Functions of Ubiquitin Specific Protease 7 (USP7) in Epstein-Barr Virus Infection and Associated Cancers

by

Feroz Sarkari

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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2010

ABSTRACT
The Epstein-Barr virus (EBV) infects over 90% of the human population and is associated with several human malignancies. The EBNA1 protein of EBV binds recognition sites in the latent origin of replication (oriP) and is important for the replication and segregation of EBV genomes in latently-infected cells. EBNA1 is also directly implicated in malignant transformation and immortalization of the host cell. EBNA1 does not have any known enzymatic activity and it employs cellular proteins to mediate its functions. One such protein is the ubiquitin specific protease, USP7, which is a key regulator of the p53 tumor suppressor. The aim of this thesis was to functionally characterize the interaction between EBNA1 and USP7. Here I show that USP7 promotes the DNA-binding activity of EBNA1 and is recruited along with an accessory protein, GMPS, to the oriP. The USP7-GMPS complex can deubiquitinate histone H2B and may enable epigenetic regulation of latent viral infection. Additionally, I present evidence for a direct role of EBNA1 in EBV-mediated carcinogenesis. EBNA1 prevents stabilization of p53 by USP7 and abrogates p53 activation by disrupting promyelocytic leukemia nuclear bodies (PML-NBs) that acetylate p53. This interferes with p53-activated gene expression and inhibits apoptosis. EBNA1-expressing cells also have impaired ability to repair DNA, but survive as well as or
better than control cells. Thus EBNA1 creates a cellular environment conducive to transformation and immortalization. These studies have also allowed me to learn more about and expand on the known functions of USP7. I provide biochemical evidence suggesting that a P/A/ExxS motif is a preferred sequence for binding the USP7 N-terminal domain. Furthermore, I show USP7 is a negative regulator of PML proteins and PML-NBs and promotes p53 DNA-binding activity. Surprisingly, neither function required the deubiquitinase activity of USP7.
ACKNOWLEDGMENTS

I would like to express my utmost gratitude for my supervisor, mentor and teacher Dr. Lori Frappier. This work would not have been possible without her insightful critique, endless encouragement and her timely feedback. Her tireless commitment to graduate training and inspirational work ethic are something I would always strive to emulate. I want to thank my supervisory committee members, Dr Jack Greenblatt and Dr James Ellis, for their critique and guidance that ensured this journey was not any longer than it had to be. I would also like to thank Dr. Peter Whyte for getting me started on the right foot in research and helping me develop an interest in oncogenic viruses. I want to thank the donors (and their families), whose contribution led to the cell lines I used in my work.

Spending the better half of the past decade in the Frappier lab has been one of the most fun life experiences, thanks partly to the company of all members of the lab. Special thanks go to Kathy Shire and Tin Nguyen, without whom the Fappier lab would not be the well-oiled machine that it is. I want to thank Dr. Vivian Saridakis and Dr. Yi Sheng for their continuing collaboration that has helped me get a promising start to my scientific career. Thanks for holding my hand while I navigated the treacherous world of protein biochemistry during my early days as a graduate student. Thanks for the generosity you have shown with time and reagents alike. You are two of the best individuals I have ever worked with and will do so again in a heartbeat. I would like to thank Jayme and Madhav for giving a much needed boost to my social life. Your friendship has truly been one of the highlights of my graduate school experience and I look for forward to it ‘spilling’ into the rest of my life.

Thank you Vicky PKH Nguyen for being in my life. Thank you for making home the best place on earth. Thank you for understanding what I mean when I say ‘the western didn’t work’. Thank you for helping me be the person I am and the citizen of the world I aspire to be.

I thank my family, particularly my parents Behram and Rukhsana Sarkari, for fostering independence and taking decisions that have so positively shaped my life. You uprooted your own lives in Pakistan to provide me a promising one in Canada. You are my heroes and I cannot thank you enough. Finally I thank my late grandparents Anis Fatima and Kaikhusru Sarkari for inspiring me with their progressive outlook, courage and compassion.
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BARF1</td>
<td>BamHI rightward frame 1</td>
</tr>
<tr>
<td>BART</td>
<td>BamHI rightward transcript</td>
</tr>
<tr>
<td>Brd4</td>
<td>Bromodomain 4</td>
</tr>
<tr>
<td>BZLF1</td>
<td>BamHI Z leftward frame 1</td>
</tr>
<tr>
<td>BPV</td>
<td>Bovine papilloma virus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Ck2</td>
<td>Casein kinase 2</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DS</td>
<td>Dyad symmetry</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating enzyme</td>
</tr>
<tr>
<td>E1</td>
<td>ubiquitin activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>ubiquitin ligase</td>
</tr>
<tr>
<td>EBER</td>
<td>Epstein-Barr expressed RNA</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein-Barr nuclear antigen</td>
</tr>
<tr>
<td>EBNA-LP</td>
<td>Epstein-Barr nuclear antigen leader protein</td>
</tr>
<tr>
<td>EBP2</td>
<td>EBNA1 binding protein 2</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ECF</td>
<td>Enhanced chemifluorescence</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>FA</td>
<td>FLAG-Protein A</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FOXO4</td>
<td>Forkhead box O4</td>
</tr>
<tr>
<td>FR</td>
<td>Family of repeats</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMPS</td>
<td>Guanosine monophosphate synthetase</td>
</tr>
<tr>
<td>H2A</td>
<td>Histone 2A</td>
</tr>
<tr>
<td>H2B</td>
<td>Histone 2B</td>
</tr>
<tr>
<td>H3</td>
<td>Histone 3</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HAUSP</td>
<td>Herpesvirus associated ubiquitin specific protease</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>ICP0</td>
<td>Infected cell polypeptide 0 (HSV protein)</td>
</tr>
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<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IM</td>
<td>Infectious mononucleosis</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma associated herpes virus</td>
</tr>
<tr>
<td>LANA</td>
<td>Latency associated nuclear antigen</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>LCMS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization – time of flight</td>
</tr>
<tr>
<td>NAP</td>
<td>Nucleosome assembly protein</td>
</tr>
<tr>
<td>ND10</td>
<td>Nuclear Domain 10</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>oriLyt</td>
<td>Origin of lytic replication</td>
</tr>
<tr>
<td>oriP</td>
<td>Origin of plasmid replication</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting gene</td>
</tr>
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<td>SCID</td>
<td>Severe combined immunodeficiency disorder</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>TRF</td>
<td>Telomere repeat factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>Ub-H2B</td>
<td>Ubiquitinated H2B</td>
</tr>
<tr>
<td>UBP</td>
<td>Ubiquitin binding protein</td>
</tr>
<tr>
<td>UCH</td>
<td>Ubiquitin C-terminal hydrolase</td>
</tr>
<tr>
<td>USP7</td>
<td>Ubiquitin specific protease 7</td>
</tr>
<tr>
<td>USP7-NTD</td>
<td>Ubiquitin specific protease 7 N-terminal domain</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>ZEBRA</td>
<td>Z EBV replication activator</td>
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CHAPTER 1

INTRODUCTION
1.1 INFECTIOUS AGENTS AND CANCER

A sizeable portion of the global cancer burden can be attributed to infectious agents. Viral, bacterial and parasitic infections cause or are linked to an estimated 20% of human cancers (Hausen, 2006; Parkin et al., 2005; Zur Hausen, 2009). Epstein-Barr Virus (EBV), human papillomavirus (HPV), hepatitis B virus and Kaposi’s Sarcoma Associated Virus (KSHV) are among the DNA viruses associated with cancers, while human T lymphotrophic virus type 1 and hepatitis C virus are the oncogenic RNA viruses. In addition to viruses, the bacterium Helicobacter pylori is tightly associated with gastric carcinomas, while several parasites are associated with cancers of various origins. The use of a vaccine against HPV as a preventive measure against cervical cancer (Harper et al., 2006; Villa et al., 2006) illustrates that a well rounded strategy to contain cancer must address infectious agents with oncogenic potential. In this thesis, I explore the molecular mechanisms underlying the oncogenic potential of one of the DNA tumor viruses, the Epstein-Barr Virus.

1.1.1 Discovery and Taxonomy of EBV

In 1964, Anthony Epstein and Yvonne Barr discovered herpesvirus-like particles while examining electron micrographs of cells derived from Burkitt’s lymphoma biopsies (Epstein et al., 1964). The virus found in those biopsies would later come to be known as the Epstein-Barr virus (EBV) or human herpesvirus 4. Soon after, the link between EBV and Burkitt’s lymphoma, which is named after the surgeon who first described it in children in equatorial Africa as a tumor involving the jaw (Burkitt, 1958), was further substantiated (Henle and Henle, 1966). Subsequently, a link between EBV and what is now known as Nasopharyngeal Carcinoma was revealed (Henle et al., 1970; zur Hausen et al., 1970). Since then the oncogenic potential of EBV has been fully acknowledged through association with several other malignancies, including gastric carcinomas, Hodgkins lymphoma, several T cell lymphomas and post-transplant lymphoproliferative disease (Rickinson, 2001).

EBV is an enveloped virus with a genome comprised of a single linear molecule of double-stranded DNA. Following initial infection the genome is circularized and maintained as an extrachromosomal episome. EBV is part of the herpesviridae family of viruses, which can be further divided into the alpha (α), beta (β) and gamma (γ) subfamilies based on parameters such
as the length of the infectious cycle and the host range (International Committee on Taxonomy of Viruses; http://www.ictvonline.com). EBV belongs to the $\gamma$ subfamily, while herpes simplex virus and cytomegalovirus are prominent examples of the $\alpha$ and $\beta$ subfamilies respectively.

1.2 BIOLOGY OF EBV INFECTION

EBV exhibits both a productive or lytic infectious cycle and a latent form of infection, as outlined below. While EBV can infect epithelial cells for both lytic infection and latent persistence, this process is inefficient and the mechanism is not completely understood (Borza and Hutt-Fletcher, 2002). EBV preferentially infects B-lymphocytes through the binding of the viral glycoproteins gp350 and gp42 to CD21 receptor and human leukocyte antigen (HLA) respectively (Borza and Hutt-Fletcher, 2002; Nemerow et al., 1987).

1.2.1 Lytic infection

EBV is virtually ubiquitous, infecting over 90% of the human population. Primary infection mostly occurs during infancy through saliva and is largely asymptomatic. Delayed exposure in adolescence, however, can lead to infectious mononucleosis (IM), which is characterized by fever, sore throat, swollen lymph nodes and the presence of atypical lymphoblasts in large numbers in the blood (Hislop et al., 2007). After oral transmission, EBV infects epithelial cells of the oropharynx and engages in the replicative, or lytic, infectious cycle. During this process, the virus amplifies itself and infects adjacent cells. EBV also infects mucosal B-lymphocytes, which leads to proliferative expansion of these cells. While the majority of these transformed cells are eliminated by antigen specific T lymphocytes, many escape this elimination process by virtue of lowered antigen expression and enter a resting state. These cells survive in the long-term memory B-cell pool, allowing EBV to persist in an asymptomatic latent infection. Through physiological and environmental signals, the latent infection can be reactivated into the lytic cycle, subsequent replication in epithelial cells and release of infectious virus (Rickinson, 2001). This process of reactivation is complex and still incompletely understood (Miller et al., 2007).

Many aspects of the lytic cycle are regulated by the EBV protein ZEBRA. For example, along with another EBV protein, RTA, ZEBRA coordinates transcriptional regulation of viral lytic genes, including those required for lytic DNA replication (Miller et al., 2007).
Additionally, ZEBRA can directly activate the viral lytic cycle by binding to the origin of lytic replication (oriLyt) and recruiting proteins important for viral replication (Miller et al., 2007).

