repeat, which contains three Pax5 binding sites based on Pax5 consensus sequence and location within the L1-TR promoter. The sequences for the three predicted Pax5 binding sites are shown below with the underlined Pax5 consensus site.

Pax5 binds to all three predicted L1-TR Pax5 sites *in vitro*

Although previous studies indicate that CpG methylation does not prevent Pax5's ability to bind a cellular B-cell specific promoter (Maier et al., 2003), it is unknown whether methylation of viral promoters, including L1-TR, affects Pax5 DNA binding. Therefore, we next examined if Pax5 can bind *in vitro* to all three predicted sites in the L1-TR promoter, and whether DNA methylation of these sites affects Pax5 DNA-binding. EMSA was performed to determine if Pax5 binds to the three binding sites (Pax5 consensus site GCGTGAC) in their methylated or unmethylated forms. As shown in **Fig. III-2**, methylation does not inhibit Pax5 binding to two sites *in vitro*, as Pax5 is able to bind equally as well to a methylated or unmethylated probes. We found the same result for an additional third Pax5 site (data not shown).

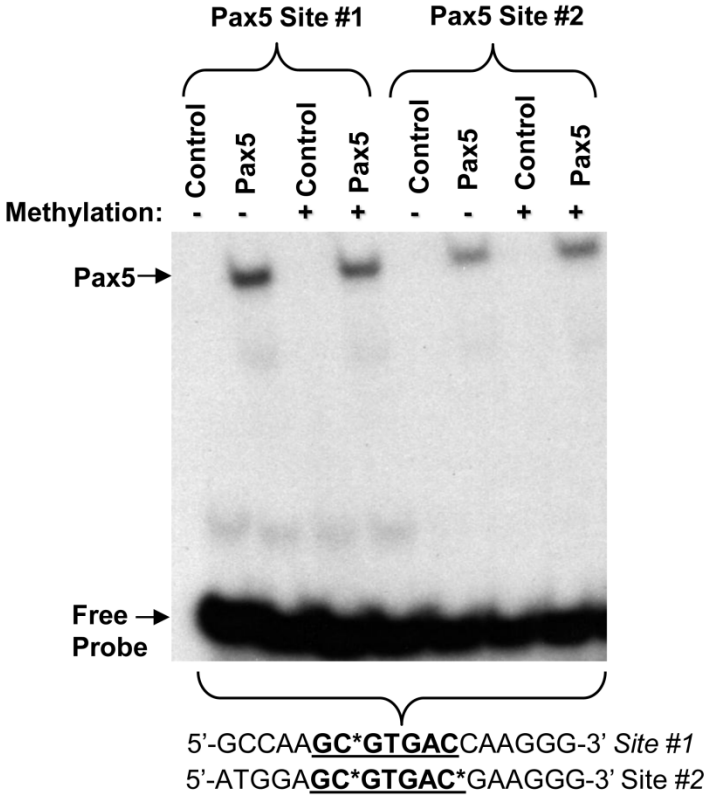

Figure III-2. **Pax5 binds to methylated and unmethylated L1-TR sites *in vitro*.** EMSA was performed using *in vitro* translated Pax5 protein and two different [γ-^{32}P] ATP-labeled L1-TR oligonucleotide probes, either methylated or unmethylated, containing the Pax5 consensus site (underlined). Arrows indicate protein-DNA complexes. Pax5 Sites #1 and #2 and corresponding sequences are shown. C* indicates methylated cytosines.

Pax5 binds the L1-TR promoter in cells with both type I and type III latency *in vivo*

Since Pax5 is able to bind *in vitro* to each of the L1-TR sites regardless of methylation status at CpG motifs, we next asked whether Pax5 is able to bind to the L1-TRp *in vivo* in either type I latency or type III latency BL lines. We performed ChIP assays utilizing a primer spanning a 206 bp region of the L1-TR (containing two of the Pax5 binding sites) as shown in **Fig. III-3A,** to determine if Pax5 is able to bind *in vivo* to the L1-TR promoter in KemI and KemIII BL lines. As shown in **Fig. III-3B**, Pax5 binds the L1-TR promoter in both KemI and KemIII, although KemIII shows relatively less binding, presumably due to the lower input DNA in this cell line. Likewise, Pax5 was recently shown to bind with high occupancy at the TR locus in MutuI (type I latency) and LCLs (type III latency) compared with other known Pax5 binding sites (EBV Wp promoter and human CD19 promoter) by ChIP-qPCR (Arvey et al., 2012). These results confirm that Pax5 binds to the L1-TR promoter in cells with both type I and type III latency.

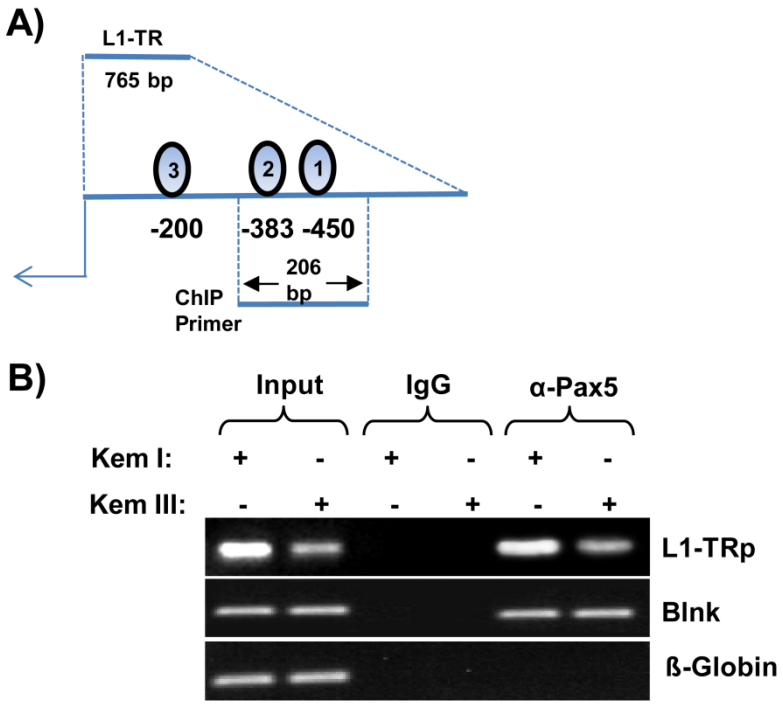

Figure III-3. Pax5 binds to the L1-TR promoter in cells with either type I or type III latency *in vivo*. (**A**) A schematic of the designed ChIP primer spanning 206 bp of the L1-TRp, containing the two Pax5 sites that lie within the major peak of the first viral terminal repeat. (**B**) ChIP assays were performed in KemI and KemIII cells, using anti-Pax5 or control mouse IgG antibodies as indicated. Antibody bound DNA sequences were subjected to PCR amplification utilizing primers spanning the EBV L1-TR promoter, the cellular Blnk promoter (known to be bound by Pax5), or β-globin (negative control).

Pax5 activates the L1-TR promoter in its unmethylated form

Since Pax5 is able to bind to the L1-TR promoter *in vivo* and *in vitro* regardless of methylation status, we next determined whether Pax5 is able to activate the L1-TR promoter in its methylated or unmethylated forms. A 609 bp L1-TRp-luciferase construct containing all three Pax5 activation sites (**Fig. III-4A**) was methylated or mock methylated *in vitro*, and then transfected into EBV-negative HONE-1 epithelial cells in the presence or absence of co-transfected Pax5. As shown in **Fig. III-4B**, Pax5 activates the unmethylated form of the L1-TR promoter, but activates the methylated form of the promoter much less efficiently. The Pax5 DM (V26G/P80R), which was previously reported for its inability to bind to and activate the CD19 promoter (Raver et al., 2013), is defective in the ability to activate either the methylated or unmethylated L1-TR promoter (**Fig. III-4B**). These results suggest that methylation of the EBV L1-TR promoter inhibits Pax5 activation. Therefore, in EBV-infected cells with type I latency, where the L1-TR promoter may be methylated, Pax5 may be unable to activate LMP1 transcription.

