Contributions of Epstein-Barr Nuclear Antigen 1 (EBNA1) to Epithelial Cell Infections

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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Doctor of Philosophy

Department of Molecular Genetics
University of Toronto
2011

Abstract

Epstein-Barr virus (EBV) latent infection is associated with lymphoid and epithelial tumours, including nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). Since EBNA1 protein is expressed in all EBV tumours, I explored whether EBNA1 alters the cellular environment in ways that would contribute to the development of these epithelial tumours. I have shown that EBNA1 disrupts nuclear bodies (NBs) formed by the PML tumor suppressor and degrades PML proteins in a proteasome dependent manner in NPC and GC cell lines. I have verified the role of EBNA1 in disrupting PML NBs through overexpression and silencing of EBNA1 and shown that EBNA1 alone is sufficient to mediate these effects. Using EBNA1 mutants I found that USP7 and protein kinase CK2 (two enzymes that negatively regulate PML NBs) are important for EBNA1-mediated disruption of PML NBs.

Furthermore, I have shown that EBNA1 localizes to PML NBs, and interacts with PML IV, which mediates the enrichment of USP7 and CK2β with PML NBs and increases CK2 phosphorylation of PML proteins, a known prerequisite for PML degradation. Consequently, functions downstream of PML were impaired in the presence of EBNA1. In particular, cells expressing EBNA1 had decreased levels of p53 acetylation, p21 and apoptosis in response to DNA damage. Furthermore, DNA repair was markedly impaired in these cells, despite the fact that they survived better after induction of DNA damage than cells lacking EBNA1.

In keeping with these observations, immunohistochemistry staining of GC biopsies showed that EBV-positive GC biopsies had lower PML staining compared to EBV-negative samples. These results show that EBNA1 directly affects host cell processes that would be expected to promote malignant transformation. Additionally, I have shown that EBNA1’s ability
to disrupt PML NBs is important for reactivation of EBV from latency; hence, is required for efficient spread of EBV from host to host.
Acknowledgments

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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<td>APL</td>
<td>Acute promyelocytic leukemia</td>
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<td>ATF-1</td>
<td>Activating transcription factor 1</td>
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<td>Avian Sarcoma virus</td>
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<td>BART</td>
<td>BamHI rightward transcript</td>
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<td>B-cell receptor</td>
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<td>Brd4</td>
<td>Bromodomain 4</td>
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<td>BZLF1</td>
<td>BamHI Z leftward frame 1</td>
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<td>BPV</td>
<td>Bovine papilloma virus</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
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<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>Ck2</td>
<td>Casein kinase 2</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CREB</td>
<td>cAMP response element-binding</td>
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<td>Daxx</td>
<td>Death associated protein</td>
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<td>DAPI</td>
<td>4′-6-Diamidino-2-phenylindole</td>
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<td>DRiPs</td>
<td>Defective ribosomal products</td>
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<td>DS</td>
<td>Dyad symmetry</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>DUB</td>
<td>Deubiquitinating enzyme</td>
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<tr>
<td>E3</td>
<td>ubiquitin ligase</td>
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<td>EBER</td>
<td>Epstein-Barr expressed RNA</td>
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<td>EBNA</td>
<td>Epstein-Barr nuclear antigen</td>
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<td>Epstein-Barr nuclear antigen leader protein</td>
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<td>EBP2</td>
<td>EBNA1 binding protein 2</td>
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<td>ECF</td>
<td>Enhanced chemifluorescence</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>FA</td>
<td>FLAG-Protein A</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FR</td>
<td>Family of repeats</td>
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<td>GA</td>
<td>Glycine alanine</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GMPS</td>
<td>Guanosine monophosphate synthetase</td>
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<td>H2A</td>
<td>Histone 2A</td>
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<tr>
<td>H2B</td>
<td>Histone 2B</td>
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<tr>
<td>HAUSP</td>
<td>Herpesvirus associated ubiquitin specific protease</td>
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<td>HCMV</td>
<td>Human cytomegalovirus</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HDACs</td>
<td>Histone deacetylases</td>
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<td>Hepatitis D virus</td>
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<td>HHV</td>
<td>Human herpes virus 6</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoproteins</td>
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<td>Human papilloma virus</td>
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<td>HSP 90</td>
<td>Heat shock protein 90</td>
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<td>IE</td>
<td>Immediate early</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<td>IM</td>
<td>Infectious mononucleosis</td>
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<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactoside</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>KSHV</td>
<td>Kaposi’s sarcoma associated herpes virus</td>
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<tr>
<td>LANA</td>
<td>Latency associated nuclear antigen</td>
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<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>LMP</td>
<td>Latent membrane protein</td>
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<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization – time of flight</td>
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<td>MCMs</td>
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<td>Myocyte-specific enhancer factor 2D</td>
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<td>Nucleosome assembly protein</td>
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<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
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<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>PAR</td>
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<td>PARP</td>
<td>Poly-ADP ribose polymerase</td>
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<td>PML</td>
<td>Promyelocytic leukemia</td>
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<td>PML NBs</td>
<td>Promyelocytic leukemia nuclear bodies</td>
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<td>PRMT</td>
<td>Protein arginine-methyl transferase</td>
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<td>Puumala virus</td>
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<td>Origin of plasmid replication</td>
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<td>Ring finger, B-box, Coiled-coil</td>
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<tr>
<td>RING</td>
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<td>RSV</td>
<td>Respiratory syncitial virus</td>
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<td>SCID</td>
<td>Severe combined immunodeficiency disorder</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>Sp100</td>
<td>Speckle pattern 100 protein</td>
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<tr>
<td>SPA</td>
<td>Sequential peptide affinity</td>
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<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
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<td>SV40</td>
<td>Simian virus 40</td>
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<td>Template activating factor 1</td>
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<td>TAP</td>
<td>Tandem affinity purification</td>
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<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TRAF</td>
<td>Tumor necrosis factor associated factor</td>
</tr>
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<td>TRF</td>
<td>Telomere repeat factor</td>
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<td>TRIM</td>
<td>Tripartite motif</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<td>Ub-H2B</td>
<td>Ubiquitinated H2B</td>
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<td>Ubiquitin binding protein</td>
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<td>Z EBV replication activator</td>
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<tr>
<td>ZRE</td>
<td>BZLF1 responsive element</td>
</tr>
</tbody>
</table>
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EBNA1-dependent segregation of EBV-based plasmids in Nasopharyngeal carcinoma 162
Chapter 1

INTRODUCTION
1.1 Epstein-Barr Virus

Denis Burkitt, a surgeon, first described in a lecture an endemic variant form of pediatric cancer, which later became known as Burkitt's lymphoma. Anthony Epstein, a pathologist, received specimens of this cancer from Uganda and analyzed it using electron microscopy. Together with Yvonne Barr, they identified and characterized virus particles in the cancer specimens. The work was published in The Lancet with Bert Achong in 1964 and the virus was later named Epstein-Barr virus (EBV) (Epstein, Achong, and Barr, 1964; Epstein, Barr, and Achong, 1964). EBV was later known to cause infectious mononucleosis in adults, also known as the kissing disease. To date, EBV has been one of the most successful human viruses. It establishes a latent infection in B lymphocytes in 95% of the adult human population (Rickinson and Kieff, 2001). It is capable of a lifelong latent infection, in part due to its ability in evading the host immune system. This latent infection is also associated with several EBV-associated diseases. Although EBV may not be the sole contributing factor to disease outcome, sufficient evidence suggests that EBV plays an important role in their onset, development and progression (Young and Rickinson, 2004). Due to its viral and genome structure, it was classified as a herpes virus of the gamma subfamily, and later became known as human herpes virus 4 (HHV-4) (Epstein, Barr, and Achong, 1964).

1.1.1 Classification and Viral Structure

There are three groups of herpes virus families (alpha, beta and gamma) each distinguished by their viral and genome structure and biological activity. EBV is a gamma herpes virus, and it and Kaposi’s sarcoma associated herpes virus (KSHV) are the only two gamma herpes viruses that infect humans.

Similar to other herpes viruses EBV has an outer envelope with glycoproteins spikes surrounding a proteinaceous matrix known as the tegument, and a nucleocapsid with 162 capsomeres containing a core with DNA and protein that is toroid-shaped (Figure 1-1) (Rickinson and Kieff, 1996; Roizmann and Sears, 1990). The EBV genome is a 172 Kbp linear, double stranded DNA molecule that has a GC content of 60%. The genome is characterized by short and long unique sequence domains known as U₅ and U₇ respectively. Although the genome is linear, once EBV infects a host cell, the linear termini are fused to form a circular, episomal DNA (Kaschka-Dierich et al., 1976; Lindahl et al., 1976; Rickinson and Kieff, 2001). Since the terminal repeats vary in length, each infection results in a specific fusion event and
determines the size of the terminal repeat. The heterogeneity in the number of joined terminal repeats can distinguish the number of clonal infection events (Raab-Traub and Flynn, 1986). EBV was the first herpes virus to be fully sequenced and encodes approximately 80 viral proteins that function in latent and lytic modes of infection (Baer et al., 1984; Rickinson and Kieff, 2001). The gene organization is shown in Figure 1-2.

Figure 1-1: Structure of Epstein-Barr virus particle (Epstein et al., 1964). (A) Cartoon schematic of EBV showing glycoprotein spikes on its envelope that incases the tegument proteinaceous matrix. The envelope also encases the nucleocapsid, the latter encases and protects the viral DNA. (B) Electron micrograph of EBV particle clearly showing the various layers of the viral structure (going from the outward to inward): viral envelope with glycoprotein spikes, tegument, capsid, and the core (the dark matter in the core is the viral DNA).
Figure 1-2: The Epstein–Barr virus genome (Adapted with permission from Murray and Young, 2001). (a) Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (oriP) is shown in orange. The large solid blocks (in purple) represent coding exons for each of the latent proteins and the arrows indicate the direction in which they are transcribed; the latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. LMP2A and LMP2B are composed of multiple exons located on either side of the terminal repeat (TR) region, which is formed during the circularisation of the linear DNA to produce the viral episome. The orange arrows at the top represent the highly transcribed non-polyadenylated RNAs EBER1 and EBER2; their transcription is a consistent feature of latent EBV infection. The outer long arrowed red line represents EBV transcription during a form of latency known as latency III (Lat III), where all the EBNAs are transcribed from either the Cp or Wp promoter. The different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript. The inner shorter arrowed blue line represents the EBNA1 transcript originating from the Qp promoter during Lat I and Lat II. Transcripts from the BamA region can be detected during latent infection, shown here are the locations of the BARF0 and BARF1 coding regions.
1.1.2 Primary Infection

EBV is acquired by exposure to oral secretions or blood and the virus persists in the oral and/or blood compartment in B lymphocytes, epithelial cells or both (Figure 1-3). Upon primary infection EBV is thought to infect epithelial cells of the orthopharynx where virions are produced and transferred to B lymphocytes where latent infection is established (Hadinoto et al., 2009; Sixbey et al., 1984; Sixbey et al., 1983). However, there is also evidence that EBV can latently infect epithelial cells as is seen in nasopharyngeal carcinoma. In most humans, the primary infection occurs within the first three years of life and results in an asymptomatic infection. However, when primary infection occurs in adolescents, it results in infectious mononucleosis (IM) after a five to seven week incubation period (Gerber et al., 1972). IM is a transient self-limiting lymphoproliferative disease distinguished by sore throat, fever, pharyngitis and lymphadenopathy (Gerber et al., 1972; Kutok and Wang, 2006). Once the acute phase of the infection terminates, EBV establishes a reservoir of latently infected B-cells within the host. In doing so, EBV persists for the life of the host in a latent mode expressing very few viral genes with sporadic bursts of lytic viral replication.
Figure 1-3. Epstein–Barr virus infection in normal healthy virus carriers (Adapted with permission from Murray and Young, 2001). Virus infection involves two cellular compartments: (1) B cells, where infection is predominantly latent and has the potential to induce growth-transformation of infected cells; and (2) epithelial cells, where infection is predominantly productive. Although the exact mode of primary and persistent EBV infection and the relative contributions of B cells and epithelial cells are uncertain, recent data point to the B-cell compartment as the main mediator of primary as well as persistent infection. Following primary infection of B cells, a chronic virus carrier state is established in which the outgrowth of EBV-transformed B cells is controlled by an EBV-specific cytotoxic T lymphocyte response re-activated from a pool of virus-specific memory T cells. At certain sites, latently infected B cells can become permissive for lytic EBV infection. Infectious virus released from these cells can be shed directly into the saliva or might infect epithelial cells and other B cells. In this way a virus-carrier state is established that is characterised by persistent, latent infection in circulating B cells and occasional EBV replication in B cells and epithelial cells.
1.1.3 Latent Infection

Latent infection is marked by the absence of virion production. The virus is maintained in an episomal state in resting or proliferating cells with limited viral gene expression. EBV has four different programs of gene usage in latency (latency 0, I, II and III). This is determined by the type of cell infected (and differentiation state for B-cells) and the viral proteins expressed (Table 1). In most of the human population EBV largely exists in latency mode 0 (also called latency program), where EBV is in a transcriptionally quiescent state (no protein expression) within a resting memory B-cell (Thorley-Lawson, 2001). Within a healthy individual this mode of latency prevents activation of any unnecessary immune response, thus infection remains silent.

<table>
<thead>
<tr>
<th>Latency Program</th>
<th>EBNAs:</th>
<th>LMPs:</th>
<th>Detected in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>---</td>
<td>---</td>
<td>Healthy individuals</td>
</tr>
<tr>
<td>I (EBNA ONLY)</td>
<td>EBNA1</td>
<td>---</td>
<td>BL</td>
</tr>
<tr>
<td>II (DEFAULT)</td>
<td>EBNA1</td>
<td>LMP1,2A</td>
<td>NPC, GC, Hodgkin's disease</td>
</tr>
<tr>
<td>III (GROWTH)</td>
<td>EBNA1,2,3A,B,C and LP</td>
<td>LMP1,2A,2B</td>
<td>EBV associated diseases in immunocompromised individuals</td>
</tr>
</tbody>
</table>

When EBV infects a naive B-cell in the resting state, it uses latency III (also called the growth program) to activate these cells to become proliferating blasts. This is a similar process to the activation of naive B-cells in response to antigen exposure. EBV latency III involves expression of six EBV nuclear antigens or EBNAs (EBNAs 1, 2, 3A, 3B, 3C and LP), the three latent membrane proteins (LMP1, LMP2A and LMP 2B). This combination of expression immortalizes the B-cells giving rise to lymphoblastoid cell lines (LCLs) (Rickinson and Kieff, 2001). The only location in the human body where latency III is detected is in the tonsils of healthy individuals, where EBV manipulates the antigen induced B-cell differentiation pathway to generate memory B-cells carrying the latent EBV genome (Babcock et al, 2000; Thorley-Lawson et al., 2004). In this manner, EBV can ensure viral persistence in the B-cell
compartment. Since the EBNA's other than EBNA1 are highly immunogenic, thus, it is observed in immunocompromised individuals or post-transplant lymphoproliferative disease. Latency III is also the form of latency that arises when B-cells are infected in vitro and therefore is the best characterized form of EBV latency (Thorley-Lawson and Allday, 2008; Young and Rickinson, 2004).

Latency III cells can shut down expression of viral proteins and enter latency I or 0, where they circulate between the peripheral blood and Waldeyer's ring (Thorley-Lawson and Gross, 2004). In latency I, infected memory cells proliferate and express Epstein-Barr Nuclear antigen 1 (EBNA1), which is the only viral protein required to replicate and partition the viral DNA in the dividing cells (Kanda, Otter, and Wahl, 2001; Rawlins et al., 1985a; Yates, 1996). This form of latency is known as "EBNA1 only" program or latency I. It is another form of latency commonly found in healthy individuals and is also characteristic of Burkitt's lymphoma (Rickinson and Kieff, 2001). It is evident that the differentiation status of the B-cells dictate the type latency program ensued by EBV.

The default (latency II) program is typically observed in nasopharyngeal carcinoma, gastric carcinoma and Hodgkin's disease (Rickinson and Kieff, 2001). In addition to EBNA1 expression, in latency II, latent membrane proteins (LMP1 and LMP2A/B) are often expressed. Furthermore, in latency II epithelial cells there is expression of a secreted protein BARF1, which may function as a growth factor (zur Hausen et al., 2000). Finally, in addition to the viral proteins outlined above, all of the EBV latencies express double-stranded, nuclear, non-transcribed RNA molecules called EBERs as well as viral micro RNAs (discussed below).

### 1.1.3.1 Epstein-Barr Nuclear Antigens

The EBNAs are the most diverse group of proteins that function in episome maintenance, immune recognition and regulate host cell immortalization and transformation. Mostly they are transcription factors or known to regulate transcription (Young and Rickinson, 2004). The six EBNAs include 1, 2, 3A, 3B,3B and LP. EBNA1 is expressed in all EBV-infected cells because it is required for the replication and segregation of the viral episome and also plays roles in controlling viral gene expression (Rickinson, 2001). These functions and more of EBNA1 will be discussed in detail in section 1.2.

EBNA2 is essential for immortalization of B-cells in vitro and is the main viral protein responsible for transactivating viral and cellular promoters required for this process (Zimber-
EBNA2 does not bind to DNA directly but is tethered to its responsive elements in promoters via cellular proteins such as RBP-Jκ (also called CBF1), a downstream target of Notch, and PU.1 (Fuentes-Panana et al., 2000; Grossman et al., 1994; Henkel et al., 1994; Hsieh and Hayward, 1995; Johannsen et al., 1995). This allows effective activation of the growth program and proliferation of the infected B-cells, giving rise to LCLs in vitro. EBNA-LP is one of the first protein expressed upon infection of B-cells. EBNA-LP interacts with EBNA2, where it functions as a transcriptional coactivator for EBNA2-mediated transcription via RBP-Jκ. This direct interaction facilitates the cell cycle transit of B-cells from $G_0$ to $G_1$ (Izumi et al., 1994; Zhao et al., 1996). Additionally, EBNA-LP has been shown to displace SP100A from PML NBs and this is important for its EBNA2 coactivation function (Ling et al., 2005).

1.1.3.2 Latent membrane proteins

EBV latent membrane proteins (LMP1, LMP2A, LMP2B) are plasma membrane proteins that span the membrane multiple times and have both their N- and C-termini in the cytoplasm. They act as constitutively active ligand-independent receptors (Gires et al., 1997). LMP1 is an oncogene, and is required for the in vitro transformation of B-cells (Wang, Liebowitz, and Kieff, 1985). LMP1 functionally mimics CD40 tumor necrosis factor receptor (TNFR) superfamily except that activation of the signalling pathways is ligand-independent. It provides growth and differentiating signals to B-cells, which allows for the establishment of a reservoir of EBV infected memory B-cells (Kilger et al., 1998; Mosialos et al., 1995). LMP1 regulates various genes including those involved in anti-apoptotic pathways (such as the upregulation of Bcl2 and A20) and cytokines (Young and Rickinson, 2004).

The LMP2 proteins are not essential for in vitro transformation of B-cells. In the absence of signalling through the B-cell receptor (BCR), LMP2A has been shown to drive proliferation and survival of B-cells (Portis and Longnecker, 2004). Expression of LMP2A in epithelial cells can transform them and increase their adhesion and motility, which may be
directed through the PI3K-Akt pathway (Scholle, Bendt, and Raab-Traub, 2000). LMP2A has been shown to play a role in EBV latent to lytic cycle switch and LMP2B is known to suppress the functions of LMP2A (Rechsteiner et al., 2008a; Rechsteiner et al., 2008b).

1.1.3.3 EBV-non coding RNA’s and BamHIA transcripts

EBERs code for two small nuclear RNAs that are abundantly transcribed and represent the single most expressed transcript for EBV (Arrand and Rymo, 1982). EBERs are suggested to contribute to the malignant phenotype of Burkitt’s lymphoma and increase their resistance to apoptosis (Iwakiri and Takada, 2010; Nanbo and Takada, 2002; Takada and Nanbo, 2001). Furthermore, they are known to promote proliferation of infected cells in nasopharyngeal carcinoma and gastric carcinoma through the induction of insulin-like growth factor (Iwakiri and Takada, 2010; Takada and Nanbo, 2001).

EBV has also been shown to express more than 40 non-coding microRNAs from two separate regions of the genome (Cosmopoulos et al., 2009). MicroRNAs negatively regulate the expression of some proteins by hybridizing to specific transcripts and inducing their degradation or inhibiting their translation. One cluster of EBV microRNAs is referred to as BamHIA rightward transcripts (BARTs), which was originally identified in NPC and is found to be expressed in all EBV latency programs (Cosmopoulos et al., 2009). Their role in regulating expression of cellular and viral genes and contribution to latent EBV infection is an area of active study. The second cluster of microRNAs is expressed from the BHRF1 region and they are selectively expressed in latency program III in B lymphocytes (Lee et al., 2011).

1.1.4 Lytic Infection

EBV can be reactivated from latency into lytic infection to allow virion production and transmission of the virus from cell to cell and from host to host. This likely occurs in vivo when the EBV infected memory B cells are triggered to differentiate. For example, in response to antigen stimulation memory B cells differentiate into plasma cells (Laichalk and Thorley-Lawson, 2005). As a consequence plasma cells are known to harbour EBV lytic replication that can be detected in peripheral B lymphocytes. Typically antigen binding is sufficient to reactivate EBV in vivo (Laichalk and Thorley-Lawson, 2005). However, in vitro study of lytic EBV infection has been permitted by chemical inducing agents, including phorbol esters, butyrate, calcium ionophores, and B-cell receptor stimulation (Faggioni et al., 1986; zur Hausen et al., 1978).
EBV lytic gene expression is regulated in a temporal manner, with immediate early (IE) gene expression followed by early and late genes. Regardless of the stimuli for EBV reactivation, it ultimately results in the activation of the two EBV IE genes, BZLF1 and BRLF1 from the latent viral genome (Biggin et al., 1987; Flemington, Goldfeld, and Speck, 1991). These proteins function as transcription factors that activate their own promoter and each other’s, thus, even the smallest stimulus is sufficient to amplify the lytic cascade (Adamson et al., 2000; Flemington, Goldfeld, and Speck, 1991; Liu and Speck, 2003; Speck, Chatila, and Flemington, 1997). Early viral genes encode the viral replication proteins, including the virally encoded DNA polymerase. Once the viral DNA is replicated, the late viral genes are transcribed, many of which encode structural proteins that make up the virion particle (Amon and Farrell, 2005).

1.1.4.1 Expression, regulation of BZLF1 - Lytic master regulator

BZLF1 is a homologue of c-jun and c-fos, and binds as a homodimer to AP-1 like motifs, which is known as Z-responsive elements (ZREs) (Chang et al., 1990; Lieberman et al., 1990). The IE transcript that encodes BZLF1 is derived from the IE promoter, Zp, and is inactive during latent infection. It is believed that epigenetic modifications such as DNA methylation and histone deacetylation contribute to the prevention of IE gene transcription in the intact viral genome (Bhende et al., 2004; Paulson and Speck, 1999; Szyf et al., 1985). Since the expression of BZLF1 can efficiently commence the EBV lytic cascade, the regulation of the Zp promoter has been very well studied. There are several cellular factors that control the activation and repression of the Zp promoter as can be seen in Figure 1-4, and the region of Zp between –475 and +12 contains the cis and trans-acting sequences required for Zp activation and repression (Kenney, 2007).

The MEF 2D transcription factor binds three elements within the Zp promoter (see Figure 1-4) (Liu et al., 1997). MEF 2D is known to preferentially interact with histone deacetylating complexes during viral latency, thus inhibiting transcription of the BZLF1 gene. However, upon lytic inducing stimuli, this switches to a preferential interaction with acetylating complexes, thereby allowing transcription of BZLF1 (Gruffat, Manet, and Sergeant, 2002). Since MEF 2D can both repress and activate Zp, it is believed that the phosphorylation status of MEF 2D may play an essential role in dictating its exact function in Zp transcription. The phosphorylated form of c-Jun by c-Jun N-terminal kinase (JNK) seems to be important for activation of BZLF1
transcription since many of the stimuli that induce lytic EBV infection \textit{in vitro} are known to activate JNKs. Also, inhibitors of JNKs reduce the effectiveness of a number of lytic-inducing stimuli (Adamson et al., 2000; Feng et al., 2004; Feng et al., 2002).

Phosphorylated CREB and ATF-1 are known to activate Zp in reporter gene assays. These two factors show increased binding to Zp CRE motif as a result of Zp activation in response to epithelial cell differentiation (Karimi et al., 1995; MacCallum, Karimi, and Nicholson, 1999).

Negative regulation of BZLF1 transcription plays a critical role in maintaining latency in EBV infected cells. A number of different negative regulatory elements in the BZLF1 promoter have been identified. The zinc-finger protein, ZEB, is of importance as it has been shown to bind the "ZV" motif located near the TATA box and represses transcription of BZLF1 (Kraus et al., 2001). Deletion of the "ZV" site greatly enhances activation of Zp (Binne, Amon, and Farrell, 2002). The cellular transcription factor YY-1 has been shown to bind the "ZIV" element within Zp, which negatively regulates BZLF1 transcription (Montalvo et al., 1995; Montalvo et al., 1991). Although much is known about cellular proteins that regulate Zp promoter, it is not clear why some cell types are more permissive than others for lytic reactivation.

![Figure 1-4](image)

**Figure 1-4:** Regulation of the EBV immediate–early BZLF1 promoter (Adapted with permission from Kenney, 2007). Regulatory motifs in the BZLF1 (Zp) promoter are shown. Cellular and viral proteins known to bind to each motif are indicated.

**1.1.4.2 Lytic DNA replication**

EBV lytic DNA replication is essential for the production of large amounts of viral genomes for packing into infectious virus particles. In addition to its role as a transcription factor, BZLF1 plays a very important role in lytic viral replication. BZLF1 directly binds to the ZREs within the lytic origin of replication, OriLyt, and this is critical for replication from OriLyt (Fixman, Hayward, and Hayward, 1992; Hammerschmidt and Sugden, 1988; Schepers et al.,
The transcription and replication functions of BZLF1 are independent of one another since there are mutants of BZLF1 competent for transcription but not for viral replication (Sarisky et al., 1996). In addition, BZLF1 can directly interact with some of the core viral replication proteins, which implicates BZLF1 as a scaffold for the formation of the replication complex (Gao et al., 1998; Zhang et al., 1996). EBV lytic replication also involves six additional viral replication proteins that are conserved in all herpesviruses (Fixman, Hayward, and Hayward, 1995) and, although not well defined, replication is thought to proceed by the same mechanism as for herpes simplex genome replication. As in herpes simplex virus, lytic replication results in the generation of head-to-tail concatamers that are cleaved within the terminal repeats into unit lengths upon packaging (Zimmermann and Hammerschmidt, 1995).

1.1.5 Diseases associated with EBV infection

Since its discovery and link with Burkitt's lymphoma there has been considerable interest focused on EBV pathogenesis. EBV is the causative agent in endemic Burkitt's lymphoma and to date EBV has also been shown to play an important role in the development and progression of a large number of diseases in humans. EBV can cause infectious mononucleosis, also known as the "kissing disease" (Henle, Henle, and Diehl, 1968). In addition to the association of EBV with Burkitt’s lymphoma, NPC, and infectious mononucleosis, EBV has now been found to be involved in a much wider range of human diseases. EBV is generally accepted to be involved with several other malignancies of lymphocyte origin, such as Hodgkin’s lymphoma, and epithelial origin, such as gastric carcinoma (Kutok and Wang, 2006). A summary of all EBV associated diseases can be reviewed in Table 2. EBV is also a factor in some T and NK cell lymphomas (Delecluse et al., 2007) and has been linked to diseases such as breast carcinoma and hepatocellular carcinoma, however, this remains controversial and will require clarification with additional research in the near future (Murray, 2006). In immuno-suppressed patients, EBV causes a variety of proliferative disorders including oral hairy leukoplakia in AIDS patients, immunoblastic lymphomas, and an unusual tumor of muscle origin in children with AIDS or who are under immune suppression for liver transplantation (Delecluse et al., 2007). In young boys with X-linked immunodeficiencies, EBV causes severe mononucleosis that results in death (Longnecker and Neipel, 2007).

There is accumulating evidence that EBV may also be associated with immune mediated
Of interest is the fact that there is a variety of auto-immune diseases that appear to have an infectious agent as a cofactor such as multiple sclerosis, rheumatoid arthritis, and diabetes (Niller, Wolf, and Minarovits, 2008). It should be emphasized that many of these studies may be controversial considering EBV's ubiquitous nature. However, in the interest of this thesis I will discuss two very important EBV-associated epithelial tumours NPC and GC, as there is substantial evidence implicating a causal role for EBV in these tumours.

Table 2: Epstein-Barr virus associated diseases

<table>
<thead>
<tr>
<th>Nonmalignant disease:</th>
<th>Malignant disease:</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Infectious mononucleosis</td>
<td><strong>Immunocompromised host B cell malignancies</strong></td>
</tr>
<tr>
<td>- Chronic active infection</td>
<td></td>
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<tr>
<td>- Oral hairy leukoplakia</td>
<td>- AIDS-associated B cell lymphomas</td>
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<td></td>
<td>- Post-transplantation lymphoproliferative disorder</td>
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<tr>
<td></td>
<td>- Lymphomatoid granulomatosis</td>
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<tr>
<td></td>
<td>- Methotrexate-associated B cell lymphoma (lymph.)</td>
</tr>
<tr>
<td></td>
<td><strong>Congenital immunodeficiency:</strong></td>
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<tr>
<td></td>
<td>- Severe combined immunodef.-associated B cell lymph.</td>
</tr>
<tr>
<td></td>
<td>- Wiskott-Aldrich syndrome-associated B cell lymph.</td>
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<tr>
<td></td>
<td>- X-linked lymphoproliferative disorder-associated Bcell lymph.</td>
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<tr>
<td></td>
<td><strong>Messenchymal malignancies:</strong></td>
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<tr>
<td></td>
<td>- Leiomyosarcoma</td>
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<tr>
<td></td>
<td><strong>Immunocompetent host</strong></td>
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<tr>
<td></td>
<td><strong>B cell malignancies:</strong></td>
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<tr>
<td></td>
<td>- Burkitt lymphoma</td>
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<tr>
<td></td>
<td>- Classical Hodgkin lymphoma</td>
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<tr>
<td></td>
<td><strong>T cell malignancies:</strong></td>
</tr>
<tr>
<td></td>
<td>- Extranodal NK/T cell lymphoma, nasal type</td>
</tr>
<tr>
<td></td>
<td>- Virus-associated hemophagocytic syndrome T cell lyphomas</td>
</tr>
<tr>
<td></td>
<td><strong>Epithelial cell malignancies:</strong></td>
</tr>
<tr>
<td></td>
<td>- Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td></td>
<td>- Gastric carcinoma</td>
</tr>
<tr>
<td></td>
<td>- Lymphoepithelioma-like carcinoma</td>
</tr>
<tr>
<td></td>
<td>(salivary, thymus, lungs)</td>
</tr>
<tr>
<td></td>
<td>- Breast carcinoma</td>
</tr>
<tr>
<td></td>
<td>- Hepatocellular carcinoma</td>
</tr>
<tr>
<td></td>
<td><strong>Mesenchymal malignancies:</strong></td>
</tr>
<tr>
<td></td>
<td>- Follicular dendritic cell sarcoma</td>
</tr>
</tbody>
</table>
1.1.5.1 *Nasopharyngeal carcinoma*

NPC is a tumour of the nasopharyngeal surface epithelium, and often presents as a mass on the neck or nasal obstruction and loss of hearing. NPC is classified by the World Health Organization (WHO) into two main histological types: keratinizing squamous cell carcinoma (WHO1) and non-keratinizing squamous cell carcinoma (WHO2/3) (Shah and Young, 2009). The latter is further subdivided into differentiated non-keratinizing (WHO2) and undifferentiated carcinomas (WHO3) (Shanmugaratnam, 1978). Up to 80% of the NPC cases are of the undifferentiated type and are universally latently infected with EBV, where the virus is exclusively in the tumour cells and absent in infiltrating lymphocytes (Chan, Teo, and Huang, 2004; Shah and Young, 2009). NPC is a monoclonal proliferation of EBV infected cells marked by the presence of clonal EBV genomes (detected by EBV terminal repeat length), suggesting that these carcinomas arise from the clonal expansion of a single EBV infected progenitor cell (Raab-Traub and Flynn, 1986).

Like BL, NPC is endemic to certain parts of the world including southern China, Indonesia and Northern Africa, where NPC accounts for up to 20% of all adult cancers in some regions. EBV-associated NPC has also been identified in Eskimos in Alaska and Greenland. NPC is very rare in developed countries including North America and Europe (Shah and Young, 2009). The association of EBV with NPC was first implicated when serological studies identified a link between virus and the development of NPC (Henle and Henle, 1976). Examination of DNA extracted from undifferentiated NPC’s revealed that cases were consistently positive for EBV, and *in situ* hybridization showed the presence of EBV DNA in virtually all tumour cells (Desgranges et al., 1975; zur Hausen et al., 1970). Using epidemiological studies it has been possible to identify other aetiological factors involved in the pathogenesis of NPC. These include a genetic susceptibility in some individuals (particular human leukocyte antigen haplotypes) and an early-age exposure to chemical carcinogens (particularly of Cantonese salted fish that contains nitrosamines) (Busson et al., 2004). The development and progression of NPC involves the accumulation of a number of genetic changes (e.g. gene amplification, deletion and mutation) and epigenetic changes (hyper methylation of tumor suppressors) (Lo and Huang, 2002). These changes can affect the development of NPC by altering the functions of genes that are critical for proliferation, apoptosis, and differentiation (Lo and Huang, 2002). In NPC, EBV adopts a type II latency program expressing EBERs, EBNA1, LMP2A and variable expression of LMP1 (Brooks et al., 1992). Expression of the secreted
BARF1 protein has also been reported (Seto et al., 2005). The exact functions of the viral proteins remains to be explored in development and progression of NPC, hence EBV's role in the pathogenesis of NPC is not very well characterized.

1.1.5.2 Gastric carcinoma

GC is the fourth most common cancer worldwide and is the second most common cause of death from cancer (Parkin et al., 2005). Unlike NPC, GC presents itself without regional or racial differences and is a major clinical problem worldwide (Uozaki and Fukayama, 2008). Up to 10% of all GC is associated with EBV, where EBV infected tumor cells are present in the proximal stomach (Imai et al., 1994; Young and Rickinson, 2004). EBV infection is known to occur mainly in the upper middle portions of the stomach rather than the lower part of the stomach (Osato and Imai, 1996). This results in two histomorphologically distinct forms of EBV-associated GC: a rare lymphoepithelioma-like carcinoma (LLC) (similar in appearance to NPC) and a more common gastric carcinoma type (glandular adenocarcinoma) (Fukayama et al., 1994). EBV associated GC can develop at multiple sites independently and, using RT PCR and in situ hybridization techniques, EBV was detected in almost 90% of gastric LLC cases (Ryan et al., 2009; Takada, 2000).

Similar to NPC, EBV- associated GC tumours display a type II-like latency program of EBV latent gene expression in that they express EBNA1, LMP2A and BARF1, however LMP1 typically seen in type II latency is not expressed (Fukayama, 2010; Fukayama, Hino, and Uozaki, 2008; Takada, 2000). EBV- positive GCs have very distinct phenotypic and clinical characteristics compared to EBV-negative GC. This includes the loss of p16 expression, p73 promoter methylation and wild-type p53 expression (Chang et al., 2006; Lee et al., 2004b).

Similar to NPC, the exact role of EBV in the pathogenesis of gastric carcinoma remains to be determined. However, the absence of EBV infection in premalignant gastric lesions supports the notion that viral infection is a relatively late event in gastric carcinogenesis (Zur Hausen et al., 2004).

1.2 Epstein-Barr Nuclear Antigen 1

EBNA1 was the first viral protein to be detected in EBV infected LCLs (Reedman and Klein, 1973). It is expressed from the BKRF1 gene, which encodes the 641 amino acid protein (Rickinson and Kieff, 2001). The exact number of amino acid can vary depending on the length of the Gly-Ala repeat found within EBNA1 protein from aa 100-324 (Baer et al., 1984). Since
its discovery, it has been identified as being essential and sufficient for the latent origin-dependent (oriP) DNA replication and EBV plasmid maintenance in latently infected cells (Yates et al., 1984; Yates, Warren, and Sugden, 1985b). In addition to maintaining constant EBV DNA copy number, EBNA1 is also known to transcriptionally regulate the expression of itself as well as other latent viral genes (Rickinson and Kieff, 2001). Increasing evidence suggests that EBNA1 can not only play an important role in maintaining EBV, but can also alter the infected cell environment.