### 1.2.2 Latent infection

The purpose of the latent cycle appears to be immune evasion and survival of the host cell to ensure long term persistence of the viral genome in host cells. To achieve this, approximately 20 copies of the EBV genome are maintained as circular extrachromosomal episomes during the latent cycle and minimal viral gene expression is observed (Rickinson, 2001). Viral latency gene products not only mediate the replication and segregation of the viral genome in host cells but, as outlined below, also contribute to transformation and immortalization to varying degrees. It is thus not surprising that latent EBV infection is associated with several human malignancies of lymphoid and epithelial origins.

The B-lymphotrophic property of EBV is exploited to infect, transform and immortalize resting B-lymphocytes *in vitro* to generate lymphoblastoid cell lines (LCLs) (Young and Rickinson, 2004). In addition to this, there are several cell lines of lymphoid and epithelial origin derived from malignancies natively associated with EBV. The study of EBV-derived cell lines and LCLs have contributed much to our understanding of latent EBV infection and helped identify at least three distinct patterns of EBV gene expression, referred to as latency types, during latent infection (Rickinson, 2001). In latency type I, also referred to as the ‘EBNA1-only’ program, primarily characterized by Burkitt’s lymphoma, expression of EBV-encoded RNAs (EBERs), the BamHI-A rightward transcripts (BARTs) and Qp promoter driven expression of EBV nuclear antigen 1 (EBNA1) is observed (Rickinson, 2001). In latency type II, also referred to as the ‘default’ program, expression of latent membrane proteins (LMP1, LMP2A and LMP2B) is observed in addition to the expression of EBERs, BARTs and EBNA1. In addition latency II epithelial cells express a secreted EBV protein called BARF1 which may function as a growth factor (Decaussin et al., 2000; zur Hausen et al., 2000). While some studies have linked BARF1 to development of NPC (Seto et al., 2008; Sheng et al., 2001), the mechanism of BARF1 activity is still under investigation. This latency type is generally characteristic of nasopharyngeal carcinoma, EBV associated gastric carcinoma and Hodgkin’s lymphoma (Rickinson and Kieff, 2001). Latency type III is characterized by LCLs and post-transplant
lymphoproliferative disease and exhibits expression of the full range of EBV latent genes that includes EBNAs 1, 2, 3A, 3B, 3C and LP in addition to the genes expressed in latency I and II (Rickinson and Kieff, 2001). This program is also known as the growth program. Expression of EBNA1 in this case however is driven by the Cp/Wp promoter (Rickinson and Kieff, 2001). The additional EBNAs expressed in latency III are highly immunogenic and therefore this latency form is only seen in immunocompromised people. It is important to note that these classifications are not definite and that further details and subtle variations are still being discovered. For example, BARTs are the major source of EBV-encoded microRNAs (Karran et al., 1992; Smith et al., 2000), but their role in regulating viral and cellular gene expression has only recently found interest (Cai et al., 2006). On the other hand, it is important to note the ubiquitous expression of EBNA1 in all EBV infected cells. This observation is compatible with the fact that EBNA1 is required for the replication of the viral genome and its partitioning or segregation in dividing cells. Indeed it is the only protein required for the persistence of latent infection. Finally, another program, known as latency 0, exists in memory B cells in which no expression of EBV RNAs or proteins is detected (Hochberg et al., 2004). This program is often simply known as the latency program.

1.2.3 EBV latent proteins and transcripts in host cell transformation

EBV tumor derived cell lines and in vitro studies using infection with recombinant EBV has helped assign roles to the different EBV latent proteins. As discussed below, these roles range from crucial contributions to an absolute requirement for EBV-mediated host cell transformation. The relative positions of genes encoding these proteins are shown in Figure 1-1.

1.2.3.1 Latent Membrane Proteins

One of the most important transforming EBV proteins is the latent membrane protein 1 or LMP1. LMP1 mimics CD40 tumor necrosis factor receptor (TNFR), but unlike CD40, LMP1 remains constitutively active in a ligand independent manner (Gires et al., 1997; Kilger et al., 1998; Mosialos et al., 1995; Uchida et al., 1999). Thus LMP1 provides growth and differentiation signals to the cell in an unregulated fashion, contributing to host cell transformation. The other latent membrane proteins, LMP2A and LMP2B are not essential for EBV-mediated transformation. However LMP2A can act as a B-cell receptor and drive survival
and proliferation of B-cells or switch to EBV lytic cycle (Rechsteiner et al., 2008), while LMP2B is known to suppress the functions of LMP2A (Rechsteiner et al., 2008).

1.2.3.2 Epstein-Barr Nuclear Antigens

Another group of latent proteins is referred to as the Epstein-Barr Nuclear Antigens or EBNAs. These include EBNAs, 1, 2, 3A, 3B 3C and EBNA-LP (Young and Rickinson, 2004). The significance of EBNA1 in EBV latent infection is highlighted by its ubiquitous expression in all latency types in proliferating cells and in all EBV-associated tumors. EBNA1’s omnipresence is compatible with its function, as it is required for the replication and stable partitioning of the EBV genome in dividing cells (Rickinson, 2001). While these are more established roles of EBNA1, relatively recent evidence also points to a more direct role of EBNA1 in EBV-induced host cell transformation and immortalization. Later in this thesis, I will discuss these roles and propose new mechanistic details of these EBNA1 functions, which have emerged from my work.

EBNA2 is absolutely required for EBV-mediated host cell transformation, as an EBV strain lacking EBNA2 fails to transform B-cells in vitro (Zimber-Strobl and Strobl, 2001). Further work in LCLs has shown that EBNA2 interacts with cellular DNA binding proteins such as RBP-Jκ, PU.1 and AUFI, which allow EBNA2 recruitment to promoters of target genes (Fuentes-Panana et al., 2000; Grossman et al., 1994; Henkel et al., 1994; Hsieh and Hayward, 1995; Johannsen et al., 1995). These interaction allow EBNA2 to initiate a gene expression program, not only activating transcription of viral latency genes, including LMP1 and LMP2, but also activating cellular genes that ultimately lead to activation and proliferation of B-cells (Zimber-Strobl and Strobl, 2001). EBNA-LP interacts and co-operates with EBNA2 to induce RBPJκ-mediated transcription activation. Furthermore, the EBNA3A, B and C proteins repress the transcriptional activities of EBNA2 and EBNA-LP to tightly modulate the gene expression program in latently infected cells (Izumi et al., 1994; Zhao et al., 1996). In addition to modulating the transcriptional activity of ENBA2, EBNA3C can play a more direct role in B-cell transformation. EBNA3C can promote cell proliferation by associating with Cyclin A and suppressing p27-mediated inhibition of cyclin A/Cdk2 activity (Knight and Robertson, 2004) and by usurping the SCFskp2 ubiquitin ligase complex to target pRB and p27 for proteasome
A. EBV genome: latent genes

B. Open reading frames for the EBV latent proteins
Figure 1-1. The Epstein-Barr virus genome (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Young and Rickinson, 2004, copyright 2004). (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 green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes encoding these proteins 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 and 2B). EBNA-LP is transcribed from a variable number of repetitive exons. LMP2A and LMP2B are composed of multiple exons, which are located on either side of the terminal repeat (TR) region, which is formed during the circularization of the linear DNA to produce the viral episome. The blue 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 long outer green arrow represents EBV transcription during a form of latency known as latency III (Lat III), in which all the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs that are generated by differential splicing of the same long primary transcript. The inner, shorter red arrow represents the EBNA1 transcript, which originates from the Qp promoter during Lat I and Lat II. Transcripts from the BamHIA region can be detected during latent infection, but no protein arising from this region has been definitively identified. The locations of the BARF0 and BARF1 coding regions are shown here. (B) Location of open reading frames for the EBV latent proteins on the BamHI restriction-endonuclease map of the prototype B95.8 genome. The BamHI fragments are named according to size, with A being the largest. Lowercase letters indicate the smallest fragments. Note that the LMP2 proteins are produced from mRNAs that splice across the terminal repeats (TRs) in the circularized EBV genome. This region is referred to as N$_{het}$, to denote the heterogeneity in this region due to the variable number of TRs in different virus isolates and in different clones of EBV-infected cells.
dependent degradation (Knight et al., 2005). Recently, EBNA3C was shown to possess deubiquitylating activity (Saha et al., 2009). EBNA3C can deubiquitinate itself and Mdm2 and additionally promote Mdm2 ubiquitin ligase activity towards p53. EBNA3C can also modulate the transcriptional and apoptotic activities of p53 (Yi et al., 2009).

1.2.3.3 EBV non-coding RNAs and the BamHI Transcripts

Latent EBV-gene expression also includes two non-coding small RNAs, EBER1 and EBER2. Since their discovery, several roles of EBERs in host cell transformation have been proposed (Swaminathan, 2008). Although it was first suggested that EBERs can rescue PKR kinase-mediated inhibition of translation (Bhat and Thimmappaya, 1983; Bhat and Thimmappaya, 1985), this is unlikely and not consistent with the nuclear localization of EBERs. Other studies using Burkitt’s lymphoma derived cell lines suggest a role of EBERs in the transformation and immortalization process (Iwakiri and Takada, 2010), for example, expression of EBERs in isolation in EBV-negative cell lines was found to contribute to resistance to apoptosis, capacity for growth on soft agar, tumorigenicity in mice and enhanced expression of the antiapoptotic BCL-2 protein (Komano et al., 1999), but the mode of their function remains controversial and the mechanistic details elusive (Swaminathan, 2008). Additionally, expression of many non-coding microRNAs (miRNAs) is possible from the EBV genome, though the expression patterns and functions of these miRNAs remain poorly understood. One cluster of EBV miRNAs, originally identified in NPC, is referred to as BamHI A rightward transcripts or BARTs. Their role in regulating expression of cellular and viral genes and contribution to latent EBV infection is only just beginning to emerge (Swaminathan, 2008).

1.3 Functions of the Epstein-Barr Nuclear antigen 1 (EBNA1) and their significance to EBV-latent infection

The EBV genome is maintained at a constant copy number as a circular double-stranded DNA episome during latent infection. This is made possible by replication of the episomes once per cell cycle and segregation of the episomes in proliferating cells by tethering them to the chromosomes of dividing cells. The absolute minimum requirements to ensure this persistence are a cis-acting element in the EBV-genome, the latent origin of DNA replication (oriP) and one viral trans-acting factor, the EBNA1 protein (Rickinson, 2001). The oriP is comprised of two
distinct regions, the dyad symmetry (DS) element and the family of repeats (FR) element, which are about 1kb apart (Reisman et al., 1985b) (Figure 1-2). The DS and FR elements have four and twenty 18bp palindromic EBNA1 recognition sites, respectively. EBNA1-binding to the DS element is required for initiation of viral DNA replication (Wysokenski and Yates, 1989), whereas binding to the FR is required for stable partitioning of the viral genome in dividing cells, transcriptional activation of viral latency genes and enhancement of replication at the DS element (Reisman and Sugden, 1986). The DNA-binding and dimerization domain of EBNA1 is absolutely required for these functions of EBNA1 (Figure 1-3).