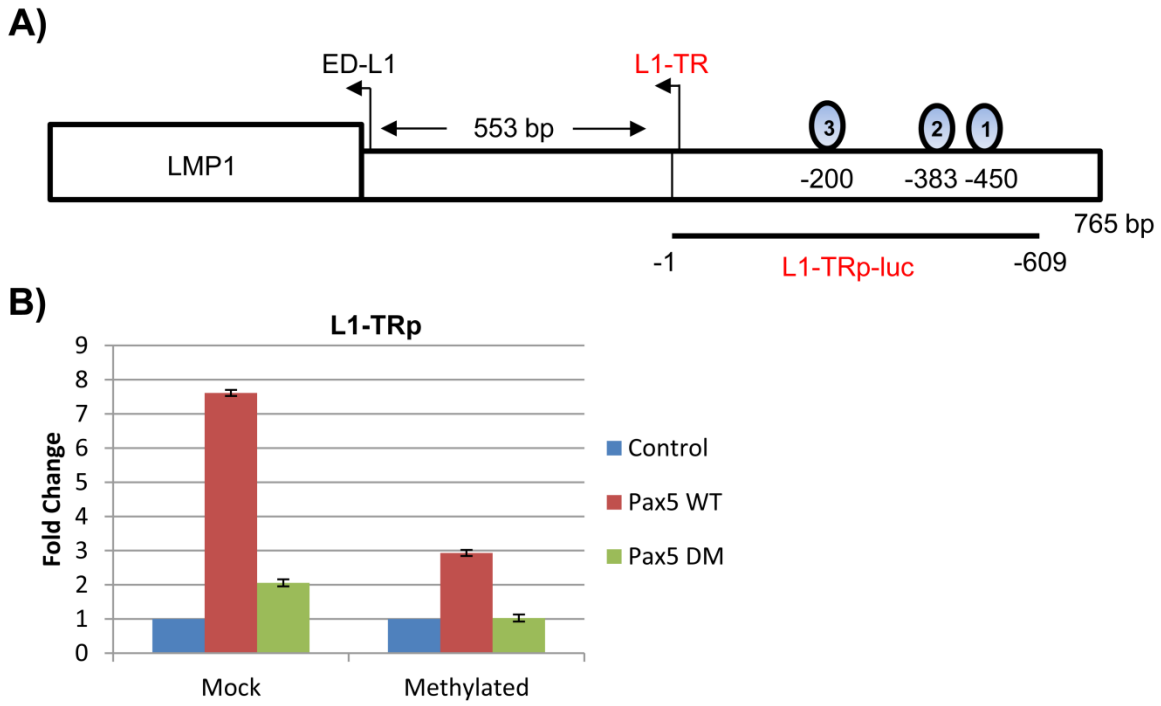

Figure III-4. Pax5 preferentially activates the unmethylated form of the L1-TR promoter. (**A**) A schematic diagram of the L1-TRp-luciferase construct which contains all three Pax5 binding sites and 609 base pairs of the promoter. (**B**) HONE-1 cells (EBV-negative) were transfected with L1-TRp-luciferase construct in the presence or absence of Pax5 (250ng), Pax5 V26G/P80R (DM) (350 ng), or vector control (up to 500 ng). Following transfection, cells were harvested two days later and luciferase activity was measured. The fold change in activity under each condition is shown relative to the activity of the promoter in the presence of SG5 control vector (set to 1). Values are given as means ± standard deviations of results from two replicates. Similar levels of transfected WT or mutant Pax5 proteins were present in each experiment (data not shown).

Pax5 cannot restore LMP1 expression in KMH2-Akata Hodgkin lymphoma cells

We next determined whether Pax5 is a negative or positive regulator of LMP1 expression in a type I latency HL cell type, as these cells do not express either LMP1 or Pax5. We asked whether either stable or transient Pax5 expression by lentiviral infection activates LMP1 expression in these cells. We utilized two different lentiviral vectors, each driven by different promoters. As shown in **Fig. III-5A**, the expression of Pax5 in these cells did not induce LMP1 expression, suggesting that Pax5 by itself is insufficient to activate LMP1 expression in this cell type. These cells have type I latency, in which the EBV genome, including the LMP1 promoters, are usually highly methylated (Altiok et al., 1992; Imai et al., 1994; Jansson et al., 1992; Li and Minarovits, 2003; Minarovits, 2006; Robertson et al., 1996; Rowe et al., 1992). Nevertheless, the amount of Pax5 expressed under the control of the murine stem cell virus (MSCV) promoter in the lentiviral vector was less than the endogenous level found KemIII BL cells. Therefore, we also utilized a vector with a stronger promoter (CMV) to drive transient Pax5 expression as shown in **Fig. III-5B**. We found that following short term infection, Pax5 was expressed at equal levels compared to that of endogenous KemIII BL cells, and that LMP1 expression still was not activated. These results suggest that Pax5 does not activate LMP1 expression in Hodgkin Lymphoma cells, and may potentially even act as a repressor.

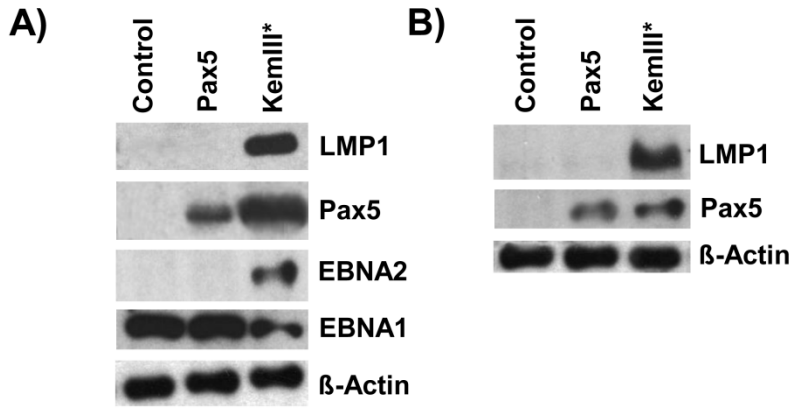

Figure III-5. Pax5 does not activate LMP1 expression in an EBV-superinfected Hodgkin's Lymphoma cell line with type I latency. (**A**) KMH2-Akata cells were infected with a lentiviral vector CDH713-Pax5, or the control vector. The cells were selected for 10 days using puromycin (0.5 μg/ml). Immunoblot analysis was performed to compare the levels of EBNA1, LMP1, Pax5, EBNA2, and β-Actin as loading control. * Indicates KemIII cells and endogenous levels of protein. (**B**) KMH2-Akata cells were transiently infected with a Pax5 expressing lentiviral vector, or the control vector. The cells were harvested 5 days after infection. Immunoblot analysis was performed to confirm the levels of LMP1, Pax5, and β-Actin. * Indicates KemIII cells and endogenous levels of protein.

Pax5 positively regulates LMP1 expression in type III latency BL cells

We next asked whether loss of Pax5 expression in a type III latency BL line affects LMP1 expression, as it was previously reported that loss of Pax5 in LCLs leads to an increase in LMP1 and LMP2 transcripts (although LMP protein levels were not examined) (Arvey et al., 2012). We infected KemIII cells with lentiviral vectors expressing two different control shRNAs or two different shRNAs directed against Pax5, selected for puromycin for 10 days, and then performed immunoblot analysis to examine the level of Pax5 and LMP1 expression. As shown in **Fig. III-6**, the expression of Pax5 was decreased in KemIII cells using either of two different shRNA constructs, and in each case loss of Pax5 was associated with a decrease in LMP1 protein expression. Additionally, loss of Pax5 in KemIII cells also decreased expression of the essential transforming EBNA2 viral protein. Therefore, the loss of LMP1 expression may also be due to the loss of EBNA2 expression, since EBNA2 is required for LMP1 expression in cells with type III latency. Pax5 may thus work in conjunction with EBNA2 to positively regulate LMP1 expression in type III latency.