1.2.1 Functional Domains and structure

There are several functional regions present in EBNA1 (Figure 1-5). First, EBNA1 forms very stable homodimers in solution and when bound to its recognition sites (Ambinder et al., 1991; Frappier and O'Donnell, 1991b; Shah et al., 1992). C-terminal sequences (aa 450–620) encode the DNA binding and dimerization domain of EBNA1. It is essential for interaction with DS, FR, and BamHI Q binding sites (Figure 1-7) (Ambinder et al., 1990; Ceccarelli and Frappier, 2000; Chen, Middeldorp, and Hayward, 1993; Goldsmith, Bendell, and Frappier, 1993; Rawlins et al., 1985a; Wu, Ceccarelli, and Frappier, 2000; Wu, Kapoor, and Frappier, 2002b). This domain of EBNA1 has been crystallized with and without DNA (Figure 1-6) (Bochkarev et al., 1996; Bochkarev et al., 1995), and was shown to consist of a core β-barrel domain that contacts the major groove of the DNA and a flanking domain that inserts in the minor groove. The core domain also mediates dimerization and shows a remarkable similarity to the structural fold of the DNA binding domain of the E2 protein of bovine papillomavirus, despite a lack of sequence similarity. The structures also revealed that EBNA1 bends the bound DNA.
Figure 1-5. EBNA1 domains (Frappier, 2010). (A) Schematic representation of wild-type EBNA1 protein (EBNA1 GA), with some of the functional elements indicated. Shown are the Gly-Ala repeat, the large Gly-Arg repeat, the USP7 binding site (USP7) and the flanking and core DNA binding and dimerization domains. A version of EBNA1 protein lacking most of the Gly-Ala repeat sequence (referred to simply as EBNA1) is used in studies discussed in this thesis. The Gly/Ala region varies in length and does not contribute to known EBNA1 functions in cell culture. (B) Deletions that abrogate indicated functions of EBNA1 are shown in black bars, where as those that partially inhibit these functions are shown in grey.
The central and N-terminal sequences of EBNA1 possess distinct activities critical for DNA replication, plasmid maintenance and transcriptional regulation as discussed below (see Figure 1-5) (Wu, Kapoor, and Frappier, 2002b; Yates and Camiolo, 1988). In this region, two RGG-rich regions (aa 34–56, and 339–377) have been identified, that resemble RGG-elements found in hnRNP RNA binding proteins (Burd and Dreyfuss, 1994). These regions of EBNA1 have been shown to confer some RNA binding ability in vitro, however, the biological significance of this function has yet to be explored (Snudden et al., 1994). In addition to binding RNA, these RGG-rich regions confer intra- and inter-molecular looping and linking between EBNA1-DNA complexes as detected by electron microscopy (Frappier and O'Donnell, 1991a; Goldsmith, Bendell, and Frappier, 1993) and electrophoretic mobility shift assay (EMSA) (Frappier, Goldsmith, and Bendell, 1994; Mackey, Middleton, and Sugden, 1995). Using electron microscopy, EBNA1 was shown to induce DNA looping between FR and DS and the region responsible was mapped to the central Gly-Arg rich region with aa 350-361 being sufficient for the interaction (Frappier, Goldsmith, and Bendell, 1994; Frappier and O'Donnell, 1991a; Goldsmith, Bendell, and Frappier, 1993; Middleton and Sugden, 1992; Su et al., 1991). The precise functional role of DNA linking remains ill-defined and is difficult to dissect since the same sequences also mediate interactions with other proteins important for transcriptional activation, segregation and metaphase chromosome attachment.

EBNA1 also contains a region that can vary in length, which contains approximately 200 residue of glycine-alanine (GA) repeat from amino acids 100-324 (in the B95-8 strain). The removal of this GA region does not affect any of the known functions of EBNA1 (DNA replication, segregation of EBV-based plasmids, or transcription regulation). Although, it was originally proposed to inhibit ubiquitin-dependent/proteosomal processing of EBNA1 (Levitskaya et al., 1995; Levitskaya et al., 1997), this has since been refuted by data from other laboratories (Tellam et al., 2004). While the GA repeat has been shown to inhibit proteosomal processing when fused to other proteins, it has not been shown to do this for EBNA1 which is very stable even without the GA repeats (Heessen et al., 2002; Levitskaya et al., 1997). The GA repeats have been shown to reduce peptide processing and HLA class I presentation, and limit cytotoxic T-cell responses to EBNA1 in latently infected cells (Blake et al., 1997). In addition, the GA repeats inhibit EBNA1 translational efficiency (Yin, Manoury, and Fahraeus, 2003), therefore limiting the production of defective ribosomal products (DRiPs), which contributes to MHC I-restricted antigen presentation (Fahraeus, 2005). Therefore the GA repeats are thought
to contribute to immune evasion by EBNA1 by decreasing EBNA1 DRiPs. Sun et al. (Sun et al., 2010) have implicated a role for heat shock protein 90 (hsp90) in regulating EBNA1 translation through the Gly-Ala region. They have shown that Hsp90 inhibitors decrease EBNA1 expression and translation, and that this effect requires the Gly-Ala repeat of EBNA1. Hsp90 inhibitors induced the death of established, EBV-transformed lymphoblastoid cell lines at doses nontoxic to normal cells, and this effect is substantially reversed when lymphoblastoid cell lines are stably infected with a retrovirus expressing a functional EBNA1 mutant lacking the Gly-Ala repeats (Sun et al., 2010).
Figure 1-6. Crystal structure of the EBNA1 DNA binding and dimerization domains bound to DNA (PDB ID 1B3T) (Bochkarev et al., 1996). The core domain is shown in light, whereas the flanking domain is in black. Also shown are the proline loop (PL), the recognition helix (RH) and the WF residues (W464 and F465).

Figure 1-7. Organization of the EBV oriP (Frappier, 2010). Relative positions of the FR and DS elements in the EBV genome are shown at the top, with organization of the DS element depicted in detail below. Indicated are EBNA1 binding sites 1 to 4 (grey boxes), nonamer repeats (black boxes) and the 65bp dyad symmetry element (inverted arrows).
1.2.2 Functions of EBNA1

1.2.1.1 DNA Replication

Testing the ability of different EBV DNA fragments to allow replication and stable maintenance of plasmids led to the identification of the latent origin of DNA replication, the oriP sequence (Yates et al., 1984). The dyad symmetry (DS) element within oriP was shown to be sufficient for replication of oriP-containing plasmids in human cells in the presence of EBNA1 (Harrison, Fisenne, and Hearing, 1994; Yates, Camiolo, and Bashaw, 2000). The DS element is a 120 base-pairs long and has four EBNA1 binding sites (see Figure 1-7) (Rawlins et al., 1985a; Reisman, Yates, and Sugden, 1985). There are also three 9 base-pair sequences within the DS element, that are bound by telomeric repeat factors (TRF) 1 and 2 (Deng et al., 2003). As long as two adjacent EBNA1 sites are present (sites 1 and 2, or sites 3 and 4) minimal replication take place, however, all four EBNA1 binding sites and the nonamers are required for efficient replication from the DS element (Deng et al., 2002; Koons, Van Scoy, and Hearing, 2001). EBV genomes have been shown to replicate once and only once in the S phase of the cell cycle (Adams, 1987). Recent work by Zhou et al. (Zhou, Snyder, and Lieberman, 2009) has shown that EBV genomes replicate in mid- to late S phase. In fact chemical agents that accelerate replication timing of EBV reduce viral genome stability. For instance, hydroxyurea (HU) treatment, a chemical that is known to eliminate EBV episomes, shifted EBV replication to earlier times in the cell cycle (Zhou, Snyder, and Lieberman, 2009). This suggests that delayed timing of EBV genome replication is important for genome stability. These observations have been consistent with oriP-containing plasmids as they also replicate once per cell cycle in EBNA1 positive cell (Yates and Guan, 1991).

EBNA1 is the only viral protein required for latent EBV DNA replication. However, since EBNA1 lacks any enzymatic activity that would facilitate DNA replication or melt DNA (Frappier and O'Donnell, 1991a), replication must rely on host cell factors. Cellular DNA replication requires the assembly of pre-replication licensing factors at origins. This includes the origin recognition complex (ORC), the minichromosome maintenance (MCM) complex, CDC 6, Geminin, and CDT1 (Bell and Dutta, 2002). Using chromatin immunoprecipitation (ChIP) assays, components of the ORC complex were shown to associate with OriP in vivo (Chaudhuri et al., 2001; Dhar et al., 2001; Schepers et al., 2001). EBNA1 itself was shown to co-immunoprecipitate with ORC2 and oriP replication was inhibited by overexpression of Geminin.
(Dhar et al., 2001). In addition, failure of oriP to replicate in cells that are haploinsufficient for ORC2 provides genetic evidence that oriP is regulated by cellular origin components (Dhar et al., 2001).

Many recent findings have added complexity to replication initiation at the DS element. For instance, TRF1 and TRF2 were shown to bind the nonamers in a cell cycle-dependent manner. ChIP analyses have shown that TRF2 binding to the DS peaks at G1/S while TRF1 peaks at G2/M (Deng et al., 2003; Deng et al., 2002). TRF2 stimulates replication initiation at the DS, perhaps via its interaction with ORC subunits and through recruitment of ORC to the DS element (Atanasiu et al., 2006). TRF1 does not to bind ORC and antagonizes the activity of TRF2, thereby inhibiting replication from the DS. TRF2 selectively binds the nonamers in the DS, which are absent in the FR element. This might explain the preferential recruitment of ORC to the DS element and its absence at the FR even though EBNA1 is constitutively bound to FR. In addition, the RGG-motifs in the N-terminal region of EBNA1 bind G-rich RNAs, which have been shown to interact with Orc1 peptides (Norseen et al., 2008). This may imply that G-rich RNA species form a bridge between EBNA1 and ORC subunits. Poly-ADP-ribosylation (PAR) has also been suggested in regulation of EBV replication at the DS. EBNA1 itself was shown to be PARylated, a modification which inhibits its replication activity (Deng et al., 2005; Deng et al., 2002). Downregulation of PARP1 increased EBNA1, ORC2 and MCM3 recruitment to oriP (Deng et al., 2002). Then, PARylation negatively regulates EBNA1 mediated EBV replication at the DS element. Finally, Chk2 kinase has also been shown to play a role in the regulation of replication at the oriP. Chk2 phosphorylates TRF2, and a phoso-mimetic mutation at the Chk2 phosphorylation site in TRF2 reduces its ability to recruit ORC, implying a negative role for Chk2 phosphorylation (Zhou et al., 2010). Consistent with these observations, shRNA-induced depletion of Chk2 results in reduced replication efficiency and maintenance of oriP-containing plasmids. Therefore, Chk2 is likely important in coordinating ORC recruitment and replication in a cell cycle-dependent manner through modulation of TRF2.

1.2.1.2 DNA Segregation

During latent EBV infection in proliferating cells, the replicated EBV genomes are equally partitioned to the daughter cells in order to maintain a constant copy number of EBV episomes. There are only two viral elements required for partitioning or segregation of EBV genomes; EBNA1 and the cis-acting family of repeat (FR) element of oriP (Krysan, Haase, and
Calos, 1989; Lee, Diamond, and Yates, 1999; Lupton and Levine, 1985b). The FR consists of 20 copies in tandem of a 30 bp sequence, each of which has an 18 bp palindromic EBNA1 binding site followed by a 12 bp AT-rich sequence (Rawlins et al., 1985a; Reisman, Yates, and Sugden, 1985) (Figure 1-7). EBNA1 binding to FR is critical for the segregation of the EBV genomes (Gahn and Sugden, 1995; Reisman and Sugden, 1986a; Sugden and Warren, 1989).

Both EBV genomes and EBNA1 associate with host metaphase chromosomes (Grogan et al., 1983; Harris, Young, and Griffin, 1985; Petti, Sample, and Kieff, 1990) during mitosis. In fact, EBNA1 tethers EBV genomes or FR-containing plasmids to host chromosome such that they are ‘piggy-backed’ into daughter cells on the host chromosomes. There are several findings in support of this model. For example, the FR region is required for association of oriP-containing plasmids with cellular chromosomes (Kanda, Otter, and Wahl, 2001; Krysan, Haase, and Calos, 1989). Second, the deletion of EBNA1 sequences 325-376 greatly reduces the interaction of EBNA1 with host mitotic chromosomes and prevents segregation of EBV-based plasmids (Wu, Kapoor, and Frappier, 2002b) (see Figure 1-5). Third, EBNA1 N-terminal sequences important for segregation can be replaced with the HMG I/Y or histone H1 chromatin binding sequences and this is sufficient for efficient plasmid maintenance (Hung, Kang, and Kieff, 2001).

Although EBNA1 directly binds to the FR, interaction is with host mitotic DNA may involve multiple mechanisms. EBNA1 could attach to cellular DNA either by direct binding or indirectly through interactions with cellular chromosome-associated proteins. Our lab has isolated a highly conserved protein that interacts with EBNA1 sequences critical for segregation function and chromosome attachment, termed EBNA binding protein 2 (EBP2) (Shire et al., 1999). EBP2 is nucleolar in interphase and associates with chromosomes in mitosis. It was shown to be important for EBNA1 segregation function through the tethering of EBNA1-bound EBV episomes to mitotic chromosomes in human cells and was shown to enable EBNA1-mediated segregation of plasmids in yeast (Kapoor and Frappier, 2003; Kapoor, Lavoie, and Frappier, 2005; Kapoor, Shire, and Frappier, 2001; Wu, Ceccarelli, and Frappier, 2000). Immunofluorescence imaging of EBNA1 and EBP2 during mitosis has revealed that EBNA1 associates with cellular chromosomes prior to EBP2 (Nayyar, Shire, and Frappier, 2009) and that EBNA1 and EBP2 co-localize on chromosomes from mid to late mitosis. This suggests that EBNA1 may initially associate with DNA via mechanisms not involving EBP2, and EBP2 may later stabilize this interaction through the various stages of mitosis. It has also been proposed that
that the chromosomal attachment sites of EBNA1 may have AT-hook activity that can bind to AT-rich DNA (Sears et al., 2003; Sears et al., 2004). Therefore, a combination of interaction with cellular factors like EBP2 and direct interaction with DNA might account for EBNA1 attachment and oriP-plasmid tethering to cellular chromosomes.

In order to faithfully segregate and maintain EBV episomes in proliferation cells it is largely believed that a mechanism must exist to ensure equal distribution of the EBV episomes on the two paired chromosomes in metaphase. Recent studies support this notion of symmetrical pairing of EBV episomes on daughter chromosomes. Kanda et al. (Kanda et al., 2007) generated a recombinant EBV baculovirus (BAC) expressing hemagglutinin (HA)-tagged EBNA1 and examined the localization of EBNA1 and the EBV BAC in human cells by immunofluorescence microscopy for the HA epitope and fluorescence in situ hybridization (FISH), respectively. They showed that EBNA1 localized with the BAC and was often symmetrically distributed on sister chromatids in mitosis. Further, this symmetrical localization of EBNA1 dots was observed in prematurely condensed G2 chromosomes as well, correlating with the presence of closely spaced double dots of EBNA1 in G2-phase-enriched cells (Kanda et al., 2007). They suggest that this pairing of replicated molecules through catenation of the newly replicated circular molecules is linked to segregation in mitosis. Similarly, Nanbo et al. (Nanbo, Sugden, and Sugden, 2007) also reported transient pairing of EBV-based plasmids in G2.

1.2.1.3 Transcription activation

EBNA1 is known to transcriptionally activate expression of several viral latency genes through binding to the FR enhancer element (Rickinson and Kieff, 2001). Specifically, EBNA1 binding to the FR enhances transcription from the Cp promoter, from which all EBNA genes are transcribed, and from the promoter controlling LMP1 expression (Sugden and Warren, 1989; Wysokenski and Yates, 1989a). EBNA1 and the FR also enhance transcription from many promoters in reporter assays, where six of the twenty EBNA1 binding sites within the FR element are sufficient for the FR to function as an enhancer (Wysokenski and Yates, 1989a). Also in reporter constructs, FR has been shown to activate transcription when placed in either orientation, upstream or downstream of the promoter (Ceccarelli and Frappier, 2000; Reisman and Sugden, 1986a; Reisman, Yates, and Sugden, 1985). There are two regions of EBNA1 that have been shown to be important for its transcriptional activity (see Figure 1-5). The first being in the N-terminal region including amino acids 61-89 (Kennedy and Sugden, 2003; Wu, Kapoor,
and Frappier, 2002b) and the second the 325-376 sequence (Ceccarelli and Frappier, 2000). It is not solely the Gly-Arg richness of the 325-376 region that is important for transactivation as mutations of the four serines repeated through this region to alanines inhibited the transactivation function of EBNA1 in reporter assays (Shire et al., 2006).

Recently the 61-89 was shown to interact with bromodomain protein 4 (Brd4) and play a role in EBNA1 mediated transcriptional activation of reporter constructs (Lin et al., 2008). Interestingly, the functional homologue of EBNA1 from bovine papillomavirus (BPV), the E2 protein, also binds Brd4 (You et al., 2004). This is important for E2’s function in transcriptional activation and may also contribute to mitotic segregation (McPhillips et al., 2006; Schweiger, You, and Howley, 2006). The second EBNA1 region involved in transcriptional activation, 325-376, is important for binding the nucleosome assembly protein, NAP1 (Holowaty et al., 2003b). NAP1 has been implicated in transcription regulation (Park and Luger, 2006) and was shown to preferentially associate with the FR element and contribute to EBNA1 mediated transcription in reporter assays (Wang and Frappier, 2009). These studies suggest a role for NAP1 in EBNA1-mediated transcriptional activation. In addition, recent work by Sarkari et al. (Sarkari et al., 2009) have shown that EBNA1 recruits USP7 and GMP synthetase (GMPS) to the FR element on oriP, where they affect ubiquitination of H2B and transcription. Down-regulation of USP7 reduced the level of GMPS at the FR, increased the level of monoubiquitylated H2B in this region and decreased the ability of EBNA1 to activate transcription from the FR (Sarkari et al., 2009). These observations indicate that USP7 can also stimulate EBNA1-DNA interactions and that EBNA1 can alter histone modification at oriP through recruitment of USP7.

Since EBNA1 can bind regulatory elements in the EBV genome to activate transcription, there is the possibility that it might modulate transcription of some cellular genes. In support of this idea is the observation that FR-like sequences have been found in the cellular genome using a nearest-neighbor positional weight matrix (d’Herouel, Birgersdotter, and Werner, 2010). Using this technique, 40 novel regions have been identified in the human genome, which contain tandemly repeated binding sites for EBNA1. Genes located in the vicinity of these regions have been suggested to be possible targets for EBNA1-mediated regulation including: IQCB1, IMPG1, IRF2BP2 and TPO. Additional cellular genes such as HDAC3, CDC7, and MAP3K1 have also been identified to be positively regulated by EBNA1 (Lu et al., 2010). Although the functional significance of these genes is yet to be elucidated, there is increasing evidence that implicates EBNA1 in regulation of cellular genome (Canaan et al., 2009; Dresang, Vereide, and
Sugden, 2009; Lu et al., 2010). Using microarray analysis with the B cell BJAB and the epithelial 293 cell lines transfected with EBNA1, Canaan et al. (Canaan et al., 2009) have shown that EBNA1 binds distinct cellular promoters in the two cell lines. They have correlated EBNA1 binding to promoters with changes in gene expression. Detailed analysis of the 100 promoters most enriched revealed a DNA motif that differs from the EBNA1 binding site in the EBV genome (Canaan et al., 2009), suggesting that EBNA1 may use different mechanisms to regulate viral and cellular gene expression. Furthermore, using the combined techniques of ChIP and microarray, additional DNA-binding sites for EBNA1 have been identified in the genomes of EBV (Dresang, Vereide, and Sugden, 2009), suggesting layers of transcriptional control mediated by EBNA1 on host and EBV genes. It seems that EBNA1 can interact with a number of cellular genes and chromosomal loci in latently infected cells, but that these sites are likely to represent a complex ensemble of direct and indirect EBNA1 binding sites.

1.2.1.4 Auto-regulation via Qp promoter

EBNA1 protein levels are very tightly regulated, in part by EBNA1 itself. EBNA1 transactivates all EBNA's from the Cp and Wp promoter, but it represses its own expression from the Qp promoter that is used in latencies I and II. The Qp promoter is used to express EBNA1 in the absence of the other five EBNAs (Nonkwelo et al., 1996; Sample, Henson, and Sample, 1992); hence, this mechanism of regulation is important for NPC and GC. Interestingly, EBNA1’s repressive effects on Qp are independent of the FR element. There are two EBNA1 binding sites downstream of Qp (Sample, Henson, and Sample, 1992) and EBNA1’s affinities for these sites are much weaker than those in the FR (Ambinder et al., 1990; Jones, Hayward, and Rawlins, 1989). This suggests that EBNA1 would only bind this site when its levels are high and both FR and DS are saturated by EBNA1. This implies that a negative feedback loop is present to maintain steady levels of EBNA1. A recent study has suggested that EBNA1 may not directly inhibit transcription from Qp but rather act post or co-transcriptionally to prevent processing of primary transcripts (Yoshioka, Crum, and Sample, 2008).

1.2.1.5 Lytic infection

It is intriguing that EBNA1 is the only latent protein that continues to be expressed in lytic infection and this occurs from the BamHI F promoter, Fp (Brink et al., 2001; Lear et al., 1992; Nonkwelo et al., 1996; Schaefer, Strominger, and Speck, 1995). This promoter is 200 base-pairs upstream of the Qp latency promoter for EBNA1. Fp is now considered an early lytic
promoter (Schaefer, Strominger, and Speck, 1995). Using reporter constructs of 146 base-pairs, three cis-acting elements of importance for Fp regulation have been identified: one Sp1 site and two tandem LR1 sites (Figure 1-8) (Bulfone-Paus, Dempsey, and Maizels, 1995). Earlier studies by Lear et al. (Lear et al., 1992) demonstrated that Fp was upregulated upon entry of EBV-infected cells into the lytic cycle. Recently, the BZLF1 protein has been shown to activate transcription from the Fp promoter via binding to an AP-1-like site in the promoter (Zetterberg et al., 2002). While the expression of EBNA1 from the Fp promoter is well established, the functional significance of this promoter switch and the requirement for EBNA1 in the lytic cycle of EBV has not been studied.

Figure 1-8: Sequences involved in the regulation of the EBV Fp (Adapted with permission from Zetterberg et al., 2000) (a) Schematic illustration of part of the BamHI F and Q fragments of the EBV genome. The sequence coordinates are from the DNA sequence of the B95-8 EBV genome (Baer et al., 1984). The bent arrows indicate the Fp and Qp transcription start sites at positions 62239 and 62429, respectively. (b) Detailed map of previously identified upstream Fp-proximal transcriptional elements and putative transcriptional elements described in this paper. Black boxes indicate AP-1-like sites. Open boxes indicate LR1 and Sp1 sites.

A role for EBNA1 in BZLF1 expression has been briefly explored using transfer infection of AdAH epithelial cells with EBNA1 knock-out green fluorescent protein (GFP) tagged virus. Cells were sorted for GFP and all infected cells were analyzed for BZLF1 expression twenty-four hours post infection. Interestingly, there was a reduction in BZLF1 protein expression and a marked decrease in progeny virion production (Shannon-Lowe et al., 2009). This suggested that EBNA1 is required for an efficient lytic cycle and may control the expression of the lytic master regulator, BZLF1. The same study showed that infection with a
BZLF1 knock-out virus reduced the number of EBNA1 positive cells and decreased virion production, which is consistent with the findings of Zetterberg et al. (Zetterberg et al., 2002) that BZLF1 can bind and transactivate Fp (Shannon-Lowe et al., 2009). This shows how two very important proteins of EBV, EBNA1 and BZLF1, may regulate each other, having potentially important consequences in the regulation of latent and lytic programs of EBV.

1.2.1.6 Cell immortalization and transformation

The role of EBV in the development of specific cancers has been well established and is due to the latent mode of infection that can result in immortalization of the host cell giving rise to tumours of lymphoid and epithelial origin. Both LMP1 and EBNA2 have been shown to have important roles in B-cell immortalization in vitro (Rickinson and Kieff, 2001). The exact role of EBNA1 in host cell immortalization has been difficult to establish, in part due to its dispensability in maintaining the EBV genome in proliferating cells. However, the observation that in NPC and GC, EBNA1 is the only latent protein expressed with very little to no LMP1 and absence of EBNA2 would suggest a more direct role of EBNA1 in host cell transformation and immortalization.

Several lines of evidence implicate EBNA1 in cellular pathways that promote genomic instability and tumorigenesis, while decreasing apoptosis of infected cells (Cheng et al., 2010; Flavell et al., 2008; Hong et al., 2006; Ian et al., 2008; Kaul et al., 2007; Lu et al., 2011; Murakami et al., 2005; Saridakis et al., 2005; Yin and Flemington, 2006). For example, a dominant-negative version of EBNA1 that lacks the N-terminal portion of EBNA1 decreased survival of Burkitt’s lymphoma cells (Kennedy, Komano, and Sugden, 2003), which suggests that EBNA1 functions to allow survival of EBV-positive or EBNA1-positive cells. Likewise, RNAi-based down regulation of EBNA1 decreased cell proliferation in EBV-positive Burkitt’s lymphoma cells and nasopharyngeal carcinoma cells (Hong et al., 2006). Since EBNA1 is required for the maintenance of EBV genomes and expression of other viral genes, the interpretation of these observations are not clear, as EBNA1 may indirectly affect survival by enabling the continued expression of other EBV components.

To avoid such concerns, many studies have investigated the function of EBNA1 in the absence of EBV genomes. The first of such studies showed that expression of EBNA1 in transgenic mice induced B-cell lymphomas (Wilson, Bell, and Levine, 1996). However, others have since shown that the same transgenic mice had similar prevalence of lymphomas with and
without EBNA1 expression (Kang et al., 2005; Kang et al., 2008). It is evident that EBNA1's ability to induce lymphomas in transgenic mice requires additional work and should be tested in mice with multiple genetic backgrounds. An alternative approach involved expressing EBNA1 in EBV-negative cell lines, followed by assessment of their tumorigenicity in immunocompromised mice. Using this method, EBNA1 expression in nasopharyngeal carcinoma cells and Hodgkin's lymphoma cells has been shown to promote tumorigenicity in nude mice and non-obese diabetic-SCID mice, respectively (Kube et al., 1999; Sheu et al., 1996). In another study, EBNA1 expressed in breast carcinoma cells promoted their growth into tumors and lung metastasis (Kaul et al., 2007). Therefore, these studies support for a direct role of EBNA1 in malignant transformation.

The presence of EBV has also been shown to induce genomic instability (Gualandi et al., 2001; Kamranvar et al., 2007). For example, direct oxidative stress caused by hydrogen peroxide enhanced the number of micronuclei (marks of genomic instability) only in an EBV-positive cells. These cells were also resistant to apoptosis. This suggests that apoptosis may efficiently eliminate cells with genetic damage if they are EBV-negative but not if they are EBV-positive (Gualandi et al., 2001). This implies that proteins encoded by EBV during latency promote genomic instability, but decrease apoptosis.

In keeping with the above observations, recent studies have shown that EBNA1 itself induces chromosomal aberrations, DNA double-strand breaks, and engages the DNA damage response (DDR). These signs of genomic instability are associated with the production of reactive oxygen species (ROS) and are reversed by antioxidants (Gruhne et al., 2009; Gruhne, Sompallae, and Masucci, 2009). In addition, EBNA1 expressed on its own is sufficient to inhibit apoptosis after exposure to DNA damage agents (Saridakis et al., 2005). These observations support a more direct role for EBNA1 in immortalization and transformation, through the accumulation of DNA damage. Furthermore, EBNA1 can alter the cellular environment in ways that may promote tumorigenesis, as microarray analyses have shown that EBNA1 increases STAT1 and decrease TGF-β levels (Wood et al., 2007). EBNA1 has also been reported to disrupt TGF-β signaling, leading to lowered SMAD2 protein levels and decreased tyrosine phosphates receptor kappa activity (Flavell et al., 2008). Regulation of this pathway by EBNA1 may allow the growth and survival of EBV infected Hodgkin lymphoma cells (Flavell et al., 2008).
1.2.3 EBNA1-interacting proteins

Since EBNA1 does not exhibit any known enzymatic activities, it has been proposed to carry out its many functions by interacting with cellular proteins. EBNA1 interacting proteins have been identified by several different methods. Yeast two hybrid screens using EBNA1 as bait have identified p32/TAP (Chen et al., 1998; Shire et al., 1999; Wang et al., 1997), importins α and β (Fischer et al., 1997; Ito et al., 2000; Kim et al., 1997) and EBP2 (Shire et al., 1999). The interaction with the importins is may be important for the nuclear import of EBNA1. The interaction with p32/TAP has been suggested to be important for transcriptional activation by EBNA1 (Chen et al., 1998), however this interaction does not correlate well with the transcriptional activity of EBNA1 mutants (Holowaty et al., 2003). Also the tendency of P32/TAP to interact with a wide variety of basic proteins draws into the question the functional significance of this promiscuous interaction. EBP2 has been shown bind EBNA1 through amino acids 325-376 and is involved in EBNA1-mediated segregation of EBV-based plasmids as discussed above (Kapoor, Shire, and Frappier, 2001; Shire et al., 1999). In addition, a telomere-associated poly-ADP ribose polymerase, tankyrase (TNKS1 and 2), has been shown to bind and modify EBNA1. This study demonstrated that TNKS proteins can interact directly with the EBNA1 protein, associate with the FR region of oriP in vivo, and inhibit oriP replication in a PARP-dependent manner (Deng et al., 2005). This may be a mechanism to finely tune latent viral replication mediated by EBNA1.

EBNA1 has been shown to interact with or alter the properties of cellular proteins involved in proliferation and apoptosis (Cheng et al., 2010; Lu et al., 2011; Saridakis et al., 2005; Sun et al., 2010). One such observation is the ability of EBNA1 to bind Nm23-H1 and counteract Nm23-H1 mediated growth inhibition and suppression of cell migration in vitro (Murakami et al., 2005), although this interaction has not been shown to be direct. This effect of EBNA1 has been proposed to promote tumor growth in xenografts of breast carcinoma cells in nude mice (Kaul et al., 2007).

EBNA1-containing protein complexes have also been identified using Tandem Affinity Purification (TAP) of EBNA1 from human 293T cells, (Holowaty et al., 2003b). The identity of co-purifying proteins was then identified by MALDI-TOF mass spectrometry. Both TAP tagging and affinity column profiling with EBNA1 led to the discovery of several cellular proteins that bind EBNA1 including NAP1, TAFI, importin, protein kinase CK2, and PRMT1/5 (Holowaty et al., 2003b). In these studies EBNA1 was also found to stably interact with
herpesvirus-associated ubiquitin specific protease (USP7 or HAUSP) (Holowaty et al., 2003b). USP7 was originally identified as an interacting partner of the ICP0 protein of herpes simplex virus (Everett et al., 1997; Meredith, Orr, and Everett, 1994). Later it was shown that, upon cellular stress, USP7 deubiquitinates the p53 tumor suppressor and protects p53 from proteasome-mediated degradation. The resulting accumulation of p53 promotes apoptosis and cell cycle arrest (Li et al., 2004). EBNA1 binding to USP7 interferes with this stabilization of p53 by USP7, thereby decreasing p53-dependent apoptosis, hence promoting cell survival (Saridakis et al., 2005). This is due to the fact that EBNA1 and p53 bind to the same pocket in the N-terminal domain (NTD) of USP7 (Saridakis et al., 2005; Sheng et al., 2006). However EBNA1 makes more extensive contacts with USP7 resulting in a ten-fold greater affinity for USP7 than p53 (Holowaty et al., 2003a). These observations provide a structural framework for understanding how EBNA1 might contribute to the survival of EBV infected cells.

In addition to USP7, EBNA1 has been shown to bind directly to protein-arginine methyl transferases PRMT1 and PRMT5 and to casein kinase 2 (CK2). These proteins can modify EBNA1 and possibly regulate its functions (Shire et al., 2006) (Cao et al., unpublished observations). Conversely, EBNA1 could potentially affect the cellular functions of these enzymes. Interestingly, PRMT5 methylates p53 and regulates its apoptotic function (Jansson et al., 2008). CK2 has many protein targets and modifies proteins in ways that promote tumourigenesis and cell proliferation (Duncan and Litchfield, 2008; Litchfield, 2003; Scaglioni et al., 2006). One of these targets is the PML tumor suppressor protein (discussed in detail below), which is targeted for polyubiquitination and degradation after CK2 phosphorylation (Scaglioni et al., 2006). It is conceivable that EBNA1 can hijack the functions of both PRMT5 and CK2, shifting the cellular equilibrium towards transformation and immortalization.
1.3 PML

The promyelocytic leukemia (PML) protein received widespread interest because of its link to several cancers, including acute promyelocytic leukemia (APL). Hence, PML is recognized as a cellular tumor suppressor protein that forms distinct structures within the nucleus of the cell, where the PML protein is essential for the nucleation of PML nuclear bodies (NBs). These foci are also known as nuclear domain 10 (ND10s) or PML oncogenic domains (PODs). These bodies can act as scaffolds for cellular reactions or functions including apoptosis, transcriptional regulation and DNA repair and they are part of the interferon-mediated anti-viral response (see Figure 1-9) (Borden, 2002; Dellaire and Bazett-Jones, 2004; Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Lallemand-Breitenbach and de The, 2010; Reineke and Kao, 2009). Hence, there is much interest in the regulation of PML and bodies formed by them.

Figure 1-9: PML Nuclear bodies and associated functions. The middle immunofluorescence image (IF) is representative of the nuclear bodies formed by the PML protein in CNE2 NPC cell line (shown as green foci) in the nucleus of the cell. The exact number of NBs formed varies from cell line to cell line. A schematic of some of the functions associated with PML NBs are shown around the IF image, which are mediated by the coordinated action of numerous cellular proteins that stably or transiently associate with the PML NBs.

Recently, Scaglioni et al. (2006) have demonstrated that CK2, a kinase associated with cancer promotion (Duncan and Litchfield, 2008), phosphorylates PML and targets it for degradation by the proteasome (see Figure 1-10).
Figure 1-10: **CK2 mediated disruption of PML NBs and degradation of PML protein.** CK2 regulates PML protein levels by promoting its ubiquitin-mediated degradation dependent on direct phosphorylation at Ser517 indicated by the orange P. Phosphorylation of this site primes it for polyubiquitination (indicated by the yellowish Ub. tails) by an unknown ubiquitin ligase and degradation by the proteasome.

Of particular interest for my thesis is the ability of EBNA1 to stably interact with all three subunits of CK2 (Holowaty et al., 2003b), since it has been well characterized for its ability to mediate PML NB disruption and target PML tumor suppressor protein for degradation. Hence, the focus of my thesis has been to determine whether EBNA1 can alter the multi-functional PML NBs (see Figure 1-11). In order to understand the remainder of my thesis, it is important that I describe PML protein and related functions that may have consequences in the development of EBV-associated epithelial tumours and regulation of EBV reactivation.

Figure 1-11: **Proposed hypothesis for regulating PML NBs by EBNA1.** EBNA1 stably interacts with all three subunits of the CK2 enzyme, which phosphorylates PML targeting it for ubiquitination and degradation. EBNA1 could potentially disrupt PML NBs through the action of CK2, thereby contributing to the tumorigenesis process.
1.3.1 Identification and characterization of promyelocytic leukemia (PML) protein

The PML gene was originally identified in APL patients as a result of a chromosomal translocation that juxtaposes the PML gene on chromosome 15 and the retinoic acid receptor (RAR)α gene on chromosome 17 (Kakizuka et al., 1991). Consequently, a chimeric protein of PML-RARα is produced, which retains the DNA-binding and ligand-binding domains of RARα and the multimerization domain of PML (Di Croce, 2005). However, the chimeric PML disrupts the localization of the wild-type PML from normal PML NBs into multiple micro speckles (Brown et al., 2009). This induces a maturation block at the promyelocytic level and initiates the onset of disease and APL phenotype (Wang and Chen, 2008).

PML is a highly conserved gene in mammals but its absence in lower eukaryotes and plants would imply that it may not be essential for viability (Borden, 2002). In fact, pml -/- mice develop normally further implying that PML is not essential for growth (Guo et al., 2000). PML is alternatively spliced to generate seven isoforms from 9 different exons that vary in size from 48 - 97 kDa (see Figure 1-12) (Jensen, Shiels, and Freemont, 2001). When analyzed on SDS-PAGE followed by Western blotting, multiple bands can be observed for PML starting from 50 to 160 kDa as a result of alternative splicing and from post-translational modification of the PML protein (Burkham et al., 2001; Jensen, Shiels, and Freemont, 2001; Pandolfi et al., 1991). The PML protein contains distinct domains that are important for protein-protein interactions and self-association/multimerization. It is a member of the tripartite motif (TRIM) family of proteins that contain the RBCC motif (made up of RING finger, B-box, and coiled-coil regions) in the N-terminal region (see Figure 1-12) (Nisole, Stoye, and Saib, 2005). All seven isoforms (I-VII) contain the TRIM domain in the N-terminal region (exons 1-4) and they differ in their cytoplasmic tails (exons 5-9), giving unique PML protein profiles (Jensen, Shiels, and Freemont, 2001). PML isoforms I through VI contain a nuclear localization signal (NLS), at amino acids 479-490 (Melnick and Licht, 1999) and thus, are found in the nucleus whereas isoform VII is cytoplasmic due to lack of the exon containing the NLS. Each PML isoform may take part in specific protein interactions dictated by their unique C-termini, which may explain the involvement of PML NBs in so many cellular processes and the many proteins that have been identified to associate with them.
Figure 1-12: Seven PML isoforms are expressed in human cells (Adapted with permission from Salomoni et al., 2008). PML proteins are comprised of six nuclear isoforms (PML I-VI) and one cytoplasmic isoform (PML VII) that results from alternative splicing. The general PML domain structure is shown on top, which indicates the positions of the RING domain (R, amino-acid residues 45-105), Beta box 1 (B1, residues 124-166), Beta box 2 (B2, residues 184-230), coiled-coil (C, residues 229-323) and exonuclease domain (E, residues ~600-750; exclusive to isoform I). The positions of the nuclear localization sequence (N) and SUMOylation sites (black arrowheads) are also indicated. Below are the alternatively spliced isoforms PML I-VII and the exact exons used to generate the unique PML isoforms are indicated. Exons 1-6 (dark grey) are common to all nuclear isoforms, however, they all vary in their C-termini being encoded by several alternatively spliced exons as indicated (light grey). The absence of exon 6 (which carries the nuclear localization signal) results in the cytoplasmic localization of PML VII.