1.3.1 EBNA1 in EBV DNA replication
The latent origin of DNA replication for EBV, oriP, was identified by testing the ability of different EBV DNA fragments to allow replication and stable maintenance of plasmids in EBV-infected human cells (Yates et al., 1984). The DS element is essential for the replication of oriP-containing plasmids (Wysokenski and Yates, 1989) and was subsequently shown to be sufficient for replication of plasmids in human cells in the presence of EBNA1 (Harrison et al., 1994; Yates et al., 2000). Two-dimensional gel electrophoresis analyses have revealed that replication forks form at or very near the DS element (Gahn and Schildkraut, 1989). The DS element is 120 bp long with four EBNA1 binding sites, two of which (sites 3 and 4) are within a 65bp dyad symmetry element (Rawlins et al., 1985b; Reisman et al., 1985b) (Figure 1-2). The DS also contains three copies of a 9 bp sequence, two on each end of the DS element and one in the middle of the DS element between sites 2 and 3 (Niller et al., 1995). The nonamers are bound by telomeric repeat factors (TRF) 1 and 2 in a cell cycle dependent manner (Deng et al., 2003; Deng et al., 2002). All four EBNA1 binding sites and the nonamers are required for efficient replication from the DS element (Koons et al., 2001). Nevertheless, minimal replication can take place in the presence of just two adjacent EBNA1 sites (either sites 1 and 2 or sites 3 and 4) and is subject to simulation.
Figure 1-2. Organization of the EBV oriP. 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).
Figure 1-3. EBNA1 domains. (A) Schematic representation of wild-type EBNA1 protein (EBNA1 GA). Indicated some of the functional elements. 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, whereas those that partially inhibit these functions are shown in grey.
by the nonamers (Atanasiu et al., 2006; Harrison et al., 1994; Koons et al., 2001; Yates et al., 2000).

EBNA1 initiates viral DNA replication at the DS by binding four recognition sites at the DS (Koons et al., 2001; Rawlins et al., 1985b) (Figure 1-2). 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, 1991b), replication must rely on host cell factors. Additionally, since the EBV genome replicates once per cell cycle (Adams, 1987), it is conceivable that viral replication is orchestrated in a manner similar to the host’s DNA replication. EBNA1 binding destabilizes the histone-DNA interactions at the region, making it accessible to the replication machinery (Avolio-Hunter et al., 2001). An important step in eukaryotic DNA replication is the recruitment of the origin recognition complex (ORC) to the origin of replication (Duncker et al., 2009), followed by the loading of the MCM helicase (Mcms 2–7) at the origin (Maiorano et al., 2006). Indeed, several studies have shown that ORC is recruited to the DS element by EBNA1 and facilitates the loading of the MCM complex to initiate replication at the DS (Chaudhuri et al., 2001; Dhar et al., 2001; Schepers et al., 2001a). The MCM complex functions as a helicase that unwinds DNA in front of the replication fork and likely plays the same role in EBV replication. EBNA1 physically interacts with ORC in immunoprecipitation assays (Dhar et al., 2001; Schepers et al., 2001a) and this interaction is required for the recruitment of Orc2 to the DS element (Julien et al., 2004).

Several recent findings have added further complexity to replication initiation at the DS element. First, TRF1 and TRF2 bind the nonamers in a cell cycle-dependent manner. Chromatin immunoprecipitation analyses have shown that TRF2 binding peaks at G1/S while TRF1 peaks at G2/M (Deng et al., 2003; Deng et al., 2002). TRF2 can stimulate replication initiation at the DS, possibly by interacting with ORC subunits and promoting recruitment of ORC to the DS element (Atanasiu et al., 2006). TRF1, however, antagonizes the activity of TRF2, inhibits replication from the DS and was shown not to bind ORC. It is interesting to note that ORC is not recruited to the FR even though EBNA1 is constitutively bound to the FR. The selective binding of TRF2 to nonamers in the DS, which are absent in the FR element, might explain the preferential recruitment of ORC to the DS element. The same group which discovered the role
of TRF2 in ORC recruitment has also proposed a mechanism for EBNA1-mediated ORC recruitment. RGG-motifs in the N-terminal region of EBNA1 bind G-rich RNAs, which also interact with Orc1 peptides (Norseen et al., 2008). This suggests that G-rich RNA species form a bridge between EBNA1 and ORC subunits. A role of Poly-ADP-ribosylation (PAR) has also been suggested in regulation of EBV replication at the DS: EBNA1 was shown to be PARylated, a modification that inhibits its replication activity (Deng et al., 2005; Deng et al., 2002) and knockdown of PARP1 increases EBNA1, ORC2 and Mcm3 recruitment to the oriP (Deng et al., 2002; Tempera et al.). These findings suggest a negative role for PARylation in EBV replication at the DS element. Finally, Chk2 kinase has also been implicated in regulation of replication at the oriP. Chk2 can phosphorylate TRF2, and a phospho-mimetic mutation at the Chk2 phosphorylation site in TRF2 reduces its ability to recruit ORC, suggesting a negative role for Chk2 phosphorylation (Zhou et al.). On the other hand, shRNA-induced depletion of Chk2 results in reduced replication efficiency and maintenance of oriP-containing plasmids. Therefore, while it might be unclear whether the role of Chk2 in replication of oriP-containing plasmids is stimulatory or inhibitory, it is likely important in coordinating ORC recruitment and replication in a cell cycle-dependent manner.

It is important to note that, while the DS is sufficient to initiate replication when cloned into unrelated plasmids and is the most efficient site of initiation of replication in the EBV genome, it is not the only site of replication initiation in EBV genomes. Replication of EBV genomes has also been observed to initiate at a second region 14 kb upstream of the oriP (Little and Schildkraut, 1995; Norio and Schildkraut, 2001). This region is devoid of EBNA1-binding sites, and replication here is not initiated at a discrete site but rather occurs from multiple sites within a broad zone analogous to initiation in eukaryotic cells. This suggests that replication initiation from this region is solely mediated by cellular proteins. Studies have shown that, at least in some EBV-positive cell lines, the DS element is not required for replication and maintenance of EBV genomes (Norio and Schildkraut, 2004; Norio et al., 2000), questioning the importance of oriP in replication initiation. However, since the cell lines studied were of different latency types, it is proposed that the preference of origin of replication may be a function of the latent viral gene expression program. Consistent with this idea, overexpression of
LMP1 in latency I cells, which only express EBNA1, resulted in decreased replication of EBV genomes in these cells (Shirakata et al., 2001). This supports the possibility that LMP1 might inhibit usage of oriP as the origin of replication.

### 1.3.2 EBNA1 in segregation of EBV genomes and oriP plasmids

Persistence of latent EBV infection requires both replication of EBV genomes and their stable partitioning during division of host cells. The only two viral elements required for partitioning of EBV genomes are EBNA1 and the cis-acting FR element of the oriP (Krysan et al., 1989; Lee et al., 1999; Lupton and Levine, 1985). 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., 1985b; Reisman et al., 1985b) (Figure 1-2).

When bound by EBNA1, the FR not only confers partitioning of the EBV genomes but also works as an enhancer for transcriptional activation of viral latency genes (Gahn and Sugden, 1995; Reisman and Sugden, 1986; Sugden and Warren, 1989). The partitioning ability of the FR is transferable when cloned into other plasmids so long as EBNA1 is also present in the cell and plasmids can autonomously replicate (Kapoor et al., 2001; Krysan et al., 1989; Simpson et al., 1996). An important distinction is that, unlike EBV genomes, oriP-containing plasmids cannot be maintained indefinitely, even in the presence of EBNA1, and are lost at a rate of 2% – 5% every cell division cycle (Kirchmaier and Sugden, 1995; Leight and Sugden, 2001; Sears et al., 2003; Vogel et al., 1998; White et al., 2001).

EBV episomes and EBNA1 associate with condensed cellular DNA during mitosis (Grogan et al., 1983; Harris et al., 1985; Petti et al., 1990), however the association of FR containing plasmids with mitotic chromosomes is dependent on EBNA1 (Kanda et al., 2001). These observations suggest a model for partitioning in which EBV genomes or FR-containing plasmids are tethered to the host chromosomes and ‘piggy-backed’ into daughter cells. Over the years, several findings have emerged in support for this model. For instance, the FR region is required for association of oriP-containing plasmids with cellular chromosomes (Kanda et al., 2001; Krysan et al., 1989). Secondly, the functional domains of EBNA1 (Figure 1-3) responsible for attaching to chromosomes and segregation of plasmids can be replaced with the
chromosome binding regions of high mobility group 1 protein (HMG1) or histone H1 (Hung et al., 2001). Also, mutants of EBNA1 that do not bind mitotic chromosomes, also fail to partition oriP-containing plasmids (Hung et al., 2001; Shire et al., 1999; Wu et al., 2000).

While EBNA1 binds the FR directly, the mechanism of its attachment to cellular DNA is a little more complex. EBNA1 could attach to cellular DNA either by direct binding or indirectly through interactions with cellular DNA-binding proteins. There is evidence supporting both possibilities. Chromosome binding cellular proteins are employed by origin binding proteins of bovine papilloma virus and Kaposi’s Sarcoma-associated Herpesvirus (KSHV) to attach viral genomes to mitotic chromosomes (Barbera et al., 2006; Brannon et al., 2005; Cotter and Robertson, 1999; Krithivas et al., 2002; Parish et al., 2006; Viejo-Borbolla et al., 2005; You et al., 2004; You et al., 2006). Given that the origin binding proteins of these viruses exhibit structural and functional similarities to EBNA1, it is conceivable that EBNA1 employs a similar mechanism for viral genome attachment to chromosomes of the host cell. Evidence that EBNA1 uses cellular chromosome binding proteins for attachment first emerged with the discovery of EBNA1 interaction with EBP2 (EBNA1-binding protein 2) in a yeast-two hybrid screen (Shire et al., 1999). Deletion of EBNA1 residues 325 – 376 not only disrupted EBNA1-mediated segregation of oriP-based plasmids, but also abolished EBP2 binding, suggesting a correlation between EBP2 binding and EBNA1 segregation function (Shire et al., 1999; Wu et al., 2000; Wu et al., 2002). The role of EBP2 was further examined in a partitioning system reconstituted in yeast (Kapoor et al., 2001). In this system, the autonomously replicating sequence (ARS), a yeast origin of replication was cloned into an FR-containing plasmid. The ARS element and the FR complement each other and facilitate the replication and partitioning of the plasmid respectively. Stable partitioning of plasmids in this system only occurred when both EBNA1 and EBP2 were present and only when the EBNA1-EBP2 interaction was intact (Kapoor et al., 2001; Wu et al., 2002). Both EBP2 and EBNA1 were attached to mitotic chromosomes and EBNA1 attachment was dependent on EBP2 (Kapoor and Frappier, 2003). The importance of EBP2 in segregation of oriP plasmids was then confirmed in human cells. Down-regulation of EBP2 not only impaired attachment of EBNA1 to mitotic chromosomes but also that of oriP plasmids (Kapoor et al., 2005).
Though it is important, EBP2 cannot solely account for EBNA1 attachment to chromosomes and partitioning of oriP plasmids. Immunofluorescence imaging of EBNA1 and EBP2 during mitosis has revealed that EBNA1 associates with cellular DNA prior to EBP2 (Nayyar et al., 2009). Thus EBNA1 may initially associate with DNA via mechanisms other than those involving EBP2, and EBP2 might subsequently stabilize this association. It is possible that other cellular factors contribute to EBNA1 binding to cellular DNA. Alternatively EBNA1 could directly attach to cellular chromosomes. In support of this mechanism, it has been shown that two N-terminal glycine-arginine-rich regions of EBNA1 similar to AT-hooks show affinity towards AT sequences in vitro and may allow EBNA1 to directly bind cellular DNA (Sears et al., 2004). Therefore, interaction with cellular factors like EBP2 and direct interaction with DNA might account for EBNA1 attachment and oriP-plasmid tethering to cellular chromosomes, though to varying degrees.