Since Pax5 is required for B-cell identity and development, and regulates the B-cell gene expression program (Cobaleda et al., 2007; Holmes et al., 2008; Revilla et al., 2012), we also examined whether the loss of Pax5 in KemIII cells would affect the expression of certain B-cell specific factors that are known to bind the ED-L1 promoter and transactivate LMP1 with EBNA2. Interestingly, the loss of Pax5 in KemIII cells decreased expression of the cellular DNA-binding protein RBP-Jκ. RBP-Jκ binding to the ED-L1 promoter has been the most established mediator of EBNA2 activation (Grossman et al., 1994; Henkel et al., 1994; Johannsen et al., 1995), and is crucial for EBNA2-mediated transactivation of LMP1. Therefore,

since loss of Pax5 leads to a decrease in both EBNA2 and RBP-Jκ expression, it is not surprising that LMP1 expression is also decreased. However, the mechanism by which Pax5 regulates RBP-Jκ expression in BL cell lines remains to be determined.

Other factors such as PU.1, TBP, Sp1, EBF-1, and TCF12 bind the ED-L1 promoter and may also be involved in EBNA2 transactivation of LMP1 (Arvey et al., 2012; Johannsen et al., 1995; Sjoblom et al., 1995; Zhao et al., 2011). We found that upon loss of Pax5 expression, two of the known EBNA2 transactivators for LMP1 expression, PU.1 and EBF-1, were unaffected. Therefore, it is unlikely that Pax5 regulates LMP1 expression via the ED-L1 promoter through either of these two cellular DNA-binding proteins or through the loss of the B-cell transcriptional program.

Our results suggest that Pax5 may be a positive regulator of LMP1 expression during type III latency, contrary to what was previously reported by another group (Arvey et al., 2012), and that additional factors may work together with Pax5 to activate either the ED-L1 and/or L1-TR LMP1 promoters in order to drive LMP1 expression during type III latency. Therefore, Pax5 may help to promote type III EBV latency and B-cell transformation by acting as an activator of LMP1 expression.

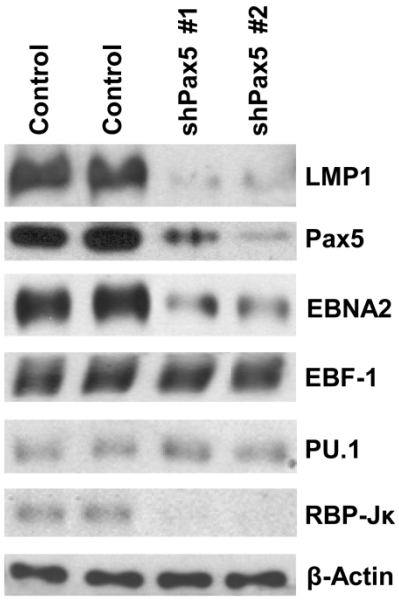

Figure III-6. Loss of endogenous Pax5 decreases LMP1, EBNA2, and RBP-Jκ expression in type III EBV-infected Burkitt Lymphoma cells. KemIII cells were infected with two different individual lentivirus vectors directed against Pax5, or control shRNAs. The cells were selected for 10 days using puromycin (0.5 μg/ml). Immunoblot analysis was performed to compare the levels of LMP1, Pax5, EBNA2, EBF-1, PU.1, and RBP-Jκ. ß-Actin was used as loading control.

DISCUSSION

In EBV-associated tumors, viral expression is limited to a small number of latency genes. EBNA1, which is essential for latent viral replication, is the only viral protein always expressed in EBV-positive cells. However, LMP1-expressing cells are more transforming than cells with type I latency, and thus, LMP1 is often expressed in EBV-positive tumors. The well-characterized ED-L1 promoter drives LMP1 synthesis in B cells with type III latency, whereas a second promoter (L1-TR), which is located upstream of ED-L1 inside the terminal repeat region of the EBV genome, directs LMP1 transcription (in the absence of EBNA2) in Hodgkin's Lymphoma and NPC tumors (Sadler and Raab-Traub, 1995; Tsai et al., 1999). Interestingly, L1-TR-initiated transcripts have also been detected in EBV-positive type I Burkitt cell lines treated with IL-6 (Chen et al., 2001). This indicates that the use of L1-TR promoter is not restricted to just epithelial cells. Despite the frequent detection of EBV in Hodgkin lymphoma, the underlying mechanisms which contribute to the development and maintenance of the transformed phenotype are not well understood (Baumforth et al., 2005). In addition, the expression of LMP1 and its regulation via B-cell specific transcription factors in EBV-infected Hodgkin lymphoma cells has not been well characterized. In this report, we investigated the role by which the B-cell specific transcription factor and master regulator, Pax5, might contribute to LMP1 regulation and expression during different types of latency in B cells.

A recent study used ChIP-seq analysis to show that Pax5 binds the viral terminal repeats directly at two sites in EBV-positive B-cell lines with type I and type III latency (Arvey et al., 2012). However, Pax5's functional role in regulating LMP1 expression via the two LMP1 promoters was not examined. Furthermore, this study showed that loss of Pax5 led to an increase

in LMP1 and LMP2a transcription in cells with type III latency. However, a confounding issue with this result is that the lytic (truncated) form of LMP1, which is upregulated during the lytic phase of viral replication (Ahsan et al., 2005; Baichwal and Sugden, 1987; Boos et al., 1987; Erickson and Martin, 1997; Hudson et al., 1985; Modrow and Wolf, 1986; Rowe et al., 1987a; Vazirabadi et al., 2003; Wang et al., 1988), may have been expressed. Since the loss of Pax5 can lead to lytic viral reactivation (Raver et al., 2013), the increase in LMP1 lytic transcripts could simply have reflected the lytic form of LMP1. Therefore, we sought to further investigate the effect of Pax5 on LMP1 expression.

In this report, analysis of four independent Pax5 ChIP-seq experiments in LCLs (mined from ENCODE data) identified multiple Pax5 binding peaks within the terminal repeats of the EBV genome (**Figure III-1**). Our analysis predicted three Pax5 binding sites that lie within the LMP1 L1-TRp, including one Pax5 binding site that was not previously identified or investigated. We report that Pax5 is able to bind to all three predicted Pax5 sites within the L1-TRp, whether methylated or unmethylated, by gel-shift assay *in vitro* (**Figure III-2**). We also used ChIP assay to examine whether Pax5 can bind to the L1-TRp *in vivo* in two Burkitt lines, KemI and KemIII. As shown in **Figure III-3**, Pax5 is able to bind to the L1-TRp in both type I and type III latency *in vivo*. However, the methylation status of the L1-TRp in both type I and type III latency has yet to be determined.

Upon confirming that Pax5 is able to bind *in vitro* and *in vivo* to the L1-TRp, we next sought to determine whether Pax5 is able to activate the L1-TRp in its methylated or unmethylated forms. Using reporter gene assays, we found that Pax5 activates the L1-TRp in its unmethylated form, but is inhibited in its ability to activate the methylated form in EBV-negative

cells (**Figure III-4**). Since Pax5 is deficient in activating the L1-TRp in the methylated form, we hypothesize that methylation may prevent Pax5's ability to turn on LMP1 *in vivo*.

To investigate whether loss of Pax5 also regulates LMP1 expression in HL, we utilized an HL line (KMH2-Akata) that has type I latency, and does not express Pax5. The impact of EBV infection on the growth and survival of this KMH2-Akata HL-derived cell-line has previously been characterized, and EBV infection has been reported to increase cellular proliferation compared to the EBV-negative parental cells (Baumforth et al., 2005). EBV-infected KMH2 HL cells have been shown to display a pattern of virus gene expression typical of type I latency, including Qp-driven EBNA1, EBERs, and the *Bam*H1A BART transcripts (Baumforth et al., 2005; Kis et al., 2005). Our findings show in **Figure III-5** that either long-term or short-term forced expression of Pax5 in KMH2-Akata HL cells does not induce LMP1 expression. Since Pax5 is unable to efficiently activate the methylated form of the L1-TR promoter in reporter gene assays (**Fig. III-4**), our results suggest that one or both LMP1 promoters may be methylated in these cells, and that this methylation acts to inhibit Pax5's ability to turn on LMP1 *in vivo*. Alternatively, Pax5 may only turn on LMP1 expression in the context of the intact viral genome in conjunction with EBNA2.