1.3.2 Architecture and composition of nuclear bodies formed by PML

Using light and electron microscopy the structure and dynamics of PML NBs has been studied extensively. The bodies are 0.1 - 1 μM in diameter and can either appear as spherical or ring-shaped, with very little protein in the core (Eskiw et al., 2003; Lallemand-Breitenbach and de The, 2010; Lallemand-Breitenbach et al., 2001). These bodies are found in the nucleus in the interchromatin domain space and are not associated with DNA or RNA, however, newly synthesized RNA has been found to associate with their periphery (Boisvert, Hendzel, and Bazett-Jones, 2000). Furthermore, the bodies seem to be connected to the surrounding...
chromatin via multiple contacts mediated by protein and chromatin like fibres (Eskiw, Dellaire, and Bazett-Jones, 2004; Eskiw et al., 2003). PML NBs can vary in both number and size in different cell types (Melnick and Licht, 1999) and they differ in composition between cell types and between bodies within the same cell (Bloch et al., 1999).

PML is post-translationally modified by SUMO-1 (Boddy et al., 1996; Ishov et al., 1999; Sternsdorf, Jensen, and Will, 1997), where the SUMO-1 moiety is covalently attached to PML protein on residues K65 (RING finger), K160 (B1 Box), and K490 (NLS) (Kamitani et al., 1998). SUMO-1 is an 11.5 kDa protein (appears as 20kDa by SDS-PAGE), hence PML appears as a ladder of proteins when analyzed by Western blotting due to 7 isoforms each capable of being modified three times by SUMO-1 (Kamitani et al., 1998). SUMO-1 modification of PML proteins is important for their interactions to form NBs (Borden, 2002) as well as for the association of some other proteins (eg. Sp100 and Daxx (Zhong et al., 2000)) with the PML NBs. In addition to sumoylation, PML has multiple sites for phosphorylation with one such site being serine 517 that is phosphorylated by CK2, which has been designated as the phospho-degron site for PML (Scaglioni et al., 2006). This modification then triggers the ubiquitination of PML, through a mechanism that is yet to be identified, and leads to proteasome mediated degradation of the PML protein (Scaglioni et al., 2006).

To date, PML NBs have been documented to stably or transiently associate with over a hundred proteins (Lallemand-Breitenbach and de The, 2010). SUMO-1, Sp100 and Daxx proteins are some of the core residents of the typical PML NB. It is important to note that SUMO-1 modification and association with Sp100 seem to regulate the protein localization dynamics with the bodies (Eskiw et al., 2003; Maul et al., 2000; Zhong et al., 2000), which directly affects the function of each PML NB. Since SUMO-1 modification is vital for PML function, the removal of SUMO-1 from PML is equally important for NB dynamics and regulation. Removal of SUMO-1 is regulated by SUMO proteases, and three such proteases have been identified in mammals; SENP-1, SENP-2, and SENP-3 (Best et al., 2002; Gong et al., 2000). The action of SENP's on PML NBs results in the redistribution and reorganization of PML NBs and associated components (Best et al., 2002; Nefkens et al., 2003). Interestingly, viruses also have evolved mechanisms to prevent the SUMOylation of PML to over-come the repressive nature of PML during lytic viral infection (to be discussed in detail in 1.3.6) (Adamson and Kenney, 2001; Bailey and O'Hare, 2002). Other environmental factors that PML NBs respond to include: heat-shock, heavy-metal stress, inhibition of transcription, exposure to
exogenous nucleases and DNA damage which affect the structure and modifications if the PML NBs (Eskiw, Dellaire, and Bazett-Jones, 2004; Eskiw et al., 2003; Nefkens et al., 2003).

1.3.3 PML NB associated functions

There are three general views on the function of PML NBs. The first is that PML NBs act as depots for nuclear proteins (Negorev, Ishov, and Maul, 2001; Negorev and Maul, 2001), waiting to be used or to be degraded. In support of this view is the observed accumulation of Daxx (proapoptotic protein) at PML NBs only when its function is repressed (Lehembre et al., 2001). This could be a mechanism to fine tune the levels of protein that function in the nucleoplasm.

In the second model, PML NBs act as sites for post-translational modifications of target proteins. This model was put forward due to the observation that proteins involved in post-translational modifications and their substrates were found at PML NBs. A well characterized example is p53. p53 is known to be acetylated and phosphorylated by CREB binding protein (CBP) and homeodomain interacting protein kinase 2 (HIPK2), respectively at PML NBs. The accumulation of acetylated p53 in PML NBs was linked to p53-mediated cell death, which lead to the identification of a functional role for PML in apoptosis (D'Orazi et al., 2002; Lain et al., 1999; Pearson et al., 2000). In addition, p53 has been shown to interact with the C-terminal sequence of PML IV (Fogal et al., 2000). The kinase responsible for the activation of p53, HIPK2, was also shown to interact specifically with PML IV (Hofmann et al., 2002; Moller et al., 2003). Hence, PML NB acts as an important scaffold for the activation of p53 upon DNA damage to maintain cellular integrity, and similar observations have made for DNA repair proteins (Boe et al., 2006; Carbone et al., 2002; Dahm and Hubscher, 2002; Dellaire et al., 2006).

In the third model, it is proposed that PML NBs associate with and maintain a subset of genes (Ching et al., 2005). PML NBs have been observed to make contacts with the surrounding chromatin (Boisvert, Hendzel, and Bazett-Jones, 2000), however, it is not evident whether these are random or specific genes associated with PML NBs. Interestingly though, PML NBs are often associated with transcriptionally active regions of the genome (Wang et al., 2004). ImmunoFISH studies have demonstrated that PML NBs associate with some specific loci, including MHC II and TP53 (Shiels et al., 2001; Sun, Durrin, and Krontiris, 2003). Hence, the chromatin contacts made by PML NBs may result in the regulation of a specific set of genes directly through the action of PML protein or through cellular proteins found at PML NBs.
Interestingly, this property of PML NBs may be exploited by viruses since replicating genomes from many DNA viruses are known to associate with PML NBs (perhaps for transcriptional regulation) and these viruses are also known to modify the PML NB structure (Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011) (to be discussed in 1.3.6).

Regardless of how it mediates its actions, PML NBs have been linked to many important cellular functions since they are made up of and interact with proteins with diverse functions. This includes, but is not limited to, transcription (Zhong, Salomoni, and Pandolfi, 2000), DNA repair (Dellaire and Bazett-Jones, 2004), apoptosis (Takahashi et al., 2004), proteolysis (Lallemand-Breitenbach et al., 2001) and, of importance for my thesis, tumor suppression (Salomoni and Pandolfi, 2002) and the antiviral response (Regad and Chelbi-Alix, 2001).

1.3.4 The role of PML in tumor suppression

PML-RARα is a chimera that prevents the formation of normal PML NBs giving rise to APL (Dyck et al., 1994), implicating PML and PML NBs in the pathogenesis of leukemia. Arsenic trioxide is a drug that shows substantial anticancer activity in patients with APL. It does this by promoting the formation of disulfide bond between molecules of PML and PML-RARα (Zhao et al., 2001). Once multimers are formed it increases the interaction with the SUMO-conjugating enzyme UBC9, resulting in enhanced SUMOylation. RNF4, a SUMO-dependent ubiquitin ligase, is recruited to PML NBs through interactions between its SIM domain and SUMO moieties of PML (Hakli et al., 2005; Lallemand-Breitenbach et al., 2008; Percherancier et al., 2009; Tatham et al., 2008). Once at the bodies, PML and PML-RARα is polyubiquitinated and subsequently degraded (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Zhang et al., 2010). Arsenic trioxide will also cause a partial differentiation and apoptosis of the APL blasts (Chen et al., 1996).

Since the discovery of its link to APL, PMLNBs have been identified to play a tumour suppressive role in several different types of human neoplasms. One of the most important barriers against tumorigenesis is cellular replicative senescence, a permanent exclusion from the cell cycle, which can be caused by induction of the DNA damage response. Markers of senescence have been detected in vivo in normal tissues and more importantly in pre-neoplastic lesions, which suggests that senescence can occur in the early events of tumorigenesis (Bartkova et al., 2006; Campisi and d'Adda di Fagagna, 2007; Mallette, Gaumont-Leclerc, and Ferbeyre, 2007). PML’s role in cellular senescence was first observed from overexpression of PML that
resulted in cell cycle arrest in cancer cell lines (He et al., 1997; Le, Yang, and Chang, 1996; Mu et al., 1997). This resulted in increased levels of pRb and consequently a $G_1$ arrest (Ferbeyre et al., 2000; Le et al., 1998; Mu et al., 1997). Furthermore, PML was shown to potentiate the repressive function of pRb, where PML overexpression allows accumulation of the active hypophosphorylated form of pRb, which prevents cell cycle progression (Khan et al., 2001). Consistent with the pRb effects, cells arrested in $G_1$ have increased levels of PML. PML levels also increased upon overexpression of p53 (another protein whose activity is important for cellular senescence) (Campisi and d'Adda di Fagagna, 2007; Mu et al., 1997; Serrano et al., 1997). PML-IV specifically has been shown to induce replicative senescence in primary human and mouse fibroblasts (Bischof et al., 2002; Mallette et al., 2004). These findings suggest an important role for PML in the induction of cellular senescence that is achieved through the regulation of the tumour suppressors, pRb and p53.

Another mechanism by which PML NBs contribute to tumour suppression is by regulating apoptosis. First, overexpression of PML is known to initiate apoptosis that can be inhibited by the use of pan-caspase inhibitor zVAD (Narita et al., 2003). Second, cells from Pml-/- mice have decreased susceptibility to apoptosis compared to cells from wild-type mice. This observation also applies to apoptosis induced by ionizing radiation (a stimulus which is primarily mediated by p53) (Guo et al., 2000; Lowe et al., 1993; Yang et al., 1997). As described earlier, PML NB-mediated activation of p53 is an important step in p53-dependent apoptosis. In addition to regulating the activity of p53, PML is known to regulate p53 levels upon cellular stress through inhibition of Mdm2 (the major E3 ubiquitin ligase for p53) (Bernardi et al., 2004; Haupt, Louria-Hayon, and Haupt, 2003; Kurki, Latonen, and Laiho, 2003; Louria-Hayon et al., 2003; Wei et al., 2003; Zhu, Wu, and Maki, 2003). PML has also been shown to sequester Mdm2 to the nucleolus, thereby promoting p53 stabilization/activation in the presence of DNA damage (Bernardi et al., 2004). The multifaceted regulation of p53 by PML marks the importance of apoptosis regulation in healthy cells, a hallmark that is absent in most tumours.

In addition, there are several other pathways that PML modulates to regulate tumour suppression. PML has been shown to bind and sequester eukaryotic initiation of translation factor 4 E (eIF4E), a potent oncogene (Cohen et al., 2001; Culjkovic, Topisirovic, and Borden, 2007). This has important implications as eIF4E is a central node in an RNA regulon which governs proliferation and survival and PML has been shown to be a potent inhibitor of this regulon (Cohen et al., 2001; Culjkovic, Topisirovic, and Borden, 2007). More importantly, PML
has been implicated in controlling genomic stability, a mechanism used by tumour suppressors to limit tumour initiation/progression. Effectors of the DNA damage pathways are regulated by PML NBs, for instance, DNA helicase bloom protein (BLM) localizes to PML NBs. In the absence of PML, increased sister chromatid exchange is observed, a process normally repressed by BLM (Zhong et al., 1999). In addition, MRE11 (involved in DNA damage response) localizes to PML NBs and interacts with PML protein (Carbone et al., 2002; Lombard and Guarente, 2000). Upon DNA damage, MRE11 is released from PML NBs and localizes to DNA damage foci, which implicates PML in regulating MRE11 availability (Carbone et al., 2002).

In addition to the roles of PML in several pathways that prevent tumour formation, the observation that PML expression is frequently lost in human cancers (see Table 3) provides strong evidence that PML suppresses tumor formation and/or progression. PML loss correlates with higher tumour grade in breast adenocarcinomas, prostate carcinomas and CNS tumours (Gambacorta et al., 1996). It is important to note that, although PML is reduced at the protein level in these tumours, expression of PML mRNA is not affected nor are there mutations found in the PML gene (Gambacorta et al., 1996; Gurrieri et al., 2004). This implies that PML is actively degraded in cancer cells (Gurrieri et al., 2004), which is mediated by the ubiquitin-proteasome system. Consistent with this conclusion, Scaglioni et al. (Scaglioni et al., 2006) have shown that CK2-dependent phosphorylation controls PML ubiquitination and that this mechanism is important for tumorigenesis. In this thesis, I will show how EBNA1 exploits this latter function of CK2 to bring about the disruption of PML NBs and degradation of the PML protein and contributes to EBV-associated epithelial tumours (Sivachandran, Cao, and Frappier, 2010; Sivachandran, Sarkari, and Frappier, 2008b)(Sivachandran et al. 2011, submitted).
### Table 3: Tumours with loss of PML Expression (Reviewed in (Salomoni et al., 2008))

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<tr>
<th>Tumour Type</th>
<th>Percentage</th>
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<tr>
<td>17% of colon adenocarcinomas</td>
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<tr>
<td>21% of lung tumours</td>
<td></td>
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<tr>
<td>27% of prostate adenocarcinomas</td>
<td></td>
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<tr>
<td>31% of breast adenocarcinomas</td>
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<tr>
<td>49% of CNS tumours:</td>
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<tr>
<td>- 100% medulloblastomas</td>
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<tr>
<td>- over 90% oligodendroglial tumours</td>
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<tr>
<td>49% of germ cell tumours</td>
<td></td>
</tr>
<tr>
<td>68% of non-Hodgkin’s lymphomas</td>
<td></td>
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<tr>
<td>- 83% diffuse large cell lymphomas</td>
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<tr>
<td>- 77% follicular lymphomas</td>
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<tr>
<td>Other cancers that show loss of PML expression:</td>
<td></td>
</tr>
<tr>
<td>- Breast carcinomas</td>
<td></td>
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<tr>
<td>- Gastric cancer</td>
<td></td>
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<tr>
<td>- Small cell lung carcinoma</td>
<td></td>
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<tr>
<td>- Invasive epithelial tumours</td>
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#### 1.3.5 The role of PML in lytic viral infection

The cell has developed various barriers and mechanisms to defend itself from foreign matter including viral infections. The role of interferons (IFNs) is to establish an antiviral state in cells, and this was the defining property that led to their discovery (Geoffroy and Chelbi-Alix, 2011). IFNs confer resistance to viral infection by the acting on target cells to prevent all stages of viral replication (entry, transcription, translation, maturation, assembly and release). IFNs mediate their effects through IFN-up regulated cellular proteins, that act to create an antiviral state within cells. PML is one such cellular protein, that is an effector of IFN action and
displays intrinsic antiviral activities (Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011). There are several lines of evidence implicating PML as part of antiviral response.

First, PML is transcriptionally regulated by IFNs upon viral infection. In fact, IFNs are the best-characterized inducers of PML. Upon treatment of cells with IFNs, there is a marked increase in PML mRNA and protein levels, which results in a dramatic increase in PML NB number and size (Chelbi-Alix et al., 1995). This induction is mediated by binding of IFN to IFN-stimulated response element in the PML promoter (ISRE; GAGAATCGAAACT-) and through the binding of IFN to the gamma-activated site (GAS; TTTACCGTAAG-) in the PML promoter (Stadler et al., 1995). The deletion of ISRE/GAS in the PML promoter is sufficient to abolish the response to IFN, suggesting that IFN upregulates PML in response to viral infection through direct binding to these sites. In addition, IFN regulatory factor 8 (IRF-8) is also known to bind the ISRE in PML and upregulate PML in response to viral infections (Dror et al., 2007). This observation suggests that IFN ensures the upregulation of PML during viral infection through multiple mechanisms. Further, this implies that the action of PML during viral infection is an important defence mechanism for the cell.

The involvement of TRIM domain-containing proteins in antiviral defence is another line of evidence implicating the importance of PML in this process. The TRIM protein family, including PML/TRIM19, is made up of greater than 70 members that have emerging roles in IFN responses (Ozato et al., 2008). Twenty four of the TRIM genes are known to be upregulated at the mRNA level by IFN (Carthagena et al., 2009). For instance, TRIM5α, TRIM1 and TRIM22 harbor a RING dependent E3 ubiquitin ligase activity on different substrates restricting lentivirus infection (Ozato et al., 2008). In addition, TRIM22 confers resistance to hepatitis B virus by acting as a transcriptional suppressor (Gao et al., 2009) and to encephalomyocarditis virus by targeting the viral 3C protease for ubiquitin-mediated degradation (Eldin et al., 2009). TRIM5α interferes with uncoating of the viral preintegration complex of human immunodeficiency virus (HIV), while TRIM11 and TRIM32 repress viral gene expression and TRIM15 and TRIM22 inhibit virus assembly (Stremlau et al., 2004; Uchil et al., 2008). Taken together, the involvement of IFN-induced TRIM proteins in antiviral defence and signalling pathways underlines the importance of the TRIM protein family in IFN responses.

Genetic approaches have also revealed evidence for PML in antiviral responses. The ablation of the PML gene via homologous recombination has demonstrated that mice are viable and develop normally, although, the loss of PML does alter hematopoietic differentiation, cell
growth and tumorigenesis (Wang et al., 1998a; Wang et al., 1998b). In addition, these mice showed increased susceptibility to lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) infections (Bonilla et al., 2002). In addition, fibroblasts derived from PML knockout mice (PML-/- MEFs) exhibit enhanced LCMV replication (Djavani et al., 2001) and are more sensitive to infection with encephalomyocarditis virus (EMCV) (El McHichi et al., 2010) and rabies virus ((Blondel et al., 2002). Furthermore, these cells were defective for IFN induced apoptosis (Wang et al., 1998b). Similarly, downregulation of PML via siRNA in human cells resulted in a block in IFN-mediated apoptosis (Crowder et al., 2005). Collectively, these observations provide very good evidence for PML in restricting virus replication via PML isoforms or through NB-associated proteins such as Sp100 or Daxx.

Evidence that PML actively targets viruses has come from live cell imaging of HSV-1 infection. This showed that PML NBs move toward the viral genomes as they enter the nucleus (Everett and Murray, 2005). In addition, during varicella zoster virus (VZV) infection, large PML-NBs have been shown to sequester newly assembled nucleocapsids in neurons and satellite cells of human dorsal root ganglia (DRG) and skin cells. Quantitative immuno-electron microscopy revealed that these distinctive NBs consisted of PML fibers forming spherical cages that enclosed mature and immature VZV nucleocapsids. Of the six PML isoforms, only PML IV promoted the sequestration of nucleocapsids. PML IV significantly inhibited viral infection and interacted with the ORF23 capsid surface protein, which was identified as a target for PML-mediated nucleocapsid sequestration (Reichelt et al., 2011).

Functions of other individual PML isoforms in restricting viral replication has been the interest of recent studies. The HSV-1 immediate-early protein ICP0 counteracts PML-mediated restriction of HSV-1 replication (Everett and Maul, 1994; Maul and Everett, 1994). HSV-1 mutants that fail to express ICP0 have a pronounced defect in viral gene expression and plaque formation (Everett, 1989; Everett et al., 2008a), however, this effect can be reversed by depleting PML (Everett et al., 2006). Stable expression of PML isoform I or II (but not other PML isoforms) can partially reverse the increase in ICP0-null HSV-1 replication in PML-depleted cells (Cuchet et al., 2011). This activity of PML isoform I is dependent on SUMO modification, its SIM domain and each element of its TRIM domain (Cuchet et al., 2011). The observation that PML isoform I and II selectively mediate this effect would suggest that complete functionality of PML is dependent on isoform specific C-terminal sequences acting in concert. Cytoplasmic forms of PML have also been observed to limit HSV-1 replication. McNally et al.
(McNally et al., 2008) have demonstrated that a PML isoform lacking exons five and six, called PML Ib, mediates an intrinsic cellular defence against HSV-1 through the cytoplasmic sequestration of the ICP0 protein of HSV-1. Restriction of viral replication by individual PML isoforms is not limited to HSV-1, as specific PML isoforms have been reported to inhibit the replication of several DNA and RNA viruses (see Table 4 below).
Table 4: PML isoforms that confer resistance to DNA and RNA viruses

<table>
<thead>
<tr>
<th>DNA Viruses:</th>
<th>PML isoform giving protective effect:</th>
<th>Mechanisms:</th>
<th>References:</th>
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<tbody>
<tr>
<td>HSV-1</td>
<td>cPML1b</td>
<td>Sequesters ICP0 to cytoplasm</td>
<td>(McNally et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>PML I, II</td>
<td>ND</td>
<td>(Cuchet et al., 2011)</td>
</tr>
<tr>
<td>HCMV</td>
<td>PML VI</td>
<td>Inhibits viral mRNA and protein</td>
<td>(Tavalai et al., 2008)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>PML VI</td>
<td>ND</td>
<td>(Doucas et al., 1996)</td>
</tr>
<tr>
<td>Varicella-Zoster</td>
<td>PML IV</td>
<td>Sequesters ORF 23 into PML cages</td>
<td>(Reichelt et al., 2011)</td>
</tr>
</tbody>
</table>

**RNA Viruses:**

<table>
<thead>
<tr>
<th>Human foamy virus</th>
<th>PML III</th>
<th>Inhibits transcriptional activity of Tas</th>
<th>(Regad et al., 2001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV</td>
<td>PML III</td>
<td>Inhibits transcription</td>
<td>(Chelbi-Alix et al., 1998)</td>
</tr>
<tr>
<td>Rabies</td>
<td>PML IV</td>
<td>Inhibits transcription</td>
<td>(Blondel et al., 2010)</td>
</tr>
<tr>
<td>Influenza A</td>
<td>PML III</td>
<td>ND</td>
<td>(Chelbi-Alix et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>PML IV</td>
<td>ND</td>
<td>(Iki et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>PML VI</td>
<td>ND</td>
<td>(Iki et al., 2005)</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>PML III</td>
<td>Activates p53 and induces p-53 target genes</td>
<td>(Pampin et al., 2006)</td>
</tr>
</tbody>
</table>

ND - Not determined
1.3.6 Viral proteins that degrade/alter PML NBs

In order to subvert the suppressive effects of PML, many viruses have evolved mechanisms to inactivate PML NBs, either through remodelling of the PML NB, interfering with SUMOylation of PML thereby preventing NB formation, or inducing the degradation of the PML proteins. The first virus reported to perform the latter function was HSV-1 (Maul, Guldner, and Spivack, 1993), in which the ICP0 protein has been well characterized for its ability to disrupt PML NBs (Maul and Everett, 1994). ICP0 is a tegument protein that acts as an E3 ubiquitin ligase, it localizes to PML NBs during infection and disrupts them by inducing the degradation of PML proteins and the SUMO-modified isoforms of Sp100 (Boutell, Sadis, and Everett, 2002; Chelbi-Alix and de The, 1999). It has been shown that newly synthesized ICP0 following infection is essential in addition to the levels found in the tegument for PML degradation (Maul et al., 2003). It mediates such effects via polyubiquitylation and degradation of the PML protein by the proteasome, thereby allowing HSV-1 gene expression and replication (Everett et al., 1998; Everett et al., 2006). To be specific, the initial localisation of ICP0 to NBs is followed within a few hours by complete disruption of these structures, a process that requires both its RING finger domain and sequences in the C-terminal region of the protein that direct efficient localisation of ICP0 to PML NBs (Everett and Maul, 1994; Maul and Everett, 1994). The orthologues of ICP0 expressed by other alpha-herpesviruses have similar biological functions and are also RING finger proteins that disrupt PML NBs (Parkinson and Everett, 2000).

There are also well-studied mechanisms of PML regulation by other herpesviruses. For example, IE1 of human cytomegalovirus (HCMV) was shown to colocalize with PML resulting in NB reorganization through preventing or removing SUMO moieties on PML (Ahn and Hayward, 1997; Lee et al., 2004a; Muller and Dejean, 1999). In addition, the gamma-herpesviruses, EBV and KSHV, are also known to modify PML NBs, through the BZLF1 and LANA2 proteins respectively (Adamson and Kenney, 2001; Marcos-Villar et al., 2009). Expression of BZLF1 was sufficient to disperse PML NBs in cell lines and the transcriptional activation domain of BZLF1 was required for this effect. Further, BZLF1 was shown to disperse PML NBs by reducing the amount of SUMO-1 modified form of PML and BZLF1 itself was shown to be SUMO modified. Therefore, BZLF may compete with PML for limiting amounts of SUMO-1 (Adamson and Kenney, 2001). For KSHV, the LANA2 protein was shown to be essential for viability and proliferation of KSHV-infected primary effusion lymphoma cells. In
addition, LANA2 was shown to increase the levels of SUMO2-ubiquitin-modified PML, which subsequently induced the disruption of the NB via a proteasome-mediated mechanism. The SIM motif in LANA2 and the lysine 160 from PML is important for this disruption (Marcos-Villar et al., 2009). Recent evidence has shown that LANA2 itself is SUMO-1 and SUMO-2 modified *in vitro* and in latently KSHV-infected B-cells (Marcos-Villar et al., 2011). LANA2 sumoylation-deficient mutant was impaired for PML NB disruption, which suggests that either directly bound or covalently conjugated SUMO moieties may act as a bridge for interaction between LANA2 and other SUMO-modified proteins required for disruption of NBs (Marcos-Villar et al., 2011).

Other DNA viruses are also armed with mechanisms to counteract PML NB-mediated repression, such as adenoviruses. An interesting observation was that PML NBs were reorganized from punctate structures into elongated nuclear tracks during infection with adenovirus type 5 (the most studied subtype) (Puvion-Dutilleul et al., 1995). Hoppe et al. (Hoppe et al., 2006) later showed that E4orf3 protein of this virus was responsible for reorganization and PML track formation, and that it specifically targeted PML II to mediate this effect. In fact, E4orf3 was shown to interact with PML II through the C-terminus (aa645-684), which is encoded by the unique exon 7b found only on PML II (Leppard et al., 2009). Mutants of E3orf3 unable to bind PML II were compromised in their ability to cause NB rearrangement (Hoppe et al., 2006), further supporting the importance of PML II for this NB rearrangement. This effect of E4orf3 is thought to be important for adenovirus replication, since an adenovirus mutant lacking the E4orf3 was unable to alter PML NBs and was compromised for efficient viral growth (Ullman, Reich, and Hearing, 2007).

In addition to DNA viruses, RNA viruses have also evolved mechanisms to counteract cellular defences by altering PML. PML confers resistance to RNA viruses in a p53-independent manner by interacting with target viral proteins and inhibiting their function, or in a p53-dependent manner through induction of apoptosis in infected cells (Blondel et al., 2010; Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Pampin et al., 2006; Regad and Chelbi-Alix, 2001). Therefore, these viruses have co-evolved to counteract these mechanisms of action. For instance, infection with lymphocytic choriomeningitis (LCMV) results in the modification of PML NBs mediated by the viral nonstructural Z protein. Z is a RING finger protein that associates with and delocalizes PML to the cytoplasm. In the cytoplasm, both PML and Z interact with the elongation factor, eIF-4E, inhibiting cellular translation (Borden et al., 1998; Chenik, Chebli, and Blondel, 1995). During rabies virus infection, PML NBs are reorganized as
they appear as large, dense aggregates. Of the five rabies viral proteins, expression of the P phosphoprotein is sufficient to delocalize PML III from the NB into cytoplasmic dots where the two proteins co-localize. In transfection assays, the P protein was shown to bind to the RING finger domain of PML III through its C-terminus. Although, P protein was shown to alter localization of PMLIII, recent evidence suggests that only PML IV confers resistance to rabies virus (Blondel et al., 2010). Three proteins from Influenza A virus (M1, NS1 and NS2) have been shown to associate with PML NBs (Iki et al., 2005). While the functional significance of these findings is unclear, over-expression of PML III (Chelbi-Alix et al., 1998), PML IV or PML VI (Iki et al., 2005; Li et al., 2009) significantly reduced influenza A viral propagation. For a detailed list of viral proteins known to alter or disrupt PML bodies see Table 5 below.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral protein</th>
<th>PML-NB protein</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCMV</td>
<td>pp71</td>
<td>Daxx</td>
<td>Degrades PML, ↑ IE genes</td>
</tr>
<tr>
<td></td>
<td>IE1</td>
<td>PML</td>
<td>Disrupts NB, ↑ IE genes</td>
</tr>
<tr>
<td>HSV-1</td>
<td>ICP0</td>
<td>PML/Sp100</td>
<td>Degrades PML, ↑ replication</td>
</tr>
<tr>
<td></td>
<td>ICP27</td>
<td>PML</td>
<td>Alters PML mRNA splicing</td>
</tr>
<tr>
<td>EBV</td>
<td>BZLF1</td>
<td>PML</td>
<td>Disrupts NB</td>
</tr>
<tr>
<td></td>
<td>EBNA-LP</td>
<td>SP100</td>
<td>Displaces SP100, ↑ EBNA2 Transcription</td>
</tr>
<tr>
<td>HHV-6</td>
<td>IE1</td>
<td>PML NB</td>
<td>ND</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>E4orf3</td>
<td>PML</td>
<td>Reorganizes PML NB</td>
</tr>
<tr>
<td>HPV</td>
<td>E6</td>
<td>PML</td>
<td>↓ PML IV, senescence</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>PML</td>
<td>↓ PML IV, senescence</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>Sp100/Daxx</td>
<td>Reorganizes PML NB</td>
</tr>
<tr>
<td>KSHV</td>
<td>LANA2</td>
<td>PML</td>
<td>Disrupts NB</td>
</tr>
<tr>
<td>Murine γHV68</td>
<td>ORF 75c</td>
<td>PML</td>
<td>Disrupts NB, degrades PML, ↑ replication</td>
</tr>
<tr>
<td>HFV - human foamy virus</td>
<td>Tas</td>
<td>PML</td>
<td>↓ TAS transactivation</td>
</tr>
<tr>
<td>Virus</td>
<td>Protein/Function</td>
<td>Daxx Effect</td>
<td>Notes</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------</td>
<td>-------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>ASV - avian sarcoma virus</td>
<td>Integrase</td>
<td>↓ viral gene expression</td>
<td></td>
</tr>
<tr>
<td>VSV</td>
<td>ND</td>
<td>PML</td>
<td>PML OE, ↓ viral replication</td>
</tr>
<tr>
<td>Influenza A</td>
<td>? M1, NS1, NS2</td>
<td>PML</td>
<td>PML OE, ↓ viral replication</td>
</tr>
<tr>
<td>HDV - hepatitis D virus</td>
<td>L-HDAg</td>
<td>PML NB</td>
<td>Reorganizes PML NB</td>
</tr>
<tr>
<td>RSV - respiratory syncytial virus</td>
<td>ND</td>
<td>PML/Sp100</td>
<td>Cytoplasmic redistribution</td>
</tr>
<tr>
<td>PUUV - puumala virus</td>
<td>N protein</td>
<td>Daxx</td>
<td>ND</td>
</tr>
<tr>
<td>Dengue</td>
<td>C protein</td>
<td>Daxx</td>
<td>Sensitization to apoptosis</td>
</tr>
<tr>
<td>HCV</td>
<td>Core protein</td>
<td>PML</td>
<td>↓ PML IV-induced apoptosis</td>
</tr>
<tr>
<td>Phage ΦC31</td>
<td>Integrase</td>
<td>Daxx</td>
<td>↓ recombination</td>
</tr>
</tbody>
</table>

ND - not determined, OE - over expression
1.4 Thesis Rationale

EBV latent infection is widely accepted as a major causative agent of the epithelial tumours, NPC and GC. In both epithelial cell backgrounds, EBNA1 is the only viral nuclear protein expressed, with little to no expression of the EBV oncoprotein, LMP1. Even though EBNA1 is implicated in EBV-mediated host cell immortalization and transformation, the mechanism(s) for this EBNA1 function has been elusive. The objective of my thesis was to better understand how EBNA1 alters the epithelial cell environment in ways that contribute to EBV-associated epithelial tumours. In particular, I tested the hypothesis that EBNA1 may contribute to cell survival and oncogenesis through disruption of PML NBs. I present data showing that EBNA1 induces the degradation of PML proteins, which impairs functions downstream of PML (Chapter 2 and 4). I also investigated the mechanism by which EBNA1 induces loss of PML NBs and showed that it involves recruitment of the cellular enzymes CK2 and USP7 (Chapter 3). Furthermore, I have assessed the functional significance of EBNA1-mediated PML NB disruption in tumorigenesis (Chapter 4) and reactivation of EBV from latency (Chapter 5).
CHAPTER 2

EPSTEIN-BARR NUCLEAR ANTIGEN 1 CONTRIBUTES TO NASOPHARYNGEAL CARCINOMA THROUGH DISRUPTION OF PML NUCLEAR BODIES

This chapter has been published as:


Feroz Sarkari performed experiments in Figures 2-7 and 2-8. I performed all other experiments in this chapter.
2.1 Introduction

Epstein-Barr virus (EBV) is widely recognized as a causative agent of nasopharyngeal carcinoma (NPC), as most NPC tumors are monoclonal proliferations of latently EBV-infected cells (Raab-Traub, 2002). Latent EBV infection in NPC involves expression of four viral proteins; two latent membrane proteins (LMP1 and LMP2), one nuclear protein (EBNA1) and one secreted protein (BARF1) (Raab-Traub, 2002; Seto et al., 2005). LMP1, LMP2 and BARF1 have all been reported to have cellular effects that may contribute to the development of NPC, although LMP1 and BARF1 are not consistently detected in all NPC tumors (Sall et al., 2004; Stewart et al., 2004; Zheng et al., 2007). EBNA1 is required for the replication and stable persistence of EBV episomes in proliferating cells and is the only EBV protein that is expressed in all EBV-associated tumors (Frappier, 2004). EBNA1 enables the expression of the other EBV latency proteins, however, whether or not EBNA1 directly contributes to the development of tumors has not been clear.

A number of observations in the literature are consistent with a role for EBNA1 in the proliferation of EBV-positive cells. For example, interference with EBNA1 function in EBV-positive Burkitt’s lymphoma cells, by overexpression of a dominant-negative EBNA1 mutant, increased cell death (Kennedy, Komano, and Sugden, 2003). Similarly, down-regulation of EBNA1 in Raji Burkitt’s lymphoma or EBV-positive epithelial cells by RNA interference decreased cell proliferation (Hong et al., 2006; Yin and Flemington, 2006). However, since EBNA1 is needed to maintain the EBV episomes and to enhance expression of other latency proteins, it is not clear from the above observations whether EBNA1 is directly affecting cell proliferation or is functioning indirectly by enabling expression of other EBV gene products.

Other studies have investigated whether expressing EBNA1 in various EBV-negative cancer cells affects tumorigenicity. EBNA1 expression in HONE-1 NPC cells was found to increase primary tumor formation as well as metastases in nude mice (Sheu et al., 1996). EBNA1 expression in Hodgkin’s lymphoma cells enhanced their ability to form tumors in non-obese diabetic-SCID mice but not in regular SCID mice (Kube et al., 1999). In addition, Kaul et al (Kaul et al., 2007) found that expression of EBNA1 in a breast carcinoma cell line promoted the rate of tumor growth in nude mice, reversed the growth inhibitory effect of the cellular Nm23-H1 protein and increased lung metastases.

The molecular basis for the observed effects of EBNA1 on cell proliferation are largely unknown, although an interaction between EBNA1 and the cellular ubiquitin specific protease, USP7 or HAUSP, has been proposed to be partially responsible (Holowaty et al., 2003b). USP7 binds and stabilizes p53 (Li et al., 2002), and EBNA1 was found to block the USP7-p53 interaction in vitro by
competing for the same binding pocket on USP7 (Holowaty et al., 2003a; Saridakis et al., 2005; Sheng et al., 2006). In keeping with these findings, expression of EBNA1 (but not a USP7-binding mutant of EBNA1) in U2OS cells was shown to protect these cells from apoptosis in response to DNA damage by interfering with p53 stabilization (Saridakis et al., 2005). However, USP7 is likely to have multiple cellular roles and the functional significance of the EBNA1-USP7 interaction remains to be determined in the context of latent EBV infection.

Few studies have investigated the role of EBNA1 in NPC. Studies on the contribution of EBV proteins to NPC in general have been hampered by the lack of EBV-positive NPC cell lines, since NPC cells tend to rapidly lose the EBV genomes when propagated in culture. The isolation of a NPC cell line (C666-1) that stably maintains EBV episomes (Cheung et al., 1999) has greatly facilitated NPC studies, enabling a comparison to EBV-negative NPC cell lines. I have compared C666-1 cells to the EBV-negative NPC cell lines CNE2 (Sun et al., 1992) and HK1 (Huang et al., 1980) in order to better understand cellular alterations caused by EBV infection that may contribute to cell transformation. Here I show that EBV latent infection in NPC cells is associated with the disruption of host PML nuclear bodies (NBs) and that EBNA1 is entirely responsible for this effect. Consistent with the known importance of PML NBs in p53 activation and DNA damage responses, I also show that EBNA1 expression in NPC impairs both of these processes.