1.3.3 EBNA1 in transcriptional activation and repression

In addition to functioning in segregation, FR also functions as an enhancer and controls expression of latent genes when bound by EBNA1 (Lupton and Levine, 1985; Reisman and Sugden, 1986). EBNA1-bound FR activates the LMP promoter and the Wp and Cp promoters, which control expression of the EBNA genes (Gahn and Sugden, 1995; Puglielli et al., 1997; Sugden and Warren, 1989). Although there are 20 EBNA1-binding sites in the FR element, only 6 sites are sufficient for the FR to function as an enhancer (Wysokenski and Yates, 1989). In reporter constructs, the FR can activate transcription when cloned in either orientation, upstream or downstream of the promoter (Ceccarelli and Frappier, 2000a; Reisman and Sugden, 1986; Reisman et al., 1985b).

Precisely how EBNA1’s activity in transcription is regulated is not fully known. Two regions in EBNA1 protein have been shown to be important for its transactivation function. One region is in the N-terminal region of the protein and maps to residues 61 – 89 (Kennedy and Sugden, 2003; Wu et al., 2002) and another region maps to residues 325 -376 (Ceccarelli and Frappier, 2000a) (Figure 1-3). The region spanning residues 61 – 89 was recently shown to be required for EBNA1 interaction with the bromodomain protein 4 (Brd4) (Lin et al., 2008). Silencing of Brd4 diminished EBNA1-dependent transcriptional activation from the FR.
Interestingly, Brd4 is also known to interact with the E2 protein of Bovine Papillomavirus (BPV) (You et al., 2004). The DNA binding domain of E2 is structurally similar to that of EBNA1, and E2 serves functions similar to EBNA1 in viral genome segregation and transcriptional activation. Not only is Brd4 important for E2’s function in tethering BPV genomes to host chromosomes, it is also important for transcriptional activation by E2 (McPhillips et al., 2006; Schweiger et al., 2006). The 325–376 region also offers potential means of regulating the transactivation ability of EBNA1. Mutation of four serines in the 325-376 region to alanines or to phosphomimetic aspartate inhibits the transactivation function of EBNA1 in reporter assays (Shire et al., 2006). These results seem most consistent with these sequences being important for transcription rather than phosphorylation playing a role in this EBNA1 function. Furthermore, the 325–376 region is also responsible for interactions with cellular proteins (Holowaty et al., 2003b). One such protein is P32/TAP. P32/TAP may play a role in EBNA1-dependent transactivation since it has been reported to be recruited to the oriP and its C-terminal fragment can activate transcription when fused to the GAL4 DNA-binding domain (Van Scoy et al., 2000; Wang et al., 1997). More importantly, the 325–376 region is also implicated in binding the nucleosome assembly protein, NAP1 (Holowaty et al., 2003b). Initially thought to function only as histone chaperones and chromatin assembly factors, the NAP family of proteins has been implicated in a host of functions including, but not limited to, transcription regulation and cell cycle regulation (Park and Luger, 2006). NAP1 was also found to be recruited preferentially to the FR element of the oriP, which is consistent with its role in transactivation by EBNA1 (Wang and Frappier, 2009). Moreover, knockdown of NAP1 diminished EBNA1 transactivation as measured in a reporter assay, further supporting NAP1’s role in transactivation.

In addition to activating transcription from the Cp promoter, EBNA1 can also repress its own expression from the Qp promoter, which is used in the absence of EBNAs other than EBNA1 (Nonkwelo et al., 1996; Sample et al., 1992). The repressive effects of EBNA1 are independent of the FR element and involve two EBNA1 binding sites downstream of the Qp (Sample et al., 1992). The affinities of these two sites for EBNA1 are much lower than those in the FR (Ambinder et al., 1990; Jones et al., 1989). This implies that EBNA1 would only bind these two sites when the levels are high enough and the FR and the DS binding sites are
saturated. This also offers a feedback mechanism for EBNA1 to keep its expression levels in check. A mechanism for EBNA1 repression from the Qp promoter has been proposed recently. This report suggests that it is not transcription but rather post or co-transcriptional processing of primary transcripts that is inhibited by EBNA1 (Yoshioka et al., 2008).

The ability of EBNA1 to bind regulatory elements in the EBV genome and modulate transcription of viral genes raises the possibility that EBNA1 can regulate transcription of cellular genes. This possibility has been tested by several groups, which have obtained conflicting results. Kang et al have found that EBNA1 cannot affect transcription from an oriP reporter integrated into the genome (Kang et al., 2001). On the other hand there have been reports of EBNA1 expression in EBV negative cell lines leading to changes in levels of several cellular genes products (Chuang et al., 2002; Kube et al., 1999; Srinivas and Sixbey, 1995; Wood et al., 2007). However, so far it has not been confirmed that these changes are a direct effect of EBNA1 on transcription by binding regulatory elements in the host genome.

1.3.4 EBNA1 in host cell immortalization and transformation

As part of the latent infectious cycle, EBV immortalizes the host cell and is tightly associated with several malignancies. As outlined above, several latent proteins, including the LMPs and the EBNAs, play important roles in this process. The importance of EBNA1 in host cell immortalization has been largely attributed to its indispensability in maintenance of the EBV genome in dividing cells and to some extent its control of latent viral gene expression. Nonetheless, these functions do not rule out a more direct role of EBNA1 in host cell transformation and immortalization. This possibility appears more intriguing given that EBNA1 is expressed in all EBV-infections associated with host cell immortalization and EBV-associated tumors and, in many instances, it is the only latent viral protein expressed. Consistent with the role of EBNA1 in host cell immortalization, Kennedy et al (Kennedy et al., 2003) have shown that inhibition of EBNA1 function, using an EBNA1 dominant negative in Burkitt’s lymphoma cells, decreases cell survival. 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). Still, interpretation of the role of EBNA1 in these studies remains
complicated, since perturbing EBNA1 function in these cells may also affect maintenance of EBV genomes and expression of other viral latency genes.

To circumvent this issue, several studies have resorted to studying the effects of expression of EBNA1 in isolation, independent of variables such as expression of viral genes other than EBNA1 and the impact on viral genome maintenance. Some studies have made use of transgenic mouse models to ascertain the direct role of EBNA1 in tumorigenesis, albeit with mixed results. In the first study of this kind, Wilson et al (Wilson et al., 1996) reported that expression of EBNA1 in transgenic mice induced B-cell lymphomas. Conversely, this finding has not been upheld by other studies using transgenic mice. Kang et al showed that FVB mice or C57BL/6 mice used by Wilson et al expressing EBNA1 in lymphocytes did not exhibit a higher prevalence of lymphomas than control mice (Kang et al., 2005; Kang et al., 2008). Therefore, the ability of EBNA1 to induce lymphomas in transgenic mice is not conclusive.

Another approach to studying the role of EBNA1 in host cell transformation is to express EBNA1 in EBV-negative cell lines and assess their tumorigenicity in immunocompromised mice. EBNA1 expression in nasopharyngeal carcinoma cells and Hodgkin's lymphoma cells promotes tumorigenicity in nude mice and nonobese 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 keep the debate on the direct role of EBNA1 in malignant transformation alive.

1.3.4.1 Cellular Effects of EBNA1 contribute to host cell transformation
Molecular events observed after expression of EBNA1 in various tumor cell lines not only continue to support EBNA1’s role in host cell transformation but also offer mechanisms for this process. Numerous studies have reported that EBNA1 can alter the levels and/or function of cellular proteins and thus affect cell survival and proliferation.

EBNA1 can counteract Nm23-H1 mediated growth inhibition and suppression of cell migration in vitro (Murakami et al., 2005). This effect has been linked to EBNA1’s ability to promote tumor growth in xenografts of breast carcinoma cells in nude mice, as mentioned above
(Kaul et al., 2007). A microarray study looking at EBNA1’s effect on expression of cellular genes reported an increase in STAT1 and decrease in TGF-β (Wood et al., 2007). Disruption of TGF-β signaling by EBNA1 was also reported in another study, which found EBNA1 expression lowered SMAD2 protein levels and led to diminished activity of the tyrosine phosphatase receptor kappa (Flavell et al., 2008).

Nonbiased proteomics approaches were used to identify EBNA1-interacting cellular proteins by passing HeLa cell extracts over EBNA1-specific affinity columns and by Tandem Affinity Purification (TAP) of EBNA1-containing protein complexes from 293 cells (Holowaty et al., 2003b). Specifically bound proteins were then identified using MALDI-TOF mass spectrometry. This led to the discovery of EBNA1 interactions with several modifying enzymes. One such protein was USP7, also known as Herpesvirus-associated USP7 or HAUSP, since it was originally identified as an interacting partner of the ICP0 protein of herpes simplex virus (Everett et al., 1997b; Meredith et al., 1994). Interaction with USP7 suggested one manner by which EBNA1 may contribute to the survival of EBV-infected cells. USP7 has emerged as a key regulator of the p53 tumor suppressor (Lee and Gu). Deubiquitination of p53 by USP7 protects p53 from proteasome-mediated degradation and promotes p53 function in apoptosis and cell cycle arrest (Li et al., 2004). EBNA1’s interaction with USP7 can block p53 stabilization by USP7, inhibit p53-dependant apoptosis and thus promote cell survival (Saridakis et al., 2005; Sivachandran et al., 2008). As additional substrates for USP7 continue to be identified (Li et al., 2004; Meulmeester et al., 2005a; Song et al., 2008a; Tang et al., 2006; van der Horst et al., 2006), the full impact of EBNA1 interaction with USP7 should be revealed.

Additionally, the proteomics approaches revealed that EBNA1 interacted with protein-arginine methyl transferases PRMT1 and PRMT5 and casein kinase 2 (CK2). These proteins can modify EBNA1 and possibly regulate its functions ((Shire et al., 2006), Kathy Shire, Jennifer Cao and Lori Frapier, unpublished observations). Reciprocally, EBNA1 can affect the cellular functions of these proteins and contribute to host cell transformation. For instance, CK2 phosphorylates the PML proteins and marks them for polyubiquitination and degradation (Scaglioni et al., 2006). PML proteins associate to form PML nuclear bodies that mitigate several key processes such as apoptosis, senescence, DNA damage repair and anti-viral
response (Salomoni et al., 2008). Interestingly, EBNA1 expression in nasopharyngeal carcinoma cells was found to lower PML protein levels and compromise PML-associated functions such as DNA-damage repair and apoptosis (Sivachandran et al., 2008). Subsequently EBNA1 expression was also found to promote phosphorylation of PML proteins, likely because of its interaction with CK2 (Niro Sivachandran and Lori Frappier, in preparation). Thus EBNA1 potentially usurps CK2 functions to misregulate PML stability. Moreover, a role for PRMT5 in methylating p53 and regulating its apoptotic function has also been documented (Jansson et al., 2008). It is conceivable that this function of PRMT5 may also be subject to hijack by EBNA1.

1.3 THE UBIQUITIN SYSTEM

Work discussed in this thesis focuses on the functions of the ubiquitin specific protease, USP7. Though the functions of USP7 I have discovered are independent of its deubiquitinating activity, the reversible process of ubiquitination remains an important aspect of USP7 function and its ability to regulate various processes. Thus I will now briefly review the process of ubiquitination and its role in regulation of protein function.

1.3.1 Ubiquitin and the ubiquitin transfer mechanism

Ubiquitin is a 76 amino acid protein with a molecular mass of about 8.5kDa. Ubiquitin is not found in archaeabacteria or eubacteria, but is ubiquitously present and conserved in eukaryotes, hence its name, ubiquitin. Covalent attachment of ubiquitin to proteins is one of the numerous forms of post-translational modification and serves to regulate the stability, cellular localization and function of modified proteins. Attachment of ubiquitin takes place in several steps and involves at least three distinct types of enzymes (Hershko et al., 1983). First, the ubiquitin is adenylated at its C-terminus by the E1 activating enzyme and then coupled to an E1 cysteine side chain via a thioester bond. The activated ubiquitin is then transferred to an E2 conjugating enzyme. Finally an E3 ligase transfers the ubiquitin from the E2 to the ε-amino group of a lysine residue in a target protein. Most organisms have just one E1 and several E2 conjugating enzyme. There are, however, numerous E3 ligases. Fittingly, this variety of E3 ligases confers substrate specificity on the final step of ubiquitin transfer to target proteins.