As shown in **Figure III-6**, Pax5 may be a positive regulator of LMP1 expression during type III latency, since loss of Pax5 in KemIII cells led to a decrease in LMP1 expression. This effect may also be mediated through effects of Pax5 on EBNA2 and RBP-Jκ expression. Interestingly, since Pax5 is able to activate the L1-TR promoter in its unmethylated form (**Fig. III-4**), it is quite possible that Pax5 may help to drive LMP1 expression via this promoter early during primary EBV infection prior to methylation of the viral genome. If so, this would be an

additional mechanism by which Pax5 promotes EBV latency in B cells. Additionally, as the EBV genome becomes more methylated following primary infection and progress towards type I latency, LMP1 expression is downregulated, while Pax5 is still present in order to maintain B-cell identity (Raver et al., 2013). Moreover, since genome-wide approaches have revealed that Pax5 can regulate expression of genes when bound to binding elements located thousands of base pairs away from the transcriptional start sites (McManus et al., 2011; Revilla et al., 2012), we think it is likely that Pax5 bound to the EBV TRs regulates both the ED-L1 and L1-TR promoters. Additionally, Pax5 may cooperate with EBNA2 to activate the ED-L1 promoter and drive LMP1 expression. However, whether Pax5 acts as a positive regulator of LMP1 expression via the ED-L1 and/or L1-TR promoter early during type III latency remains to be determined. We conclude that Pax5 may potentially act as either a positive regulator of LMP1, perhaps dependent on latency type and promoter methylation status. The results from our studies propose a new idea and mechanism by which Pax5 may help to further promote and drive EBV latency.

We conclude that Pax5 is able to promote EBV latency by multiple mechanisms, including activating the essential latency promoter, Wp (Tierney et al., 2007), inhibiting Z function (Raver et al., 2013), and as shown in this report, through positively regulating LMP1 expression in cells with type III latency. Here we provide a possible mechanism for which Pax5 may help promote EBV latency by activating the unmethylated form of the terminal repeat promoter, which may be used to drive LMP1 expression during primary EBV infection. In addition, Pax5 bound to the TRs may also enhance EBNA2-mediated activation of the ED-L1 promoter. These results suggest that EBV has established multiple different mechanisms to ensure that it can efficiently enter latency in B cells.

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Chapter IV

General Discussion

This work has focused on novel mechanisms by which Pax5 promotes B-cell specific viral latency by inhibiting Z transcriptional function and DNA-binding activity, thereby preventing viral reactivation. Furthermore, Pax5 binds to the terminal repeat region of the EBV genome and may positively regulate expression of the major EBV oncogene, LMP1, during type III latency. In this chapter, I will further discuss this work and how it relates to the EBV field at large, and address future directions and questions that remain to be answered.

Promotion of EBV Latency in B cells by Pax5 and other Cellular Factors

As discussed in detail in **Chapter I**, the establishment of latency is important for EBV-positive malignancies to develop, and involves several different EBV proteins. However, the virus must also reactivate to the lytic form of infection in order to infect other hosts. The viral immediate-early (IE) protein, BZLF1 (Z), is a transcription factor that mediates the switch from latent to lytic infection, and binds to and activates the promoters of early EBV genes (Chevallier-Greco et al., 1986; Cox et al., 1990; Giot et al., 1991; Holley-Guthrie et al., 1990; Kenney et al., 1989; Quinlivan et al., 1993). Expression of the IE Z protein is sufficient to induce activation of the entire lytic gene cascade in most EBV-positive cell lines. Since EBV is latent in some cells and is able to undergo lytic reactivation in others, regulation of the Z promoter and/or protein is presumably different between latent and lytic cells. Cellular factors that activate Z gene expression and/or function may allow for the virus to leave the latency program and reactivate. On the other hand, cellular factors that prevent Z gene expression and/or function presumably promote viral latency. In this thesis, I have characterized Pax5 as a factor that promotes viral

latency in B cells by inhibiting Z function via a direct protein-protein interaction (**Figure IV-1**). Previous work by another graduate student in our lab (Amanda R. Panfil) identified Oct-2 as another such B-cell specific factor. Additionally, the Mertz group and others have shown that the ZEB1 and/or ZEB2 proteins inhibit Z promoter activity in B cells (Ellis et al., 2010; Feng et al., 2007; Kraus et al., 2003; Yu et al., 2007), and a cellular transcription factor Ikaros (which is abundantly present in B cells) also contributes to the maintenance of EBV latency in B cells by directly interacting with the R protein (unpublished data, Tawin Iempreedee). Therefore, EBV has multiple mechanisms for retaining viral latency in B cells.

Furthermore, Pax5 and/or Oct-2 may also promote viral latency *in vivo* by inhibiting Z function. Recent work from our lab utilizing a humanized mouse model to study EBV infection of B cells has confirmed that activation of Z expression in B cells does not always lead to early lytic protein expression (Ma et al., 2012). In this paper, the infection of B cells with a "superlytic" (SL) virus (in which the ZEB sites in the Zp were deleted) led to high levels of Z protein expression, but very little early lytic viral proteins and essentially no late viral proteins, and thus resulted in an abortive lytic form of EBV infection. Thus, EBV lytic replication can be inhibited in B cells even when Z expression is very high. Since most Z-expressing B cells do not express early or late lytic viral proteins in this mouse model, this further supports the idea that Pax5 and/or Oct-2 inhibit Z transcriptional function *in vivo*. Since the SL mutant can establish a long-term viral latency in some mice, this suggests that the activation of Z transcription requires not only the inhibition of negative regulators, but also the presence of positive regulators. Future studies could determine if cellular expression of early lytic EBV proteins in the humanized mouse model is inversely correlated with Oct-2 and/or Pax5 expression.

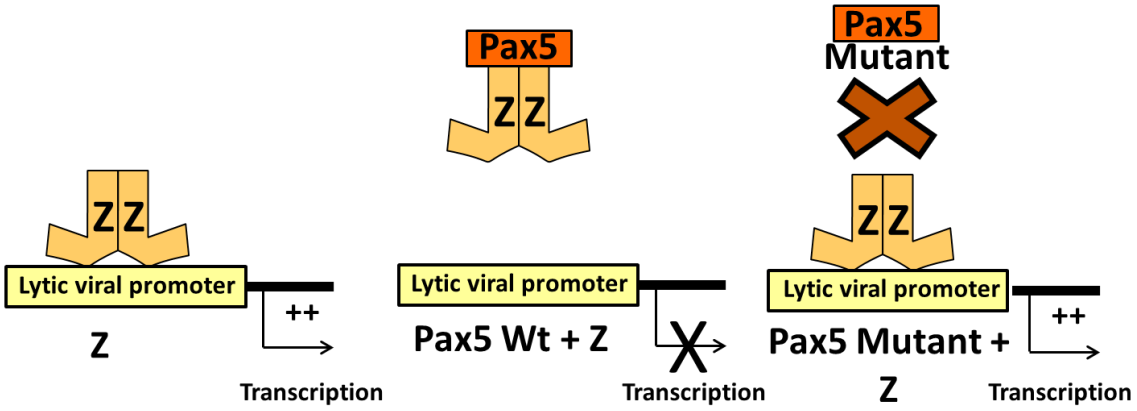

Figure IV-1. Pax5 prevents lytic reactivation in B cells and inhibits Z function via a direct protein-protein interaction. (Left) Z alone (in the absence of Pax5) is able to bind to early lytic viral promoters and activate their transcription. (Middle) Pax5 promotes EBV latency by interacting with Z and inhibiting Z DNA-binding to early lytic viral promoters. (Right) Disruption of the Pax5/Z protein-protein interaction (using a Pax5 mutant that is deficient for interaction with Z) prevents Pax5's ability to inhibit Z DNA-binding and transcriptional activation of early lytic viral promoters.