2.2 Materials and Methods

2.2.1 Cell lines

The Saos-2 p53-negative, human osteoblast cell line was cultured in DMEM (Sigma) supplemented with 10% fetal calf serum. The EBV-positive NPC C666-1 cell line and the EBV-negative NPC cell lines HK1 and CNE2Z (CNE2) (both of which lost the EBV genomes after growth in culture) have been previously described (Cheung et al., 1999; Huang et al., 1980; Sun et al., 1992). C666-1 and HK1 were grown in HEPES-modified RPMI 1640 (Sigma), while CNE2 was maintained in alpha minimal essential media (αMEM, Gibco), in all cases supplemented with 10% fetal calf serum. To generate the CNE2E and HK1E cell lines, EBNA1 cDNA was cloned into pcDNA3.1/hygro (-/-; Invitrogen), and CNE2 and HK1 cells were transfected with 10 μg of linearized plasmid. Transfected cells were maintained at low densities in medium supplemented with hygromycin B (Invitrogen; 0.75mg/mL for CNE2 and 0.5mg/mL for HK1) to allow growth of individual colonies. Colonies were examined for EBNA1 expression by IF microscopy, then picked and propagated for
further studies in media containing 0.5 mg/mL (CNE2E) and 0.35 mg/mL (HK1E) of hygromycin B, respectively.

### 2.2.2 Transfections and RNA interference

To generate CNE2 or Saos-2 cells transiently expressing EBNA1, 1.5 x 10^5 cells were transfected with 2 μg of an EBNA1 expressing plasmid containing EBV oriP, pc3OriPE (Wu, Kapoor, and Frappier, 2002a), unless otherwise indicated, using lipofectamine 2000 (Invitrogen). Where indicated, the same plasmid expressing EBNA1 mutants Δ325-376 or Δ395-450 was used (Holowaty et al., 2003b; Shire et al., 1999), and the same plasmid lacking EBNA1 cDNA (pc3OriP) was used as a negative control (Wu, Kapoor, and Frappier, 2002a). 48 hrs later, cells were fixed for IF imaging as described below. For RNA interference experiments, 1.5 x 10^5 CNE2E or C666-1 cells were transfected with 50 pmol of siRNA against GFP (GCAAGCUGACCCUGAAGUUCAU), against EBNA1 (GGAGGUUCCAACCGAAUUTT) or against USP7 (UCAAGAUGACUACCAGCUG) using 2 μL of lipofectamine 2000. For C666-1 and one CNE2E sample (Figure 2-3A and 2-3B), cells underwent an identical second round of siRNA transfection 72 hours after the first transfection. For Figure 2-5C, cells were subjected to three rounds of transfection with siRNA against USP7 or GFP prior to transfection with pc3OriPE.

### 2.2.3 Immunofluorescence microscopy

Cells grown on coverslips were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 20 min, rinsed twice in PBS and permeabilized with 1% Triton X-100 in PBS for 5min. Samples were blocked with 4% BSA in PBS followed by incubation with primary antibodies against EBNA1 (R4 rabbit serum at 1:300 dilution (Holowaty et al., 2003b)) and PML (Santa Cruz PG-M3 at 1:50 dilution) and incubation with the secondary antibodies goat anti-rabbit Alexafluor 555 (Molecular Probes) and goat anti-mouse Alexafluor 488 (Molecular Probes) in 4% BSA. Coverslips were mounted onto slides using ProLong Gold antifade medium containing DAPI (Invitrogen). Images were obtained using the 40 x oil objective on a Leica inverted fluorescent microscope and processed using OpenLAB (ver.X.0) software. PML nuclear bodies were quantified by counting all visible PML foci in 100 cells.

### 2.2.4 Western blots

Cells were lysed in 9 M urea, 5 mM Tris-HCl (pH 6.8) and briefly sonicated. 100 μg of total protein was subjected to 10% SDS-PAGE and transferred to nitrocellulose. Where indicated, CNE2E
cells were treated with 10 μM MG132 for 8 or 10 hours prior to lysis. Membranes were blocked in 5% non-fat dry milk in PBS, then incubated with antibodies against PML (Chemicon AB1370; 1:2000 dilution), EBNA1 (OT1X at 1:2000; kindly supplied by Japp Middeldorp), actin (Ab-1, Oncogene Research Products; 1:20 000), Sp100 (Chemicon 1380, 1:1000 dilution) or USP7 (rabbit serum against full-length USP7). After washing, blots were probed with goat anti-mouse peroxidase (1:3000) or goat anti-rabbit peroxidase (1:5000) from Santa Cruz, then developed using chemiluminescence reagents (ECL, Perkin Elmer). Membranes were stripped in 0.1 M glycine pH 2.9 for 30 min, washed in PBS-Tween, blocked and re-probed with the next antibody as described above.

Experiments in Figure 2-7 were performed as above except that some cells were treated with 10 μg/mL of etoposide 24 hrs prior to harvesting and 80 μg (p53 blot) or 60 μg (acetyl-p53 blot) of total protein was analyzed by Western blotting using the following antibodies: PAb 1801 for p53 (Banks, 1986) (a gift from Sam Benchimol) and antibody 2525 for acetyl-p53 K382 (Cell Signaling Technologies). For acetyl-p53 blots, membranes were blocked and incubated with primary antibody in 5% BSA, 50 mM Tris pH7.4, 150 mM NaCl, 0.1% Tween-20. The same experiment was also performed in Hela cells transfected with pc3OriP or pc3OriPE. In the later case, EBNA1 expression was confirmed in approximately 80% of the cells by IF prior to etoposide treatment (10 μg/mL) 48 hours post-transfection. 100 μg of cell lysate was Western blotted as described above except that the anti-p53 antibody was DO-1 from Santa Cruz (sc-126).

### 2.2.5 Co-immunoprecipitation

Log phase C666-1 cells were lysed in IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM MgCl2, 10% glycerol, 1% Triton X-100, and protease inhibitors) on ice for 30 min. After centrifugation, the supernatant was pre-cleared with protein A/G agarose (Santa Cruz) and then equal amounts (4 mgs) were incubated for 4 hr at 4°C with either mouse IgG coupled to agarose (Santa Cruz; negative control) or with OT1X EBNA1 monoclonal antibody followed by protein A/G agarose overnight at 4°C. Beads were collected by centrifugation, washed in IP buffer then boiled in SDS loading buffer. Immunoprecipitated proteins were separated by SDS-PAGE and Western blotted as described above. The positions of PML I and PML IV isoforms on Western blots was determined by transfecting C666-1 cells with constructs expressing FLAG-PML I or FLAG-PML IV (Beech et al., 2005) and analyzing 30 μg of lysate by Western blotting using anti-FLAG antibody (Abcam AB21536; 1:10,000 dilution). For the experiment in Figure 2-4C, CNE2 cells were co-transfected with pc3OriPE and the FLAG-PML I or FLAG-PML IV expression constructs and 16 hours later, IPs
were performed using 2 mg of lysate and anti-FLAG M2 agarose beads (Sigma). 30 μg of lysate was used for input and post-IP signal.

### 2.2.6 FACS analysis

CNE2 and CNE2E cells (before and after siRNA treatment) seeded at 60% confluence in 10 cm dishes were treated with UV (50x10² μJ/cm²) or 10 μg/mL of etoposide. 24 hours later, adherent cells were harvested, fixed in 70% ethanol, treated with RNAse (50 μg/mL) at 37°C for 1 hour and stained with propidium iodide. DNA content was analyzed immediately after propidium iodide treatment on a FACS calibur (Becton Dickinson, USA) and cell cycle analysis was performed using Modfit LT 3.1 (Verity Software House).

### 2.2.7 Apoptosis Assay

CNE2 and CNE2E cells were treated with etoposide as stated above. 48 hours later, cells were processed for TUNEL staining using the APO-BrdU TUNEL Assay Kit (Invitrogen, MP23210) according to the manufacturer’s instructions. Cells were then mounted on coverslips, counter-stained with DAPI and analyzed by fluorescence microscopy. Apoptotic index was calculated as the number of TUNEL-positive cells divided by the total number of cells. The experiment was done in triplicate and at least 100 cells were counted for each sample.

### 2.2.8 Cell viability assay

Cell viability was measured using Trypan blue (Gibco) exclusion assay as follows: CNE2 and CNE2E cells were seeded in 12-well plates such that they reached 80-90% confluence at time of harvesting. 24 hours later, cells were treated with etoposide (10 μg/mL) or UV (50x10² μJ/cm²). After growing for 24, 48 and 72 hours, floating and adherent cells were harvested, washed in PBS, stained with 0.04% Trypan blue in PBS and counted immediately. Experiments were done in triplicate and at least 200 cells were counted for each replicate.
2.3 Results

2.3.1 EBNA1 disrupts PML NBs in NPC cells

In initial studies comparing EBV-positive and EBV-negative NPC cell lines, I examined the host PML NBs, which have the PML protein as the main constituent and have been implicated in many important cellular processes (Bernardi and Pandolfi, 2007). I was particularly interested in examining PML NBs in the context of NPC because the disruption of PML NBs, or lack of the PML protein, is a factor in the development of several types of cancer (Gurrieri et al., 2004; Wang et al., 1998a) and because DNA viruses are known to have mechanisms to disrupt PML NBs (Everett, 2001). Immunofluorescence (IF) microscopy for PML revealed that C666-1 cells have considerably fewer PML NBs than do CNE2 or HK1 cells (average number per cell of 4, 16 and 11 respectively; Figure 2-1A and 2-1B), suggesting that some aspect of EBV infection disrupts PML NBs. I investigated whether this involved the EBNA1 protein by down-regulating EBNA1 expression with siRNA. This treatment greatly decreased EBNA1 expression in some but not all of the C666-1 cells allowing a direct comparison of silenced and non-silenced cells in the same culture (Figure 2-1A bottom row). The number of PML NBs was found to increase 2-3 fold upon EBNA1 silencing, as compared to non-treated cells or cells treated with siRNA against green fluorescence protein (GFP), indicating that EBNA1 contributes to PML disruption in C666-1.

To determine whether EBNA1 expression was sufficient to disrupt PML NBs, I generated CNE2E and HK1E cell lines in which EBNA1 was constitutively expressed in CNE2 and HK1 NPC cells from an integrated cassette. EBNA1 expression levels in HK1E and CNE2E were shown by immunoblot to be approximately 2-fold and 3-fold higher than in C666-1, respectively (Figure 2-S1). EBNA1 expression in both cell lines resulted in a notable decrease in the number of PML NBs per cell (Figure 2-1B). To further verify that this effect was caused by EBNA1, CNE2E cells were treated with siRNA to down-regulate EBNA1 expression. EBNA1 silencing restored the number of PML NBs to the level seen in the parent CNE2 cells (Figure 2-1B). I also examined whether transient expression of EBNA1 was sufficient to cause PML disruption. To this end, CNE2 cells were transfected with an EBNA1 expression plasmid and examined by IF 48 hrs later (Figure 2-2A). EBNA1 expression lowered the number of PML NBs in a dose-dependent manner, where PML NBs were decreased 5-fold in cells staining brightly for EBNA1 and decreased 3-fold in those with lighter EBNA1 staining (Figure 2-2B). Therefore EBNA1 expression has an immediate effect on PML NBs.
**Figure 2-1. Immunofluorescence imaging of PML NBs in NPC cell lines.** Log phase cells were fixed and stained for EBNA1 (red) and PML(green). The number of PML foci seen per cell was counted for 100 cells for each sample in three separate experiments and the average number with standard deviation is shown in the histograms, where *** denotes p values less than 0.0001 relative to the parental cell line. Exposure times of image capture were constant for all samples with the same antibody treatment. (A) EBV-positive C666-1 cells before and after treatment with siRNA against GFP (siGFP) or EBNA1 (siEBNA1) are shown. Arrowheads indicate a siEBNA1 treated cell that continued to express EBNA1 and can be used for comparison to neighbouring silenced cells. (B) EBV-negative CNE2 and HK1 cell lines with (CNE2E, HK1E) and without stable EBNA1 expression are shown. CNE2E are also shown after silencing of EBNA1 expression where one of the three cells shown continues to express EBNA1 (arrowhead).
Figure 2-2. Transient expression of EBNA1 and EBNA1 mutants in CNE2 cells. (A) CNE2 cells were transiently transfected with a plasmid expressing EBNA1 or EBNA1 mutants Δ325-376 or Δ395-450, then stained for EBNA1 and PML. Both EBNA1-expressing and non-expressing cells are shown 48 hrs post transfection. Exposure times of image capture were constant for all samples with the same antibody treatment. (B) Numbers of PML NBs per cell were counted 48 hours after expression of wildtype EBNA1. Cells were categorized into low and high EBNA1 expression depending on the intensity of EBNA1 staining. ** indicates 0.0001<p<0.001 and *** indicates p<0.0001 relative to untransfected cells. (C) The number of PML NBs were counted for all cells in (A) expressing wildtype or mutant EBNA1 proteins.
2.3.2 EBNA1 lowers PML protein levels

Viral proteins have been found to decrease PML NBs either by inducing the degradation of the PML protein or by disrupting the interaction of PML proteins required to form NBs (Everett, 2001). To address the mechanism by which EBNA1 expression disrupts PML NBs, I compared the levels of PML isoforms in CNE2 before and after stable (CNE2E) or transient expression of EBNA1 by Western blotting. PML is known to exist as several isoforms (comprised of alternative spliced and modified forms), resulting in multiple bands migrating between 60 and 200 Kda on PML immunoblots (Condemine et al., 2006; Everett et al., 1999). Down-regulation of EBNA1 expression in C666 cells resulted in increased expression of all PML isoforms (Figure 2-3A). Similarly, EBNA1 expression in CNE2E resulted in a dramatic decrease in all PML isoforms (Figure 2-3B compare lanes 1 and 2), which was restored by silencing EBNA1 expression (Figure 2-3B lanes 3 and 4). In addition, transient EBNA1 expression in CNE2 decreased the level of all PML isoforms in a dose-dependent manner (Figure 2-3C). Therefore EBNA1 expression results in the loss of PML protein, as opposed to dispersal of PML from the foci. However the level of another PML NB component, Sp100, was unaffected by EBNA1 demonstrating the specificity of this effect (Figure 2-3C). Effects of EBNA1 expression on the level of PML mRNA was also examined by RT-PCR to rule out potential effects on PML transcription. As expected, no change in the level of PML transcripts was evident, indicating that the EBNA1-mediated PML effects were occurring at the protein level (Figure 2-S2).

The EBNA1-induced loss of PML protein suggests that EBNA1 might be increasing the degradation of PML isoforms by the proteasome. I tested this possibility by examining the effect of blocking the proteasome in CNE2E cells with MG132 (Figure 2-3D). This treatment was found to increase the levels of all PML isoforms, and higher molecular weight forms suggestive of polyubiquitination also became visible. Therefore the loss of PML protein caused by EBNA1 is proteasome dependent.
Figure 2-3. EBNA1 expression diminished PML protein levels. (A) C666-1 cells were treated with siRNA against EBNA1 (siEBNA1) or GFP (siGFP) then equal amounts of whole cell lysates were Western blotted and probed with an antibody recognizing all PML isoforms, EBNA1 and actin. (B) Equal amounts of whole cell lysates from CNE2 and CNE2E cells were Western blotted and probed as in A. Lysates from CNE2E cells after one (+) or two (++) rounds of transfection with siRNA against EBNA1 are also shown (lanes 3 and 4). (C) Lysates from CNE2 cells 48 hrs after transfection with the indicated amounts of an EBNA1 expression plasmid (OriPE) or the empty plasmid (OriP) were Western blotted for PML, EBNA1, actin or Sp100. (D) CNE2E cells were treated with MG132 proteasomal inhibitor for 0, 8 or 10 hours then equal amounts of lysates were blotted for PML, EBNA1 and actin.
2.3.3 EBNA1 associates with PML NBs through a specific PML isoform

In most cells, EBNA1 is found throughout the nucleus making it difficult to assess whether some of the EBNA1 localizes to PML NBs. However, some of the transiently transfected CNE2 cells expressed very low levels of EBNA1 and, in these cells, discreet EBNA1 foci were observed, many of which localized to PML NBs (Figure 2-4A). In addition, EBNA1 foci are frequently seen in C666-1, which naturally express low levels of EBNA1, and these foci often correspond to or overlap with PML NBs, even though few PML NBs are present in C666-1 (Figure 2-4A).

To further assess the interaction of EBNA1 with PML in the context of a latent infection, EBNA1 was immunoprecipitated from C666-1 and co-precipitating proteins were analysed for PML (Figure 2-4B). One PML isoform was consistently found to co-immunoprecipitate with EBNA1 (Figure 2-4B lane 3). Interestingly this did not correspond to the most prevalent PML band in the lysate (presumably isoforms I and II according to its size and abundance) but rather corresponded to a less abundant form consistent with the size of PML isoform IV (Condemine et al., 2006). I further tested the apparent preferential association of EBNA1 with PML IV by transfecting CNE2 cells with constructs expressing EBNA1 and either FLAG-tagged PML I or PML IV followed by immunoprecipitation with anti-FLAG antibody. I found that more EBNA1 co-immunoprecipitated with PML IV than PML I. It is unclear whether the small amount of EBNA1 recovered with PML I is the result of a lower affinity interaction of EBNA1 with PML I or is due to an indirect interaction with PML I through endogenous PML IV, since the PML isoforms interact with each other.
Figure 2-4. Interaction of EBNA1 with PML. (A) Immunofluorescence images of CNE2 cells transfected with pc3OriPE and of C666-1 cells are shown after staining for EBNA1 and PML. The transfected CNE2 cells shown are those expressing very low levels of EBNA1. (B) EBNA1 was immunoprecipitated from C666-1 cells with anti-EBNA1 antibody (IP:EBNA1). The starting lysate (Input) and protein remaining after IP (Post IP) are also shown, in each case representing 1/40th of the lysate used in IP. The same lysate was also treated with IgG beads as a negative control (IP:IgG). All samples were Western blotted using antibodies against EBNA1 or all PML isoforms. In the right panel, the positions of FLAG-tagged PML isoform I (FLAG-PML I) and PML isoform IV (FLAG-PML IV) expressed in C666-1 cells are shown by Western blotting with anti-FLAG antibody.
To gain insight into the mechanism by which EBNA1 induces loss of PML NBs and protein, I tested the ability of EBNA1 mutants to disrupt PML NBs after transfection in CNE2 cells (Figure 2-2A and 2-2C). Initially I tested the EBNA1 Δ325-376 mutant, as this mutation disrupts the interaction of EBNA1 with cellular chromatin and abrogates the transcriptional activation function of EBNA1 (Ceccarelli and Frappier, 2000; Wu, Ceccarelli, and Frappier, 2000). However EBNA1 Δ325-376 disrupted PML NBs to the same degree as wildtype EBNA1 indicating that neither transcriptional activation nor strong chromatin interactions are required for the observed effects. An EBNA1 mutant, Δ395-450, that is fully functional for all of the known functions of EBNA1 (replication, segregation and transcriptional activation) but fails to bind the cellular USP7 protein was also tested for PML effects (Holowaty et al., 2003b). USP7 is known to be partially associated with PML NBs and associates with another herpesvirus protein (ICP0 or Vmw110 from herpes simplex virus) that also disrupts PML NBs through loss of PML protein (Everett et al., 1997; Everett et al., 1998; Maul and Everett, 1994). Unlike wildtype EBNA1, Δ395-450 caused no obvious change in the number of PML NBs or the level of PML protein, suggesting that USP7 binding might be important for PML disruption.

The role of USP7 in EBNA1-mediated disruption of PML NBs was further investigated by silencing USP7 by siRNA treatment in the CNE2E cells that are stably expressing EBNA1. USP7 silencing restored the number of PML NBs and the level of the PML protein (Figure 2-5A and 2-5B), indicating that EBNA1 does not disrupt PML NBs in the absence of USP7. Similar experiments were conducted in which CNE2 cells were transfected with siRNA against USP7 (or GFP as a negative control) prior to transient expression of EBNA1. The siUSP7 treatment was confirmed by IF to silence USP7 expression in virtually all of the CNE2 cells prior to transfection of the EBNA1 expression plasmid (Figure 2-5C, left panel). Cells pretreated with siUSP7 had numerous PML NBs regardless of whether or not EBNA1 was expressed, whereas EBNA1 continued to diminish PML NBs in cells pretreated with siGFP (Figure 2-5C, right panel). Similarly, pretreatment with siUSP7 but not siGFP interfered with EBNA1-induced loss of PML protein as determined by Western blotting (Figure 2-5D). Therefore EBNA1-mediated disruption of PML NBs requires USP7.

Since USP7 can alter p53 levels (Li et al., 2004; Li et al., 2002) and p53 induces PML transcription (Chan et al., 1997; de Stanchina et al., 2004), I wanted to ensure that the observed effects of EBNA1 on PML were not due to interference with p53 stabilization by USP7. Therefore I examined whether the EBNA1 effects on PML were independent of p53 by expressing EBNA1 in the p53-null
Saos-2 cells. As shown in Figure 2-6, EBNA1 reduced the number of PML NBs per cell and the level of PML protein to a similar degree as in the NPC cells lines. Therefore the disruption of PML NBs by EBNA1 does not involve p53.

Figure 2-5. Effect of USP7 silencing on PML degradation by EBNA1. (A) CNE2E cells expressing EBNA1 were transfected with siRNA against USP7 or GFP (negative control) then stained for USP7 and PML. Exposure times of image capture were constant for all samples with the same antibody treatment. (B) Equal amounts of cell lysates from (A) were analysed by Western blotting with the indicated antibodies. siUSP7-1 and siUSP7-2 are duplicate samples treated with siRNA against USP7. (C) CNE2 cells were transfected with siRNA against GFP or USP7 (left panel) then were transfected with EBNA1 expression plasmid pc3OripE and stained for EBNA1 and PML (right panel). Exposure times of image capture were constant for all samples with the same antibody treatment. (D) Equal amounts of cell lysates from (C) were analysed by Western blotting after pretreatment with siGFP or siUSP7 followed by EBNA1 expression.
Figure 2-6. **EBNA1-induced disruption of PML NBs in Saos-2 cells.** p53-null Saos-2 cells were transiently transfected with the expression plasmid with or without the EBNA1 gene as in Figure 2. (A) Cells were stained for EBNA1, PML and DNA (DAPI) and visualized by fluorescence microscopy. The number of PML NBs per cell were counted and average numbers with standard deviations are shown in the histogram, where *** indicates p<0.0001. (B) Equal amounts of lysates from the transfected cells were analysed by Western blotting as in Figure 2-3.
2.3.5 *EBNA1 interferes with p53 activation, DNA repair and apoptosis*

Considerable data indicate that PML NBs are important for p53 activation, apoptosis and DNA repair which would have important consequences for the development of NPC. Therefore I asked whether the effect of EBNA1 on PML NBs was sufficient to disrupt these processes. PML NBs are required for the activation of p53 through acetylation (Guo et al., 2000; Pearson et al., 2000), and therefore I compared p53 activation in CNE2 and CNE2E cells after treatment with the DNA damaging agent etoposide (Figure 2-7A). I consistently observed that the EBNA1-expressing cells had an impaired ability to acetylate p53 (at K382) while the induction of p53 was affected to a lesser degree. In Hela cells, EBNA1 has no obvious effect on PML NBs (data not shown) and therefore I examined the effect of EBNA1 expression on p53 activation in Hela cells to verify that this effect involved PML NB disruption. As shown in Figure 2-7B, p53 acetylation in Hela cells occurred in response to etoposide treatment at least as efficiently in the presence of EBNA1 as in its absence. Therefore PML disruption by EBNA1 appears to be responsible for the lack of p53 acetylation.

We examined the effect of EBNA1 expression on DNA repair by comparing FACS profiles of CNE2 and CNE2E after inducing DNA damage with UV or etoposide treatment (Figure 2-8A). Previous studies have shown that unrepaired DNA damage is reflected by the accumulation of cells in S-phase, while cells that have repaired the damage pass through S and accumulate either in G2/M or G1 depending on which DNA damage checkpoint has been activated (Biard, 2007; Boe et al., 2006; Callegari and Kelly, 2007). Hence silencing of PML or a number of DNA repair proteins has been found to increase the percentage of cells in S phase after DNA damage (Biard, 2007; Boe et al., 2006). Similarly, I consistently observed that CNE2E cells had a higher fraction of cells in S phase after UV or etoposide treatment as compared to CNE2 cells (compare profiles ii and v, and profiles iii and vi in Figure 2-8A) even though the cell cycle distribution of the two cell lines was indistinguishable prior to treatment. In multiple experiments the percentage of CNE2 cells in S phase after UV or etoposide treatment was 55.8 ±2.5 and 55.4±1.5, respectively, while the same treatments in CNE2E cells resulted in S-phase percentages of 66.5 ±2.2 and 91.4 ±0.2, respectively. In both cases, differences with and without EBNA1 are statistically significant with p values < 0.001. This effect was confirmed to be due to EBNA1 expression, as down-regulation of EBNA1 in CNE2E with siRNA reduced the S-phase accumulation after DNA damage as compared to the control siRNA treatment against GFP (Figure 2-8A, compare profiles viii and xi, and profiles ix and xii). Therefore EBNA1 expression results in an impaired ability to repair DNA damage, consistent with the disruption in PML NBs.
The effect of EBNA1 expression in CNE2 cells on apoptosis was also examined by TUNEL assay. The percentage of cells that became TUNEL-positive after etoposide treatment was decreased two-fold in the presence of EBNA1 (Figure 2-8B), showing that EBNA1 also interferes with apoptosis.

Figure 2-7. Effects of EBNA1 on p53 activation. (A) CNE2 and CNE2E cells were treated with etoposide (+) or left untreated (-) and equal amounts of total cell lysates were analysed by SDS-PAGE and Western blotting for p53 acetylated on K382 and total p53. Actin loading controls are also shown. (B) Hela cells were transfected with a plasmid lacking (oriP) or expressing (oriPE) EBNA1 then were treated with etoposide (+) or left untreated (-). Equal amounts of cell lysate were then analysed by Western blotting as in A.
2.3.6 *EBNA1 increases cell survival after DNA damage*

We compared the viability of CNE2 and CNE2E cells after etoposide or UV treatment and found that CNE2E cells had a somewhat higher survival rate than CNE2 cells (particularly after etoposide treatment), despite their reduced ability to repair DNA damage (Figure 2-8C). This is consistent with the known importance of PML NBs in apoptosis (Takahashi et al., 2004) and the observed inhibition of apoptosis by EBNA1. The results suggest that EBNA1 promotes the survival of cells even though they contain DNA damage, which has important implications for tumorigenesis.
Figure 2-8. Effects of EBNA1 on DNA repair, apoptosis and cell survival. (A) CNE2 and CNE2E cells, before and after siRNA treatment for GFP (siGFP; negative control) or EBNA1 (siEBNA1), were treated with UV or etoposide or left untreated. 24 hrs later cells were fixed, stained with propidium iodide and analysed for DNA content by FACS. The percentage of cells in each cell cycle stage was determined using Modfit and is shown for each sample. (B) CNE2 and CNE2E cells were treated with etoposide then analysed by TUNEL assay. Average percentage of TUNEL-positive cells are shown from three experiments with standard deviation and $0.0001 < p < 0.001$ (**). (C) CNE2 (grey) and CNE2E (black) cells were treated with etoposide (top graph) or UV (bottom graph) then grown for the indicated number of days. At each time point, cells were incubated with Trypan blue and the percentage of cells that excluded Trypan blue was determined. Experiments were performed in triplicate and average numbers with standard deviations are shown. The difference in cell survival with and without EBNA1 3 days post etoposide treatment is statistically significant with a p value of 0.05.
2.4 Discussion

I have identified a major effect of EBNA1 expression on host cell PML NBs in NPC, where EBNA1 expression results in pronounced loss of PML NBs and the PML protein itself. This effect has important biological implications due to the strong association between PML disruption and tumor development. While initially identified as a gene whose rearrangement leads to promyelocytic leukemia, it has since been found that loss of the PML protein is associated with cancer development for a variety of human tumors (Gurrieri et al., 2004). In addition, mice lacking PML develop normally but their cells are more prone to malignant transformation (Wang et al., 1998a).

Unlike the results in NPC cells, I have not seen any notable disruption of PML NBs when EBNA1 is expressed in Hela or 293 cells, suggesting that this effect is specific to particular cell backgrounds. Indeed a previous study examined PML NBs in B-cells latently infected with EBV (both latency and I and latency III forms of infection) and found no obvious difference from uninfected cells (Bell, Lieberman, and Maul, 2000). This ability of EBNA1 to disrupt PML NBs in cells of the nasopharynx could be part of the reason that these cells are particularly susceptible to malignant transformation by EBV.

I found that EBNA1 is partly associated with PML NBs in a native latent infection in NPC cells and can physically associate with at least one PML isoform that appears to be PML IV. I have also shown that disruption of the PML NBs by EBNA1 is due to loss of multiple isoforms of the PML protein and that this effect is proteasome-dependent. This suggests that EBNA1 is targeted to PML bodies through an interaction with PML IV but, once there, can promote the degradation of all PML isoforms.

The disruption of PML NBs by EBNA1 requires EBNA1 binding to USP7, a cellular ubiquitin specific protease that is known to associate with PML NBs (Meredith, Orr, and Everett, 1994). There are several possible scenarios of the role of the EBNA1-USP7 interaction in PML-disruption as depicted in Figure 2-9. In scenario I, EBNA1 mediates the interaction between a specific PML isoform (ie. PML IV) and USP7 thereby increasing recruitment of USP7 to PML NBs. USP7 could then promote PML degradation either through its catalytic activity or through recruitment of additional cellular proteins. It is not intuitively obvious why deubiquitination would lead to PML destabilization, however approximately three quarters of USP7 is comprised of protein interaction domains, so recruitment of additional cellular enzymes to PML is a viable possibility (Holowaty et al., 2003a; Hu et al., 2006; Oh, Yoo, and Seol, 2007; Sheng et al., 2006; van der Horst et al., 2006; van der Knaap et al., 2005). In scenarios II and III, the interaction of EBNA1 with PML NBs depends entirely (scenario
II) or partly (scenario III) on USP7. In these cases, loss of PML may require the recruitment of additional cellular proteins by EBNA1, since EBNA1 itself is not known to have any enzymatic activities. For example, our laboratory has previously shown that EBNA1 forms a stable complex with casein kinase 2 (CK2) (Holowaty et al., 2003b) and Scaglioni et al (Scaglioni et al., 2006) showed that phosphorylation of PML by CK2 targets PML proteins for ubiquitination and subsequent degradation. Therefore it is possible that increased recruitment of CK2 to PML NBs by EBNA1 might give the observed effect.

While additional experiments are required to distinguish the above scenarios, the involvement of USP7 in PML disruption by EBNA1 has an interesting parallel with studies of PML disruption by herpes simplex virus type 1 (HSV-1). In HSV-1 infection, ICP0 (also called VMW110) plays a major role in disrupting PML NBs by promoting the degradation of PML protein through its action as an ubiquitin ligase (Boutell, Sadis, and Everett, 2002; Everett, 2001). Although unrelated to EBNA1 in its sequence, ICP0 also binds tightly to USP7 (albeit through a different USP7 domain than EBNA1(Holowaty et al., 2003a)) and this interaction is required for PML disruption, at least in part because USP7 stabilizes ICP0 by preventing its autoubiquinitation (Canning et al., 2004; Everett et al., 1997). While EBNA1 and ICP0 may utilize USP7 in different ways, it is striking that the only two viral proteins known to bind USP7 both require this interaction to facilitate PML disruption.

PML NBs have been shown to be important for p53 activation and DNA repair (Boe et al., 2006; Pearson et al., 2000), prompting us to investigate whether the degree of disruption of PML NBs by EBNA1 was sufficient to impair these processes. Indeed both the acetylation of p53 and the repair of DNA lesions after UV or etoposide treatment were found to be impaired by EBNA1. EBNA1-expressing cells, however, survived these treatments as well or better than parental cells, due to an inhibition of apoptosis which also requires PML NBs (Guo et al., 2000; Wang et al., 1998b). The data as a whole support a model in which EBNA1 contributes to the development of NPC through the disruption of PML NBs, thereby increasing the accumulation of DNA damage while promoting cell survival. This model is consistent with reports of frequent but varying chromosomal aberrations in NPC tumors (Li et al., 2006; Shao et al., 2001). PML disruption by EBNA1 also provides a mechanistic basis for the observation that EBNA1 expression increases the tumorigenicity of EBV-negative NPC cells (Sheu et al., 1996). While several viral proteins are known to promote lytic viral infection through disruption of PML NBs, my results indicate that viral proteins can also contribute to carcinogenesis through PML disruption.
Figure 2-9. Models of the EBNA1, USP7 and PML interactions. Three possible interpretations of the data on EBNA1-PML and EBNA1-USP7 interactions at PML nuclear bodies are shown.
CHAPTER 3

EPSTEIN-BARR NUCLEAR ANTIGEN 1 DISRUPTS PML NUCLEAR BODIES BY TRIGGERING CK2-MEDIATED PML DEGRADATION

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Jennifer Cao performed experiments in Figures 3-2, 3-3 and 3-6. I performed all other experiments in this chapter.
3.1 Introduction

PML protein and its role as a tumor suppressor was first identified in the oncogenesis of acute promyelocytic leukemia (APL). PML forms distinct nuclear structures called PML nuclear bodies (NBs), also known as PODS or ND10s and the formation of these bodies are essential to mediate the known functions of PML (Everett and Chelbi-Alix, 2007; Salomoni et al., 2008). PML exists as six nuclear and one cytoplasmic isoform that are generated by alternative splicing (Bernardi and Pandolfi, 2007; Jensen, Shiels, and Freemont, 2001; Salomoni et al., 2008). The 6 nuclear isoforms are modified by the addition of the small ubiquitin-like modifier SUMO, which enables their interaction to form the structural basis of the PML NB with which many additional proteins then interact (Bernardi and Pandolfi, 2007). PML NBs can be regarded as nuclear organizing centers that govern many important cellular events including cell cycle progression, DNA damage response/repair, transcriptional regulation, apoptosis, and activation of p53 (Bernardi and Pandolfi, 2007; Everett, 2006; Everett and Chelbi-Alix, 2007; Salomoni et al., 2008; Takahashi et al., 2004). In APL, the translocation of the PML gene results in the expression of a fusion protein, PML-RARα, that disrupts the function of PML NBs and is the driving force for the development of APL (Salomoni et al., 2008). The loss of PML NBs is also associated with the development or progression of several additional types of tumours (Gurrieri et al., 2004; Salomoni et al., 2008).

In addition to the above cellular functions, PML NBs are part of the innate immune response to suppress lytic viral replication and transcription (Everett and Chelbi-Alix, 2007; Everett et al., 2006). As a result, many viruses encode proteins that disrupt PML NBs thereby enabling lytic infection (Ahn and Hayward, 1997; Hoppe et al., 2006; Salsman et al., 2008; Ullman and Hearing, 2008). These include the ICP0 protein of herpes simplex virus, which triggers the degradation of PML proteins (Chelbi-Alix and de The, 1999; Everett et al., 1998), and the BZLF1 protein of Epstein-Barr virus (EBV) that interferes with the interaction of the PML proteins to form NBs (Adamson and Kenney, 2001). In addition, viral proteins may disrupt PML NBs in order to promote cell survival by inhibiting apoptosis, as appears to be the case with Epstein-Barr Nuclear Antigen 1 (EBNA1) during Epstein-Barr virus (EBV) latent infection in nasopharyngeal carcinoma cells (Sivachandran, Sarkari, and Frappier, 2008b).

EBV is a widespread herpesvirus that induces cell proliferation and survival as part of its normal latent infection. As a result, EBV is strongly associated with a growing list of cancers including nasopharyngeal carcinoma (NPC), a tumour that is endemic in several parts of the world (Raab-Traub, 2002). Latent EBV infection of NPC cells involves expression of one viral nuclear
protein, EBNA1 (Raab-Traub, 2002). EBNA1 is required for the replication and stable persistence of EBV episomes in proliferating cells and is the only EBV protein that is expressed in all EBV-associated tumors (Raab-Traub, 2002). In addition, increasing evidence suggests a role for EBNA1 in the development and/or progression of EBV-associated tumors. For example, down-regulation of EBNA1 by RNA interference in several types of EBV-positive cells has been shown to decrease cell proliferation and survival (Hong et al., 2006; Yin and Flemington, 2006). Consistent with these observations, overexpression of a dominant-negative EBNA1 mutant increased cell death in EBV-positive Burkitt’s lymphoma cells indicating an anti-apoptotic role for EBNA1 (Kennedy, Komano, and Sugden, 2003). In addition, expression of EBNA1 in breast carcinoma cells increased metastasis in nude mice via inhibition of a known suppressor of cell migration and tumor metastasis, Nm23-H1 (Kaul et al., 2007; Murakami et al., 2005).