1.3.1.1 Monoubiquitination
Monoubiquitination is the attachment of a single ubiquitin molecule to one or more lysines in a protein. Unlike polyubiquitination which controls a protein’s stability (see below), monoubiquitination is a reversible signaling modification and regulates other aspects of protein function, such as cellular localization or activity.

The diverse consequences of monoubiquitination make it akin to phosphorylation in signaling. For example, attachment of a single ubiquitin to one lysine or multiple lysines (mult ubiquitination) not only regulates trafficking of membrane proteins but also their internalization into the endocytic pathway and lysosomal degradation (d’Azzo et al., 2005). Studies in yeast show an important role for monoubiquitination in translesion DNA synthesis after DNA damage (Stelter and Ulrich, 2003). Proliferating cell nuclear antigen (PCNA), which is a processivity factor for DNA replication, is monoubiquitinated at K164 in both yeast and humans. This initial modification primes K164-conjugated ubiquitin for addition of multiple ubiquitin moieties through K63 linkages, which are important for PCNA function in error free repair (Hoege et al., 2002). Furthermore, the p53 transcription factor is not only polyubiquitinated by its predominant ubiquitin ligase, Mdm2, but also monoubiquitinated (Li et al., 2003). Monoubiquitination leads to accumulation of p53 in the cytoplasm, thereby restricting its function as a transcription factor in the nucleus. On the other hand, nuclear localization and transcriptional activity of another transcriptional factor, FOXO4, is induced by monoubiquitination after oxidative stress (van der Horst et al., 2006). Interestingly, monoubiquitination of both p53 and FOXO4 is reversible by USP7. Thus monoubiquitination mirrors phosphorylation in a signaling pathway in that it is not only reversible but can have both stimulatory or inhibitory effects on protein function.

1.3.1.2 Modification of histones by Monoubiquitination

One of the most commonly known examples of regulation by monoubiquitination is that of histones H2A and H2B. In Saccharomyces cerevisiae, H2B is monoubiquitinated by the Rad6 E2 conjugase and the Bre1 E3 ligase (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003). In mammals HR6A and HR6B have been implicated to fulfill a role similar to Rad6 in S. cerevisiae (Koken et al., 1991; Roest et al., 1996; Sun and Allis, 2002). Homologs of Bre1 in Drosophila and human cells have also been identified as H2B-specific E3 ligases (Bray et al.,
Ubiquitin conjugase UbcH6 is implicated in H2B ubiquitination and works with the RNF20/RNF40 complex, a major E3 ligase complex in mammalian cells (Zhu et al., 2005). Although the ubiquitin conjugases and ligases are essential for H2B monoubiquitination, studies suggest they are not sufficient. Several accessory proteins involved in transcription initiation and elongation are required for recruitment of the ubiquitin attachment complexes (Weake and Workman, 2008). H2B monoubiquitination by Bre1 ligase on K123 promotes dimethylation and trimethylation of histone H3 on K4 and K79, but not monomethylation (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Osley, 2006; Shahbazian et al., 2005; Sun and Allis, 2002). This series of histone modifications is conserved among eukaryotes and correlates with increased transcriptional activation. H2B monoubiquitination can promote transcription initiation and elongation and interestingly can do so in a methylation-independent manner (Fleming et al., 2008; Minsky et al., 2008; Tanny et al., 2007; Zhu et al., 2005). Conversely, evidence also exists for an inhibitory role of H2B monoubiquitination in transcription (Minsky and Oren, 2004; Shema et al., 2008; Wyce et al., 2007; Zhao et al., 2008).

Histone H2A is monoubiquitinated by the Ring1B ubiquitin ligase in human cells, and this modification is associated with gene silencing (Cao et al., 2005; Wang et al., 2004; Zhou et al., 2008). Ring1B is part of at least three distinct complexes, the polycomb complex PRC1, E2F-6 and FBXL10-BcoR, which may repress expression in a gene-specific manner (Cao et al., 2005; Gearhart et al., 2006; Ogawa et al., 2002; Sanchez et al., 2007; Wang et al., 2004). Ring1B-containing complexes are not the only ones involved in H2A ubiquitination. Another H2A ubiquitin ligase, 2A-HUB, is part of another complex formed on a portion of chemokine genes and represses their expression by hindering RNA polymerase II elongation (Zhou et al., 2008).

Like other ubiquitination events, histone monoubiquitination is also reversible, and several histone deubiquitinating enzymes have been discovered. In yeast, one deubiquitinating enzyme for ubiquitinated H2B (ubH2B) is Ubp8, which works as part of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (Daniel et al., 2004; Henry et al., 2003). Orthologs of Ubp8 in Drosophila and humans are Nonstop and USP22, respectively, and work with their
corresponding STAGA HAT complexes (Weake et al., 2008; Zhang et al., 2008). Interestingly, deubiquitination of ubH2B by Ubp8 and its orthologs is associated with transcription activation, consistent with a negative role for monoubiquitinated H2B in transcription, at least at certain loci. Another yeast H2B deubiquitinase is Ubp10, which works independently of the SAGA complex (Emre et al., 2005; Gardner et al., 2005) and, unlike Ubp8, is involved in gene silencing. Interestingly, it may not be the deubiquitinase activity of Ubp10 that is responsible for gene silencing, as certain mutations in Ubp10 compromise its gene silencing function and result in increased H2B ubiquitination without affecting its deubiquitinase activity (Gardner et al., 2005). Additionally, deletion of both UBP8 and UBP10 showed increased expression of nontelomeric genes compared to when either of the two was deleted individually. This suggest that Ubp8 and Ubp10 may work complementarily at certain loci to inhibit gene expression (Gardner et al., 2005). In Arabidopsis thaliana SUP32/Ubp26 is implicated in H2B deubiquitination, DNA and H3 K9 methylation and heterochromatic silencing (Sridhar et al., 2007). Recently in Drosophila, the product of the scrany gene (SCNY) was shown to encode an H2B deubiquitinase, which shows homology with yeast Ubp10 and human USP3, deubiquitinates ubH2B and is required to maintain Drosophila stem cells (Buszczak et al., 2009). Additionally, the Drosophila USP7 deubiquitinase forms a complex with GMP synthetase (GMPS) that deubiquitinates ubH2B and is implicated in Polycomb-mediated gene silencing (van der Knaap et al., 2005). In humans the process of histone deubiquitination is only beginning to be understood and both H2B and H2A have been found to be deubiquitinated by USP3 (Nicassio et al., 2007). As discussed later, observations made during the course of this thesis will further define the role of the deubiquitinase USP7 in H2B deubiquitination in human cells.

1.3.1.3 Polyubiquitination
A remarkable aspect of modification with ubiquitin is the ability to form a chain of sequentially linked ubiquitin moieties. Once a ubiquitin molecule is attached to a target protein, lysines in the attached ubiquitin can serve as sites for subsequent conjugation of more ubiquitin molecules. There are up to 7 lysines in ubiquitin from budding yeast and humans and at least five of these, K6, K11, K29, K48 and K63, are known to form linkages (Arnason and Ellison, 1994;
Baboshina and Haas, 1996; Chau et al., 1989; Koegl et al., 1999; Mastrandrea et al., 1999). K48 of a conjugated ubiquitin is covalently linked to G76 of an incoming ubiquitin in an isopeptide bond (Chau et al., 1989). This chain acts as a signal for degradation of the modified protein in an ATP-dependent manner by a multisubunit and multicatalytic protein complex known as the proteasome (Hershko and Ciechanover, 1998). This process of protein degradation was initially thought to recycle only defective and misfolded proteins, but later proved to be a mechanism that regulates diverse cellular processes including the cell cycle (Koepp et al., 1999), induction of the inflammatory response (Ghosh et al., 1998) and antigen presentation (Rock and Goldberg, 1999). While K48-conjugated polyubiquitin chains may work as a universal degradation signal, it is not the only configuration of ubiquitin conjugation. Polyubiquitin chains generated by linkages of lysines other than K48 are implicated in processes other than proteolysis (Pickart, 2001).

1.3.1.4 E3 Ligases
There are potentially 2 E1 and 30 E2 enzymes encoded by the human genome (Li et al., 2008). By contrast over 600 ubiquitin ligases are thought to be encoded by the human genome, even surpassing even the number of protein kinases (Deshaises and Joazeiro, 2009; Li et al., 2008). This large number of ubiquitin ligases is not only consistent with their ability to provide specificity for diverse targets but also with the involvement of ubiquitination in diverse cellular processes. Based on the presence of conserved domains, ubiquitin ligases can be classified into two major types: the HECT (homologous to E6-AP carboxy terminus) E3 ligases (Huibregtse et al., 1995) and the RING (really interesting new gene)-finger domain E3 ligases (Barlow et al., 1994; Borden et al., 1995; Freemont et al., 1991). Only about 28 of the estimated ubiquitin ligases belong to the HECT family, whereas the majority are RING E3 ligases. Not all RING domain-containing proteins function as ubiquitin ligases, a prominent example is the MdmX protein (Shvarts et al., 1996). Found at the C-terminus of proteins, the HECT domain consists of about 350 amino acids and was first discovered in the human papilloma virus E6-associated protein (E6AP) (Huibregtse et al., 1995). In HECT E3ligases a conserved cysteine residue forms an intermediate thioester bond with ubiquitin before a transfer is made to the target protein (Scheffner et al., 1995). The HECT domain comprises two lobes connected by a hinge loop, where one lobe (N-terminal) binds the E2 and the other lobe (C-terminal) contains the conserved
cysteine that conjugates with ubiquitin (Huang et al., 1999). Substrate recognition takes place via domains other than the HECT domain in the E3. HECT domain E3s can either work alone or in complex with accessory or adaptor proteins. The RING domain E3 ligase activity depends upon binding two zinc ions and while ubiquitin transfer can involve accessory proteins, RING E3s transfer the ubiquitin from the E2 to the substrate without an intermediate thioester linkage (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999). The RING domain, in addition to having intrinsic ligase activity, also binds the E2 conjugase (Joazeiro et al., 1999; Lorick et al., 1999; Zheng et al., 2000). The two zinc coordination sites separated by a helix provide a shallow groove for E2 binding (Zheng et al., 2000). How exactly RING E3s ubiquitinated the substrates is not precisely known, though it is believed that instead of an active catalytic role of the RING domain, it is the proximity of the ub-E2 and the substrate and the activating conformational changes upon binding the RING E3 that facilitate the process (Deshaies and Joazeiro, 2009).

1.3.1.5 Deubiquitinating Enzymes

Ubiquitination, like phosphorylation, is a reversible modification. Reversal of ubiquitination, or deubiquitination, is carried out by deubiquitinating enzymes (DUBs), which belong to the metallo-cysteine families of proteases (Nijman et al., 2005). About 100 putative DUBs are encoded by the human genome and can be roughly grouped into five subfamilies (Reyes-Turcu et al., 2009). Four of these are cysteine proteases, including the ubiquitin C-terminal hydrolases (UCHs), the ubiquitin specific proteases (USPs/UBPs), the ovarian tumor domain (OTU) DUBs and the Josephin domain (MJD) DUBs. The fifth subfamily is the JAMM domain zinc-dependent DUBS, which are metalloproteases.