Inhibition of Pax5 expression by various physiological stimuli may contribute to EBV reactivation in B cells

As discussed in **Chapter II**, Pax5 expression is lost during plasma cell differentiation. This effect is due to Blimp-1, which binds to the Pax5 promoter and inhibits Pax5 function in plasma cells. Another study suggests there is yet another correlation between the level of Pax5 expression and the stringency of EBV latency. In this study, cellular genes shown to be overexpressed in non-permissive (latent) LCLs include EBF-1 and Pax5, whereas overexpressed genes in permissive (high level of EBV reactivation) LCLs include XBP-1 and Blimp-1 (which promote plasma cell differentiation) (Davies et al., 2010). Therefore, this further supports the idea that Pax5 helps to maintain latency in B cells and that loss of Pax5 leads to plasma cell differentiation and viral reactivation.

Since Pax5 function is known to decrease during plasma cell differentiation, we asked whether other lytic inducing stimuli also lead to loss of Pax5. As shown in **Figure IV-2**, treatment of a Type I BL line (MutuI) with TGF-β led to EBV reactivation and loss of Pax5 (unpublished data). This result is similar to what was shown in **Chapter II**, where treatment of Raji cells with TPA and sodium butyrate strikingly decreased Pax5 protein levels, while BMRF1 (EAD) and Z increased. As shown in **Chapter II**, this effect is mediated at the level of Pax5 RNA. However, the precise cellular transcription factors that are responsible for these effects are unknown. It is also possible that these agents decrease Pax5 RNA stability rather than its transcription. Therefore, future studies could examine this further to determine if lytic agents affect Pax5 by similar or different mechanisms.

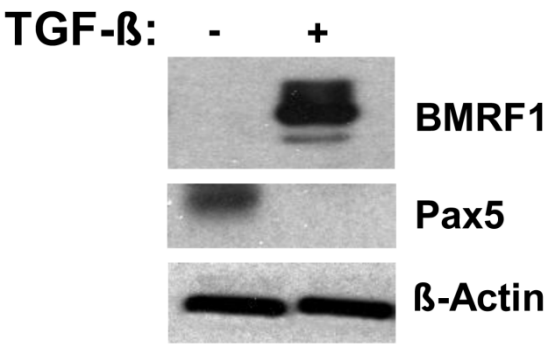

Figure IV-2. **The lytic inducing agent, TGF-β, decreases endogenous Pax5 expression**. MutuI cells were lytically induced with TGF-β (5 μg/ml), and immunoblot analysis was performed two days later to compare levels of endogenous Pax5, lytic viral protein BMRF1 (EAD), and β-Actin as a loading control.

The EBV latent protein, LMP1, promotes EBV latency

As discussed in this chapter, there are numerous cellular factors which help to promote viral latency in B cells. However, there are also viral factors which help to promote viral latency in B cells. Interestingly, LMP1 has been shown to inhibit lytic viral reactivation *in vitro* (Adler et al., 2002; Miller et al., 1994; Miller et al., 1993; Prince et al., 2003). Since LMP1 has been shown to inhibit ATM transcription, as well as p53 function (Hagemeier et al., 2012; Ma et al., 2012), this suggests that LMP1 may inhibit lytic EBV reactivation through these mechanisms. Additionally, since our lab previously showed that NF-κB directly interacts with Z and inhibits its function (Gutsch et al., 1994; Morrison and Kenney, 2004), LMP1 could inhibit Z through its well-known ability to activate NF-κB (Johansson et al., 2009; Luftig et al., 2003; Saito et al., 2003). Additionally, since LMP1 has been shown to downregulate Blimp-1α, and disrupt the plasma cell differentiation program, this could be yet another mechanism by which LMP1 prevents viral replication in B cells (Vrzalikova et al., 2011).

Viral factor LMP2A has complex effects on viral reactivation

The LMP2A viral protein, which mimics a partially activated BCR, has complex effects on viral reactivation. On the other hand, LMP2A has been shown to maintain latency by blocking high level IgG-mediated stimulation of the BCR from inducing lytic reactivation (Fukuda and Longnecker, 2005). Nevertheless, since LMP2A induces low level constitutive BCR-like signaling required for B-cell survival known as “tonic” BCR activation (Portis et al., 2004; Portis and Longnecker, 2004a, b), expression of LMP2A in type I BL cells actually

enhances lytic reactivation (Engels et al., 2012; Schaadt et al., 2005). Interestingly, LMP2A has been shown to decrease E2A and Pax5 expression in B cells from LMP2A transgenic mice (Portis and Longnecker, 2003). LMP2A expression also correlates with permissivity of spontaneous lytic reactivation in LCLs (type III latency) and low Pax5 expression (Davies et al., 2010). Therefore, this indicates that EBV may utilize LMP2A in LCLs to negatively regulate Pax5 and activate the Z promoter during plasma cell differentiation and spontaneous lytic reactivation.

Maintenance of EBV latency in Hodgkin's Lymphoma by viral and cellular factors in the absence of Pax5

Since Pax5 promotes EBV latency in B cells, but HL cells do not express Pax5, what protects Hodgkin's lymphoma cells from entry into the EBV lytic cycle in the absence of Pax5? Surely, there must be additional cellular and/or viral factors in B cells that act to keep the virus latent. Hodgkin's lymphoma is unusual among B-cell lymphomas in that these cells (known as Hodgkin/Reed-Sternberg or HRS cells) lack not only a functional B-cell receptor, but also show loss of expression of BCR signaling molecules such as Syk, Lyn, and SLP65, as well as the B-cell specific transcription factor OBF-1 (Schwering et al., 2003; Stein et al., 2001).

One recent study showed that a cellular defect may contribute to HL pathogenesis and help to promote EBV latency. This particular study showed that LMP2A-expressing HRS cells are defective for inducing EGR1 (early growth factor 1) (Vockerodt et al., 2013). The host gene EGR1 encodes an essential transcription factor which is induced following EBV lytic

reactivation, by Z, where Z binds to the EGR1 promoter and activates its expression (Chang et al., 2006; Heather et al., 2009; Zalani et al., 1995). EGR1 also binds to Rp at two sites and activates its transcription (Zalani et al., 1995). These two EGR1 binding sites are required for activation of the Rp by several EBV lytic inducing agents, suggesting that EGR1 may play an important role in the regulation of viral latency (McMahon and Monroe, 1995; Zalani et al., 1995). Furthermore, EGR1 expression is upregulated by LMP2A and BCR signaling in BCR-competent B cells followed by induction of Z expression (Vockerodt et al., 2013). Additional studies suggest that EGR1 is upregulated by LMP2A in a BL mouse model (Bieging et al., 2011). Although HRS cells have high levels of LMP2A expression necessary for BCR-like survival signals, the expression of Z is rarely seen in EBV-positive HRS cells, which indicates LMP2A does not commonly activate the lytic cycle in this cell type (Brauninger et al., 2006; Hamilton-Dutoit and Pallesen, 1992; Pallesen et al., 1991; Vockerodt et al., 2013). Therefore, one mechanism by which HL cells could maintain viral latency in the absence of Pax5 may be through their inability to upregulate LMP2A-mediated EGR1 expression, which in turn prevents entry into the viral lytic cycle.

Additionally, cellular factors such as STAT3 and NF-κB may play a role in helping to maintain latency in HL. One study showed that upregulation of STAT3 in Burkitt cells prevented lytic reactivation and is correlated with EBV latency (Daigle et al., 2010). Interestingly, HL express high, constitutive levels of STAT3 (Chen et al., 2001; Kube et al., 2001; Kuppers, 2009; Skinnider et al., 2002), which is correlated with LMP1 expression (Chen et al., 2003). HL cells also express high, constitutive levels of the NF-κB transcription factor, which is a hallmark of HL (Bargou et al., 1997; Bargou et al., 1996; Gruss et al., 1997; Hinz et al., 2001; Jost and

Ruland, 2007; Krappmann et al., 1999; Kuppers, 2009; Nishikori, 2005). Inhibition of NF-κB results in apoptosis in HRS cells indicating that NF-κB is essential for HL survival and resistance to apoptosis (Izban et al., 2001; Mathas et al., 2003). Since NF-κB inhibits Z transcriptional function (Brown et al., 2003; Gutsch et al., 1994; Morrison and Kenney, 2004), it may be another mechanism of maintaining B-cell-specific viral latency in HL in the absence of Pax5. These studies suggest that although Pax5 is not present in these cells, there are multiple factors and possible B-cell environment defects which help EBV maintain latency in HL.