The role of EBNA1 in inhibiting apoptosis can be partly explained by its interaction with the host ubiquitin specific protease 7 (USP7), also called HAUSP (Holowaty et al., 2003b; Saridakis et al., 2005). In response to genotoxic stress, USP7 binds and stabilizes p53 by removing polyubiquitin chains (Cummins et al., 2004; Li et al., 2002). EBNA1 was found to interfere with USP7 binding to p53, thereby resulting in the destabilization and degradation of p53 (Saridakis et al., 2005). However the effects of EBNA1 on apoptosis are not limited to alteration of p53 levels, as I have found that EBNA1 disrupts PML NBs in NPC cells (Sivachandran, Sarkari, and Frappier, 2008b). More specifically, EBV-positive NPC cells were observed to have fewer PML NBs than their EBV-negative counterparts and silencing EBNA1 restored the PML NBs to the level of EBV-negative cells. Furthermore, expression of EBNA1 on its own in EBV-negative NPC cells dramatically decreased the number of PML NB and the cellular level of PML proteins without affecting PML transcript levels, indicating that EBNA1 induced the degradation of the PML proteins. In keeping with the known functions of PML NBs, EBNA1 expression in this system lead to impaired DNA repair, decreased p53 activation and apoptosis, and increased cell survival after treatment with DNA damaging agents. Unexpectedly, the ability of EBNA1 to disrupt PML NBs was found to require its interaction with USP7, in that PML NBs were not disrupted by an EBNA1 USP7-binding mutant nor were they disrupted by wildtype EBNA1 when USP7 was silenced (Sivachandran, Sarkari, and Frappier, 2008b). These observations suggested that USP7 itself is a regulator of PML NBs that is utilized by EBNA1, a hypothesis that was further substantiated by showing that PML NB numbers are increased when USP7 is silenced and decreased upon USP7 overexpression (Sarkari et al., 2011).

The proteomics approaches that identified USP7 as a binding partner of EBNA1 also showed
that EBNA1 stably interacted with protein kinase CK2 (formerly known as casein kinase 2) (Holowaty et al., 2003b; Saridakis et al., 2005). CK2 is a tetramer consisting of two catalytic subunits (CK2α and/or CK2 α’) and two regulatory subunits of CK2β (Litchfield, 2003). All three of these subunits were recovered in complex with EBNA1 showing that EBNA1 sequesters or recruits the holoenzyme (Holowaty et al., 2003b). CK2 is known to regulate many cellular processes through phosphorylation of a variety of cellular targets including PML proteins. Scaglioni et al. (Scaglioni et al., 2006; Scaglioni et al., 2008) have shown that CK2 phosphorylates PML at serine 517 which primes PML for polyubiquitylation and degradation by the proteasome. The loss of this CK2 phosphorylation site in PML results in stabilization of PML and increases PML-induced apoptosis and senescence. The importance of CK2-mediated PML degradation in cell transformation is reflected in the observations that there is an inverse relationship between CK2 activity and PML expression in several human tumours (Duncan and Litchfield, 2008).

In this chapter, I have examined the significance of the EBNA1-CK2 interaction for PML disruption, the mechanism of EBNA1-CK2 interaction and the relationship between the CK2 and USP7 interactions with EBNA1. We show that EBNA1 interacts directly with CK2β through EBNA1 residues 387-394, thereby increasing CK2 association with and phosphorylation of PML. Furthermore, we show that EBNA1-CK2 interaction occurs independently of the EBNA1-USP7 interaction which is also required for PML disruption.
3.2 Materials and Method

3.2.1 Cell lines

The EBV-negative nasopharyngeal carcinoma cells CNE2 (also called CNE2Z) and the CNE2E cells that stably express EBNA1 have been previously described (Sivachandran, Sarkari, and Frappier, 2008b; Sun et al., 1992). Both cell lines were grown at 37°C in alpha minimal essential media (αMEM, Gibco) supplemented with 10% fetal calf serum (Sigma), and 0.5 mg/mL of hygromycin B was added to CNE2E to stably maintain EBNA1 expression.

3.2.2 EBNA1 expression plasmids

C-terminally SPA-tagged EBNA1 (lacking most of the Gly-Ala repeat) and EBNA1 mutants, Δ41-376, Δ395-450, 31-641 and 452-641, were generated by PCR amplification of these EBNA1 sequences from pc3oriPE or pc3oriPEΔ41-376 (Shire et al., 1999) or pc3oriPEΔ395-450 (Holowaty et al., 2003b) and insertion between the Not I and Xho I sites of pMZS3F (Zeghouf et al., 2004). EBNA1 Δ387-394 in pMZS3F was generated by QuickChange site-directed mutagenesis (Stratagene, La Jolla, Calif.) of pMZS3F EBNA1. In experiments studying the effect of EBNA1 on PML NBs, EBNA1 was expressed (without tags) from the pc3oriP plasmid (referred to as pc3oriPE), which contains the EBV oriP element. Pc3oriP and pc3oriPE are described in Shire et al 1999 (Shire et al., 1999), while pc3oriP expressing EBNA1Δ395-450 is described in Holowaty et al 2003 (Holowaty et al., 2003b). Pc3oriP expressing Δ387-394 was generated by PCR amplification of EBNA1 Δ387-394 coding sequences in pMZS3F and insertion between the Hind III and Bam HI sites of pc3oriP. The sequences of all of the EBNA1 mutants were verified by DNA sequencing.

3.2.3 Transfections and RNA interference

To generate CNE2 cells transiently expressing EBNA1, 1.5 x 10^5 cells were transfected with 2 μg or 8 μg of pc3OriPE using lipofectamine 2000 (Invitrogen) and pc3OriP was used as a negative control. Where indicated, the same plasmid expressing EBNA1 Δ395-450 or EBNA1 Δ387-394 was used in place of pc3OriPE. Cells were fixed 48 hrs later for immunofluorescence microscopy as described below, except for experiments designed to determine the ability of EBNA1 proteins and USP7 to localize to PML NBs in which cells were fixed 12 hours post transfection. For RNA interference experiments, 1 x 10^6 CNE2 or CNE2E cells in 10 cm dishes were transfected with 100 pmol of siRNA against GFP (GCAAGCUGACCCUGAAGUUCAU) or against CK2α (UAGAUGAACCCAUUCGAGCCUUGC) using 2 μL of lipofectamine 2000. Both cell lines were
treated with an identical second and third round of siRNA transfection 24 hours after the previous transfection. Samples were harvested 24 hours later and processed for microscopy or Western blotting as described below.

3.2.4 Immunofluorescence microscopy

Cells grown on coverslips were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 20 min, rinsed twice in PBS and permeabilized with 1% Triton X-100 in PBS for 5 min. Samples were blocked with 4% BSA in PBS followed by incubation with primary antibodies against either EBNA1 (R4 rabbit serum at 1:300 dilution (Holowaty et al., 2003b)), PML (Santa Cruz PG-M3 at 1:50 dilution), phosphoserine 517 in PML (generous gift from P.P. Pandolfi) or CK2α (Abcam cat #2), then incubation with the secondary antibodies goat anti-rabbit Alexafluor 555 (Molecular Probes) and goat anti-mouse Alexafluor 488 (Molecular Probes) in 4% BSA. Coverslips were mounted onto slides using ProLong Gold antifade medium containing DAPI (Invitrogen). For triple labeling experiments, PML was conjugated to goat anti-rabbit Alexafluor 488, EBNA1 to goat anti-rabbit Alexafluor 555 and USP7 to goat anti-rabbit Alexafluor 647 as per manufacturer’s instructions (Molecular probes, Z25360). Coverslips were mounted onto slides as described above. Images were obtained using the 40 x oil objective on a Leica inverted fluorescent microscope and processed using OpenLAB (ver.X.0) software. PML nuclear bodies were quantified by counting all visible PML foci in 100 cells.

3.2.5 Western blot

Cells were lysed in 9 M urea, 5 mM Tris-HCl (pH 6.8) and briefly sonicated. 50 µg of total protein was subjected to 10% SDS-PAGE and transferred to nitrocellulose. Where indicated, CNE2 and CNE2E cells were treated with 10 µM emodin for 3 days prior to lysis. Membranes were blocked in 5% non-fat dry milk in PBS, then incubated with antibodies against PML (Bethyl A301-167A; 1:2000 dilution), phosphoserine 517 in PML (1:2000, kindly supplied by P.P. Pandolfi), EBNA1 (OT1X at 1:2000 kindly supplied by Jaap Middeldorp, or R4 at 1:2000 (13)), actin (Ab-1, Oncogene Research Products; 1:20 000), CK2α (Abcam ab10466 – 50, 1:5000 dilution), CK2β (Bethyl A301-984A, 1:2500) or USP7 (rabbit serum against full-length USP7) (Sivachandran, Sarkari, and Frappier, 2008b). After washing, blots were probed with goat anti-mouse peroxidase (1:3000) or goat anti-rabbit peroxidase (1:5000) from Santa Cruz, and developed using chemiluminescence reagents (ECL, Perkin Elmer). Membranes were stripped in 0.1 M glycine pH 2.9 for 30 min, washed in PBS-Tween, blocked and re-probed with the next antibody as described above.
3.2.6 Co-immunoprecipitation of endogenous proteins

CNE2 cells were transfected with either 10 µg OriP or OriPE using lipofectamine 2000 (Invitrogen). Cells were harvested 12-14 hours post transfection and nuclei were isolated using hypotonic lysis buffer (50 mM sodium bisulfate, 8.6% sucrose, 20 mM Tris pH 8.0, 2 mM MgCl₂, 0.1% Triton and protease inhibitors) followed by dounce homogenization. Nuclei were lysed in IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% Triton X-100, and protease inhibitors) on ice for 30 min. After centrifugation, 1.5 mg of the supernatant was added to rabbit ExactaCruz beads (SantaCruz) that were pre-coupled to either PML (0.5 µg) or USP7 (1.0 µg) antibody (Bethyl A300-033A) and mixed for 4 hour at 4°C. Beads were collected by centrifugation, washed in IP buffer then boiled in SDS loading buffer. Immunoprecipitated proteins were separated by SDS-PAGE and Western blotted as described above.

3.2.7 Protein purification

GST-fusion plasmids expressing C-terminally tagged CK2α or CK2β were kindly provided by Dr. David Litchfield. GST-CK2α or CK2β proteins were expressed in E. coli BL21 pLysS cells and purified as described previously (Bosc et al., 1995). EBNA1 (lacking most of the gly/ala repeat) and USP7 was expressed in SF9 insect cells and purified as described previously (Holowaty et al., 2003b). Prior to glycerol gradient sedimentation, GST-CK2α or CK2β fusion proteins were incubated with 1 µg Factor Xa protease (New England Biolabs) for 50 µg protein at room temperature for 6 hrs to remove the GST fusion. Dansyl-glu-gly-arg-chloromethyl ketone (CALBIOCHEM) was then added to a final concentration of 5 µM to inactivate Factor Xa protease, and the mixture was passed through a glutathione-sepharose column to remove the GST fragment.

3.2.8 GST pull-down assay

Purified EBNA1 (55 µg) was incubated with GST (27 µg), GST-CK2α (60 µg) or GST-CK2β (48 µg) at a 1:1 molar ratio at 37°C for 1 hr in a 300 µl of assay buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 5% glycerol). The mixture was passed through a 0.2 ml glutathione-sepharose column. After extensive washing with assay buffer minus glycerol, bound proteins were eluted with 20 mM reduced glutathione and detected by SDS-PAGE and Coomassie staining.

3.2.9 Glycerol gradient sedimentation assays

Purified EBNA1 (55 µg), CK2α (45 µg), CK2β (27 µg) and USP7(100 µg) were incubated
alone or in the indicated combinations at a 1:1 molar ratio at room temperature for 1 hr in assay buffer with 10% glycerol in a final volume of 300 µl. A 12 ml 10 to 20% glycerol gradient in assay buffer was generated using a Gradient master apparatus (Biocomp), and the protein mixture was layered onto the gradient. After centrifugation at 34,000 RPM in a SW41 rotor (Beckman) for 20 hrs at 4°C, twenty-four 500 µl fractions were collected from the top of the gradient. Equal volumes of each fraction (5 µl for individual proteins; 10 µl for protein combinations) were then subjected to SDS-PAGE and analyzed by Western blotting.

3.2.10 Mapping of CK2-binding region on EBNA1

SPA-tagged EBNA1, EBNA1 mutants or LacZ were expressed in 293T cells by transfecting one 10 cm plate of cells with 4 µg of the corresponding pMZS3F plasmid using 8 µl of 1 µg/µl PEI (Polysciences). 48 hrs later, the cells were harvested, washed in PBS and lysed in 4 volumes of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.1% sodium deoxycholate and protease inhibitors) on ice for 15 min followed by sonication and centrifugation. Equal amounts of clarified lysate (1.5 mgs) were incubated with 30 µl of M2 FLAG resin (Sigma) for 4 hrs at 4°C with mixing. FLAG resin was harvested by centrifugation, washed in RIPA buffer then boiled in SDS loading buffer. Immunoprecipitated proteins were separated by SDS-PAGE and analysed by Western blotted as described above.

3.3 Results

3.3.1 CK2 plays an important role in EBNA1-mediated disruption of PML NBs

Since EBNA1 interacts with CK2 and both proteins negatively regulate PML NBs, I investigated the possible role of CK2 in EBNA1-mediated PML NB disruption. This was done initially using siRNA targeted to the catalytic subunit of CK2 to silence CK2α in NPC cells that stably express EBNA1 (CNE2E) or in the parental NPC cells that lack EBNA1 expression (CNE2). As previously shown (Sivachandran, Sarkari, and Frappier, 2008b), as a result of EBNA1 expression, CNE2E cells treated with negative-control siRNA (siGFP) have fewer PML NBs (Figure 3-1A, rows 1 and 3 and Figure 3-1B) and less PML protein (Figure 3-1C, compare lanes 1 and 3) as compared to CNE2 with the same treatment. siRNA treatment against CK2α greatly reduced the levels of CK2α (Figure 3-1A, middle column and Figure 3-1C, bottom panel) and resulted in increased levels of PML NBs (Figure 3-1A, right panels and Figure 3-1B) and PML proteins (Figure 3-1C, top panel) relative to siGFP treatment in both CNE2 and CNE2E cell lines. The difference in numbers of PML NBs in
siGFP and siCK2α treated cells is statistically significant for both CNE2 (p<0.01) and CNE2E cells (p<0.001). The data suggest that EBNA1 is less able to disrupt PML NBs when CK2 is lacking and that EBNA1 does not interfere with the ability of CK2 to negatively regulate PML NBs. The observation that CK2α silencing in CNE2 cells increased PML NBs and protein levels is consistent with the known mechanism for CK2-mediated disruption of PML NBs.

To further verify the importance of CK2 in EBNA1-mediated PML degradation and to determine if the catalytic activity of CK2 is required for this contribution (as opposed to the presence of the CK2α subunit), emodin was used to inhibit the kinase activity of CK2. Emodin treatment of CNE2 and CNE2E cells decreased phosphorylation of a known CK2 target, serine 517 of PML (Scaglioni et al., 2006), without affecting the level of CK2α (Figure 3-1D), showing that this inhibitor was functioning as expected. In addition, emodin treatment of CNE2 increased PML protein levels (Figure 3-1D, top left panel), demonstrating the role for CK2 is regulating PML. Interestingly, emodin treatment of CNE2E also resulted in increased PML levels despite the presence of EBNA1 in these cells (Figure 3-1D, top right panel). This supports the CK2α silencing results and further indicates that EBNA1-mediated PML degradation requires CK2 kinase activity.

CK2 has been shown to trigger PML degradation through the phosphorylation of PML proteins at serine 517 (p517) (Scaglioni et al., 2006). Since EBNA1 induces PML degradation by a mechanism that requires CK2, I asked whether EBNA1 increased CK2-mediated phosphorylation of PML S517, using an antibody specific to the phosphoserine 517 of PML (Scaglioni et al., 2006). Western blots of whole cell extracts of CNE2 and CNE2E cells showed that, although CNE2E had considerably less PML protein, it had increased levels of p517 compared to CNE2 (Figure 3-1E). This trend was also apparent in the experiment in Figure 3-1D (compare lanes 1 and 3). I also examined changes in p517 by immunofluorescence microscopy and observed an increase in the intensity of p517 staining in the EBNA1-expressing CNE2E cells as compared to the CNE2 cells (Figure 3-1F). In CNE2E cells, the p517 staining also appeared to be more dispersed throughout the nucleoplasm, as compared to CNE2 where p517 was mostly concentrated at the PML NBs; a pattern that is likely the result of disruption of the PML NBs. Taken as a whole, the data strongly suggest that EBNA1 triggers degradation of PML proteins by increasing their phosphorylation by CK2.
Figure 3-1. EBNA1-mediated PML degradation requires CK2 and involves increased PML phosphorylation. (A) CNE2 cells and CNE2E cells were transfected with siRNA against CK2α (siCK2α) or GFP (siGFP; negative control) then stained for CK2α and PML. Images were captured using the same exposure time for both cell lines. (B) Quantification of the number of PML NBs per cell in each of the samples in A. P value is <0.01 for siGFP and siCK2 in CNE2 and <0.001 for siGFP and siCK2 in CNE2E. (C) Equal amounts of cell lysates from (A) were analysed by Western blotting with the indicated antibodies, where (-) samples are treated with siGFP and (+) are treated with siCK2α. (D) CNE2 and CNE2E were treated with 10 μM emodin (in DMSO; +) or DMSO alone (-) or for three days, then equal amount of cell lysates were analysed by Western blotting with the indicated antibodies, including one that recognizes only PML phosphorylated at S517 (p517). (E) Equal amount of CNE2 and CNE2E cell lysates were analysed by Western blotting with the indicated antibodies. (F) CNE2 and CNE2E cells were stained with antibodies against total PML and PML phosphorylated at serine 517 (p517). Images were captured using the same exposure time for both cell lines.
3.3.2 EBNA1 directly interacts with CK2β

CK2 was found to interact with EBNA1 in both in vivo TAP-tagging experiments and in vitro affinity column experiments using human cell lysates (Holowaty et al., 2003b). However, since several other interactions were also identified in these assays, it was not clear whether EBNA1 interacted directly with CK2 or indirectly through another cellular protein. We tested whether EBNA1 could bind directly to CK2 subunits using purified proteins in both GST-pull down assays and glycerol gradient sedimentation analyses. For the GST-pull down assays, GST-CK2α, GST-CK2β or GST alone was mixed with equimolar amounts of EBNA1 then bound to gluthathione resin and, after washing, retained proteins were eluted with glutathione. Samples of the input protein, resin flow-through and eluted proteins were then analyzed by SDS-PAGE followed by Coomassie staining (Figure 3-2A). Little to no EBNA1 was retained on the resin by GST-CK2α or GST alone relative to the amount of the eluted GST proteins, whereas a considerably higher ratio of EBNA1 to GST-CK2β was recovered in the eluates of the resin containing GST-CK2β, suggesting that EBNA1 directly interacts with the regulatory subunit of CK2.

The interaction of EBNA1 with CK2α or CK2β (lacking tags) was also assessed using equimolar amounts of purified protein by glycerol gradient sedimentation. To this end, EBNA1, CK2β or CK2α were subject to glycerol gradient sedimentation either individually or in combination, then protein fractions were collected and equal volume aliquots were analyzed by western blotting (Figure 3-2B). When run individually on the gradient, EBNA1 peaked at fractions 5-7, CK2α at fraction 4 and CK2β at fraction 5 (Figure 3-2B, top three panels). However, when EBNA1 and CK2β were mixed, both proteins shifted to the position of a larger complex and co-migrated, peaking at fraction 8 (Figure 3-2B, middle panels). The formation of a larger complex was not seen when EBNA1 was mixed with CK2α, as neither protein shifted towards the bottom of the gradient nor did the two protein peaks correspond closely to each other (Figure 3-2B, bottom panels). These results support those of the GST-pull down assays and indicate that EBNA1 binds directly to the CK2β subunit of CK2.
Figure 3-2. EBNA1 directly interacts with CK2β. (A) Purified EBNA1 was incubated with purified GST-tagged CK2α or CK2β or with GST alone at equimolar ratios, then mixed with glutathione-Sepharose. After washing, proteins were eluted with glutathione and analyzed by SDS-PAGE and colloidal Coomassie staining. Sample shown are the input protein mixture (I; 3% of the total), the flow through that was not retained on the resin (F; 3% of the total) and protein that was retained by the resin and eluted (E; 50% of the total). The positions of EBNA1, CK2α-GST, CK2β-GST and molecular weight markers (M) in kDa are indicated. (B) Purified EBNA1, CK2α or CK2β proteins were analyzed by glycerol gradient sedimentation individually (top panels), or after pre-incubation of equal molar ratios of EBNA1 and CK2β or CK2α (middle or bottom panels.) Equal volume fractions were collected from top of each gradient, and equal volumes of each fractions were analyzed by Western blotting using antibodies against EBNA1, CK2α or CK2β.
### 3.3.3 Identification of the CK2-binding site of EBNA1

To further investigate the mechanism of the EBNA1-CK2 interaction and its functional significance, we tested the ability of a series of EBNA1 mutants (Figure 3-3A) to interact with CK2 in human cells. These EBNA1 proteins were expressed by transient transfection of CNE2 cells, fused to a C-terminal SPA tag that includes a triple FLAG epitope (Zeghouf et al., 2004). Initially EBNA1 and three EBNA1 mutants, 452-641, Δ41-376 and 31-641, were expressed in CNE2, immunoprecipitated with anti-FLAG antibody and examined for recovery of endogenous CK2 by western blotting for CK2α. CK2α co-immunoprecipitated with all of these EBNA1 proteins except for 452-641, even though all of the EBNA1 proteins were expressed and recovered at similar levels (Figure 3-3B). In addition, the negative control of SPA-tagged lacZ failed to recover detectable levels of CK2α. These results indicated that EBNA1 binds CK2 through internal sequences located between residues 376 and 451.

The 376-451 region of EBNA1 includes the nuclear localization sequence, a serine-rich region and a region known to bind USP7. I have previously shown that an EBNA1 mutant Δ395-450 fails to recover USP7 in TAP-tagging experiments but still recovers CK2 (Holowaty et al., 2003b). In keeping with this observation, Δ395-450 co-immunoprecipitated both CK2α and CK2β as did EBNA1 but, unlike EBNA1, failed to recover USP7 (Figure 3-3C). This indicated that the CK2 binding site was between residues 376-395, prompting the generation of EBNA1 mutant Δ387-394, which lacks a serine-rich sequence but retains the nuclear localization sequence. Δ387-394 was expressed and recovered at similar levels as EBNA1 and Δ395-450 in FLAG immunoprecipitations (Figure 3C, bottom panel), yet failed to co-immunoprecipitate either CK2α and CK2β (Figure 3-3C, top 2 panels). However, Δ387-394 bound USP7 as efficiently as EBNA1, indicating that this mutation did not disrupt the structure of the EBNA1 internal region or EBNA1 nuclear localization. Furthermore the results indicate that CK2 and USP7 bind independently to EBNA1 and that the Δ387-394 and Δ395-450 mutants are useful for functional studies to specifically address roles EBNA1-CK2 and EBNA1-USP7 interactions, respectively.
Figure 3. Mapping of CK2-binding region of EBNA1 by co-immunoprecipitation. (A) Schematic representation of EBNA1 and the EBNA1 mutants used in this study, showing the glycine and arginine-rich regions (G/R), the glycine and alanine-rich region (G/A), the nuclear localization signal (NLS) and the DNA binding domain (DNA BD). Note that the EBNA1 used in this study contains a short version of the G/A repeat. The ability of the EBNA1 proteins to bind endogenous CK2 by co-immunoprecipitation is also indicated (as determined in parts B and C). (B and C) 293T cells were transfected with plasmids expressing the indicated FLAG-tagged EBNA1 protein or FLAG-tagged lacZ and tagged proteins were immunoprecipitated from cell lysates using anti-FLAG resin. Proteins recovered from the FLAG resin were analyzed by Western blotting using antibodies against EBNA1 (B) or the FLAG epitope (C), CK2α (B and C), CK2β (C) and USP7 (C). Western blots are also shown for 10% of the starting cell lysates (Input).
3.3.4 An EBNA1 CK2-binding mutant does not disrupt PML NBs

I have shown that the mechanism by which EBNA1 disrupts PML NBs involves CK2 and results in increased CK2-mediated phosphorylation of PML, however it was not clear whether these effects were due to EBNA1 binding to CK2 or involve a less direct mechanism. To address this question, I asked whether the EBNA1 mutant that is defective in CK2 binding, Δ387-394, was able to disrupt PML NBs. To this end, EBNA1 and Δ387-394 (lacking any tags) were expressed in CNE2 cells by transient transfection of oriP-based expression plasmids and compared to transfection with the oriP plasmid lacking EBNA1. Cells were then stained with anti-EBNA1 and anti-PML antibodies to identify EBNA1-expressing cells as well as any differences in PML NBs in neighbouring cells expressing or not expressing EBNA1 (Figure 3-4A). As previously reported (Sivachandran, Sarkari, and Frappier, 2008b), cells expressing wildtype EBNA1 had fewer PML NBs than their neighbours lacking EBNA1 expression (Figure 3-4A, top panel) and the number of PML NBs in these EBNA1-expressing cells was on average 3-fold lower than the same cells transfected with the oriP control plasmid with a p value of <0.005 (Figure 3-4B). However expression of Δ387-394 did not result in any noticeable differences in the abundance, size or shape of the PML NBs when compared to neighbouring cells lacking any EBNA1 expression (Figure 3-4A, bottom panels) or to cells transfected with the negative control plasmid (Figure 3-4B).

I also examined the effect of Δ387-394 expression on PML protein levels by performing western blots on lysates of the above cells. As expected, transient expression of wildtype EBNA1 resulted in a large decrease in the amount of PML proteins as compared to control cells transfected with the empty oriP plasmid (Figure 3-4C, compare lanes 1 and 2). However, despite being expressed at the same level as EBNA1, Δ387-394 did not induce the loss of PML proteins (Figure 3-4C, lane 3). This result is similar to that with EBNA1 mutant Δ395-450 (Figure 3-4C, lane 4), which does not bind USP7 and which I previously showed does not disrupt PML NBs. The results indicate that EBNA1 must bind CK2 in order to disrupt PML NBs and induces the degradation of PML proteins. In addition, this in conjunction with my previous work, indicates that EBNA1 must bind to both CK2 and USP7 to disrupt PML NBs.
Figure 3-4. The EBNA1 CK2-binding mutant fails to disrupt PML NBs. (A) CNE2 cells were transiently transected with a plasmid expressing EBNA1 or EBNA1 mutant Δ387-394, then stained for EBNA1 and PML 24 hrs post transfection. Both EBNA1-expressing (red) and non-expressing cells are shown within the same image. (B) The numbers of PML NBs per cell were counted 24 hours after expression of EBNA1 or Δ387-394 and compared to control cells transfected with the empty expression plasmid (OriP). (C) CNE2 cells were transiently transfected with a plasmid expressing EBNA1, Δ387-394, Δ395-450 or no protein (OriP) and, 48 hrs later, equal amounts of cell lysates were analysed by Western blotting with the indicated antibodies.
3.3.5 EBNA1 increases the interactions of CK2β and USP7 with PML

I imagined two different scenarios in which the interaction of EBNA1 with CK2 and USP7 might be important for EBNA1-mediated disruption of PML NBs. First, since a proportion of USP7 and CK2 is associated with PML NBs, EBNA1 binding to one or both of these proteins might be required for EBNA1 to localize to PML NBs. Second, EBNA1 might associate with PML NBs independently of CK2 or USP7 and bring additional CK2 and USP7 to the PML NBs where they can trigger PML degradation. To test the first possibility, I examined the nuclear localization of the Δ387-394 and Δ395-450 EBNA1 mutants that fail to bind CK2 and USP7, respectively, soon after their expression when PML NBs are largely intact. As shown in Figure 3-5A, both EBNA1 mutants were observed to form foci that localize to PML NBs in addition to giving more diffuse nuclear staining, a pattern that is typical of wildtype EBNA1 (Sivachandran, Sarkari, and Frappier, 2008b) and Figure 3-5B, rows 2 and 6). Therefore EBNA1 binding to CK2 or USP7 does not appear to be required for EBNA1 to associate with PML NBs.

I then examined whether EBNA1 affected the degree to which USP7 and CK2 were associated with PML NBs. These experiments were performed by expressing EBNA1 in CNE2 cells by transient transfection and imaging or harvesting cells soon after EBNA1 expression, at which time the PML NBs are still largely intact (Figure 3-5B-D). Immunofluorescence imaging of these cells for EBNA1, USP7 and PML, showed that, in cells lacking EBNA1 expression, USP7 gave mostly diffuse nuclear staining and formed a small number of foci that were associated with PML NBs (Figure 3-5B). This is consistent with previous reports of partial association of USP7 with PML NBs (Everett et al., 1997). However in EBNA1-expressing cells present on the same slides, there was a noticeable increase in the number of USP7 foci (Figure 3-5B, row 4, compare neighboring cells in the same panel), most of which were closely associated with PML NBs (Figure 3-5B, row 5). Quantification of these results indicated that EBNA1 caused a 2-3 fold increase in the percentage of PML bodies with associated USP7 with a p value of <0.001 (Figure 3-5C). In addition, more PML protein was found to co-immunoprecipitate with USP7 from CNE2 cells after transfection with the EBNA1-expression plasmid than after transfection with the empty plasmid (Figure 3-5D), further indicating that EBNA1 increases the association of USP7 with PML NBs. The finding that USP7 preferentially immunoprecipitates one PML isoform is consistent with our previous observations (Sarkari et al., 2011). As expected, a fraction of the EBNA1 also co-immunoprecipitated with USP7 (Figure 3-5D, bottom panel). Co-immunoprecipitations were also done in reverse, where total PML was immunoprecipitated and the amount of associated USP7 was compared in the presence and absence of
EBNA1 (Figure 3-5E). In keeping with the above experiments, more USP7 was recovered with PML when EBNA1 was present.

I also examined the effect of EBNA1 expression on the association of CK2 with PML NBs. Antibodies against either CK2α or CK2β both result in bright pan-nuclear staining making it difficult to assess the degree of their association with PML NBs by immunofluorescence imaging (data not shown). However, the effect of EBNA1 on the association of CK2 with PML NBs could be assessed by the amount of CK2 that co-immunoprecipitated with PML in the presence and absence of EBNA1. I consistently observed that more CK2β was recovered with PML in the presence of EBNA1 than in its absence (Figure 3-5F, compare lanes 5 and 6). Taken together the data suggest that EBNA1 is able to increase the occupancy of both CK2 and USP7 at PML NBs.
Figure 3-5. EBNA1 increases association of CK2β and USP7 with PML NBs. (A) EBNA1 mutants Δ387-394 or Δ395-450 were expressed in CNE2 by transient transfection and, 12 hours later the cells were stained for EBNA1 and PML. (B) CNE2 cells were transiently transfected with the EBNA1 expression plasmid and 12 hours later, cells were stained for EBNA1, USP7 and PML using specific antibodies directly conjugated to Alexafluor dyes. Overlays of USP7 with PML and EBNA1 with PML are also shown in which USP7 and EBNA1 have been colorized red and PML is green. (C) Quantification of the percentage of PML NBs that have associated USP7 foci with and without EBNA1 expression (p<0.001). (D-F) CNE2 cells were transfected with a plasmid expressing EBNA1 (+) or the empty plasmid (-) and, 14 hours later, immunoprecipitations were performed from nuclear lysates using anti-USP7 (D) or anti-PML antibody (E and F). IgG is a negative control in which immunoprecipitations were performed with rabbit IgG beads. Samples were western blotted using the indicated antibodies. One fifteenth of the starting nuclear extracts (input) is also shown. In E, the blot was initially probed for USP7 (bottom panel) then stripped and reprobed for PML (top panel). Due to incomplete stripping, some residual USP7 is still visible as indicated in the top panel.
3.3.6 EBNA1, USP7 and CK2β form a ternary complex

Our data indicate that EBNA1 can directly bind USP7 and CK2β and recruit them to PML NBs. There are two scenarios by which this may occur. First, since multiple EBNA1 molecules are likely to associate with any given PML NB, it could be that some of these EBNA1 molecules bind and recruit USP7 while other EBNA1 molecules bind and recruit CK2 to the same PML NB. In this scenario USP7 and CK2 would not be bound to the same EBNA1 protein. Second, a single EBNA1 molecule might bind to both USP7 and CK2, bringing both of these enzymes to the PML NB. If this is the case then we should be able to detect EBNA1, USP7 and CK2 in a ternary complex.

We examined whether EBNA1, USP7 and CK2β could form a ternary complex by combining these purified proteins in various combinations and subjecting them to glycerol gradient sedimentation analyses. When analysed individually, USP7, EBNA1 and CK2β peaked at fractions 6, 5 and 3 respectively (Figure 3-6A). When USP7 and EBNA1 were pre-incubated prior to analyses, the proteins co-migrated on the glycerol gradient and shifted to the position of a larger complex peaking at fraction 8 (Figure 3-6B, middle panel), confirming that USP7 forms a stable complex with EBNA1. Similarly, when combined, EBNA1 and CK2β co-migrated and shifted to a peak at fraction 6 and 7 (Figure 3-6B, bottom panel), confirming their interaction. However, no direct interaction was detected between USP7 and CK2β, as when combined, the migrations of these proteins were unchanged from their migration when analysed individually and co-migration was not observed (Figure 3-6B, top panel). Finally, when USP7, EBNA1 and CK2β were combined in equimolar amounts, a significant proportion of each protein was observed to co-migrate at a position of a complex larger than that of EBNA1-USP7 or EBNA1-CKβ, peaking at fractions 8 and 9 (Figure 3-6C). Most notably, most of the CK2β shifted two fraction from its position as an EBNA1-CK2β complex (fractions 6-7) to a larger complex including USP7 (fractions 8-9). These results indicate that EBNA1 can bind USP7 and CK2β simultaneously forming a ternary complex.
Figure 3-6. EBNA1, USP7 and CK2β form a ternary complex. Glycerol gradient sedimentation was performed with purified EBNA1, CK2β, and USP7 on 10-20 % glycerol gradients. Equal volume fractions were collected from top of each gradient and equal volumes of each fraction were analyzed by Western blotting using antibodies against each protein (indicate on the left of each panel). Fraction 14 is the pellet fraction from the bottom of the tube. (A) The sedimentation of USP7, EBNA1 or CK2β proteins was analyzed individually (top, middle and bottom panels, respectively) (B) Equal molar ratios of USP7 and CK2β (top panel), or USP7 and EBNA1 (middle panel), or EBNA1 and CK2β (bottom panel) were pre-incubated then analyzed by glycerol gradient sedimentation. (C) Equal molar ratios of USP7, CK2β and EBNA1 were pre-incubated, then analyzed by glycerol gradient sedimentation.
I have previously shown that EBNA1 disrupts PML NBs in NPC cells by inducing the degradation of PML proteins (Sivachandran, Sarkari, and Frappier, 2008b). PML NB play critical roles in p53 activation, DNA repair and apoptosis and, accordingly, EBNA1 was found to disrupt all of these processes (Saridakis et al., 2005; Sivachandran, Sarkari, and Frappier, 2008b). The combination of these EBNA1 effects would be expected to contribute to the development of this tumour, suggesting a direct role for EBNA1 in oncogenesis. In addition, it is interesting that PML disruption by EBNA1 occurs in epithelial cells, which are the main sites of lytic infection, but has not been observed in B-lymphocytes (Bell, Lieberman, and Maul, 2000) which are predominantly responsible for latent infection. Since PML NBs are known to be repressive for lytic infection of at least some herpes viruses (Everett et al., 2006; Tavalai et al., 2006), these observations raise the possibility that EBNA1 might promote the switch to lytic infection in epithelial cells through PML disruption. Here I have extended our initial studies on EBV infection and PML NBs by providing the mechanism of EBNA1-induced PML disruption.

I have shown that the ability of EBNA1 to induce the degradation of PML proteins depends on the presence of the host CK2, on the catalytic activity of CK2 with EBNA1. CK2 has been previously shown to phosphorylate PML at S517 which enables the polyubiquitylation of PML and its subsequent degradation by the proteasome (Scaglioni et al., 2006; Scaglioni et al., 2008). Our studies support this important role of CK2 in regulating PML levels and show that this pathway is being usurped by EBNA1. In particular, EBNA1 was shown to increase the occupancy of CK2 at PML NBs and the subsequent phosphorylation of S517. Experiments performed with EBNA1 mutants showed that the EBNA1-CK2 interaction was critical for PML disruption but was not sufficient for this effect as the EBNA1 interaction with USP7 was also required to trigger PML degradation through a mechanism that is not yet well understood (Sivachandran, Sarkari, and Frappier, 2008b).

Our data indicate that the EBNA1-CK2 interaction is mediated by EBNA1 residues between 387-394. The region 387-394 of EBNA1 is a serine rich stretch that includes a phosphserine at position 393 (Duellman et al., 2009) and that has no previously assigned function. One reason that EBNA1 might interact with CK2 is that EBNA1 may be a target of CK2 phosphorylation. This possibility is supported by the finding that EBNA1 is phosphorylated at serine 21 (Duellman et al., 2009), which is a predicted CK2 phosphorylation site. However, neither deletion of the 387-394 CK2 binding site nor deletion of N-terminal sequences spanning S21 disrupted the ability of EBNA1 to
function in EBV replication, plasmid maintenance or transcriptional activation (Mackey and Sugden, 1999; Yates and Camiolo, 1988) and our unpublished data), nor is the S21 site conserved in EBNA1 homologous in the EBV-like cynomolgus monkey virus and herpesvirus papio (Duellman et al., 2009). These findings suggest that EBNA1 binding to CK2 may be for the purpose of altering the host cell environment rather than for modifying EBNA1 to affect its functions at the EBV genome.