DUB activity regenerates ubiquitin to maintain the ubiquitin pools. Since ubiquitin is expressed as a proprotein, either fused to two ribosomal proteins or as a linear chain of polyubiquitin, DUBs activate ubiquitin by processing the proprotein to generate ubiquitin monomers and removing additional residues at the C-terminus (Baker and Board, 1987; Ozkaynak et al., 1987; Wiborg et al., 1985). DUBs rescue ubiquitin molecules sequestered by sporadic amides and thio-esters and small ubiquitin peptides generated as a byproduct of protein degradation (Pickart and Rose, 1985). DUB activity on proteins arriving at the proteasome
allows for recycling ubiquitin molecules, and a number of DUBs from various subfamilies form components of the 19S regulatory complex of the proteasome. These include UCH-L5, USP14 and the JAMM protease POH1 (Borodovsky et al., 2001; Lam et al., 1997; Park et al., 1997; Verma et al., 2002). In fact, deubiquitination at the proteasome is required to allow proteins access to the catalytic core and thus DUB activity is tied to the degradation process.

By reversing the actions of ubiquitin ligases, DUBs offer a way to fine tune the effects of ubiquitination as a post-translational modification. Thus the role of DUBs, often found in multiprotein complexes, extends beyond housekeeping of the ubiquitin system and extends to a range of processes such as cell cycle progression, DNA repair, apoptosis, chromatin structure and transcription, kinase cascades in signal transduction and endocytosis (Reyes-Turcu et al., 2009). For instance, USP28 stabilizes Chk2 kinase and 53BP in response to double-stranded DNA breaks and is important for DNA damage-induced apoptosis (Zhang et al., 2006). Dismantling of K63-linked polyubiquitin chains from TRAF2 and TRAF6 by the DUB CYLD and from IκB-kinase subunits by A20, inhibits activation of the NFKβ (Kovalenko et al., 2003; Trompouki et al., 2003; Wertz et al., 2004). Moreover, deubiquitination of epidermal growth factor receptor (EGFR) by USP8 inhibits its endocytosis (Mizuno et al., 2005; Row et al., 2007). As mentioned above, several DUBS have been identified that can deubiquitinate both H2A and H2B indiscriminately or either H2A or H2B specifically (Reyes-Turcu et al., 2009). Finally, USP7, the DUB which is the focus of this thesis, is involved in many key cellular processes which I will outline in detail below.

1.4 UBIQUITIN SPECIFIC PROTEASE 7 (USP7)

USP7 was first discovered as a 135kDa protein associated with the herpes simplex virus (HSV) protein ICP0, also known as Vmw110 (Meredith et al., 1995; Meredith et al., 1994). Subsequent sequence analysis revealed the ICP0-associated protein to have conserved motifs belonging to the ubiquitin specific protease (USP) family (Everett et al., 1997b) and hence the 135kDa protein was termed herpes virus associated ubiquitin specific protease, or HAUSP. ICP0 expression leads to transcriptional activation of viral genes and degradation of several cellular proteins, including PML (Everett et al., 1998), the catalytic subunit of DNA protein kinase (Parkinson et al., 1999) and the kinetocore protein CENP-C (Everett et al., 1999a). ICP0
is important for the onset of lytic HSV replication and the switch from latent to lytic infection. ICP0 is a ubiquitin ligase that ubiquitinates itself, and interaction with USP7 protects ICP0 from autoubiquitination and degradation (Boutell et al., 2005; Boutell and Everett, 2003; Boutell et al., 2002; Canning et al., 2004).

Although full length USP7 migrates at the expected size of 135kDa in SDS-PAGE analysis, a slower migrating form that reacts with USP7-specific antibodies also exists, though the nature of this form is presently unknown (Antrobus and Boutell, 2008). USP7 is predominantly pan-nuclear, with a fraction of nuclear USP7 associating with nuclear bodies formed by the PML proteins (PML-NBs) (Everett et al., 1997b; Holowaty et al., 2003b). A small pool of USP7 is also found in the cytosol (Marchenko et al., 2007).

1.4.1 Deubiquitination by USP7

USP7 is a 1102 amino acid protein with four independent domains resistant to trypsinization (Figure 1-4) (Holowaty et al., 2003a). Residues 208 to 560 were identified as the catalytic core domain and crystallized in isolation and in combination with ubiquitin aldehyde (Ubal) (Hu et al., 2002). The catalytic domain unbound by Ubal resembles an extended right hand consisting three structural elements: the fingers, the palm and the thumb (Figure 1-5). The palm and the thumb meet to form the catalytic cleft. All USPs have Cys and His boxes which contain the conserved triad of Cys, His and Asp residues that facilitate the catalytic reaction. The deprotonated Cys nucleophilically attacks the carbonyl carbon of G67 in ubiquitin at the scissile bond. Deprotonation is facilitated by the His, which is further stabilized by the Asp. In USP7 this triad is found within the catalytic cleft and formed by C223S and likely H464 and D481. A binding groove is formed between the tip of the fingers and the palm-thumb scaffold and appears ideal for binding a ubiquitin molecule, orienting its C-terminus perfectly towards the catalytic cleft. This groove is indeed shown to be bound by Ubal in the structure of the catalytic core in complex with Ubal.

Domains upstream and downstream of the catalytic domain are not involved in catalysis but mediate protein-protein interactions and provide substrate recognition (Figure 1-4).
Figure 1-4. A schematic of domain organization of USP7. Four trypsin resistant independent domains of USP7 were identified by Holowaty et al (Holowaty et al., 2003a). Pertinent regions of USP7 including the N-terminal domain (NTD), the catalytic domain (CAT) and the point mutation that abrogates catalytic activity (C223S) are indicated. Also indicated are some of the proteins known to interact with individual domains, prior to completion of this thesis.
Figure 1-5. Structure of the catalytic domain of USP7. Crystal structure of the 40 kDa catalytic domain of USP7 is shown without ligand (PDB ID 1NB8) (Reprinted from Hu et al., 2002, with permission from Elsvier). Comparison to an extended palm is illustrated with the indication of the three structural elements: fingers (green), palm (blue) and the thumb (red). Also indicated are the catalytic cleft and the ubiquitin binding groove.
The N-terminal domain (NTD), is a TRAF domain and spans residues 1 – 208 (Saridakis et al., 2005; Zapata et al., 2001). This domain not only interacts with EBNA1, but also with several proteins in the p53 pathway (see below). Sequences downstream of the catalytic domain form two independent domains, spanning residues 560 – 800 and 800 – 1102. While no structural information exists for these domains, the one immediately downstream of the catalytic domain is known to bind ICP0 (Holowaty et al., 2003a), whereas the domain spanning residues 800 to 1102 is required for binding Ataxin 1 (Hong et al., 2002).

1.4.2 USP7 is a key regulator of the p53 molecular network

Since the identification of USP7 as an ICP0-interacting cellular protein, several USP7-interacting proteins have been discovered, though not all appear to be substrates for deubiquitination. USP7 has been shown to regulate the p53 pathway in multiple ways. Initially, USP7 was found to interact specifically with and deubiquitinate p53, resulting in its stabilization (Li et al., 2002). This promoted p53-mediated growth arrest and apoptosis in response to DNA damage. Subsequently, however, the role of USP7 in p53 regulation was found to be more complex than originally thought. Whereas decreased USP7 expression levels had the expected effect of destabilizing p53, ablation of USP7 expression was found to have the opposite effect, resulting in p53 stabilization (Li et al., 2004). This stabilization of p53 seems to be due to the increased ubiquitination and destabilization of Mdm2, the E3 ligase largely responsible for the ubiquitination of p53 (Cummins et al., 2004; Cummins and Vogelstein, 2004; Li et al., 2004). This implicated Mdm2 as a second direct target of USP7, an assumption supported by co-immunoprecipitation of the two proteins, in vitro deubiquitination of Mdm2 by USP7 and stabilization of Mdm2 in vivo by USP7 (Li et al., 2004). Another connection of USP7 to the p53 pathway has been reported with the identification of MdmX as a USP7 target; MdmX inhibits transcription activation by p53 and is targeted for degradation by Mdm2 (Meulmeester et al., 2005a; Meulmeester et al., 2005b). Under steady state conditions, Mdm2 and MdmX are predominantly bound by and stabilized by USP7 (Meulmeester et al., 2005a). Only after post-translational modifications of Mdm2 and MdmX is their interaction with USP7 disrupted, leaving USP7 free to interact with and stabilize p53 (Meulmeester et al., 2005a; Meulmeester et al., 2005b). USP7 also interacts with the human Daxx protein, an interaction that stabilizes the
USP7-Mdm2 interaction and facilitates the deubiquitination and stabilization of Mdm2 (Tang et al., 2006). Additionally the deubiquitinase activity of USP7 and ubiquitin ligase activity of Mdm2 reciprocally regulate Daxx stability (Tang et al.). Another protein, RASSF1A, antagonizes the function of Daxx, disrupts the USP7-Daxx-Mdm2 complexes and thus leads to destabilization of Mdm2 (Song et al., 2008b).

1.4.3 USP7 regulates diverse processes outside the p53 pathway

USP7 function is not limited to its involvement in stress-induced p53 function. For instance, the ability of Daxx to function as a facilitator of USP7 activity towards substrates extends beyond Mdm2 deubiquitination. Daxx also coordinates removal of monoubiquitin by USP7 from specific lysines in another tumor suppressor, PTEN (Song et al., 2008a). This removal leads to the exclusion of PTEN from the nucleus, thus inhibiting its function, but is antagonized by the PML protein and PML-NBs, where interestingly all three proteins partially colocalize. USP7 also regulates trafficking of the FOXO4 (Forkhead box O) transcription factor (van der Horst et al., 2006). In response to oxidative stress, FOXO4 is initially monoubiquitinated, promoting its nuclear translocation and transcriptional activity. This is subsequently counteracted by USP7, which removes monoubiquitin from FOXO4, promotes nuclear exclusion and inhibits FOXO4 transcriptional activity, possibly as a feedback mechanism. Interestingly, FOXO4 is also ubiquitinated by Mdm2 (Brenkman et al., 2008). Thus a trend has emerged in which USP7 works with ubiquitin ligases to either coordinate ubiquitination of that ligase or of a common substrate. Indeed, USP7 also counteracts ubiquitination of at least two more ubiquitin ligases besides Mdm2 and ICP0. Once such E3 ligase is MARCH7 (Nathan et al., 2008), which plays a role in neural development and the immune system (Baker et al., 1997; Metcalfe, 2005). Another E3 ligase deubiquitinated and stabilized by USP7 is Chfr, which plays an important role in cell cycle progression (Oh et al., 2007). Chfr, in-turn, polyubiquitinates Plk1 and Aurora A proteins, which regulate mitotic progression. Finally, in two independent studies, USP7 was found associated with the proteasome complex (Besche et al., 2009; Bousquet-Dubouch et al., 2009).
1.4.4 The role of USP7 in Epstein-Barr virus-induced carcinogenesis

The discovery that another herpes viral protein, EBNA1, also interacted with USP7 not only further justified the title of USP7 as a herpes virus associated USP (HAUSP), but also offered a possible mechanism for EBV-mediated immortalization of cells. Biochemical characterization of the EBNA1-USP7 interaction revealed that EBNA1 bound the N-terminal domain of USP7 (USP7-NTD), residues 1 – 205. Although p53 also binds this domain, EBNA1 interacts with USP7-NTD with 10-fold higher affinity than does p53 (Holowaty et al., 2003a; Hu et al., 2002). Indeed, EBNA1 could compete with p53 for USP7 and displace p53 from the USP7-NTD, suggesting that binding of EBNA1 and p53 to the USP7-NTD was mutually exclusive (Holowaty et al., 2003a). These biochemical observations were further substantiated by studies that revealed the structure of the USP7-NTD bound by an EBNA1 peptide and a p53 peptide (Hu et al., 2006; Saridakis et al., 2005) (Figure 1-6). These structural studies confirmed previous bioinformatics analysis that predicted the USP7-NTD to be a TRAF domain (Zapata et al., 2001). Like other TRAF domains, the USP7-NTD forms an eight-stranded antiparallel β sandwich. EBNA1 and p53 peptides bind the same groove in USP7-NTD and in a manner analogous to other TRAF-peptide interactions. However, since they bind the same groove, EBNA1 and p53 binding would be mutually exclusive. Interestingly however, while other TRAF-binding peptides generally bind in a perpendicular manner cutting across the β sandwich, EBNA1 peptide deviates in conformation from other TRAF-binding peptides and almost runs parallel along strand β7, making specific contacts, as shown in comparison to TRAF6 bound to a CD40 peptide (Figure 1-6). As such, EBNA1 makes more extensive contacts than p53 with the USP7-NTD, an observation supported by NMR-chemical shift analysis of the two peptides bound to the USP7-NTD (Saridakis et al., 2005). This likely explains the higher affinity of EBNA1 for the USP7-NTD.