Maintenance of EBV Latency in Epithelial Cells by Cellular Transcription Factors in absence of Pax5

EBV tends to reside as a latent infection in B cells, and undergoes lytic infection during plasma cell differentiation as well as in normal differentiated oropharyngeal epithelial cells. Our lab has therefore hypothesized that cellular transcription factors which are specific to epithelial, plasma cell, or B-cell environments may provide a key to understanding why EBV preferentially establishes latency in B cells and undergo lytic infection in differentiated epithelial cells and plasma cells. One question that arises is how does EBV maintain latency in NPC or epithelial cell tumors where Pax5 is not present? Could there be other cellular factors or contributing factors that help promote and maintain EBV latency in epithelial cells?

One possibility is that ZEB1 and/or ZEB2, which inhibit Z promoter activity, promote viral latency in epithelial cells (Ellis et al., 2010; Kraus et al., 2003). The latent to lytic switch in epithelial cells has been difficult to study due to the lack of normal (non-transformed),

undifferentiated epithelial cells which can be latently infected with EBV. EBV infection of most transformed human epithelial cells (with exception of the gastric carcinoma line AGS Akata) *in vitro* leads to the establishment of cells containing the latent form of EBV (Feng et al., 2007; Raab-Traub, 2005). EBV infection of normal oropharyngeal epithelial cells occurs in the differentiated layers, and leads to lytic infection (Lau et al., 1993; Li et al., 1992; Niedobitek et al., 1991; Young et al., 1991), whereas undifferentiated NPCs are commonly associated with latent EBV infection (Hording et al., 1993; Raab-Traub, 2005). The establishment of a predominantly latent form of EBV infection in NPC tumors helps to provide a selective growth advantage and ensure survival of the virus in tumor cells. One recent study demonstrated that the dysregulation of cyclin D1 expression may contribute to NPC pathogenesis by enabling persistent infection of EBV, through promoting cell-cycle progression and suppression of terminal differentiation (Tsang et al., 2012). The suppression of Zp and Rp transcription is required for the maintenance of EBV latent infection in undifferentiated epithelial cells such as in NPC. However, the mechanisms which regulate EBV infection during the early stages of this disease, and cellular factors which help maintain viral latency in epithelial tumor cells are not well-characterized.

A graduate student in our lab, Dhananjay Nawandar, recently found that ΔNp63 may also play a role in inhibiting EBV reactivation in telomerase-immortalized normal oral keratinocytes (NOKs) (unpublished data). P63 is considered the master regulator of stratified epithelial development (Mills et al., 1999; Yang et al., 1999), and the loss of p63 leads to loss of epidermis and other epithelia in humans and mice (Zhang et al., 2013). ΔNp63 is also used as a marker for undifferentiated basal epithelial cells (Signoretti et al., 2000; Xin et al., 2007), is required for

normal development of several epithelial tissues (Mills et al., 1999; Truong et al., 2006; Yang et al., 1999), and is highly expressed in NPCs (Crook et al., 2000). The expression of ΔNp63 is also lost upon differentiation of epithelial cells (Crook et al., 2000). Our lab has demonstrated that knockdown of ΔNp63 in EBV-positive NOKs cells triggers lytic EBV gene expression, and that overexpression of ΔNp63 in EBV-positive AGS cells (which lack p63 expression) inhibits Z-mediated and R-mediated lytic gene reactivation (unpublished data). Although it is still not totally clear how EBV maintains latency in NPC, high level expression of ΔNp63 in this tumor may play a role in promoting viral latency. Therefore, whereas Pax5 promotes latency in a B-cell specific manner, ΔN-p63 may be transcription factor that helps to promote latency in an epithelial cell specific manner.

Z Negatively Regulates Pax5 Function

EBV has multiple mechanisms for retaining latency; however, downregulation of certain cellular factors, like Pax5, is essential in order to undergo plasma cell differentiation and efficiently reactivate from the latency program. The EBV Z protein directly interacts with several cellular proteins via direct protein-protein interactions and in some cases, disrupts their function, including p53 and NF-κB (Adamson and Kenney, 1999; Gutsch et al., 1994; Mauser et al., 2002). The Adamson group was the first to demonstrate that Z physically associates with Pax5 *in vivo*, and disrupts Pax5's transcriptional activity and function. The ability of Z to inhibit Pax5 activity may be important in helping Z to promote lytic replication in newly infected B cells, since it may prevent Pax5's ability to activate the Wp (EBNA2 promoter) during primary

infection. Although Z inhibits Pax5-mediated transactivation, the mechanism to this effect is still unclear. The previous report did not find that Z inhibits Pax5's ability to bind to DNA, as we have also confirmed by ChIP assay (unpublished data). However, the ability of Z to inhibit activation of B-cell specific target genes by Pax5, as well as the Wp, have not been investigated in the context of lytically infected B cells.

Future work will need to identify the mechanisms by which Z inhibits Pax5 function and determine if this is biologically relevant during EBV infection of B cells. Since long term Z expression is toxic to B cells, a double-stable cell line with inducible Z expression (that is physiologically relevant during normal EBV infection) could be utilized to determine if Z decreases expression of known Pax5 responsive genes. Using a commercially available Tet-On gene expression system, where Z is turned on in the presence of Doxycycline (Dox), stable transfection of a Z-inducible expression vector in MutuI, MutuIII, and Mutu(-) BL cell lines could be obtained. Following this, cells could be harvested to examine the effect of Z on Pax5 responsive genes, such as CD19, Blnk, or Ebf1 by western blot analysis. Alternatively, a B-cell cDNA microarray analysis could be performed to compare gene expression in the various BL lines in the presence of absence of Z. There are currently over 100 Pax5-activated target genes that have been identified by cDNA microarray in mature B cells (Pridans et al., 2008). Overall, these experiments would help to determine if Z affects Pax5 function in B cells by decreasing expression of B-cell specific genes (either at the protein or RNA level). Ultimately, the effect that Z has on Pax5 could correlate with the ability of Z to inhibit viral latency, since Pax5 is known to activate the essential Wp viral latent promoter and the expression of critical B-cell proteins required for B-cell identity. Furthermore, future studies could determine if the

interaction between Z and Pax5 is required for inhibition, and whether Z is complexed to promoters bound by Pax5 (by ChIP assay).

The Adamson group also showed that in HeLa cells, Z expression seems to stabilize Pax5 protein levels, since these cells have higher levels of Pax5 protein in the presence of Z (Adamson et al., 2005). In addition, this group showed that Pax5 colocalizes with Z on these host chromosomes during mitosis, but Pax5 is not localized to mitotic chromosomes when expressed alone (Adamson, 2005). I have performed preliminary experiments to determine if Z affects Pax5's localization in cells. Since a direct protein-protein interaction is required for the Pax5 inhibitory effect on Z, I also compared the ability of Z to re-localize wild-type Pax5 (and Pax5 DM V26G/P80R) versus a mutant Pax5 (Δ106-110) that does not interact with Z (as indicated in **Chapter II**). Pax5 has been shown to associate heavily with the nuclear matrix, and since both Pax5 mutants are nuclear and are stable, this allowed us to ask if this association in the nucleus changes in the absence or presence of Z. Both Pax5 mutants were investigated in comparison to Pax5 WT, by cotransfecting Pax5-YFP expression plasmids (YFP-Pax5- WT, V26G/P80R, and Δ106-110) in the presence or absence of Z.