CK2 is a tetramer comprised of two catalytic isoforms (either CK2α, CK2α’ or one of each) and two regulatory subunits of CK2β (reviewed in (Litchfield, 2003)). CK2β is important for enhancing the catalytic activity and stability of the enzyme and can mediate interactions with CK2 substrates, as has been shown for p53, DNA topoisomerase II and the CD5 receptor (Appel et al., 1995; Bojanowski et al., 1993; Raman et al., 1998). In addition, protein interactions with CK2β can redirect CK2 to increase CK2 activity towards specific substrates. For example the interaction of CK2β with fibroblast growth factor-2 has been shown to increase CK2 phosphorylation of nucleolin (Bonnet et al., 1996). Similarly, we have clearly shown that CK2β mediates the interaction of CK2 with EBNA1 and that this interaction results in increased phosphorylation of PML proteins by CK2.

Our data point to a model of PML disruption by EBNA1 in which an EBNA1 protein associates with PML NBs by a mechanism that is independent of its interaction with USP7 or CK2. I have previously shown that EBNA1 preferentially associates with a single PML isoform that appears to be PML IV, however it is not clear if this interaction is direct or mediated by another protein (Sivachandran, Sarkari, and Frappier, 2008b). Interestingly, USP7 also preferentially interacts with what appears to be the same PML isoform as EBNA1. I have shown that a single EBNA1 protein can interact with both CK2 and USP7 to form a ternary complex and that the interaction with CK2 occurs through the β regulatory subunit. EBNA1 is known to form stable dimers via a β-barrel structure formed by its C-terminal DNA binding domain and has never been detected in the monomeric form (Ambinder et al., 1991; Bochkarev et al., 1995; Frappier and O'Donnell, 1991b). Therefore the USP7-EBNA1-CK2 ternary complex could either result from each EBNA1 monomer in the dimer binding to both USP7 and CK2 (Figure 3-7A) or from one monomer within the dimer binding to USP7 and the other monomer binding to CK2 (Figure 3-7B). The former would result in a larger complex than the later, but due to limitations in resolution on the glycerol gradient and the contribution of shape to a molecule’s migration, I cannot differentiate these two possibilities by the sedimentation rate of the ternary complex alone. We have previously shown that EBNA1 binds USP7 through EBNA1 residues 442-448 (Saridakis et al., 2005) and here we show that EBNA1 binds CK2
through residues 387-394. The proximity of these sites raises the possibility that the binding of CK2 or USP7 to one EBNA1 monomer might sterically hinder the interaction of the second protein with the same monomer (as shown in Figure 3-7B). However, depending on the orientation of the bound proteins, it is also possible that interactions with both proteins could occur simultaneously on the same monomer. Indeed, comparison of the glycerol gradient sedimentation of the EBNA1-USP7 complex (Figure 3-6B) to that of the ternary complex (Figure 3-6C) supports the model in which USP7 and CK2β both bind to each monomer within the dimer (Figure 3-7A) as opposed to separate monomers, since the EBNA1-USP7 complex appears to get bigger in the presence of CK2β, shifting towards the bottom of the gradient. If the model in Figure 3-7B was correct, I would expect the ternary complex to be smaller than the EBNA1-USP7 complex, since the 135 kDa USP7 protein bound to one monomer would be replaced by the smaller (25 kDa) CK2β protein.

The increased recruitment of CK2 to PML NBs by EBNA1 results in increased phosphorylation of PML by CK2 as evidenced by the EBNA1-induced increase in phosphoserine 517. CK2 is known to phosphorylate this site on PML triggering the polyubiquitylation and degradation of PML by an as yet unidentified ubiquitin ligase (Scaglioni et al., 2006; Scaglioni et al., 2008). Therefore it is likely that the increase in p517 in the presence of EBNA1 results in increased degradation of PML. CK2 is not strictly localized to PML NBs but rather is found throughout the nucleus, suggesting that it transiently associates with PML NB in order to maintain these structures at a specific level. By increasing the occupancy of CK2 at PML NBs, EBNA1 would shift this equilibrium resulting in lower levels of PML NB and PML proteins.

A similar scenario appears to be occurring with USP7, another protein that is only partly associated with PML NBs, in that EBNA1 increases its occupancy at PML NBs. While USP7 is normally associated with the stabilization of specific proteins through its ability to cleave ubiquitin from them, we have shown that USP7 promotes the degradation of PML proteins by a mechanism that does not involve the catalytic activity of USP7 (Sarkari et al.). Rather this involves the protein interaction domains of USP7, suggesting that USP7 recruits another protein(s) to PML NBs that may polyubiquitylate PML (Sarkari et al.). In addition, the ability of USP7 to trigger PML degradation was shown to be independent of CK2 (Sarkari et al.). Taken together the results indicate that EBNA1 uses two independent pathways to trigger the degradation of PML proteins, one that involves CK2 and the other that involves USP7.

EBNA1 forms a stable complex with CK2 and I have now shown that this interaction is significant for the EBNA1-mediated disruption of PML NBs. However given the numerous roles
reported for CK2 in cellular processes, this is unlikely to be the only CK2 function affected by EBNA1. For example CK2 has been shown to be important for transitions through several stages of the cell cycle and to play multiple roles in regulating apoptosis (Duncan and Litchfield, 2008; Litchfield, 2003). In addition CK2 regulates both tumour suppressor proteins and oncogenes in ways that promote tumorigenesis and, as a result, elevated levels of CK2 are associated with malignant transformation of many cell types and aggressive tumour behaviour (reviewed in (Duncan and Litchfield, 2008)). The possibility that EBNA1 affects additional CK2 functions to promote the cell survival critical for latent infection and/or tumourigenesis associated with EBV infections merits further investigation.

**Figure 3-7. Models of the CK2-EBNA1-USP7 ternary complex.** An EBNA1 dimer (black) associated with a PML NB is shown where the black oval represents the dimerized dimerization/DNA binding domain. CK2 ($\alpha$ and B) and USP7 proteins are shown bound to the extended EBNA1 sequences outside of the dimerization domain. (A) CK2 and USP7 associate with the same monomer within the EBNA1 dimer. (B) CK2 and USP7 interact with opposite monomers within the EBNA1 dimer due to steric hindrance.
CHAPTER 4

CONTRIBUTIONS OF THE EPSTEIN-BARR VIRUS EBNA1 PROTEIN TO GASTRIC CARCINOMA

This chapter has been submitted for publication as:


Jaap Middeldorp provided the EBERish staining for figure 4-5. I performed all other experiments in this chapter.
4.1 Introduction

Epstein-Barr virus is a gamma herpes virus whose latent infection is reported to be associated with approximately 10% of all gastric carcinomas (GC) (Fukayama, Hino, and Uozaki, 2008; Takada, 2000), although a recent study using a more sensitive Q-PCR EBV detection method suggested that this percentage might be much higher (Ryan et al., 2009). GC is the fourth most common cancer worldwide and is the second most common cause of death from cancer. GC presents itself without regional or racial differences and is a major clinical problem worldwide (Catalano et al., 2009). There are several lines of evidence suggesting a causative role for EBV in the onset of viral-associated GC. First, EBV is present in every tumour cell, but absent in the surrounding normal epithelium (Shibata and Weiss, 1992). Second, the presence of clonal EBV in every tumour cell suggests that EBV infection preceded the final transforming event giving rise to the tumour (Imai et al., 1994). Third, EBV-associated GC has unique morphological features that distinguishes it from EBV-negative tumours, again suggesting a role for EBV in the pathogenesis of this tumour (Uemura et al., 1994).

In EBV-associated GC, EBV establishes type I latency expressing the EBNA1, LMP2A and secreted BARF1 proteins but does not express the LMP1 oncoprotein (Fukayama, 2010; Imai et al., 1994). The lack of LMP1 expression suggests that other viral proteins likely play important roles in the development of this tumour. Although LMP2A is known to alter host DNA through hypermethylation of tumour suppressor genes (Fukayama, 2010; Fukayama, Hino, and Uozaki, 2008), the molecular events that lead to tumour formation still remain unclear. EBNA1 is the only viral nuclear protein expressed in GC. It is also the only viral protein required to maintain latency, due to its roles in the replication and segregation of the EBV episomes (Frappier, 2004). However, the role of EBNA1 is not limited to EBV genome maintenance as there is increasing evidence that EBNA1 alters the cellular environment in ways that promote genomic instability, thereby leading to tumourigenesis (Cheng et al., 2010; Gruhne et al., 2009; Kennedy, Komano, and Sugden, 2003). For example, EBNA1 can lower p53 levels by sequestering the ubiquitin specific protease, USP7 (Saridakis et al., 2005). In addition, several EBV-positive cell lines have been shown to become dependent on EBNA1 expression, such that silencing of EBNA1 leads to decreased proliferation or apoptosis (Ian et al., 2008; Yin and Flemington, 2006).
I recently demonstrated that EBNA1 disrupts PML (promyelocytic leukemia) nuclear bodies (NBs) in the context of nasopharyngeal carcinoma (NPC) cells, by inducing the degradation of the PML proteins that form the structural basis for these NBs (Sivachandran, Sarkari, and Frappier, 2008b). PML NBs (also called ND10s) are important for several processes associated with tumour suppression, including p53 activation, apoptosis and DNA repair (Bernardi and Pandolfi, 2007; de Stanchina et al., 2004; Zhong et al., 1999). The PML NBs also play a role in suppressing lytic viral replication; consequently, many viruses encode proteins targeting the disruption of PML NBs, in some case by inducing the degradation of PML proteins (Everett and Chelbi-Alix, 2007). Accordingly, pml knockout mice confirm the loss of PML, in turn demonstrating increased susceptibility to both malignant transformation and viral infections (Trotman et al., 2006; Wang et al., 1998a). In addition, loss of PML NBs has been associated with the development of several types of human malignancies, but to date, has not been examined in gastric carcinoma (Gurrieri et al., 2004). While our studies in NPC cells suggest that disruption of PML NBs is a mechanism by which EBNA1 may contribute to this tumour, EBNA1 was not found to alter PML NBs in HEK293 or HeLa cells (our unpublished data); hence it remained unclear as to whether this was an NPC-specific phenomenon. In addition, since most NPCs are EBV-positive, I could not definitively prove that EBNA1 itself was causative in altering PML NBs in NPCs.

In this study, I investigated the effect of EBNA1 on PML NBs in gastric carcinoma cell lines and demonstrated that EBNA1 dramatically affected the number and function of PML NBs by inducing degradation of PML proteins. As a result, EBNA1 expression inhibited p53 activation and apoptosis, leading to cell survival. Importantly, the availability of both EBV-positive and EBV-negative GC tumour biopsies enabled a comparison of PML NBs in the primary tumours, demonstrating for the first time that EBV-positive GC tumours have reduced PML staining compared to EBV-negative samples.
4.2 Materials and Methods

4.2.1 Cell lines

The human adenocarcinoma cell line, AGS, has been described previously (Valentine et al., 2010). AGS-EBNA1 cells stably expressing EBNA1 were generated as described in Valentine et al. (Valentine et al., 2010). AGS-EBV was generated by co-culturing AGS cells with Akata Burkitt's lymphoma cells carrying recombinant neomycin-resistant EBV as described previously (Stewart et al., 2004). All three cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum and 1% L-glutamine. The AGS-EBNA1 and AGS-EBV were maintained in G418 (Invitrogen; 400 ug/mL) to select for cells containing the EBNA1 expression cassette and EBV respectively.

4.2.2 Transfections

For transient EBNA1 expression, 5x 10^5 AGS cells were transfected with 2 µg of pc3OriPE (Wu, Kapoor, and Frappier, 2002b), or the negative control plasmid lacking EBNA1 (pc3Orip) (Wu, Kapoor, and Frappier, 2002b) using lipofectamine 2000 (Invitrogen). Cells were fixed 24 hrs later for immunofluorescence microscopy or harvested for Western blotting as described below. AGS-EBNA1 and AGS-EBV were transfected with siRNA against GFP or EBNA1 as described previously (Sivachandran, Sarkari, and Frappier, 2008b) and analysed by immunofluorescence staining (IF) and Western blotting.

4.2.3 Immunofluorescence microscopy

Cells grown on coverslips were processed for imaging as described previously (Sivachandran, Sarkari, and Frappier, 2008b) Samples were incubated with primary antibodies against EBNA1 (R4 rabbit serum at 1:300(Holowaty et al., 2003b)), PML (Santa Cruz PG-M3 at 1:50), p21 (Santa Cruz sc-817, 1:50) and acetyl-p53K382(Cell Signaling 2525S, 1:200) followed by secondary antibodies goat anti-rabbit Alexafluor 555 (Molecular Probes) and goat anti-mouse Alexafluor 488 (Molecular Probes) in 4% BSA. Coverslips were mounted onto slides using ProLong Gold antifade medium containing DAPI (Invitrogen). Images were obtained using the 40x oil objective on a Leica inverted fluorescence microscope and processed using OpenLAB (ver.X.0) software. PML nuclear bodies were quantified by counting all visible PML foci in 50-100 cells. For detection of apoptotic bodies by microscopy, cells were treated with 10 µg/mL etoposide for 48 hours, then were harvested and placed onto coverslips. Coverslips were dried in
a fumehood then mounted on slides as described above. Apoptotic bodies were quantified by counting >100 cells for each sample using multiple planes of view.

4.2.4 Western blots
Cells were lysed in 9 M urea, 10 mM Tris-HCl (pH 6.8) and briefly sonicated. 50 µg of total protein was subjected to SDS-PAGE and transferred onto nitrocellulose. Membranes were blocked in 5% non-fat dry milk in PBS, then incubated with antibodies against PML (Bethyl, A301-167A, 1:2000), EBNA1 (OT1X at 1:2000 or K67-3 at 1:1000), actin (Oncogene Research Products, Ab-1; 1:10 000), p53 (Santa cruz, sc-126, 1:2000), acetyl-p53 K382 (Cell Signaling, 2525S, 1:2000), p21 (Santa Cruz, sc-817, 1:500) or caspase-3 (Cell signaling, 9665, 1:2000). After washing, membranes were probed with goat anti-mouse peroxidase (1:3000) or goat anti-rabbit peroxidase (1:5000) (Santa Cruz), and then developed using chemiluminescence reagents (ECL, Perkin Elmer). Membranes were stripped in 0.1 M glycine pH 2.9 for 30 min, washed in PBS-Tween, blocked and re-probed with the next antibody as described above. For Western blots in Figure 4, cells were treated with 50 µg/mL etoposide for 5 hrs (Figure 4A) or 24 hours (Figure 4C) prior to harvesting. For acetyl-p53 and caspase-3 blots, membranes were blocked in 5% non-fat milk for 1 hr, rinsed briefly with PBS-Tween and incubated with primary antibody in 4% BSA in PBS-Tween overnight at room temperature.

4.2.5 Cell Proliferation and Viability Assays
Proliferation of AGS-EBNA1 and AGS-EBV cells was monitored after transfection of 6 x 10^5 cells in 10 cm dishes with siRNA against GFP or EBNA1 as described previously (Sivachandran, Sarkari, and Frappier, 2008b) Cells were harvested and counted 3 and 4 days post siRNA treatment. Cell viability after DNA damage was measured by plating 2 x 10^5 cells in 12-well plates followed 24 hours later by treatment with 50 µg/mL of etoposide for 5 hrs. Cells were then rinsed in PBS and grown in fresh medium for 24, 48 and 72 hours prior to harvesting floating and adherent cells. Harvested cells were stained with 0.04% Trypan blue in PBS to identify dead cells and viable (unstained cells) were counted immediately. Each experiment is conducted in triplicate; all experiments have been performed three independent times.

4.2.6 Immunohistochemistry
GC tumour biopsies were obtained from the archives of the VU University medical center, Amsterdam and have been described in earlier studies (van Beek et al., 2004; van Beek et al.,
Sections of these tumours were analyzed for the presence of EBV EBER RNAs by EBER-RISH as described before. (van Beek et al., 2006) Sections of the same tumours were also processed for immunohistochemical detection of PML as described previously (Shi et al., 2003). Blocking was carried out using a DAKO LSAD kit (DAKO cat. no. K0679) as per manufacturer’s instructions, then samples were incubated in a diluent containing PML antibody (Bethyl, A301-167A) at 1:1000 in a humidified chamber at 4°C overnight. Slides were then rinsed and further processed according to the DAKO kit. After counter staining in hematoxylin, slides were dried and mounted using permamount and analysed using the 40x objective on a LEICA DMLB microscope.
4.3 Results

4.3.1 EBNA1 disrupts PML NBs in gastric carcinoma cells

To determine if EBNA1 affected PML NBs in GC, I initially compared the parental gastric adenocarcinoma cell line, AGS, to AGS cells containing an integrated EBNA1-expression cassette (AGS-EBNA1), or AGS cells carrying a recombinant EBV (AGS-EBV). Comparison of the PML NBs by immunofluorescence microscopy (IF) revealed that AGS-EBV cells had significantly fewer PML NBs (on average 3 NBs per cell) compared to the parental AGS cells (9 NBs per cell) (Figure 4-1A), suggesting that the presence of EBV in these cells led to the loss of PML NBs (Figure 4-1A). Comparison of AGS-EBNA1 to AGS cells revealed that EBNA1 alone was sufficient to cause this loss of PML NBs in GC cells, as AGS-EBNA1 cells had only 2 PML NBs per cell on average (Figure 4-1A). I further verified that the decreased level of PML NBs in AGS-EBNA1 and AGS-EBV cells were due to EBNA1 expression by down-regulating EBNA1 in these cells using siRNA. SiRNA treatment against EBNA1 reduced EBNA1 levels, which resulted in a 4-fold increase in PML NBs in both AGS-EBNA1 and AGS-EBV cells as compared to the negative control siRNA against GFP (Figure 4-1B). In the AGS-EBV cells, it was also noted that the PML NBs increased in size after EBNA1 silencing (Figure 4-1B, bottom panels), suggesting that some aspect of the EBV infection induced PML protein levels and that this effect was counteracted by EBNA1.

I next examined whether disruption of PML NBs by EBNA1 occurred immediately or was a consequence of long-term expression. To this end, I transiently transfected AGS cells with an EBNA1 expression plasmid and examined PML NBs 24 hours later (Figure 4-1C). EBNA1 expression lowered the number of PML NBs by 3-fold compared to mock transfected cells (Figure 4-1C), thereby demonstrating that EBNA1 expression has an immediate effect on PML NBs.
Figure 4-1. PML NBs in GC cells are reduced by EBV and EBNA1. Log phase cells were fixed and stained with anti-EBNA1 (red) and anti-PML (green) antibody. PML foci were counted for 50-100 cells for each sample in three separate experiments and the average number with standard deviation is shown in the histograms. Images with the same antibody treatment were captured using the same exposure times. (A) The AGS cells were compared to AGS-EBNA1 and AGS-EBV cells. (B) AGS-EBNA1 and AGS-EBV cells are shown after treatment with siRNA against GFP (siGFP) or EBNA1 (siEBNA1). (C) AGS cells were transfected with a plasmid expressing EBNA1 (oriPE) or empty plasmid (oriP) then stained 24 hours later with antibody against EBNA1 (red) and PML (green). Both EBNA1-expressing and non-expressing cells are shown for the oriPE transfection.
**4.3.2 EBNA1 lowers PML protein levels**

Disruption of PML NBs can occur either through interference with PML protein-protein interactions or due to degradation of the PML proteins. The two mechanisms can be distinguished since only the latter will reduce levels of PML proteins. I therefore determined whether EBNA1 induced loss of PML proteins by comparing the levels of PML isoforms in AGS, AGS-EBNA1 and AGS-EBV by Western blotting. PML is known to exist as several isoforms (comprised of alternative spliced and modified forms), resulting in multiple bands migrating between 60 and 200 KDa on immunoblots (Everett and Chelbi-Alix, 2007). Cells containing EBV or expressing EBNA1 alone had decreased expression of all PML isoforms (Figure 4-2A) compared to the parental AGS cells. In addition, EBNA1 silencing in AGS-EBNA1 and AGS-EBV increased PML proteins compared to control siGFP treatment (Figure 4-2B). In particular, I observed a robust increase in all PML isoforms in AGS-EBV after EBNA1 silencing, which is consistent with the larger PML NBs previously observed with this treatment by IF (Figure 4-1B). Finally, I also observed that transient EBNA1 expression was sufficient to degrade PML proteins in AGS cells (Figure 4-2C). Therefore the data are consistent with EBNA1 disrupting PML bodies by inducing the degradation of PML proteins.
Figure 4-2. EBNA1 expression decreases PML protein levels. (A) Equal amounts of cell lysates from AGS, AGS-EBNA1 and AGS-EBV were compared by Western blotted using an antibody that recognizes all PML isoforms. Blots for EBNA1 and actin are also shown. (B) AGS-EBNA1 and AGS-EBV cells were transfected with siRNA against EBNA1 (+) or GFP(-) and then equal amounts of cell lysate were analysed by Western blotting as in A. (C) AGS cells were transfected with a plasmid expressing EBNA1 (OriPE), or with the empty plasmid (OriP). 24hrs later whole cell lysates were compared by Western blotting for PML, EBNA1, and actin.
4.3.3 EBNA1 interferes with p53 activation and apoptosis

PML NBs are known to be important for p53 activation by acetylation and for apoptosis (Guo et al., 2000; Pearson et al., 2000). Since both of these functions would be expected to impact GC, I examined whether EBNA1 expression inhibited p53 acetylation and apoptosis as a consequence of PML disruption. The AGS cells express wild-type p53 protein (Muller et al., 1998), therefore should be activated in response to DNA damage. First, I compared levels of total and acetylated p53 (at K382) in response to etoposide-induced DNA damage in AGS, AGS-EBNA1 and AGS-EBV cells. Although etoposide induced p53 stabilization in all three cell lines, Western blots using an antibody specific for acetylated p53 showed that the presence of EBV or EBNA1 decreased p53 acetylation (Figure 4-3A, second panel, compare lane 2 to 4 and 6). I also observed that basal p53 levels were lower in cells expressing EBNA1 or containing EBV (Figure 4-3A, top panel, compare lane 1 to lanes 3 and 5), consistent with our previous findings that EBNA1 can decrease p53 levels (Saridakis et al., 2005). To verify that the reduced levels of acetylated p53 detected in response to etoposide resulted in compromised p53 function, I examined p21 protein expression, which is a direct down-stream transcriptional target of p53. Consistent with the effects on p53 acetylation, I observed a significant decrease in p21 induction by etoposide in AGS-EBNA1 and AGS-EBV cells compared to parental AGS cells (Figure 4-3A, third panel, compare lanes 2 to lanes 4 and 6). In addition to the Western blots, such changes were also observed using IF (Figure 4-3B). I found that upon induction of DNA damage, cells expressing EBNA1 or carrying EBV showed an overall decrease in staining for p53 acetylated at K382 (Figure 4-3B, right panels), and p21 (Figure 4-3B, middle panels).

I also compared the induction of apoptosis in AGS, AGS-EBV and AGS-EBNA1 cells, first by detecting apoptotic bodies after etoposide treatment by their intense DAPI staining in fluorescence microscopy (see Figure 4-3C). I observed that the number of apoptotic cells was decreased 5-10 fold in AGS-EBNA1 and AGS-EBV cells as compared to parental AGS cells. These results were supported by a second assay for apoptosis, caspase-3 cleavage, wherein the parental AGS cells had higher levels of cleaved caspase-3 after etoposide treatment compared to the other two cell lines (Figure 4-3D, compare lanes 2 to 4 and 6). Hence, all these data demonstrate that EBNA1 impairs PML function in terms of reduced p53 acetylation and apoptosis.
Figure 4-3. EBNA1 decreases p53 activation and apoptosis  (A and D) AGS, AGS-EBNA1 and AGS-EBV cells were treated with etoposide (+) or DMSO (−) for 5 (A) or 48 (D) hours then equal amounts of cell lysates were analysed by Western blotting using antibodies against p53 acetylated on K382, total p53, p21, actin and caspase-3, as indicated. In D, the positions of molecular weight markers are shown for caspase bands. (B) Cells treated with etoposide as in A were stained with antibodies against p21 (green) and acetylated p53 (red). All images were captured at the same exposure time. (C) Cells treated with either DMSO or etoposide as in D were stained with DAPI to show apoptotic bodies, indicated by the white arrowheads. The apoptotic bodies were quantified by counting over 300 cells in 3 separate experiments and average numbers with standard deviations are shown in the histogram.
4.3.4 EBNA1 provides a survival advantage to GC cells

I next examined whether EBNA1 had affected cell proliferation and survival that might therefore contribute to development of GC. During the maintenance of these cell lines, I did not detect a difference in the basal rate of proliferation in AGS-EBNA1 or AGS-EBV compared to the parental AGS cells. However, after EBNA1 silencing, proliferation of both AGS-EBNA1 and AGS-EBV cells were significantly decreased compared to siGFP-treated cells (Figure 4-4A and 4-4B), suggesting that over time, cells expressing EBNA1 can become dependent on EBNA1 for growth. In addition, I examined whether the presence of EBNA1 affected survival after DNA damage. Both AGS-EBNA1 and AGS-EBV were consistently found to more resistant to etoposide compared to parental cells (Figure 4-4C). The data indicates that EBNA1 confers a survival advantage to AGS cells, implying an important role for EBNA1 GC development.
Figure 4-4. Effects of EBNA1 on cell proliferation and viability (A and B) AGS-EBNA1 (A) and AGS-EBV (B) were treated with siRNA targeted against GFP or EBNA1. Cells were harvested immediately (day 0) or at the indicated days after silencing and counted. (C) AGS, AGS-EBNA1 and AGS-EBV were treated with etoposide and cells were harvested and counted 0, 1, 2 and 3 days post treatment. For all experiments, average numbers with standard deviation from 3 separate experiments are plotted.
4.3.5 Decreased PML staining in EBV-positive GC biopsies

While I have shown that EBNA1 expression can decrease PML NBs in GC cells lines, it is important to determine whether this effect translates to primary GC tissues. To this end, I examined biopsies from several GC samples that were classified as either EBV-positive or EBV-negative based on fluorescent in-situ hybridization for the EBV-expressed EBER RNAs, resulting in red staining (Figure 4-5, EBERish panels). Immunohistochemistry was then utilized to stain all tumour samples for PML. I observed that EBV-negative biopsies had intense, dark brown nuclear staining for PML for both intestinal (I), and diffuse (II, III) types of GC (Figure 4-5). However, a noticeable reduction in PML staining was observed in all five of the EBV-positive biopsies (see three examples in Figure 4-5). The decreased PML staining was confined to the carcinoma cells, while the infiltrating lymphocytes (L) in the same tumours exhibited strong (brown) PML staining. The results demonstrate a remarkable difference in PML expression in EBV-positive versus EBV-negative GCs, consistent with the EBNA1-induced loss of PML as observed in cell line studies.
**Figure 4-5. PML staining is diminished in EBV-positive GC biopsies.** Sections of EBV-positive and EBV-negative human gastric carcinoma biopsies were stained for PML using standard immunohistochemistry (brown stain). Sections of the same biopsies were also subjected to *in situ* hybridization staining for EBV EBER RNA (EBERish; red stain). All images were captured at the same exposure time.
4.4 Discussion

In recent years, EBV has become increasingly recognized as a causative agent in a significant proportion of GC cases. Herein I provide a mechanism by which EBV could contribute to the development of this cancer mediated by its EBNA1 protein. I have shown that EBNA1 alters nuclear bodies formed by the PML tumour suppressor protein and induces the degradation of all PML isoforms in GC cells that contain EBV or just expressing EBNA1. Previously I reported that EBNA1 alters PML NBs in NPC, implicating EBNA1 as a contributing factor in the development of NPC. However, since virtually all undifferentiated NPCs are EBV-positive, these tumours could not be used to address the important question of whether EBNA1-induced loss of PML observed in NPC cell lines also occurred in primary NPCs. Using GC samples, I was able to compare EBV-positive and EBV-negative biopsies to demonstrate that the presence of EBV leads to significantly decreased PML protein levels in these tumours.

GC can be classified into conventional gastric adenocarcinomas (intestinal type) or lymphoepithelioma-like carcinomas (diffuse type) (Fukayama, 2010; Fukayama, Hino, and Uozaki, 2008). I observed that the presence of EBV decreased PML staining in both types of GC. In the EBV-positive diffuse type, the EBER staining is not uniformly detected in the tumour cells but instead is observed in a percentage of the cells scattered throughout the tumour. In contrast, I observed decreased PML staining more extensively throughout the tumour (but not in the infiltrating lymphocytes). A recent study by Ryan et al. (Ryan et al., 2009) indicated that EBERs were only detected in GC cells containing very high EBV load. They detected EBV in 64% of GC samples from the United States using a quantitative PCR assay, whereas only 17% of GC scored as EBV-positive based on EBER staining, and those that stained positive for EBERs contained very high levels of the EBV genome. Therefore cells in my EBV-positive tumours that were not stained by EBERish might simply have had lower levels of EBV. Since I had previously shown that even low levels of EBNA1 (such as is present in the EBV-positive NPC cell line C666-1) is sufficient to disrupt PML NBs (Sivachandran, Sarkari, and Frappier, 2008b), the EBER-negative cells that contain EBV likely still express sufficient amount of EBNA1 to disrupt PML NBs.

PML has long been recognized as a tumour suppressor protein, stemming from the initial observations that a rearrangement of the PML gene occurs in promyelocytic leukemia resulting in a chimeric protein that disrupts the formation of normal PML NBs (Miller et al., 2002). A
correlation between loss of PML NBs and tumour development and/or progression was subsequently reported for prostate, colorectal, breast and lung carcinomas, lymphomas, central nervous system and germ cell tumours (Gurrieri et al., 2004). In addition, fibroblasts from PML knockout mice grew faster as monitored by $^3$H-labeled thymidine incorporation, leading to enhanced colony formation (Wang et al., 1998a). Furthermore, these mice had an increased frequency of tumour formation following injection with a tumor initiating compound, dimethybenzanthacene (Wang et al., 1998a). Hence, the EBNA1-mediated disruption of PML NBs that I have observed in gastric carcinoma cells would be expected to contribute to the onset and/or progression of this malignancy.

The reason that PML NBs suppress oncogenesis likely stems from their known importance in several processes that govern genomic integrity and cell survival, including apoptosis and DNA repair. Moreover, PML NBs have been found to be required for p53 activation, which becomes acetylated only after association with PML NBs. I have shown that EBNA1 impairs p53 acetylation, p53-dependent transcription (of p21), apoptosis and leads to cell survival following DNA damage. These effects indicate that the degree by which EBNA1 decreases PML proteins and NBs is sufficient to significantly impede PML function. These findings are consistent with the observations of Cheng et al. (Cheng et al., 2010) reporting that EBNA1 expression reduced cisplatin-induced cell death in gastric cancer cells, and enhanced tumorigenicity in LL/2 cells.

Recent work by Wei et al. (Wei et al., 2010) on H. pylori provides an interesting parallel between the two major microorganisms associated with gastric carcinoma: H. pylori and EBV. They demonstrated that the H. pylori CagA protein induces the degradation of p53 in gastric cells and increases their survival even with damaged DNA. Therefore these two very different pathogens that contribute to the development of GC both affect p53 functions. EBNA1 affects p53 in two ways; by inhibiting its activation through the disruption of PML NBs as discussed above, and by lowering the steady state levels of p53 (as seen in Figure 4-3A). The lowering of the basal p53 levels is likely due to the ability of EBNA1 to block the interaction between p53 and the de-ubiquinating enzyme USP7, which normally stabilizes p53 (Saridakis et al., 2005).

I also observed that silencing of EBNA1 in AGS cells that had been stably expressing EBNA1 (AGS-EBNA1) or contained EBV (AGS-EBV) decreased cellular proliferation. This is despite the fact that I did not observe any differences in growth rates between the parental AGS cells and AGS cells expressing EBNA1 (data not shown). These observations suggest that,
although EBNA1 might not be required for proliferation initially, over time, cells expressing EBNA1 might become dependent on its presence. This could therefore serve as an example of “oncogene addiction”, as described by others, in which the continued expression of a protein changes the cellular environment such that the cell becomes dependent on that protein for proliferation (Luo, Solimini, and Elledge, 2009). On that note, Oh et al. (Oh et al., 2007) reported that a spontaneous EBV-positive GC cell line was dependent on EBV for its survival. It is currently unclear as to whether the cellular changes induced by EBNA1 that lead to EBNA1 dependency are related to PML disruption or other effects of EBNA1.

In summary, I have shown for the first time, that the EBNA1 protein increases the survival of gastric carcinoma cells, at least in part due to its ability to induce the loss of PML NBs resulting in impairment of p53 function and apoptosis. The decreased apoptotic response in combination with the impairment of DNA repair associated with loss of PML NBs would be expected to promote the survival of cells with DNA damage thereby contributing to the development of human gastric cancer.
I performed all other experiments in this chapter.
5.1 Introduction

EBV is a successful human pathogen that latently infects greater than 90% of the world's population. Reactivation into the lytic replication is necessary for the viral progeny to pass from host to host. This occurs in infected individuals at low levels, allowing shedding of the virus into the saliva, permitting transmission (Rickinson and Kieff, 1996). However, it is yet unclear how reactivation occurs in vivo. The induction or exogenous expression of BZLF1 (also known as ZEBRA, ZTA, Z and EB1) is sufficient to commence the cascade of lytic protein expression and DNA replication. BZLF1 is a key player in the switch from EBV latency to lytic replication (Countryman et al., 1987; Feederle et al., 2000; Sinclair et al., 1991).

BZLF1 is an essential protein for amplification of infectious EBV particles. It is a multifunctional DNA-binding protein belonging to the bZIP family of transcription factors (Chang et al., 1990), that transactivates its own promoter (Zp) and the other immediate early promoter Rp, inducing the production of BRLF1 transcription factor (Adamson and Kenney, 1998; Kolman et al., 1996). BZLF1 and BRLF1 act synergistically to induce the expression of early and late EBV lytic genes. This induction includes all viral proteins required for lytic viral DNA replication (Darr, Mauser, and Kenney, 2001; Feederle et al., 2000; Kieff, 1996; Rooney et al., 1989). BZLF1 can trans activate these viral genes through direct binding to the origin of lytic replication, oriLyt (Hammerschmidt and Sugden, 1988; Lieberman and Berk, 1990; Rickinson and Kieff, 2001; Schepers, Pich, and Hammerschmidt, 1993; Schepers et al., 1993b). In addition, direct binding of BZLF1 to oriLyt recruits the virus-encoded DNA polymerase/primase and polymerase processivity factors that are essential for lytic replication (Fixman, Hayward, and Hayward, 1992; Gao et al., 1998; Lieberman et al., 1990; Schepers, Pich, and Hammerschmidt, 1993). There are also several cellular proteins that affect the activities or localization of BZLF1 and therefore contribute to EBV reactivation. Many cellular regulatory proteins, such as ubiquitous transcriptional factors Sp1 and Sp3 (Liu et al., 1997a), phosphorylated c-Jun (Feng et al., 2007), ZEB-1 (Yu, Wang, and Mertz, 2007) and CCAAT/enhancer binding protein (C/EBP) (Huang et al., 2006; Wu et al., 2004), have been shown to associate with the BZLF1 promoter region and contribute to the regulation of the promoter. Although, much is known about the function of BZLF1 in viral gene expression and EBV DNA replication, it is not clear what viral conditions trigger the expression of BZLF1 or what controls the on/off state of the cellular proteins that are known to control BZLF1 expression.
During latency, EBNA1 is the only EBV-encoded protein required for the replication, retention and partition of plasmid DNA in proliferating cells (Yates et al., 1984; Yates, Warren, and Sugden, 1985b). EBNA1 is detected in all type of EBV-infected cells and can directly bind the latent origin of replication, oriP, as a homodimer (Frappier and O'Donnell, 1991b). EBNA1 binds to two distinct regions in oriP, referred to as the family of repeats (FR) and the dyad symmetry (DS) element (Frappier, 2008). The DS element consists of four EBNA1 recognition sites with lower affinity than those in FR and is required for initiation of oriP-dependent DNA replication (Gahn and Schildkraut, 1989; Rawlins et al., 1985b; Yates, Warren, and Sugden, 1985a). FR is a cluster of twenty EBNA1-binding sites and essential for plasmid maintenance (Lupton and Levine, 1985a; Reisman and Sugden, 1986b). In addition, FR acts as an transcriptional enhancer to activate the expression of other EBV latency genes (Reisman and Sugden, 1986b; Wysokenski and Yates, 1989b). EBNA1 bound to FR can activate transcription of the EBV BamHI-C and latent membrane protein-1 promoters (Gahn and Sugden, 1995; Sugden and Warren, 1989). Thus, the ability of EBNA1 to bind to DNA is essential for its activation of replication and transcription through oriP.

After induction of the lytic program, transcription of EBNA1 mRNA switches from the Qp latency promoter to the Fp lytic promoter, enabling continued expression of EBNA1 in lytic infection (Schaefer, Strominger, and Speck, 1995; Zetterberg et al., 1999). This suggests that EBNA1 has a function in lytic infection, although this function remains unknown. In fact, EBNA1 and BMRF1 (a DNA polymerase processivity factor) have been shown to colocalize in replication compartments during the lytic cycle (Daikoku et al., 2004). Shannon-Lowe et al. (Shannon-Lowe et al., 2009) have recently shown that infection of cells using an EBNA1-KO virus resulted in decreased BZLF1 protein expression, with a marked decrease in progeny virion production. This suggests that EBNA1 expression during the lytic cycle is important for efficient viral amplification and therefore virion production. However, the exact functions and interactions of EBNA1 in the lytic cycle have yet to be explored.