Thus, the biochemical and structural analysis suggest that EBNA1 could potentially compete with p53 in vivo for USP7 binding and interfere with p53 stabilization and function. In support of this, it was shown that WT EBNA1, but not a USP7-binding mutant of EBNA1, Δ395 – 450, could inhibit p53-induced apoptosis in small cell lung carcinoma cells, H1299 and the osteosarcoma cell line U2OS (Saridakis et al., 2005). My work during the course of this thesis
Figure 1-6. Comparison of the USP7-EBNA1 interaction with the USP7-p53 and TRAF6-CD40 interactions. Transparent surface representation of USP7 bound to EBNA1 peptide (PDB ID 1YY6) (A), USP7 bound to a p53 peptide (PDB ID 2KVR) (B) and TRAF6 bound to CD40 peptide (PDB ID 1LB6) (C).
has substantiated the initial observations that EBNA1 can alter the levels and function of p53 through USP7 and shown that this occurs in a cell background relevant to latent EBV infection.

1.5 THESIS RATIONALE

This thesis aims to i) understand how the USP7-EBNA1 interaction affects EBNA1 function, ii) understand how EBNA1 exploits USP7 for the benefit of latent EBV infection and iii) better understand and build on the known functions of USP7. Replication of the EBV genome, segregation of viral episomes and transactivation of viral latency genes are some of the established functions of EBNA1 that involve its DNA-binding activity. Nevertheless, it is not fully understood how EBNA1 interactions with cellular proteins contribute to or regulate these functions. Additionally, even though EBNA1 is implicated in EBV-mediated host cell immortalization and transformation, the mechanism for this EBNA1 function has been elusive. Like EBNA1 functions on the EBV genome, EBNA1 contributions to cell immortalization may also involve recruitment and/or usurping of cellular proteins. In this regard, the interaction of EBNA1 with USP7 has been one of the most exciting. In chapter 2 I explored how USP7 influences the DNA-binding activity of EBNA1 and its functions reliant on the EBNA1-DNA interaction. In chapter 3, I demonstrate how EBNA1 can alter the function of cellular proteins like p53 through its interaction with USP7, making the cellular environment more conducive to transformation. In chapter 4 I show a new function for USP7 in the regulation of PML proteins and PML-NBs. Finally, in chapter 5 I describe biochemical approaches I undertook to characterize USP7 interactions with Mdm2 and MdmX that helped gain structural and molecular insights into substrate recognition by USP7.
CHAPTER 2

EBNA1 RECRUITS A HISTONE H2B DEUBIQUITINATING COMPLEX TO THE EPSTEIN-BARR LATENT ORIGIN OF DNA REPLICATION

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Shang Wang performed chromatin immunoprecipitation experiments in Figure 2-5. I performed all other experiments in this chapter.
2.1 INTRODUCTION

Epstein-Barr virus (EBV) is a gamma herpesvirus that infects over ninety percent of people worldwide. As part of its latent life cycle, EBV efficiently immortalizes the host cell and predisposes it to a number of malignancies, including Burkitt’s lymphoma, nasopharyngeal carcinoma, gastric carcinoma, Hodgkin’s disease and a variety of lymphomas in immunosuppressed patients (Rickinson and Kieff, 2001). In latently infected cells, replication and maintenance of the viral genome require the latent origin of replication, oriP, and the EBNA1 protein. OriP is comprised of two functional elements, the dyad symmetry (DS) and the family of repeats (FR), which contain four and twenty copies of an 18 bp palindromic EBNA1 binding site, respectively (Rawlins et al., 1985a; Reisman et al., 1985a). Replication of oriP-containing plasmids requires EBNA1 binding to the DS (Gahn and Schildkraut, 1989). EBNA1 binding to the FR is required for the mitotic segregation of the oriP-containing plasmids and transactivation of several latency genes (Krysan et al., 1989; Reisman and Sugden, 1986).

EBNA1 binds DNA through residues 459-607, which form the DNA binding and dimerization domain (EBNA1-DBD; Figure 2-1) (Ambinder et al., 1991; Chen et al., 1993; Summers et al., 1996). High resolution structures of the EBNA1-DBD, alone and in complex with its DNA binding site, have revealed details of the interaction of EBNA1 with DNA (Bochkarev et al., 1996a; Bochkarev et al., 1995; Bochkarev et al., 1998). EBNA1-DBD comprises two subdomains: residues 504-604, referred to as the core-domain; and residues 461-503, referred to as the flanking domain. The core domain is a β-barrel structure that forms the dimerization interface and makes transient sequence-specific contacts with the DNA through an α-helix (Bochkarev et al., 1995; Cruickshank et al., 2000). The flanking domain consists of an α-helix (residues 477-489) oriented perpendicular to the axis of the DNA, which contacts the major groove through Lys 477, and an extended chain (amino acids 461-469) that runs along the base of the minor groove of the DNA, making sequence-specific contacts through Lys-461, Gly-463 and Arg-469 (Bochkarev et al., 1996a).

In addition to binding specific DNA sequences, EBNA1 is also known to interact with several host-cell proteins, which in some cases have been shown to mediate EBNA1 functions at
Figure 2-1. Crystal structure of the EBNA1 DNA binding and dimerization domains bound to DNA (PDB ID 1B3T) (Bochkarev et al., 1996b). 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).
oriP (Holowaty et al., 2003b; Lin et al., 2008; Shire et al., 1999b; Van Scoy et al., 2000; Wang et al., 1997). EBNA1 can also affect cellular processes through sequestration of cellular proteins, as best exemplified by the EBNA1 interaction with the ubiquitin specific protease USP7, also referred to as Herpesvirus Associated Ubiquitin Specific Protease (HAUSP). USP7 was originally identified as a binding partner of the ICP0 protein of herpes simplex virus (HSV) (Everett et al., 1997a) and, since then, several cellular targets of USP7 have been identified, including the p53 tumour suppressor protein (Cummins et al., 2004; Li et al., 2004; Li et al., 2002; Nathan et al., 2008; Song et al., 2008a). In response to genotoxic stress, USP7 binds and deubiquitinate p53, thereby protecting it from proteasome-mediated degradation. In addition to cleaving polyubiquitin chains, USP7 has been reported to reverse monoubiquitination in some proteins (example. p53 and FOXO4), thereby affecting their subcellular localization (Marchenko et al., 2007; van der Horst et al., 2006). Similarly, the Drosophila homologue of USP7 was found to contribute to epigenetic silencing by reversing monoubiquitination of histone H2B, and this activity required USP7 to be in complex with guanosine 5’ monophosphate synthetase (GMPS) (van der Knaap et al., 2005).

Studies on the EBNA1-USP7 interaction have shown that EBNA1 binds the N-terminal domain of USP7 (USP7-NTD), which is distinct from its catalytic domain and is the same domain that is bound by p53 (Holowaty et al., 2003b). EBNA1 and p53 bind the same pocket in this domain but EBNA1 does so with an affinity that is approximately 10-fold higher than that of p53 (Holowaty et al., 2003a; Saridakis et al., 2005). As a result, EBNA1 interferes with the binding and stabilization of p53 by USP7 and with p53-mediated apoptosis in response to DNA damage (Saridakis et al., 2005; Sivachandran et al., 2008). In addition, it was recently found that EBNA1 disrupts promyelocytic leukemia (PML) nuclear bodies (also called ND10s) in nasopharyngeal carcinoma cells by inducing the degradation of the PML proteins (Sivachandran et al., 2008). This activity required USP7 and the EBNA1-USP7 interaction, indicating that this interaction can modulate cellular events in addition to p53 levels.

EBNA1 deletion analysis showed that the USP7 binding sequence in EBNA1 was just N-terminal to the flanking DNA binding domain and subsequent peptide binding assays identified EBNA1 residues 436-450 as sufficient for this interaction (Holowaty et al., 2003b; Saridakis et al., 2005). A crystal structure of an EBNA1 peptide bound to the USP7-NTD revealed multiple
interactions of EBNA1 residues 442-448 with amino acids in a shallow groove of the TRAF domain formed by the USP7-NTD (Saridakis et al., 2005). In particular interactions mediated by Ser447 in EBNA1 were shown to be critical for USP7 binding. Given the large size of USP7 (135kDa) and the proximity of its binding site to the EBNA1-DBD residues that are inserted in the DNA minor groove (amino acids 461-469), I wondered whether the USP7 interaction interfered with EBNA1 binding to DNA. Here I report that, contrary to expectations, USP7 had a large stimulatory effect on the DNA binding activity of EBNA1 in vitro and can form a ternary complex with DNA-bound EBNA1. Furthermore, USP7 can bind GMPS, forming a complex active in histone H2B deubiquitination, and this complex was recruited to oriP in EBV-infected cells, resulting in decreased H2B ubiquitination.

2.2 MATERIALS AND METHODS

2.2.3 EBNA1 purification

EBNA1\textsubscript{395-641} was expressed fused to a hexahistidine tag at the N-terminus in Escherichia coli from plasmid pET15b. This construct was generated by PCR amplification of EBNA1 sequences encoding amino acids 395-641 from pc3\textit{oriP}EBNA1 and ligation between the NdeI and BamH1 sites of pET15b. BL21 pLysS cells containing pET15b-EBNA1\textsubscript{395-641} were grown to OD\textsubscript{600nm} of 0.5 then induced for 3hrs at 37°C by the addition of IPTG (0.1 mM final concentration). After cells were lysed in 50 mM NaH\textsubscript{2}PO\textsubscript{4} pH 8.0, 300 mM NaCl, 10 mM imidazole, 20mM β-mercaptoethanol, 0.5 mM PMSF and 1mM benzamidine, EBNA1\textsubscript{395-641} was purified on Ni-NTA Agarose resin (Qiagen) and then dialyzed against 50mM Tris pH 7.5, 300 mM NaCl, 20 mM β-mercaptoethanol, 1 mM PMSF. EBNA1\textsubscript{452-641} was purified from \textit{E.coli} as previously described (Barwell et al., 1995). EBNA1 (lacking most of the Gly-Ala repeat) was purified from insect cells as described previously (Frappier and O'Donnell, 1991b).