As shown in **Figure IV-3**, our preliminary results indicate that Pax5 has distinct nuclear localization when co-expressed with Z in comparison to Pax5 alone. These results are contrary to what was reported by the Adamson group, who showed no difference in Pax5/Z nuclear localization compared to Pax5 alone (Adamson, 2005; Adamson et al., 2005). Specifically, we found that both Pax5 WT and the Pax5 V26G/P80R mutant are diffusely localized in the nucleus in the absence of Z, but are localized to nuclear dots in the presence of Z. Each HeLa cell varies in number of these structures, having up to six dots in the nuclei. This indicates that Z may

change Pax5 nuclear localization and this may functionally contribute to lytic reactivation. Furthermore, the Pax5 Δ106-110 mutant, which is inhibited for its ability to interact with Z, has a strikingly different nuclear localization pattern in the presence of Z. Instead of having very bright, small nuclear dots, Pax5 Δ106-110 has more of a dense intermediate and large size speckling, with widespread localization throughout the nucleus. I hypothesize that the ability of Z to alter Pax5 localization may play an important role in Z's ability to inhibit Pax5 function. Additionally, we found that Pax5 localizes to mitotic chromosomes in the absence of Z (data not shown). Future experiments such as immunofluorescence need to be done to confirm the identity of these nuclear dot structures; for example it would be interesting to determine if Pax5 is being shuttled by Z into PML bodies.

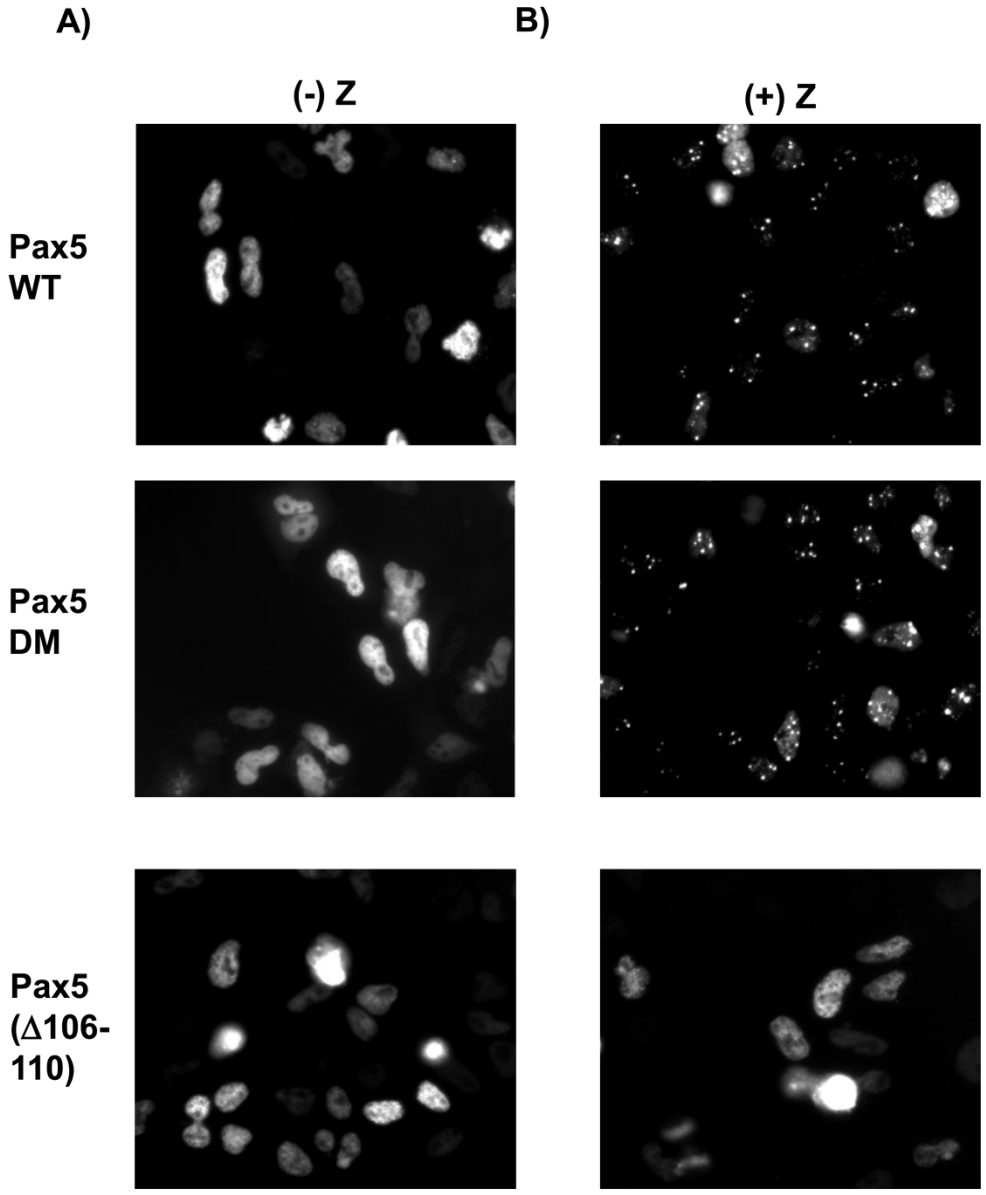

Figure IV-3. Z changes the localization of Pax5 in HeLa cells. (A) HeLa cells were transfected with Pax5-YFP WT (top), DM V26G/P80R (middle), or Δ106-110 (bottom) in the absence of Z (- Z) shown in Panel A, or presence of Z (+ Z) shown in Panel B. YFP protein was then visualized by confocal microscopy at 20X.

Does Pax5 Binding to the Terminal Repeats Regulate EBV latency?

A previous report showed that Pax5 promotes type III latency in B cells by binding to the essential EBV latency promoter, Wp, which drives expression of EBNA2 and EBNA-LP during primary infection (Tierney et al., 2007). Mutation of Pax5 binding sites within the Wp in the context of the viral genome completely inactivates the transforming function of EBV B cells and abrogates EBNA-LP, EBNA2, and LMP1 expression (Tierney et al., 2007). Plasmacytic differentiation of LCLs has been linked to suppression of Wp/Cp activity (Crawford and Ando, 1986; Rochford et al., 1993), and both Cp and Wp are active to some extent in low-passage LCLs (Elliott et al., 2004). Latency in LCLs is also correlated with over-expression of Pax5 (which positively regulates Wp activity) (Davies et al., 2010). Thus, the Wp/Pax5 connection is important for understanding how EBV exploits Pax5 to achieve B-cell specificity of its growth-transforming program and promote type III latency.

We hypothesized that Pax5 may also regulate LMP1 expression through its binding to the viral terminal repeats. Our data from **Chapter III** suggest that Pax5 may act as a positive regulator of LMP1 during type III latency (when both promoters are unmethylated), since the loss of Pax5 in KemIII BL cells led to a decrease in LMP1 expression, and Pax5 preferentially activates the unmethylated form of the terminal repeat promoter. Thus, these results suggest there is an additional mechanism by which Pax5 may help to promote viral latency and drive B-cell transformation during primary infection. However, when viral infection converts to type I infection, the presence of Pax5 in B cells no longer acts to induce LMP1 expression, and could potentially even repress it, further promoting the establishment of long-term latency of EBV.

Expression of LMP1 in HL in absence of Pax5

A second paradox in HL lines is the question of how HL cells express LMP1 *in vivo* in the absence of Pax5, since our results suggest Pax5 is a positive regulator of LMP1. In the case of HL, there are multiple possible mechanisms (and constitutively expressed cellular factors) by which LMP1 promoter activity and expression is increased independent of EBNA2.

As mentioned previously, HL often express high levels of STATs, including STAT3. Interestingly, although STATs have been shown to inhibit viral reactivation, they have been shown to induce activation of the LMP1 promoter and protein. STATs bind to both LMP1 promoters, and STAT3 positively regulates the L1-TR promoter (Chen et al., 2001). L1-TR promoter activity is also completely abolished in the presence of a dominant negative STAT3 inhibitor (STAT3β) (Chen et al., 2001). Furthermore, expression of endogenous LMP1 mRNA and protein is upregulated in cells transfected with a constitutively activated STAT3 (Chen et al., 2003). Since LMP1 also activates STAT3 (Chen et al., 2003; Shair et al., 2007), these results suggest that there is a positive autoregulatory loop between STAT3 activation and LMP1 expression in HL. Therefore, the high levels of STATs that occurs in HL may actually serve as an important activator of type II latency.