Interestingly, I have shown through my studies that EBNA1 disrupts PML NBs and degrades the PML protein in cells latently infected with EBV (Sivachandran, Sarkari, and Frappier, 2008a). PML is part of the interferon-mediated anti-viral response and there are several lines of evidence supporting its intrinsic antiviral activities (Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011). For instance, PML NBs have been shown to move towards incoming HSV-1 genomes, to repress lytic virus replication (Everett and Murray, 2005).
In order to alleviate the suppressive effects of PML, many viruses have evolved mechanisms to inactivate PML NBs. (Geoffroy and Chelbi-Alix, 2011). The first virus reported to perform the latter function was HSV-1 (Maul, Guldner, and Spivack, 1993), where the ICP0 protein has been well characterized for its ability to disrupt PML NBs (Maul and Everett, 1994). In keeping with this role of ICP0, HSV-1 viruses lacking ICP0 are defective in lytic infection in the presence but not in the absence of PML (Everett et al., 2008a; Everett et al., 2006).

Like other DNA viruses, EBV lytic replication has been shown to be associated with PML NBs (Bell, Lieberman, and Maul, 2000). For EBV, high levels of BZLF1 has been reported to be important to overcome the repressive nature of PML to allow lytic replication to take place. In this case, BZLF1 disrupts the PML NBs, by inhibiting interactions between PML proteins, but does not induce the degradation of PML proteins (Adamson and Kenney, 2001). However, in Chapter 2 I have shown that even low levels of EBNA1 are sufficient to disrupt PML NBs and degrade PML protein. Therefore, it is possible that EBNA1 is required in conjunction with BZLF1 to efficiently disrupt PML NBs, thereby promoting EBV reactivation and allowing efficient lytic infection. In this chapter, I investigated whether EBNA1 contributed to EBV reactivation. Here I present data supporting roles for EBNA1 in EBV reactivation and lytic infection. In particular, I show that EBNA1 is needed for efficient reactivation but only in cells containing PML. In addition, I have identified a role for PML in repressing EBV reactivation.
5.2 Materials and Methods

5.2.1 Cell culture and siRNAs

AGS-EBV was generated by co-culturing AGS cells with Akata Burkitt’s lymphoma cells carrying recombinant neomycin-resistant EBV as described previously (Stewart et al., 2004). These cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum and 1% L-glutamine. They were also maintained in G418 (Invitrogen; 400 ug/mL) to select for cells containing recombinant EBV. Small interfering RNA (siRNA) for EBNA1 (GGAGGUUCCAACCCGAAAUTT) was synthesized by Invitrogen. For control siRNA against green fluorescent protein (GFP) (GAACUUCAG GGUCAGCUUUGCCG) was used from Invitrogen.

5.2.2 Transfections and induction of EBV reactivation

Approximately 5 x 10^5 AGS-EBV cells were plated in 5 mLs of RPMI medium in 10 cm dishes. They were immediately transfected with 100 pmol of siRNA targeted against either EBNA1 or GFP (negative control) using 2 µL of lipofectamine 2000 (Invitrogen) (day1). These cells were subject to the same transfection protocol on day 2 and day 3. They were treated with 3 μM sodium butyrate (NaB, dissolved in dH₂O) and 20 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA, dissolved in DMSO) to induce EBV reactivation or with an equivalent amount of DMSO as a negative control. Floating and adherent cells were harvested 24 hours post drug treatment and either analyzed by Western blotting (described below) or processed for immunofluorescence microscopy.

5.2.3 Immunofluorescence microscopy and antibodies

Cells grown on coverslip were fixed using 3% formaldehyde for 15-20 mins at room temperature, then rinsed briefly using phosphate-buffered saline (PBS). Cells were permeabilized using 1% Triton in PBS for 5 mins followed by two 5 mins rinses in PBS. Coverslips were blocked for 20 mins in 4% BSA in PBS. Samples were incubated with primary antibodies against EBNA1 (R4 rabbit serum at 1:300 (Holowaty et al., 2003b)), PML (Santa Cruz PG-M3 at 1:50), or BZLF1 (1:100, a generous gift from Jaap Middeldorp) for 30 mins in the humidifying chamber and rinsed twice for 5 mins in PBS. Samples were incubated with the secondary antibodies goat anti-rabbit Alexafluor 555 (1:800, Molecular Probes) and goat anti-mouse Alexafluor 488 (1:800, Molecular Probes) in 4% BSA for 30 mins and washed twice for 5
Cover slips were mounted onto slides using ProLong Gold antifade medium containing DAPI (Invitrogen). Images were obtained using the 40x oil objective on a Leica inverted fluorescence microscope and processed using OpenLAB (ver.X.0) software. BZLF1 positive cells were quantified by counting greater than 100 cells per sample and experiments were carried out in triplicate.

5.2.4 Western blotting and antibodies

Cells were lysed in either 9 M urea, 10 mM Tris pH 6.8 followed by sonication or in RIPA (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% Triton X-100, and protease inhibitors) for 20 mins on ice. 30-50 µg of clarified lysates were loaded onto 10% SDS-PAGE and transferred onto nitrocellulose. Membranes were blocked in 5% non-fat dry milk in PBT-T (0.1% Tween) for 1 hour, followed by incubation of the listed primary antibodies in blocking buffer over night at room temperature. These include EBNA1 K67-3 (1:1000, a generous gift from Jaap Middeldorp), BMRF1 (1:10K, Chemicon, MAB8186), BZLF1 (1:500, a generous gift from Jaap Middeldorp), PML (1:2K, Bethyl, A301-167A) and actin (1:10K, Oncogene Research Products, Ab-1). Membranes were washed three times with PBS-T buffer and then incubated with goat-anti-mouse (1:3K) or goat-anti-rabbit (1:5K) conjugated to horseradish peroxidase secondary antibodies (Santa Cruz Biotechnology) for 1 hour. Membranes were washed three times with PBS-T buffer and signals were detected by enhanced chemiluminescence (ECL) assay (Perkin Elmer Life and Analytical Sciences).

5.2.5 EBV copy number

AGS-EBV samples treated with siGFP or siEBNA1 followed by treatment with DMSO or NaB/TPA for EBV reactivation (as described above) were assayed for lytic DNA replication. Approximately 1×10⁶ AGS-EBV cells were processed to perform EBV copy number assays as described previously (Zhou, Snyder, and Lieberman, 2009). Briefly, cells were resuspended and lysed in RIPA buffer on ice for 20 mins. Clarified lysates were diluted to a final volume of 400 µL and incubated with 8 µL of proteinase K (20 mg/mL) for 2 hours at 50°C. The DNA was extracted with phenol/chloroform (1:1), followed by cholorform. After centrifugation, 400 µL of the supernatant was removed and subjected to ethanol precipitation. Precipitated DNA was quantified by real-time PCR using primers for the DS region of EBV (Deng et al., 2005) and normalized to the cellular DNA signal at the GAPDH locus (Yuan et al., 2006).
5.2.6 Isolation of total RNA and Reverse-Transcriptional PCR

AGS-EBV cells were snap-frozen for total RNA isolation. Total RNA was isolated using Qiagen RNeasy mini kit (cat. no. 74 104) according to manufacturer's instructions. An alternative method was also used to isolate total RNA, using the TRIzol reagent (Invitrogen). Samples were resuspended in 1ml of TRIZol and homogenized by vortexing. To this, 200 µl of chloroform was then added, mixed vigorously and incubated at room temperature for 15 min. Samples were subjected to centrifugation for 15 min at 15,000 rpm at 4°C. The upper aqueous layer (~ 500 µl) was transferred to a new tube containing 500 µl of isopropanol, and the RNA was precipitated at -20°C for 1 hour. The RNA was pelleted by centrifugation for 30 min at 15,000 rpm at 4°C, washed once with 70% ethanol, and resuspended in RNase-free water. The quantity and quality of the extracted RNA were determined by NanoDrop Spectrophotometer (Thermo Scientific). One µg total RNA was reverse transcribed in a 20 µl reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche, cat. no. 04379012001) with random hexamer primers according to the manufacturer’s instructions. Quantitative real-time PCR was performed using 1µL of the cDNA to analyze the levels of BZLF1 and GAPDH (endogenous control) along with the LightCycle SYBR green I Master mix (Roche) on a Rotorgene qPCR system (Corbett Research). Primers used for quantification of BZLF1mRNA was described previously (Wen et al., 2007).

5.2.7 Generation of AGS-EBVshPML cell line and light microscopy

pLKO.shPML1 expressing anti-PML shRNA (Everett et al., 2006) was kindly provided by Dr. Roger Everett and are described in detail elsewhere (Cuchet et al., 2011). These plasmids were used to generate lentiviruses as previously described (Everett et al., 2008b). One ml of filtered culture medium containing the shRNA-lentivirus was added to 1x10⁵ AGS-EBV cells with polybrene (Sigma) at a final concentration of 8 µg/µl and, after 24h, was replaced with medium containing 2 µg/ml puromycin. 72 hours later, puromycin was removed and cells were processed for immunofluorescence microscopy and Western blotting as described above to verify the knockdown of PML. Light microscopy images of AGS-EBV and AGS-EBVshPML were obtained using the 40x objective on a Leica inverted fluorescence microscope and processed using OpenLAB (ver.X.0) software.
**5.2.8 Sensitivity of AGS-EBVshPML cells to drugs that induce EBV reactivation**

AGS-EBV and AGS-EBVshPML were compared for their ability to respond to DMSO, NaB, TPA or NaB/TPA. Approximately $5 \times 10^6$ cells from both cell lines in 10cm dishes were treated for 24 hours with the above-mentioned drugs. Floating and adherent cells were harvested and processed for Western blotting and EBV DNA copy number as described above.

**5.3 Results**

**5.3.1 EBNA1 expression inhibits spontaneous EBV reactivation**

To investigate the possible role of EBNA1 in the EBV lytic cycle, I used a gastric carcinoma cell line containing recombinant EBV, AGS-EBV, as these cells are permissive for EBV reactivation. Immunofluorescence microscopy (IF) for the BZLF1 protein showed that 2-3% of the cells naturally expressed this lytic master regulator protein after treatment with negative control siRNA against GFP (Figure 5-1A, top panel). When EBNA1 was downregulated with siRNA treatment, the percentage of cells expressing BZLF1, and therefore entering lytic cycle, increased to 8-9% (Figure 5-1A, bottom panel). These effects were quantified from multiple experiments and shown as a histogram on the right of the IF images, where downregulation of EBNA1 consistently increases the number of AGS-EBV cells entering the lytic cycle as compared to the control siRNA treatment. The downregulation of EBNA1 was confirmed by Western blotting as shown in Figure 5-1B (compare lanes 1 and 2, top panel). Consistent with the IF results, Western blotting also showed that EBNA1 downregulation increased BZLF1 (Figure 5-1B, compare lane 1 to 2 in second panel bottom bands). In addition, an increase in the expression of the BMRF1 early gene (DNA polymerase processivity factor) was also observed upon EBNA1 siRNA treatment (Figure 5-1B, compare lanes 1 and 2 in second panel top bands). These observations suggest that EBNA1 represses reactivation in order to maintain latency.

Since whether or not the EBV lytic proteins are expressed is controlled at the level of transcriptional activation, starting with the transcription of BZLF1, I then examined how EBNA1 silencing affected the level of BZLF1 transcripts. To this end, total mRNA was isolated and BZLF1 transcripts were quantified using quantitative RT-PCR. EBNA1 downregulation was found to cause a 2-fold increase in BZLF1 transcript levels as compared to the siGFP control (Figure 5-1C). This implies that EBNA1 decreases EBV reactivation by repressing the expression of BZLF1, as opposed to affecting the stability of the BZLF1 protein once it is made.
I also examined whether the effect of EBNA1 on immediate early and early gene expression also affected lytic DNA replication, which would be expected if the complete lytic cycle was activated but not if an abortive lytic cycle was induced. Since EBV lytic replication results in amplification of the viral genomes, lytic replication was assessed by quantification of the level of the EBV genomes. To this end, DNA was isolated from cells treated with siGFP or siEBNA1 and EBV genomes were quantified by quantitative RT-PCR using primers specific for the DS element present in the latent origin of replication, oriP, and these results were normalized to the levels of GAPDH. This revealed a consistent two-fold increase in lytic EBV DNA replication in the absence of EBNA1 compared to siGFP treatment, suggesting that a complete productive cycle is launched in the absence of EBNA1. However, it is important to keep in mind the level of EBV reactivation that takes place in the absence of EBNA1 is significantly lower than when these cells are treated with drugs known to induce the lytic cycle such as NaB and TPA. Nonetheless, downregulation of EBNA1 increases the basal level of EBV reactivation in AGS-EBV cells, hence EBNA1 appears to be a factor in promoting EBV latency.
Figure 5-1: EBNA1 expression inhibits spontaneous EBV reactivation. AGS-EBV cells were treated with siRNA against GFP or EBNA1. (A) Cells post siRNA treatment were fixed and stained with anti-EBNA1 (red) and anti-BZLF1 (green) antibody. BZLF1 positive green cells were counted (greater than 100 cells per sample in each replicate). The average numbers from three separate experiments with standard deviations are shown in the histogram on the right. Images with the same antibody treatment were captured using the same exposure times. (B) Equal amounts of cell lysates from AGS-EBV treated with siGFP and siEBNA1 were compared by Western blotting using antibodies for EBNA1, BMRF1, BZLF1 and actin. (C) Steady state transcript levels of BZLF1 were monitored by isolating total RNA, synthesizing cDNA and quantifying transcript levels using primers specific to BZLF1 and normalized to GAPDH housekeeping gene. Average levels of the BZLF1 transcript levels are shown with standard deviation after siEBNA1 as compared to siGFP treatment (set to one). (D) EBV genomic DNA quantified by isolating total DNA, amplifying the EBV DS sequence and normalizing to GAPDH. Average levels are shown with standard deviation after siEBNA1 as compared to siGFP treatment (set to one).
5.3.2 EBNA1 positively contributes to drug induced EBV reactivation

The percentage of cells spontaneously entering the lytic cycle in the above studies is very low and EBV reactivation is typically studied by treating cells with drugs that increase histone acetylation (eg. sodium butyrate or TSA) or activate protein kinase C (TPA) thereby overcoming chromatin suppression and enabling the efficient expression of BZLF1 and other lytic genes. Therefore I also examined the role of EBNA1 in EBV reactivation by drug treatment. To this end, AGS-EBV cells were transfected with siRNA against EBNA1 or GFP, then the lytic cycle was induced by the addition of sodium butyrate (NaB) and TPA. I first examined the number of cells that entered the lytic cycle after drug treatment by staining the cells for BZLF1 (Figure 5-2A, top panel). I found that on average 45% of the cells treated with siGFP entered the lytic cycle (quantified in three separate experiments and shown in the histogram). However, silencing EBNA1 markedly reduced the number of cells entering the lytic cycle after drug treatment as shown by the decreased number of cells with BZLF1 staining (Figure 5-2A, bottom panel). On average 22% of the cells entered the lytic cycle in the absence of EBNA1 in contrast to the 45% that entered it in the presence of EBNA1 (see histogram). This suggests that EBNA1 positively contributes to EBV reactivation after NaB/TPA treatment.

This effect was also investigated by Western blotting for EBV lytic proteins. Consistent with the previous observation, in the absence of drug treatment EBNA1 silencing increased the expression of both BZLF1 and BMRF1 proteins (Figure 5-2B, compare lanes 1 and 2, third panel bottom and top bands respectively). As expected, NaB/TPA treatment resulted in a robust induction of the lytic cycle proteins BZLF1 and BMRF1 (Figure 5-2B, compare lanes 1 and 3, third panel bottom and top bands respectively). However, when EBNA1-silenced cells received the same NaB/TPA treatment, the expression of both the induction of BZLF1 and BMRF1 proteins were reduced as compared to the cells expressing EBNA1 (Figure 5-2B, compare lanes 3 and 4 third panel bottom and top bands respectively). This confirms the IF data that EBNA1 positively contributes to drug induced EBV reactivation. The effect of EBNA1 silencing on drug-induced EBV lytic DNA replication was also examined by comparing the levels of the EBV genomes (quantified by RT-PCR) with siGFP and siEBNA1 treatments. Consistent with the effect on the lytic protein levels, downregulation of EBNA1 resulted in decreased lytic EBV DNA replication compared to the siGFP treatment (Figure 5-3C), further suggesting that EBNA1 contributes to the productive cycle under these conditions.
Since PML proteins are known to be suppressive for the replication of some herpesviruses and EBNA1 can induce degradation of PML proteins in latent infection, I also examined the effect of EBNA1 silencing on PML proteins after lytic induction. I found that silencing EBNA1 resulted in increased levels of the PML proteins both before (Figure 5-2B, compare lanes 1 to 2 second panel) and after (Figure 5-2B, compare lanes 3 and 4) NaB/TPA treatment. This suggests that EBNA1 lowers PML levels in both latent and lytic modes of infection, raising the possibility that this may be how EBNA1 promotes lytic infection after NaB/TPA treatment. I also noticed that a single PML isoform or modified form between 55 and 70 kDa was consistently upregulated in EBNA1-silenced cells that were induced for EBV reactivation. The nature of this PML protein is unknown but its size is consistent with PML III, V or VI (Condemine et al., 2006).
Figure 5-2: EBNA1 positively contributes to drug induced EBV reactivation. AGS-EBV cells were treated with siRNA against GFP or EBNA1 followed by induction of EBV lytic using NaB/TPA. (A) After these treatments, cells were fixed and stained with anti-EBNA1 (red) and anti-BZLF1 (green) antibody. BZLF1 positive green cells were counted (greater than 100 cells per sample in each replicate). The average numbers from three separate experiments with standard deviations are shown in the histogram on the right. Images with the same antibody treatment were captured using the same exposure times. (B) Equal amounts of cell lysates from AGS-EBV treated with siGFP and siEBNA1 with and without NaB/TPA induction were compared by Western blotting using antibodies for EBNA1, PML, BMRF1, BZLF1 and actin. (C) EBV genomic DNA quantified post NaB/TPA treatment by isolating total DNA, amplifying the EBV DS sequence and normalizing to GAPDH. Average levels are shown with standard deviation after siEBNA1 as compared to siGFP treatment (set to one).
5.3.3 PML represses EBV reactivation

Since PML levels were higher in cells treated with siEBNA1 compared to siGFP after induction of the lytic cycle, I tested the possibility that PML repressed reactivation of EBV. To this end, AGS-EBV cells were infected with a lentivirus carrying shRNA against PML, generating a stable cell line without any PML, AGS-EBVshPML. The absence of PML using was verified by both IF (Figure 5-3A, compare top panel AGS-EBV to bottom panel AGS-EBVshPML) and Western blotting (Figure 5-3B, compare lanes 1 to 2) using antibodies that recognize all PML isoforms. The IF and Western blots showed that no PML NBs or PML proteins, respectively, were detected in these cells. Interestingly, I observed that the PML-silenced cells had a higher percentage of cells that were enlarged and rounded, as is typical of cells undergoing lytic viral replication, as compared to the parental AGS-EBV cells (Figure 5-3C). To verify that these cells were indeed spontaneously reactivating EBV, BZLF1 and BMRF1 levels were examined by Western blotting. Both of these lytic protein markers were upregulated in the absence of PML compared to cells with intact PML protein and NBs (Figure 5-3D, compare lanes 1 and 3). However the level of induction was not as great as that seen in AGS-EBV cells after NaB/TPA treatment (Figure 5-3D, compare lanes 2 and 3). Taken together, the data shows the negative effect that PML elicits on EBV reactivation.

I also examined whether PML-silenced cells were more sensitive to EBV reactivation following drug treatments. To this end, AGS-EBV and AGS-EBVshPML cells were treated with NaB or TPA or both NaB and TPA and compared to the negative control DMSO treatment. The effects of these treatment on BZLF1 and BMRF1 expression was then examined by Western blotting. While treatments with NaB or TPA alone had little effect on lytic protein expression in AGS-EBV each single treatment resulted in some induction of BZLF1 and BMRF1 in AGS-EBVshPML cells, including phosphorylated forms of BMRF1 (Figure 5-3E, compare lanes 3 and 4, and lanes 5 and 6). In addition, the AGS-EBVshPML cells also produced more of the lytic proteins when treated with both NaB and TPA as compared to AGS-EBV cells (Figure 5-3E, compare lanes 7 and 8). I also examined the effect of these treatments on amplification of the EBV genomes (Figure 5-3F). In keeping with the lytic protein levels, the AGS-EBVshPML cells showed increased EBV amplification compared to AGS-EBV under all conditions tested. These data show that PML is a negative regulator of EBV reactivation.
**Figure 5-3: PML represses EBV reactivation.** AGS-EBV cells infected with a lentivirus carrying shRNA targeted against all isoforms of PML and were selected for cells infected with lentivirus generating the stable cell line, AGS-EBVshPML. (A) Parental AGS-EBV and AGS-EBVshPML were fixed and stained with anti-EBNA1 (red) and anti-PML (green) antibody to ensure PML was efficiently silenced. Images with the same antibody treatment were captured using the same exposure times. (B) Equal amounts of cell lysates from AGS-EBV and AGS-EBVshPML were compared by Western blotting using an antibody that recognized all isoforms of PML and actin. (C) Light microscopy images are shown for AGS-EBV and AGS-EBVshPML, indicating the spontaneous reactivation of EBV in PML silenced cells. They are marked by the enlarged, puffed cells in the bottom panels. Two independent sets of images are shown for each cell line. (D) Equal amounts of cell lysates from AGS-EBV and AGS-EBVshPML were compared by Western blotting using antibodies that recognize BMRF1, BZLF1 and actin, showing increased lytic protein expression in AGS-EBVshPML compared to AGS-EBV. (E) Equal amounts of cell lysates from AGS-EBV and AGS-EBVshPML were compared post treatment with DMSO, NaB, TPA and NaB/TPA by Western blotting using an antibody that recognized all isoforms of PML and detected for EBNA1, BMRF1, BZLF1 and actin. There are two exposures shown for BMRF1, a light exposure (LE) and dark exposure (DE). (F) EBV genomic DNA was quantified post treatment with DMSO, NaB, TPA or NaB/TPA by isolating total DNA, amplifying the EBV DS sequence and normalizing to GAPDH. Average levels are shown with standard deviation after each treatment where AGS-EBV DMSO treatment was set to one and used to normalize all other samples from both cell lines.
5.3.4 EBNA1 contributes to EBV reactivation through disruption of PML NBs

I next examined whether the ability of EBNA1 to disrupt PML NBs could account for the effect of EBNA1 in increasing virus reactivation after NaB/TPA treatment. I rationalized that, if this was the case, then EBNA1 should not increase EBV reactivation after drug treatment in cells that lacked PML. To test this hypothesis, EBNA1 was silenced in AGS-EBVshPML and the effect of NaB/TPA or control treatment on BZLF1 and BMRF1 protein induction was examined by Western blotting. Consistent with the results in AGS-EBV cells, in the absence of NaB/TPA treatment, EBNA1 silencing increased the spontaneous reaction of EBV as detected by increased levels of BZLF1 and BMRF1 (Figure 5-4A, compare lanes 1 and 2 second panel). This shows that the role of EBNA1 in maintaining latency in the absence of drug treatments, is independent of PML. However, the effect of EBNA1 silencing after NaB/TPA treatment in AGS-EBVshPML cells was considerably different than that observed in AGS-EBV cells. Specifically, in the AGS-EBVshPML cells, downregulation of EBNA1 did not decrease the expression of BZLF1 and BMRF1 (Figure 5-4A, compare lanes 3 and 4 second panel). In fact, EBNA1-silenced samples had slightly higher levels of lytic protein expression post drug induction, particularly for BMRF1 (Figure 5-4A, compare lanes 3 and 4 second panel). Similar results were obtained when the level of the EBV genomes was quantified, in that EBNA1 silencing in AGS-EBVshPML cells promoted genome amplification after NaB/TPA treatment (Figure 5-4B). Taken together, the data support the hypothesis that the role of EBNA1 in activating lytic infection, that is revealed after NaB/TPA treatment, is due to the ability of EBNA1 to overcome the suppressive effects of PML NBs. Moreover, the results imply that EBNA1 functions to repress EBV reactivation except when counteracted by cellular defense, such as PML, where it contributes to EBV reactivation through disruption of the PML NBs and degradation of the PML protein.
Figure 5-4: EBNA1 contributes to EBV reactivation through disruption of PML NBs. (A) Equal amounts of cell lysates from AGS-EBVshPML treated with siGFP and siEBNA1 with and without drug induction were compared by Western blotting using antibodies for EBNA1, BMRF1, BZLF, PML and actin. (B) EBV genomic DNA quantified in the above-mentioned samples by isolating total DNA, amplifying the EBV DS sequence and normalizing to GAPDH. Average levels are shown with standard deviation after siEBNA1, siGFP treatment both treated with DMSO or NaB/TPA. The AGS-EBVshPML sample treated with siGFP and DMSO was set to one and used to normalize all other samples.
5.4 Discussion

Here I examined the effects of EBNA1 and PML in reactivating EBV in the gastric carcinoma cell lines, AGS-EBV and AGS-EBVshPML, carrying a recombinant EBV. To my knowledge, this is the first report of a systematic analysis of EBNA1 and its role in EBV reactivation with and without chemical induction. Downregulation of EBNA1 was found to have important implications on the EBV lifecycle. Unexpectedly, downregulation of EBNA1 was found to increase the spontaneous expression of BZLF1 at levels sufficient to reactivate the virus into the lytic cycle, with synthesis of BMRF1 and DNA amplification typical of lytic replication. This effect was independent of PML, as it was observed in cells with and without PML expression. This indicates that the EBNA1 protein functions to maintain latency by repressing reactivation. EBNA1 is not the only latent protein with such a function. It has been shown that LMP2A represses reactivation of EBV in B-cells and appears to function in part by inhibiting B-cell receptor (BCR) signaling through its ITAM motifs (Cooper and Longnecker, 2002; Fruehling and Longnecker, 1997; Longnecker and Miller, 1996; Portis, Ikeda, and Longnecker, 2004). It is conceivable that EBNA1 could act in concert with LMP2A to maintain latency since both proteins are expressed in GC and NPC, although a role for LMP2A in maintaining latency has not been well characterized in epithelial cells. Alternatively, LMP2A could function exclusively in maintaining latency in B cells, while EBNA1 fulfills this role in epithelial cells.

The key event in the initiation of EBV reactivation is the transcription and translation of the BZLF1 gene, which starts a cascade of reactions that result in infectious virion production. To date, there are several cellular factors known to repress and activate the Zp promoter, which controls the transcription of the BZLF1 gene (Adamson et al., 2000; Countryman, Gradoville, and Miller, 2008; Gruffat, Manet, and Sergeant, 2002; Kraus et al., 2001; Liu et al., 1997a; Liu et al., 1997; Montalvo et al., 1995; Montalvo et al., 1991; Yu, Wang, and Mertz, 2007). EBNA1 could potentially mediate the latent to lytic switch by simply controlling the transcriptional state of BZLF1, a hypothesis that is supported by the observation that silencing EBNA1 increases BZLF1 transcript levels. One of the potential targets of EBNA1 is ZEB1 (also called δEF1, TCF8, AREB6, ZFHEP, NIL-2A, ZFHX1A, and BZP), a key cellular factor in repressing transcription from Zp via binding to the ZV element (Kraus, Perrigoue, and Mertz, 2003). Recent studies from our laboratory implicate EBNA1 in regulating ZEB1, where EBNA1 upregulates ZEB1 levels by altering cellular microRNAs (Sheila Mansouri, unpublished). In addition, Shinozaki et al. (Shinozaki et al., 2010) have shown that all three latency protein in GC
(BARF0, EBNA1 and LMP2A) led to a downregulation of mature miR-200 family of microRNAs and the subsequent upregulation of ZEB1/ZEB2. This raises the possibility that EBNA1 might regulate the latent to lytic switch by modulating ZEB1 levels. Furthermore, this suggests the possibility that LMP2A may contribute to the repression of EBV reactivation in epithelial cells, albeit through a different mechanism from those employed in B-cells.

EBNA1 might not only upregulate cellular proteins that repress BZLF1 transcription, but might also downregulate cellular factors that activate BZLF1 transcription or sequester them from binding to Zp and activating BZLF1. In support of this hypothesis, our laboratory has found that TAF1-β binds Zp and enhances transcription of BZLF1 (Shan Wang, unpublished). Interestingly, EBNA1 has been shown to bind TAF1-β during the latent mode of infection and has been implicated in regulating EBNA1 mediated replication and transcription (Holowaty et al., 2003b; Wang and Frappier, 2009). Therefore, EBNA1 could potentially redirect the functions of TAF1-β by preventing its binding to Zp. In support of this possibility, preliminary ChIP experiments (performed by Shelia Mansouri in our laboratory) have shown that, in the absence of EBNA1, there is increased binding of TAF1-β to the BZLF1 promoter. This is consistent with my observations that BZLF1 transcript levels are increased in the absence of EBNA1, suggesting that EBNA1 is able to repress activation of BZLF1 by sequestering TAF1-β and preventing it from binding the BZLF1 promoter. Interestingly, TAF1-β was shown to affect histone modifications at Zp and thus is thought to activate Zp through chromatin alterations (Shan Wang, unpublished). Therefore EBNA1 may ultimately affect the chromatin structure at Zp, promoting a repressive state.

Although EBNA1 seems to repress BZLF1 during latency, I found that EBNA1 is required for reactivation of EBV after NaB and TPA treatment. NaB inhibits the activity of histone deacetylases (HDACs), resulting in increased levels of acetylated histones, which promotes transcriptional activation (Davie, 2003). TPA is a multi-functional inducer, activating the cellular protein kinase C pathway in B cells (Gradoville et al., 2002). This induces phosphorylation of the BZLF1 protein (Baumann et al., 1998) which can then activate the expression of BRLF1 (the other immediate early transcription factor) (Adamson and Kenney, 1998). Downregulation of EBNA1 after NaB and TPA treatment decreased the number of cells entering the lytic cycle, decreased lytic protein expression and resulted in a 2-fold reduction in lytic DNA replication. EBNA1 downregulation also resulted in an increase in PML protein levels in the context of lytic infection, consistent with my previous data that EBNA1 induces
PML loss in latent infection.

Interestingly, a specific isoform or modified form of PML was consistently disproportionally upregulated (relative to other PML isoforms) in the absence of EBNA1 in reactivated cells. This suggests that this PML protein might impart anti-viral functions to prevent EBV reactivation but is normally counteracted by EBNA1 which induces its degradation. There is accumulating evidence that specific PML isoforms can inhibit infection by certain viruses. For example, Everett et al. (Cuchet et al., 2011) has shown that PML isoform I and II can inhibit HSV-1 replication. In contrast, PML isoform IV has been shown to inhibit varicella zoster virion production by entrapping the viral capsid protein in PML cages (Reichelt et al., 2011). The size of the PML isoform or modified form seen after NaB/TPA induction in the absence of EBNA1 (55-70 kDa) is smaller than PML I, II or IV but could be PML isoform III, V or VI. Conversely, it could be a highly modified form of the cytoplasmic PML isoform, PML VII, which has been shown to inhibit HSV-1 replication (McNally et al., 2008).

My data suggest that the role of EBNA1 in inducing the EBV lytic cycle can be entirely attributed to the ability of EBNA1 to disrupt PML NBs and degrade PML proteins. This was made evident, when EBNA1 downregulation in PML-silenced cells did not decrease EBV reactivation. This suggests that the function EBNA1 expression from the Fp promoter in the EBV lytic cycle is mainly to disrupt PML NBs and prevent the anti-viral functions mediated by these NBs, hence allowing efficient EBV replication and infectious virion production. Under conditions of EBNA1 silencing and NaB/TPA induction in AGS-EBVshPML cells, I observed an increase in lytic protein expression (BMRF1) and lytic DNA. Since this is similar to the effect that we observed in AGS-EBV cells with EBNA1 silencing (without NaB/TPA treatment), it most likely reflects the underlying role of EBNA1 in suppressing lytic reactivation that is only revealed in the lytic cycle in the absence of PML.

While PML NBs are well known to inhibit the lytic infectious cycle of many viruses, they have not been previously studied for their effect on viral reactivation. Here I show that PML proteins suppress the reactivation of EBV suggesting that they function in a similar manner in viral reactivation as they do in initial lytic infection. In addition I have shown that EBNA1 can counteract the suppressive effect of PML on reactivation by inducing the loss of PML proteins. Interestingly, while EBNA1 has been clearly shown to disrupt PML NBs in NPC and GC epithelial cells, it has not been observed to do so in B lymphocytes (Bell, Lieberman, and Maul, 2000). Epithelial cells are known to be the principal site of lytic EBV infection in the body,
whereas EBV infection of B lymphocytes is most often latent. This leads to the possibility that the purpose of EBNA1-induced PML protein degradation in epithelial cells is to facilitate lytic infection and that other consequences of PML loss maybe unintentional byproducts.
CHAPTER 6

THESIS SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTION
6.0 Thesis Summary, General Discussion and Future Directions

6.1 Thesis Summary

The study of infectious agents can be used as a tool not only to understand host-pathogen relationships, but also to enrich our knowledge of biologically important processes. My work documented in this thesis has highlighted one such important interaction mediated by the Epstein-Barr virus protein EBNA1, with the host tumor suppressor protein PML, which is required for the formation of multiprotein nuclear structures known as PML NBs. This has important consequences for the host and the virus alike. I have shown that EBNA1 mediates disruption of PML NBs in epithelial cells (NPC and GC) latently infected with EBV. I later showed that this function of EBNA1 is important for the survival of these cells. EBNA1 stably interacts with the cellular proteins USP7 and CK2 to mediate the disruption of PML NBs. Both cellular enzymes are now known to negatively regulate PML NBs, hence EBNA1 simply increases their function at PML NBs. Overall, this impairs several of the functions downstream of PML allowing the survival of EBV infected cells.

PML is involved in several important cellular processes including DNA repair, p53 activation, apoptosis, tumour suppression and inhibition of viral infection. The decreased number of PML NBs via EBNA1 impairs DNA damage induced activation of p53, resulting in decreased apoptosis. Hence, even though DNA repair was likely impaired in EBNA1-expressing cells, they survived better than non-expressing cells after DNA damage. Therefore, the regulation of PML by EBNA1 directly affects host cell processes that would be expected to promote malignant transformation. Fittingly, immunohistochemistry staining of GC biopsies showed that EBV-positive GC biopsies had significantly lower PML staining compared to EBV-negative samples. Hence, EBNA1-mediated disruption of PML NBs has important consequences in EBV-associated epithelial tumours, in both NPC and GC.

Interestingly, these studies led me to discover a new function of EBNA1 in the regulation of latent to lytic switch, otherwise known as EBV reactivation. Initially, EBNA1 represses activation of the EBV BZLF1 protein, the lytic master regulator, thereby promoting latency. However, once a cell has committed to reactivation, EBNA1 is required to disrupt PML NBs that would otherwise inhibit lytic EBV replication. Understanding the latent to lytic switch is very important as this switch has obvious implications for the pathogenesis of malignant and nonmalignant diseases associated with EBV. Being able to manipulate the transition between latency and lytic cycle offers the promise of translational application to virus associated cancers. In particular, studies
have been done to determine the efficacy of tumour cell death once EBV has been reactivated (Meng et al., 2010).

6.2 General Discussion

6.2.1 EBNA1 disrupts PML NBs in epithelial tumours

The investigation of the effect of EBNA1 on PML NBs stemmed from the observation that protein kinase CK2, a known stable protein interacting partner of EBNA1, was a potent negative regulator of PML NBs. In addition, I knew that EBNA1 sequestered USP7 from stabilizing the tumor suppressor protein p53 in response to DNA damage. This is due to the fact that EBNA1 binds USP7 in the same binding pocket as p53 but with ten times higher affinity, leading to p53 destabilization and degradation by the proteasome and increased survival of EBV infected cells. Given these observations, I postulated that EBNA1 may redirect the function of CK2 to bring about the degradation of another very important tumor suppressor protein, PML, that forms multiprotein complexes called PML NBs. Indeed, I observed a striking difference in the number of PML NBs in the EBV-positive C666-1 cell line and the EBV-negative CNE2 cell line, where the latter had at least five times more NBs than C666-1. This was an important observation and served as the platform for the remainder of this thesis. Through various techniques (overexpression, silencing, stable cell lines) I was able to show that EBNA1 alone was sufficient to disrupt PML NBs in NPC and GC cell lines. This effect has important biological implications due to the strong association between PML disruption and tumor development. While initially identified as a gene whose rearrangement leads to promyeloctic leukemia, it has since been found that loss of the PML protein is associated with cancer development for a variety of human tumors (Gurrieri et al., 2004; Salomoni et al., 2008). In addition, mice lacking PML develop normally but their cells are more prone to malignant transformation (Wang et al., 1998a).