Furthermore, as previously discussed, a hallmark of HL line and tumors is that they express high levels of NF-κB, which inhibits viral reactivation. The expression of NF-κB in HL may also play an important role in activating LMP1 expression in HL. The NF-κB transcription factors p50-p65 and p50-p50 can bind to and activate the LMP1 ED-L1 promoter *in vitro* and *in vivo*, and exogenous expression of NF-κB induces LMP1 protein expression in BL cells

(Johansson et al., 2009). LMP1 also activates NF-κB signaling and expression (Lavorgna and Harhaj, 2012; Shair et al., 2007; Soni et al., 2007; Thornburg et al., 2006), which indicates that there is a positive regulatory circuit between NF-κB activation and LMP1 expression in HL (Johansson et al., 2009).

Nearby cytokines may also further amplify the positive effect on LMP1 in HL. For example, NF-κB (a known activator of LMP1) is induced by various cytokines *in vivo.* CD40 is a cytokine receptor, and upon ligation activates NF-κB and the cytokine IL-6 (Annunziata et al., 2000; Eliopoulos et al., 1997; Hsing et al., 1997). The activation of NF-κB and IL-6 by CD40 may then act to turn on the LMP1 promoters. IL-6 (also an activator of STAT3) is a known activator of the L1-TR promoter (Chen et al., 2001). Following LMP1 promoter activation, expression of the LMP1 protein further serves to activate NF-κB, which ensures its expression is continued (Johansson et al., 2009). An additional cytokine, IL-4, has also been shown to increase LMP1 expression (Kis et al., 2005). Therefore, there are multiple mechanisms by which LMP1 is turned on and positively regulated, and it is not particularly surprising that LMP1 could be induced in HL in the absence of Pax5.

Potential future experiments to investigate the role of Pax5 in regulating LMP1 expression

In order to determine if Pax5 is a positive or negative regulator of LMP1 expression in different types of EBV-infected B cells, more studies need to be performed in KMH2-Akata HL cells. This EBV-positive KMH2-Akata HL line is Pax5- and LMP1-negative, and has type I latency (Baumforth et al., 2005). These cells could be transfected with Pax5 alone, Pax5 with

EBNA2, or EBNA2 alone, and in the presence or absence of a demethylating agent (5-azacitidine), to determine if Pax5 and/or EBNA2 are able to turn on LMP1 and/or LMP2A expression when the LMP1 promoters are methylated or unmethylated. These studies would help to determine if Pax5 ever acts as a negative regulator of LMP1 expression, depending upon the promoter methylation status and latency type. Furthermore, since the loss of Pax5 in KemIII cells also leads to a decrease in RBP-Jκ expression (**Figure III-6**), future studies could examine whether this occurs in an EBV-dependent manner utilizing EBV-negative and EBV-positive BL lines. Also, it would be interesting to examine the effect of restoring RBP-Jκ expression in Pax5-negative cells on LMP1 expression. Since RBP-Jκ is one of the main EBNA2 transactivators of the LMP1 promoter, restoration may counteract the Pax5 effect.

Additional experiments could also determine the methylation status of the L1-TR promoter in various EBV-positive cell lines, since the L1-TR methylation status has not been well studied in cells with various different latency types. Additionally, it is not clear whether either of the LMP1 promoters are fully or partially methylated in Hodgkin's Lymphoma. Bisulfite sequencing could be performed in order to detect methylated cytosine (MeC) and determine the methylation status of the LMP1 promoters. This information would help us to determine whether Pax5 and/or EBNA2 regulation of LMP1 expression is dependent on promoter methylation status and/or latency type.

An important unanswered question is whether Pax5 is able to activate the ED-L1 promoter in the context of the viral genome (alone or in combination with EBNA2). Although we were not able to clone a full length ED-L1 LMP1 promoter construct containing the upstream Pax5 binding sites (due to its rich GC content), if this were possible in the future, ED-L1

activation by Pax5 and/or EBNA2 could be examined by reporter gene assays. In addition, future studies could examine which LMP1 promoter is used during primary EBV infection. RT-PCR could be done at different time points following B-cell infection. Since the ED-L1 transcript has a 2.8 kb mRNA, and the L1-TR transcript has a transcript size of 3.5 kb, northern blot analysis could also determine which promoter is being utilized and if LMP1 promoter usage switches as EBV infection progresses.

If the L1-TRp is found to be used during primary infection, and the crucial Pax5 site(s) for L1-TRp activation is identified by site-directed mutagenesis of each site and reporter gene assays, recombinant EBVs utilizing the EBV bacterial artificial chromosome (BAC) cloning system could also be created containing mutated Pax5 binding site(s). Since LMP1 is essential for EBV-driven B-cell immortalization, and Pax5 may be required for activation of the L1-TR promoter and subsequent LMP1 expression during primary infection, cell proliferation assays would be expected to show inhibited outgrowth of transformed cells (LCLs). This result was seen when the Pax5 binding sites were deleted or mutated within the Wp, as there was no outgrowth of transformed cells (Tierney et al., 2007). Overall, these experiments may help to determine another significant mechanism by which Pax5 promotes EBV latency.

Lytic Induction Therapy

Almost all cells in an EBV-positive tumor contain the virus. Therefore, activating the lytic form of gene expression in EBV-positive tumors in order to induce EBV-dependent killing, may help to treat these tumors. Stemming from this idea, “lytic induction therapy” seeks to

identify drugs or other treatments that can induce lytic infection in EBV-positive tumors, without undue toxicity to uninfected cells (Feng et al., 2002; Israel and Kenney, 2003; Meng et al., 2010; Westphal et al., 2000; Westphal et al., 1999). There are two different virally encoded kinases, EBV thymidine kinase (EBV-TK; BXLF1 gene product) and EBV protein kinase (EBV-PK; BGLF4 gene product) (Chang et al., 2012; Feederle et al., 2009; Feng et al., 2004; Meng et al., 2010), that are expressed in lytically (but not latently) infected tumor cells. These kinases are able to convert nucleoside analogues such as ganciclovir (GCV) and FIAU into cytotoxic drugs that induce death of EBV-positive tumor cells, and also inhibit the release of virus (Meng et al., 2010). Additionally, the phosphorylated or activated form of GCV is also able to induce "bystander killing" where nearby tumor cells are also killed.

Identifying cellular factors which trigger the latent-to-lytic switch of EBV may be important for treating EBV-positive tumors, since targeting these factors could potentially be useful in lytic induction therapy. Since the loss of Pax5 (such as by treatment with certain lytic inducing agents like HDAC inhibitors) triggers EBV reactivation in B cells, this may have a therapeutic role. The use of HDAC inhibitors may prove useful in triggering the lytic form of EBV infection in patients. HDAC inhibitors reactivate EBV via multiple mechanisms not only by inhibiting Pax5 and Oct-2 expression, but also through increasing acetylation of viral chromatin (Jenkins et al., 2000) and activating ATM and p53 function (Jang et al., 2010). Therefore, FDA-approved HDAC inhibitors such as Vorinostat could be useful for inhibiting Pax5 (or Oct-2) expression. Future studies could further investigate the role of HDAC inhibitors, when combined with GCV, in the treatment of EBV-positive B-cell tumors *in vivo*.

Concluding Remarks

EBV has established multiple different mechanisms in order to establish and maintain latency in B cells. Pax5 is intricately tied to the EBV life cycle, is required for the establishment of long term latent infection, and helps to maintain EBV latency by blocking Z function. On the other hand, Z also inhibits Pax5 function, which helps to ensure that the virus can sporadically undergo lytic reactivation and infect other host cells. Furthermore, an additional mechanism by which Pax5 may also help to establish and maintain latency is by regulation of LMP expression in type III latency. Thus, EBV efficiently utilizes multiple redundant mechanisms to establish and promote viral latency, followed by the exit from the latency program, in order to infect the host for life.

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