This study is the first example of a latent viral protein, EBNA1, that is shown to disrupt PML NBs and induce the degradation of PML proteins in a proteasome dependent manner. Amongst their many functions, PML NBs are known to suppress lytic viral replication, hence many viruses encode proteins that alter PML NBs by remodelling them, disrupting the NB or degrading the PML protein itself (Adamson and Kenney, 2001; Carvalho et al., 1995; Hoppe et al., 2006; Marcos-Villar et al., 2009; Maul, Guldner, and Spivack, 1993; Muller and Dejean, 1999). However, this generally occurs during primary infection of host cells and has not been
shown for latency-associated proteins. This observation is of importance for a medically important virus such as EBV, as it sheds insights into the mechanism by which EBV may cause transformation and immortalization of host epithelial cells, leading to the onset of NPC and GC. EBV’s main reservoir is B-cells and is associated with various B-cell disorders including Burkitt’s lymphoma and Hodgkin’s lymphoma (Young and Rickinson, 2004). Whether EBNA1 contributes to these lymphomas through the disruption of PML NBs is yet to be explored, and these studies are hampered by difficulties in efficiently delivering EBNA1 to B-cells. However, analysis of EBV positive B-cells (Raji) to EBV-negative B-cells (BL41, BJAB) shows marked difference in PML NBs by IF and PML protein by Western blotting (my preliminary observations). Although interesting, this does not directly show a role for EBNA1 in PML NB disruption in B −cells and this question would require further study. Unlike the results in NPC and GC cells, I and Jayme Salsman in our laboratory have not seen any notable disruption of PML NBs when EBNA1 is expressed in Hela or 293 cells, suggesting the specificity of this effect to particular cell backgrounds. A previous study has examined PML NBs in B-cells latently infected with EBV (both latency and I and latency III forms of infection) and found no obvious difference from uninfected cells (Bell, Lieberman, and Maul, 2000). This study, however, did not quantify PML NBs or attempt to manipulate EBNA1. This ability of EBNA1 to disrupt PML NBs in cells of the nasopharynx and upper stomach could be part of the reason that these cells are particularly susceptible to malignant transformation.

6.2.2 Mechanism of PML NB disruption by EBNA1

Viruses have evolved several mechanisms to inactivate PML NBs, either through their reorganization (such as track formation induced by adenovirus E4orf3 protein (Ullman and Hearing, 2008; Yondola and Hearing, 2007)), interference with the interactions between PML proteins needed to form NBs (as observed for the Epstein-Barr virus BZLF1 protein (Adamson and Kenney, 2001)) or inducing the degradation of the PML proteins. EBNA1 mediates many of its functions through the action or modification of cellular proteins and EBNA1 itself has no known enzymatic activities. Thus, its ability to disrupt PML NBs was thought to involve cellular enzymes.

As part of my investigation of the PML effect of EBNA1, I showed for the first time that EBNA1 partly localizes to PML NBs in a native latent infection in NPC cells, where it can physically associate with at least one PML isoform that is suggested to be PML IV. This is
interesting since EBNA1 induces the proteasome-dependent loss of multiple isoforms of the PML protein and is not limited to PML IV. This implies that EBNA1 is targeted to PML bodies through an interaction with PML IV but, once there, can promote the degradation of all PML isoforms. EBNA1 itself is not known to have enzymatic activities; thus, it may require the recruitment of additional cellular proteins to mediate the loss of PML NBs. Through the use of EBNA1 mutants, I have identified a role for both USP7 and CK2 in EBNA1-mediated disruption of PML NBs. These two cellular enzymes have been shown to negatively regulate PML NBs, where downregulation of either enzyme leads to increased PML NBs and protein (Sarkari et al., 2011; Scaglioni et al., 2006).

USP7 is a cellular ubiquitin specific protease that was first identified as a result of its interaction with the ICP0 protein of HSV-1 infection and, like ICP0, USP7 was found to be partly associated with PML NBs (Meredith, Orr, and Everett, 1994). It is interesting to note that the involvement of USP7 in PML disruption may not be unique to EBNA1, as it also appears to play a role in HSV-1 infection. In HSV-1 infection, ICP0 disrupts PML NBs via its own ubiquitin ligase activity (Boutell, Sadis, and Everett, 2002; Everett, 2001). Although ICP0 and EBNA1 do not share similar sequences, they both stably bind USP7 through different USP7 domains (Holowaty et al., 2003a). For ICP0, this interaction with USP7 is important to prevent autoubiquitination of ICP0 and degradation of the protein (Canning et al., 2004; Everett et al., 1997). The stability of ICP0, mediated through the actions of USP7, allows ICP0 to disrupt PML NBs and induce the degradation of PML proteins. While EBNA1 and ICP0 may utilize USP7 in different ways, it is striking that the only two viral proteins known to bind USP7 both use this interaction to facilitate PML disruption. In addition, recent studies on the human cytomegalovirus (another herpesvirus) UL35 protein by Jayme Salsman in our laboratory, has shown that this protein remodels PML NBs (without degrading PML proteins) and also binds to USP7, although it is not clear that USP7 is required of PML remodelling by UL35 (Salsman, 2011). It will be interesting to explore whether other viral proteins that mediate PML disruption also interact with USP7. This will provide further insight into the functions of USP7 and how it may be modulated to mediate PML disruption.

One of the important outcomes of my studies, was the discovery that USP7 was required for EBNA1-mediated PML disruption as this was the first evidence that USP7 itself had a role in PML disruption. As discussed above, although USP7 was known to be PML associated and to bind a PML-disrupting viral protein (ICP0), it had not been recognized that USP7 itself may
regulate PML NBs. Based on my findings, another graduate student in the laboratory, Feroz Sarkari, further investigated the role of USP7 as a negative regulator of PML NBs (Sarkari et al., 2011). Silencing of USP7 was found to increase the number of PML NBs, to increase the levels of PML protein and to inhibit PML polyubiquitylation in NPC cells. Interestingly, CK2α and RNF4, which are known regulators of PML, were dispensable for USP7-associated PML NB disruption (Sarkari et al., 2011). In addition, the catalytic domain of USP7 was not important to mediate PML NB disruption (Sarkari et al., 2011), indicating that the ubiquitin protease activity is not required. Hence, USP7 may recruit or affect stability of other negative regulators of PML NB through protein-protein interactions.

The other cellular enzyme that is important for PML NB disruption by EBNA1 is protein kinase CK2. Our laboratory has previously shown that EBNA1 forms a stable complex with all three subunits of CK2 (Holowaty et al., 2003b). Recently, Scaglioni et al. (2006) have demonstrated that CK2, a kinase associated with cancer promotion (Duncan and Litchfield, 2008), phosphorylates PML and targets it for degradation by the proteasome (see Figure 11). PML contains a CK2 phosphorylation site and loss of this site results in stabilization of the PML protein, enhancement of PML-induced apoptosis and senescence, and prevents sensitivity to CK2 inhibitors. Furthermore, an inverse relationship between PML and CK2 activity is observed in human non-small cell lung cancers (Scaglioni et al., 2006). Consistently, frequent loss of PML expression is observed in multiple human tumours as reviewed in Salomoni et al, 2008 (Salomoni et al., 2008). Perhaps, PML degradation upon CK2 activation could account for the observed phenotype. In addition, this loss of PML could promote tumour formation because in the absence of PML Ras is activated, hence this will promote cancer. This is consistent with the observations that Pml-deficient mice had more aggressive lesions in the lungs compared to wild-type animals (Scaglioni et al., 2006).

My studies support this important role of CK2 in regulating PML levels and show that this pathway is being usurped by EBNA1. I have shown that the ability of EBNA1 to induce the degradation of PML proteins depends on the presence of the host CK2, on the catalytic activity of CK2, and on the direct interaction of CK2 with EBNA1. In particular, EBNA1 was shown to increase the occupancy of CK2 at PML NBs and the subsequent phosphorylation of S517. EBNA1's ability to alter the activity of CK2 towards PML NBs has severe consequences for EBV-associated cancers, not only due to the disruption of a tumor suppressor but because CK2
itself is known to promote cancer (Duncan and Litchfield, 2008). Below is a model by which EBNA1 mediates the disruption of PML NBs through the recruitment of CK2 and USP7.

Figure 6-1: Model for EBNA1-mediated effects on PML NBs. On the left side of the model, is the observation that EBNA1 alters PML NBs through the modulation of CK2 activity directed at PML, thereby increasing phosphorylation and polyubiquitination of the tumor suppressor and degradation by the proteasome. On the right side of the model, EBNA1 utilizes USP7's ability to negatively regulate PML through an undefined mechanism. EBNA1 exploits both pathways to mediate disruption of PML NBs in latently infected cells and impairs important functions including p53 activation and apoptosis, allowing survival of EBV infected cells.

6.2.3 Functional significance of PML NB disruption by EBNA1

As explained previously, PML disruption by EBNA1 has great implications for the onset of EBV-associated diseases. PML acts as a scaffold for many important cellular reactions that
are abrogated or impaired in the presence of EBNA1, hence, promotes the survival of EBNA1 expressing or EBV infected cells. For example, PML NBs have been shown to be important for p53 activation and DNA repair (Boe et al., 2006; Pearson et al., 2000). Indeed both the acetylation of p53 and the repair of DNA lesions after etoposide treatment were found to be impaired by EBNA1 in both NPC and GC cell lines. EBNA1-expressing cells, however, survived these treatments as well or better than parental cells, due to an inhibition of apoptosis which also requires PML NBs (Guo et al., 2000; Wang et al., 1998b). The data as a whole support a model in which EBNA1 contributes to the development of NPC and GC through the disruption of PML NBs, thereby increasing the accumulation of DNA damage while promoting cell survival. This model is consistent with reports of frequent but varying chromosomal aberrations in NPC and GC tumours (Chang et al., 2006; Kang et al., 2002; Li et al., 2006; Shao et al., 2001). PML disruption by EBNA1 also provides a mechanistic basis for the observation that EBNA1 expression increases the tumorigenicity of EBV-negative NPC and GC cells (Cheng et al., 2010; Sheu et al., 1996) and in GC cells decreases sensitivity to cisplatin induced cell death (Cheng et al., 2010). These observations correlate well with my work and provide a basic foundation of the molecular events that are altered upon expression of EBNA1 in NPC and GC cells. In addition, using GC biopsy samples I was able to directly compare EBV-positive and EBV-negative biopsies to demonstrate that the presence of EBV leads to significantly decreased PML protein levels in these tumours. While several viral proteins are known to promote lytic viral infection through disruption of PML NBs, my results indicate that viral proteins can also contribute to carcinogenesis through PML disruption.

A correlation between loss of PML NBs and tumour development and progression has been reported for prostate, colorectal, breast and lung carcinomas, lymphomas, central nervous system and germ cell tumours (Gurrieri et al., 2004). Consistent with my observation of PML loss in EBV-associated GC biopsies, Lee et al. (Lee et al., 2007) have reported the loss of PML in gastric cancers, suggesting that the loss of the PML tumour suppressor may be a key event in the onset of various cancers including those associated with EBV. Hence, the EBNA1-mediated disruption of PML NBs that I have observed in NPC and GC cells would be expected to contribute to the onset and/or progression of this malignancy.

As mentioned, most viral proteins promote lytic viral infection through disruption of PML NBs. PML NBs are part of an interferon-mediated anti-viral response and are upregulated in response to viral infections (Chelbi-Alix et al., 1995). EBNA1 is the only latent protein that
continues to be expressed during the lytic cycle using a unique promoter, Fp (Lear et al., 1992; Nonkwelo et al., 1996). Since, EBNA1 can promote the disruption of PML NBs, I postulated that EBNA1 can contribute to the lytic cycle of EBV by disrupting PML NBs. Surprisingly, I found that EBNA1 repressed reactivation of EBV and functions to maintain latency, consistent with its essential role in latent EBV DNA replication and segregation of EBV episomes in proliferating cells (Young and Rickinson, 2004). This was observed by the increased spontaneous reactivation of EBV when EBNA1 was downregulated. This is a novel observation and provides further insight into the regulation of latent to lytic switch. Since EBNA1 is expressed in all EBV-associated latency programs and is the only viral protein expressed in some latencies, it suggests that EBNA1 can indeed act as the viral trigger for latent to lytic switch. EBNA1 may contribute to the transcriptionally repressive state of the EBV genome, thereby preventing transcription of the BZLF1 gene. This may tie in with the observation that EBNA1 can regulate cellular HDAC3 (Lu et al., 2010), whose activity can be targeted to the Zp promoter and repress Zp activity.

However, consistent with my hypothesis, upon drug-induced reactivation of EBV, I found that EBNA1 is required for efficient lytic protein expression and DNA replication. This is mostly likely to overcome the repressive nature of PML proteins that are upregulated in the lytic cycle in the absence of EBNA1. This implies that EBNA1 is required to disrupt PML NBs that would otherwise inhibit EBV lytic replication in the lytic cycle. Further, these observations show that EBNA1 may act in concert with BZLF1 to disrupt PML NBs, since low levels of BZLF1 is not able to mediate such effects in the absence of EBNA1. The mechanism of PML NB disruption in the lytic cycle is yet to be characterized. Although, data from our laboratory indicates that it may involve both USP7 and CK2, since EBNA1 continues to interact with these enzymes in the lytic cycle (Natasha Malik, unpublished). In addition, through these studies I have identified a negative role for PML in EBV reactivation, since PML silencing dramatically increased the spontaneous reactivation of EBV. In particular, a specific isoform of PML was consistently upregulated in EBV reactivated cells that were silenced for EBNA1. Implying that PML isoform specific functions may exist to restrict EBV reactivation, which of course are disarmed by EBNA1. A role for PML in repressing viral reactivation has not previously been shown, however, isoform-specific functions of PML in restricting lytic replication have been shown with HSV-1 and VZV (Cuchet et al., 2011; McNally et al., 2008; Reichelt et al., 2011). Taken together, EBNA1 clearly fine tunes the latent to lytic switch of EBV, where it represses
reactivation during unfavorable conditions within the host. However, once the conditions are appropriate it supports lytic viral replication by disarming PML NBs mediated repression, thereby promoting viral replication and spread from host to host.

![Figure 6-2: Model for EBNA1 mediated disruption of PML NBs contributing to EBV reactivation.](image_url)

Interferons may upregulate PML and NBs upon latent to lytic switch and EBNA1 expression is turned on from the Fp promoter to alleviate the repressive effects mediated by PML NBs, thereby, allowing amplification of infectious EBV particles.

6.3 Future Directions
6.3.1 EBNA1 mediated PML disruption in B-cells

EBV’s main reservoir is B-cells and EBV is associated with various B-cell disorders including Burkitt's lymphoma and Hodgkin's lymphoma (Young and Rickinson, 2004). Whether EBNA1 contributes to these lymphomas through the disruption of PML NBs has yet to be explored due to the lack of techniques available to efficiently deliver EBNA1 in B-cells. However, my preliminary analysis of EBV-positive B-cells (Raji) and EBV-negative B-cells (BL41, BJAB) showed a marked difference in PML NBs by IF and PML protein by Western blotting. Although interesting, this does not directly show a role for EBNA1 in PML NB disruption in B-cells. Recently, I was able to show that adenovirus can infect and deliver EBNA1 to EBV negative B-cells, hence, it would be interesting to determine whether it can disrupt PML NBs in this background. One needs to design and generate adenoviruses that can...
deliver an EBNA1 without an epitope tag, since I have observed that C-terminally SPA-tagged EBNA1 is not efficient at degrading PML as compared to untagged EBNA1 protein. This experiment will be useful in determining the effects of EBNA1 and EBV in specific cell types. Further, this will answer the question whether EBNA1 uses similar or distinct mechanisms to contribute to EBV-associated diseases.

6.3.2 Mechanism of PML disruption

EBNA1 localizes to and binds PML IV, where it mediates the enrichment of CK2β and USP7 proteins at PML NBs. Both EBNA1 mutants, Δ3787-394 and Δ395-450, which abrogate binding to CK2 and USP7 respectively, can localize to PML NBs but are not able to mediate PML disruption. To date, I have not identified any sequence on EBNA1 that targets it to PML NBs, perhaps having either of the binding sequences, to CK2 or USP7, is sufficient to mediate localization to PML NBs but not enough induce PML NB disruption. One approach I would take to address this question is to make an EBNA1 mutant with the sequences 387-450 removed, therefore Δ 387-450 and assay for localization to PML NBs, to determine the exact sequences required for targeting to PML. It is also possible that the PML interacting sequence of EBNA1 is outside of this region, so the localization of EBNA1 proteins lacking sequences N-terminal or C-terminal to this region could also be examined.

In addition, it would be interesting to investigate whether EBNA1 interacts directly with PML IV. To this end, several protein interaction assays could be applied to these two proteins, including yeast 2-hybrid assay, co-expression in insect cells (via baculoviruses) followed by co-purification and GST pull down assays of purified proteins. Any positive results would be compared to results with other PML isoforms to verify the specificity. Also PML C-terminal tails alone could be examined for EBNA1 binding since these are the only unique sequences among the pML isoforms.

6.3.3 Contribution of PML to associated functions

EBNA1 expressing or EBV carrying cells do not respond properly to DNA damage, as they have decreased p53 acetylation, p21 expression and apoptosis, all of which are functions that could be mediated by PML NBs. In order to ensure that the effects seen in response to DNA damage are a direct consequence of PML NB loss, the assays should be repeated in cells without PML such as AGS-EBVshPML, where the lack of PML would act as a positive control. This would confirm that the effects seen are consistent with loss of PML NBs. In addition, PML could
be silenced in CNE2Z cells with and without EBNA1 expression and the effects of EBNA1 on p53 acetylation and apoptosis could be compared to determine if all of these effects of EBNA1 are due to the presence of EBNA1. Also, EBV-positive cells could be downregulated for EBNA1, which would increase PML NBs, and one could determine whether this resulted in increased acetylation of p53, p21 expression and apoptosis, as expected. These experiments would clarify the importance of PML NBs in response to DNA damage and their impairment in the presence of EBNA1.

6.3.4 Regulation of spontaneous reactivation of EBV by EBNA1

Since EBNA1 seems to repress spontaneous reactivation of EBV, it is important to understand the mechanism that mediates this repression since the latent to lytic switch has important consequences for the treatment of EBV associated diseases. One of the key cellular players that is already known to repress EBV reactivation is ZEB1. It represses transcription from Zp by binding to the ZV element (Kraus, Perrigoue, and Mertz, 2003). Recent studies from our laboratory implicate EBNA1 in regulating ZEB1, where EBNA1 upregulates ZEB1 levels by altering cellular microRNAs (Sheila Mansouri, unpublished). This raises the possibility that EBNA1 might regulate the latent to lytic switch by modulating ZEB1 levels. To investigate this possibility, one could downregulate EBNA1 and use chromatin immunoprecipitation to determine the amount of ZEB1 present at the ZV element of Zp. This will indicate whether EBNA1 can not only alter the levels of ZEB1 but can direct its activity at the Zp promoter preventing inappropriate reactivation of EBV. Likewise, a similar approach can be taken with the other potent negative regulator of Zp, MEF2D (Gruffat, Manet, and Sergeant, 2002). On the other hand, the possibility that EBNA1 prevents the binding of activating factors such as Sp1/Sp2 or TAF1-β should be investigated. TAF1-β is of particular relevance because it is known to bind EBNA1 (Holowaty et al., 2003b) and EBNA1 affects its association with other viral sequences, namely at oriP (Wang and Frappier, 2009). All of these targets could be addressed using the above mentioned chromatin immunoprecipitation assay.

6.3.5 Regulation of drug induced reactivation by EBNA1

I have shown that EBNA1 uses both USP7 and CK2 to disrupt PML NBs. Silencing either of these enzymes in EBNA1-expressing cells was shown to increase PML NBs and protein, suggesting that EBNA1 requires the functions of these proteins to maintain low levels of PML. First, it would be good to confirm this effect in other EBV-positive cells epithelial cell
lines, for example by silencing USP7 or CK2 in AGS-EBV. Second, the effect of silencing these enzymes on EBV lytic protein expression and lytic DNA replication should be determined with and without drug induced EBV reactivation. If EBNA1 still uses the same mechanism to disrupt PML NBs in EBV lytic cycle, I would suspect that both USP7 and CK2α would be required. Hence, a decrease in lytic protein expression and DNA replication would be expected when these enzymes are down regulated. In support of this idea is the observation EBNA1 stably interacts with both USP7 and all three subunits of CK2 enzyme during EBV lytic cycle (Natasha Malik, unpublished data).

Also in the drug-induced reactivation of EBV, the absence of EBNA1 consistently leads to the up regulation of a single PML isoform, which migrates on SDS-PAGE between 55 - 70 kDa. Recent evidence suggests that, upon infection with herpes simplex virus-1, there is an increase in cytoplasmic PML and this PML isoform was sufficient to suppress lytic virus replication (McNally et al., 2008). It would be interesting to determine the repressive nature of the individual PML isoforms during EBV reactivation. To this end, AGS-EBV cells already silenced for PML, AGS-EBVshPML, could be infected with lentiviruses carrying individual shRNA-resistant PML isoforms. Once stable cell lines are made, one could determine the levels of lytic protein expression (BZLF1, BMRF1) and lytic DNA replication and compare to the parental AGS-EBVshPML, which has increased EBV reactivation even in the absence of chemical treatment. This would show whether any single PML isoforms are sufficient to suppress EBV reactivation and, if so, if their size corresponds to the reactivation-induced PML isoform mentioned above. In addition, it should be determined whether the reactivation-induced PML isoform is caused by transcriptional activation, as opposed to stabilization of the existing protein. To this end, total mRNA should be isolated from the AGS-EBV cells with and without EBNA1 silencing and with and without NaB/TPA drug induction. Then the amount of each PML isoform transcript could be quantified using primers specific to each unique fragment present on the seven isoforms. The combination of the two experiments should shed some insight into the repressive nature of PML and the role of each isoform in lytic viral replication, in particular, reactivation of EBV.

6.4 Conclusions

The EBNA1 protein has established functions in replication of the EBV genome, tethering of viral episomes to mitotic chromosomes for partitioning, and transcriptional regulation of viral
latency genes. The discovery that EBNA1 interacts with cellular enzymes that alter the infected cell environment provides a foundation for understanding how EBNA1 manipulates the host cell that consequently results in disease. One such manipulation is of the cellular tumour suppressor PML and the nuclear bodies formed by them. This regulation has important consequences for EBV-associated epithelial tumours as well as EBV reactivation from latency.

Surprisingly, EBNA1 was found to localize to PML NBs in cells latently infected with EBV and associates with PML IV, the isoform that is known to bind p53 and activate p53 by CBP. Once at PML NBs, EBNA1 increases the association of both CK2β and USP7 at PML NBs. This increases CK2-mediated phosphorylation of PML, priming it for polyubiquitylation and degradation by the proteasome. In addition, EBNA1 increases USP7-mediated disruption of PML NBs through an unknown mechanism. Both USP7 and CK2 work independently of each other, however, EBNA1 requires both enzymes to mediate the disruption of PML NBs.

The loss of PML NBs abrogates many functions important for tumor suppression, including activation of p53 and apoptosis. Accordingly, EBNA1-expressing or EBV-infected cells better survive after exposure to DNA damaging agents even though they contain unrepaired DNA damage. EBNA1 therefore impairs the functions downstream of two tumour suppressors, PML and p53, the latter of which is affected both by inhibiting its activation and decreasing its stability (Saridakis et al., 2005). These effects would lay the foundation for the onset of both NPC and GC. Furthermore, through these studies I was able to identify a new role for EBNA1 in EBV reactivation, where it plays the dual role of both repressing and activating the lytic cycle depending on the nature of reactivation. Hence, it acts as a molecular sensor to initiate EBV reactivation in response to appropriate stimuli. This is key to the survival of EBV-infected cells, as inappropriate activation may stimulate cellular defense mechanisms and result in the clearance of the virus, while lack of appropriate reactivation would limit cell to cell and host to host spread. In summary, my findings have greatly increased our understanding of the multiple roles of EBNA1 in the EBV life cycle.
7.0 APPENDIX

EPSTEIN-BARR NUCLEAR ANTIGEN 1 REPLICATION AND SEGREGATION FUNCTIONS IN NASOPHARYNGEAL CARCINOMA CELL LINES

This chapter has been submitted for publication as:


Natalia Thawe performed experiments in Figures A-3B,C,D and A4-B,C,D. I performed all other experiments in this chapter.
7.1 - Introduction, Materials and Methods, Results and Discussion

Epstein-Barr virus (EBV) is causatively associated with the development of nasopharyngeal carcinoma (NPC) as almost all undifferentiated NPC is comprised of monoclonal expansions of cells latently infected with EBV. These tumour cells express the viral Epstein-Barr nuclear antigen 1 (EBNA1) and the secreted BARF1 protein and sporadically express latent membrane protein 1 (LMP1) (Raab-Traub, 2002; Tao and Chan, 2007). EBNA1 is essential for the persistence of the EBV episomal genomes due to its important roles in both the replication and the mitotic segregation of the EBV episomes (reviewed in (Frappier, 2010; Hirai and Shirakata, 2001; Lindner and Sugden, 2007)). EBV persistence also requires the OriP sequence that contains both an origin of DNA replication (DS element) and a segregation element (FR). DNA replication requires EBNA1 binding to the DS element, while segregation requires EBNA1 binding to the FR as well as EBNA1-mediated tethering of the episomes to the host mitotic chromosomes. While virtually all undifferentiated NPC tumours are EBV-positive, studies of the persistence of EBV in these cells have been hampered by the fact that these cells lose the EBV genomes when grown in culture. In fact, while EBV episomes are stably maintained in many B-cell lines, they are generally not stably maintained in epithelial cell lines (Dittmer et al., 2008; Shannon-Lowe et al., 2009). A notable exception to this rule is the C666-1 cell line, derived from a xenograft of an undifferentiated NPC, which stably maintains the EBV episomes (Cheung et al., 1999; de Jesus et al., 2003). Here we examine whether the ability of C666-1 to maintain EBV episomes better than other NPC cell lines reflects differences in OriP-mediated functions in replication or segregation.

I began by comparing the ability of various cell lines to maintain a plasmid containing OriP. Two EBV-negative NPC cell lines, CNE2Z (Sun et al., 1992) (Fig. A-1A and A-1B) and HK-1 (Hu et al., 2005) (Fig. A-1C), were transfected with an OriP plasmid that expressed EBNA1 (OriPE) and then propagated without selection for the plasmid for the indicated numbers of cell doublings (one cell doubling is one day for these cell lines). Plasmids were then recovered from the cells, linearized and treated with Dpn I in order to digest any of the input plasmid that had not undergone DNA replication (the majority of the plasmid in the 3 day samples). As expected, neither of these cell lines were able to replicate or maintain the negative control OriP plasmid that lacked EBNA1 expression (Fig A-1B, lanes 1-5; Fig. A-1C, lanes 1-3). In contrast, OriPE plasmids replicated, resulting in DpnI-resistant bands in all the cell lines after three cell doublings (Fig. A-1A, lane 13; Fig A-1B, lane 10; Fig. A-1C lane 8). Comparison of
the amount of DpnI-resistant OriPE at various time points showed that these plasmids were not maintained past 14 doublings and that there was a noticeable loss of plasmid between the 7 and 14 doubling time points for CNE2Z. This plasmid loss rate was consistent with that observed in HeLa cells (Fig. A-1A).

I also followed the maintenance of an OriP plasmid in the EBV-positive C666-1 cell line (Fig. 1D). Since this cell line already expressed EBNA1, EBNA1 expression was not supplied on the OriP plasmid. The OriP plasmid was seen to persist at stable levels for at least 28 doublings and was still detectable after 42 doublings (equivalent to 84 days since the C666-1 doubling time is approximately 48 hours). The pCAN plasmid lacking OriP sequences was used as a negative control and, as expected, showed no replication or maintenance (Fig. A-1D, lanes 1-4). These results show that C666-1 can maintain an OriP plasmid considerably better than any of the other epithelial cell lines.

EBNA1 contains a Gly-Ala repeat region that is variable in length in different isolates. C666-1 expresses a version of EBNA1 with a long Gly-Ala repeat region whereas the EBNA1 expressed on the OriPE plasmid has very little of this Gly-Ala repeat. In order to determine if the length of the Gly-Ala repeat affected plasmid maintenance, we repeated the plasmid loss experiments in CNE2Z and HK1 using a version of OriPE that expressed EBNA1 with the long Gly-Ala repeat (Fig. A-1B and 1C, OriPE,GA lanes). However OriP plasmid maintenance was not improved with the larger EBNA1.

The stable maintenance of OriP plasmids requires a combination of two EBNA1-mediated functions; DNA replication and mitotic segregation. The replication of the OriP plasmids can be measured directly by determining the level of Dpn I-resistant plasmid after 2-3 cell doublings (because at such early time point plasmids are maintained in the cells regardless of whether they have a segregation mechanism) (Shire et al., 1999). To determine whether the increased plasmid maintenance observed in C666-1 was due to increased efficiency of OriP plasmid replication, the levels of Dpn I-resistant plasmids were compared in C666-1, CNE-2Z and HeLa cells after 3 cell doublings and quantified relative to the amount of total OriP plasmid recovered at the same time point (Input), to account for any differences in initial transfection efficiencies (Fig. A-2A and A-2B). The results show that C666-1 cells do not replicate OriP plasmids more efficiently than the other cell lines, indicating that the increased ability to maintain plasmids is due to more efficient mitotic segregation.
To more directly compare the ability of CNE2Z and C666-1 cells to maintain OriP plasmids, I integrated an expression cassette for EBNA1 in CNE2Z to generate CNE2E cells. These cells express EBNA1 at levels similar to C666-1 (Sivachandran, Sarkari, and Frappier, 2008a), enabling me to follow the maintenance of the same OriP plasmid used for assays in C666-1. The OriP plasmid was maintained in CNE2E cells considerably longer than was OriPE in CNE2 cells, as it was still detected after 42 days (Fig. A-3A, lanes 10-18). These results are similar to those seen in C666-1 (Fig. A-1D) indicating that CNE2Z cells are not inherently unable to maintain OriP plasmids.

A major difference in the experiments comparing the maintenance of OriP plasmids and OriPE plasmids is that the EBNA1 levels are considerably higher when expressed from OriPE as compared to the levels expressed in CNE2E or C666-1 cells. Therefore we asked whether the higher levels of EBNA1 expressed from OriPE were detrimental for plasmid maintenance. This was tested by comparing the maintenance of OriP and OriPE plasmids in CNE2E cells (Fig. A-3A) as well as in C666-1 cells (Fig. A-4A), both of which already express low levels of EBNA1. In both cell lines, we found that the OriP plasmid was maintained for longer periods of time than the OriPE plasmid. However comparison of the replication of OriP and OriPE showed that the higher EBNA1 levels did not decrease replication efficiency in either cell line (Fig A-3B and A-4B). In fact, relative to the total plasmid taken up by the cell (Input), OriPE plasmid replicated somewhat more efficiently than the OriP plasmid in C666-1 cells (see histogram in Fig. A-4B). EBNA1 overexpression from OriPE was confirmed in the Western blots shown in Fig. A-3C (CNE2ZE) and Fig. A-4C (C666-1). In addition, the proliferation rates of CNE2E (Fig. A-3D) and C666-1 (Fig. 4D) cells were not significantly affected by the presence of OriPE as compared to OriP. Taken together the results indicate that higher EBNA1 levels are detrimental to plasmid segregation resulting in decreased plasmid maintenance.

My results show that NPC cells that have lost EBV genomes through growth in culture are able to support EBNA1-mediated replication and segregation from OriP, and therefore are not fundamentally different in that regard than C666-1 cells. However, EBNA1-mediated segregation was found to be sensitive to high levels of EBNA1, suggesting that the level of EBNA1 expression in the initial EBV-positive cells may be a factor in whether or not the episomes are stably maintained when cells are placed in culture. The requirement of a precise EBNA1 level for segregation may reflect the need for the formation of a ternary protein complex, which would be disrupted by excess EBNA1, and may be one reason that EBNA1
expression from Qp (the EBNA1 promoter used in epithelial cell infection) is subject to autoregulation (Sample, Henson, and Sample, 1992; Yoshioka, Crum, and Sample, 2008).

![Figure A-1: Comparison of maintenance of OriP plasmids in multiple cell lines.](image)

(A) CNE2 and HeLa cells were compared in their ability to replicate and maintain the OriPE plasmid. The cells grown in a 10 cm dish were transfected with 8 µg of pc3oriP plasmid expressing EBNA1 (oriPE) (Shire et al., 1999) using Lipofectamine 2000 (Invitrogen) and 24 hours later, were moved to a 15 cm dish where they were allowed to grow for 48 hours. At this point, 5 x 10^6 cells were harvested (3 day or 3 cell doublings time point) to monitor replication of the plasmid. The remaining cells were re-plated and harvested after 7, 14, 21 and 28 cell doublings to monitor plasmid loss. In all cases, cells were lysed by the Hirt method and low-molecular-weight DNA was isolated as previously described (Ceccarelli and Frappier, 2000; Shire et al., 1999). Recovered plasmids were linearized with Xho I and, where indicated, were also incubated with Dpn I to digest unreplicated plasmids. DNA was separated by agarose gel electrophoresis, Southern blotted, and probed with ^32^P-labeled pc3oriP. The position of DpnI-resistant plasmid is indicated by the asterisk. A similar experiment performed with an OriP plasmid lacking EBNA1 after 3 cell doublings is shown for
HeLa cells in lanes 1 and 2 as a negative control. Markers of the positions of linear OriP and OriPE plasmids are shown in lanes 2 and 3. (B) CNE2 cells were monitored in their ability to replicate and maintain plasmids OriP, OriPE and OriPE,GA (expressing a version of EBNA1 with long Gly-Ala repeat described in (Shire et al., 1999)) as in A. Markers of the positions of linear OriP and OriPE are shown in lanes 7 and 8. (C) Replication and maintenance of OriP, OriPE and OriPE,GA plasmids in HK-1 cells were followed as in B. (D) Replication and maintenance of pc3oriP and the parental pCAN plasmid without OriP (negative control) was assayed in C666-1 (EBV-positive) cells as described above. Plasmid maintenance was monitored for 3 to 42 cell doublings as indicated (corresponding to 6 to 84 days).

Figure A-2: Comparison of the replication of OriPE plasmids in HeLa, CNE2 and C666-1 cells. (A) Cells were transfected with OriPE plasmids and harvested after 3 cell doublings (3 days for HeLa and CNE2 and 6 days for C666-1) as described in Fig. 1. Plasmid DNA was isolated and linearized as in Fig. 1 and one-tenth of the sample was analysed directly as a recovery control (Input; bottom panel). The remaining sample was incubated with Dpn I, to digest any plasmid that was not replicated in the human cells (+DpnI; top panel). Samples were then analysed by Southern blotting as in Fig. 1. Each experiment is shown in triplicate (1,2,3). Only the Dpn I-resistant plasmid is shown in the top panel. A marker of the position of linearized OriPE is shown in the first lane. (B) Quantification of the replicated plasmid in the three cell lines from A. Linearized plasmid bands were quantified by phosphorimager analysis using ImageQuant software (Molecular Dynamics) and the intensity of the Dpn I-resistant bands were quantified relative to the Input band for the same sample. Average values are shown relative to values from CNE2 cells.
Figure A-3. Plasmid maintenance and replication assays in CNE2E cells. (A) CNE2E cells were transfected with pCAN (negative control), OriP or OriPE plasmids as indicated, harvested after 3 to 56 cell doublings (3-56 days) and processed as in Fig. 1. The amount of DpnI-resistant plasmid at each time point (marked by the asterisk) was quantified by phosphorimager analysis from three experiments and plotted relative to the 3 cell doubling amount, which was set to one (histogram). Average values with standard deviation are shown. (B) Transient replication assays comparing the bands from OriP and OriPE plasmids after 3 cell doublings before (Input) and after Dpn I digestion (+ DpnI). The positions of Dpn I-resistant bands are indicated by the asterisk. The histogram shows quantification of Dpn I-resistant bands relative to input bands from three experiments. Results are plotted relative to the signal from the OriP plasmid (set to one). (C)
Western blot confirming the overexpression of EBNA1 in OriPE samples as compared to endogenous EBNA1 in CNE2ZE. 30 µg of total cell lysates are shown from CNE2ZE 3 days post-transfection with pCAN, OriP or OriPE plasmids. Blots were probed with antibodies against EBNA1 (a mix of R4 rabbit serum (Holowaty et al., 2003b) and OT1X monoclonal antibody) and actin (CALBIOCHEM CP01). (D) CNE2ZE cells were transfected with OriP or OriPE plasmids as for replication assays. 1 x 10^5 cells were then plated in four 6 cm dishes and harvested and counted 1, 3, 5 or 7 days later. Average cell numbers from three independent experiments are plotted with standard deviations.
Figure A-4: Plasmid maintenance and replication assays in C666-1 cells. (A) C666-1 cells were transfected with pCAN (negative control), OriP or OriPE plasmids as indicated, harvested after 3 to 42 cell doublings (6 to 84 days) and processed as in Fig. 1. DpnI-resistant bands were quantified as in Fig 3A and shown in the histogram. (B) Transient replication assays comparing the bands from OriP and OriPE plasmids after 3 cell doublings (6 days) before (Input) and after Dpn I digestion (+ DpnI). The positions of Dpn I-resistant bands are indicated by the asterisk. The histogram shows quantification of Dpn I-resistant bands relative to input bands from three experiments. Results are plotted relative to the signal from the OriP plasmid (set to one). (C) Western blot confirming the overexpression of EBNA1 in OriPE samples (EBNA1) as compared to endogenous EBNA1 in C666-1 (EBNA1,GA), which is larger due to having a long Gly-Ala repeat region. 30 µg of total cell lysates, from C666-1 cells 6 days post-transfection with pCAN, OriP or OriPE plasmids, were Western blotted as in Fig 3C. (D) C666-1 cells were transfected with OriP or OriPE plasmids as for replication assays. 3 x 10^5 cells were then plated in four 6 cm dishes and harvested and counted 4, 8, 12 and 14 days later. Average cell numbers from three independent experiments are plotted with standard deviations.
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