2.2.4 Purification of USP7 and GMPS

Full length USP7 and the USP7-NTD containing amino acids 56-205 were purified according to Holowaty et al (Holowaty et al., 2003a). GMPS was expressed in insect cells from a baculovirus. The GMPS baculovirus was constructed by PCR amplification of a full-length GMPS cDNA in pOTB7 (ATCC number 7515509) using the primers: GCAGGATCCCATATGGCTCTGTGCAACGGAGAC (N-terminus) and
GCACTCGAGTTACTCCCACCTAGTTCC (C-terminus). The amplification product was digested with BamHI and XhoI and cloned between the same sites of pFastBac HT B (Invitrogen). Bacmids were obtained by transformation of competent DH10Bac *E. coli* (Invitrogen) with GMPS pFastBac HT B, then Spodoptera frugiperda (SF9) insect cells were transfected with the bacmids to generate the baculovirus according to the manufacturer’s specifications. Culture medium containing the baculovirus was harvested 5 days post-transfection and amplified twice. To generate GMPS for purification, ten 15 cm plates of High Five cells at 80% confluency were infected with the GMPS baculovirus. Cells were harvested 50 hrs post-infection, washed with PBS and lysed in 10 ml of 20 mM Tris-HCl pH 8, 0.5 mM DTT, 0.5 mM EDTA, 10% glycerol and complete protease inhibitor cocktail (Roche). The lysate was sonicated, incubated 30 min on ice, then clarified by centrifugation at 64,000 x g for 15 min at 4°C. The clarified lysate was incubated with 250 μl of a nickel resin (Sigma) for 1h (with rotation) then transferred to a column. The resin was washed 3 times with 4 column volumes of column buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl and 10 mM imidazole) and the His-tagged GMPS was eluted from the column with column buffer containing 250 mM imidazole. EDTA and DTT were added to the elutions to a final concentration of 10 mM and the eluted protein was dialyzed overnight against 50 mM HEPES pH 7.9, 50 mM NaCl, 10% glycerol, 0.1 mM EDTA and 0.1 mM DTT, then stored in aliquots at -80°C.

2.2.5 Electrophoretic mobility shift assays (EMSAs)

DNA probes for EBNA1 EMSAs were generated by end-labeling a 20-mer oligonucleotide corresponding to site 1 of the DS element (5'-CGGGAAGCATATGCTACCCG-3') with γ-32P-ATP and annealing it to its complementary sequence. In assays containing EBNA1 and either USP7 or GMPS, EBNA1 was preincubated with USP7 or GMPS at room temperature (RT) for 10 minutes prior to adding the labeled DNA, except in Figure 2-3C, where EBNA1 was incubated with labeled DNA for 10 minutes at RT first, followed by addition of increasing amounts of USP7 and further incubation at RT for 10 minutes. In Figures 1 and 2A, 10 pmols of USP7 was used along with the indicated amounts of EBNA1. For samples containing EBNA1 and both USP7 and GMPS, USP7 and GMPS were preincubated together at 4°C for 5 minutes before the addition of EBNA1 and further incubation at RT for 10 minutes.
The EMSAs in Figure 2-6 used 2 pmol EBNA1 dimer and 64 pmols of USP7 and GMPS. Protein mixtures were incubated with 10 fmols of labeled DNA at RT for 10 minutes in the presence of 1 μg salmon sperm DNA in 20 μl binding buffer (20 mM Tris pH 7.5, 200 mM NaCl). 4 μl of 6x DNA Loading Dye (10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA; MBI Fermentas, R0611) was then added to the reactions prior to electrophoresis on a 10% polyacrylamide gel. Bands were visualized by autoradiography.

2.2.6 Chromatin immunoprecipitation (ChIP) assays performed on EBV genomes.

ChIP assays were performed for GMPS and USP7 in the EBV-positive, Raji Burkitt’s lymphoma cells as previously described (Lin et al., 2008) using anti-USP7 rabbit antibody (Bethyl Laboratories, Inc) or rabbit antiserum raised against full length recombinant GMPS purified from insect cells. Rabbit IgG (Santa Cruz) and anti-EBNA1 R4 rabbit antibody (Holowaty et al., 2003b) were also used as negative and positive controls, respectively. Quantitative real-time PCR was performed with a Platinum SYBR Green qPCR superMix-UDG (Invitrogen) in a Rotorgene qPCR System (Corbett Research), using 1/50th of the ChIP samples or 1/2500th of DNA samples prior to immunoprecipitation (input) and the previously described primer sets for the DS and FR elements and the BZLF1 promoter region (Lin et al., 2008). Values obtained for ChIP samples were normalized to input samples with the same primer sets. For ChIP assays involving USP7 depletion, D98/Raji cells (Glaser and Nonoyama, 1974) were subjected to three rounds of transfection (every 24 hours) with siRNA against USP7 or with siRNA against GFP as described above. Samples were prepared as for the ChIP experiments in Raji cells except that antibodies against EBNA1, histone H2B (Upstate Biochemicals) and mono-ubiquitinated histone H2B (MediMabs Inc, Montreal) were used. Primer sets used to assess recovery of the LMP1 promoter region were CAATCAGAAGGGGGAGTGCG and ACAGCCTTGCCCTCACCTGAAC, of Cp promoter region were AACCTTGTGCGGGAGAAC and GGCGAATTAACTGAGCTTGCG, and of oriLyt region were CGTCTTACTGCCCAGCCTACT and AGTGGGAGGGCAGGAAAT. Experiments examining EBNA1 binding to region III used the primer sets
GACCAGCTGAGGTGTACGAGGAGTGTTCCACAG and ACACCGTGCGAAAAGAAGCAC described in Yoshioka et al (Yoshioka et al., 2008).

### 2.2.7 EBNA1 ChIP assays performed on transfected plasmids

CNE2Z cells (Huang et al., 1980) were plated in 6 cm dishes and transfected with 50 pmols of siRNA against GFP or siRNA against USP7. siRNA transfections were repeated twice at 24 hour intervals for a total of 3 rounds of siRNA transfection over 72 hours. Cells were then moved to 10 cm dishes and transfected with 5 μg of pc3OriP, pc3OriPEBNA1 or pc3OriPΔ395-450 and 250 ng pLacZ plasmid containing LacZ cDNA. 24 hours post-transfection, cells were fixed with 1% formaldehyde, lysed in RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.1% Sodium Deoxycholate, 1 mM PMSF) containing protease inhibitor cocktail (Sigma, P8340) and sonicated briefly to shear the DNA. Clarified lysates were precleared with Protein A/G beads (Santa Cruz, SC-2003) prior to immunoprecipitation with EBNA1 R4 antibody and normal rabbit IgG (Santa Cruz, SC-2345). Protein cross links were reversed in the immunoprecipitated DNA by incubating at 65°C for 16hrs. DNA was purified using QIAquick Gel Extraction Kit (Qiagen, 28704) and analyzed by quantitative RT-PCR using LightyCycler 480 DNA SYBR Green I Master (Roche, 04707516001) and a Rotorgene Q-PCR system (Corbett Research). Primers used for DS are as described above. Primers used for FR and lacZ quantification were CCCGGATACAGATTAGGATAGC and TGTTGCCATGGGTAGCATA for FR and ATATTGAAACCACGGCATGGTGC and TTTGATGGAGCAGCAGC for lacZ.

### 2.2.8 Transcription activation assay

EBNA1 transactivation assays were performed as described previously (Cecarelli and Frappier, 2000b) with the following modifications. CNE2Z cells were transfected with siRNA against GFP or USP7 as described above, then were moved to 10 cm dishes 24 hour prior to transfection with 2 μg of pFRTKCAT reporter construct (kindly provided by Bill Sugden) and 180 ng of pc3OriP or pc3Orip containing expression cassettes for EBNA1 (Shire et al., 1999b) or EBNA1Δ395-450. 48 hrs later, cells were harvested and lysed using three rounds of freezing and thawing. 15 μg of total protein from each sample was assayed for chloramphenicol
acetyltransferase activity using several reaction times and results from a point in the linear range were reported.

2.3 RESULTS

2.3.1 Effect of USP7 on DNA binding by EBNA1 in vitro

I initially assessed the effect of USP7 on the DNA binding activity of EBNA1 using electrophoretic mobility shift assays (EMSAs) with a version of EBNA1 that has a shortened Gly-Ala repeat but has wildtype activity for all known EBNA1 functions (referred to as EBNA1; Figure 2-2A). Purified EBNA1 was incubated with radiolabeled DNA containing a single EBNA1 recognition site (site 1 from the DS element) in the presence and absence of excess purified full length USP7. I consistently observed that USP7 stimulated the DNA binding activity of EBNA1 as shown in the representative experiment in Figure 2-2B (left panel), while no obvious effects on EBNA1-DNA interactions were seen with nonspecific proteins such as BSA (Figure 2-2B, right panel). Results from multiple experiments showed a 20-fold increase in the DNA binding affinity of EBNA1 in the presence of USP7, resulting in a shift in the dissociation constant \((K_d)\) from 85 ± 7nM for EBNA1 alone to 4.3 ± 0.4 nM for EBNA1 in presence of USP7. This increase in DNA binding affinity was largely dependent on the ability of EBNA1 to bind USP7, as the DNA binding ability of a truncation mutant of EBNA1 (EBNA1\(_{452-641}\)) containing the DNA binding and dimerization region but lacking the USP7 binding site was much less affected by USP7 (on average showing a 4-fold increase in DNA binding in the presence of USP7; Figure 2-2C).

EBNA1 dimers bound to DNA are known to interact with each other, resulting in the crosslinking of multiple DNA fragments through large EBNA1 complexes (referred to as looping or linking interactions) (Frappier and O'Donnell, 1991a; Goldsmith et al., 1993; Su et al., 1991). These complexes are retained in the wells of the gel in EMSAs as shown in Figure 2-2B, precluding analysis of the effect of USP7 on the migration of the DNA complexes. The linking interactions of EBNA1 are mediated largely by amino acids 325-376 and to a lesser degree by EBNA1 N-terminal residues (Avolio-Hunter and Frappier, 1998; Goldsmith et al., 1993). To further evaluate the effect of USP7 on the DNA binding ability of EBNA1 without the confounding effects of DNA linking, I repeated the EMSAs with the EBNA1 truncation mutant...
Figure 2-2. EBNA1 binding to DNA is stimulated by USP7. (A). Schematic representation of EBNA1 and the EBNA1 mutants used in this study. Shown are the Gly-Ala repeat (GA), the large Gly-Arg repeat (GR), the USP7 binding site (USP7) and the flanking and core DNA binding domains. (B). and (C). EMSAs showing titrations of EBNA1 (B) or EBNA1_{452-641} (C) with a fixed amount of DNA recognition site in the presence or absence of 10 pmols of USP7 or in the presence or absence of 10 pmols BSA as a negative control (B, right panel).
395-641 (Figure 2-2A), which contains the USP7 binding site and the DNA-binding region but lacks sequences that cause DNA linking. When the DNA binding affinity of EBNA1395-641 was measured in the presence and absence of excess USP7, USP7 was consistently found to stimulate DNA binding by EBNA1395-641 (Figure 2-3A, left panel), resulting in a 50-fold decrease in the calculated $K_d$ from 233 ± 76 nM for EBNA1395-641 alone to 4 ± 1.8nM for EBNA1395-641 in the presence of USP7. This experiment also showed that the bound DNA migrated more slowly in the presence of EBNA1395-641 and USP7 than with EBNA1395-641 alone, suggesting that USP7 formed a ternary complex with EBNA1395-641 and DNA.

Since EBNA1 is known to bind to the N-terminal TRAF domain of USP7 (USP7-NTD) (Holowaty et al., 2003a; Saridakis et al., 2005), I examined whether this domain was sufficient to stimulate EBNA1395-641 binding to DNA. When EBNA1395-641 titrations were performed in the presence of excess USP7-NTD, the DNA binding activity was increased 8 to 16-fold in multiple experiments, (Figure 2-3A, right panel), indicating that the USP7-NTD was partially, but not completely, responsible for the stimulatory effect of USP7 on EBNA1 DNA binding activity. Consistent with the USP7 result, the USP-NTD was found to decrease the migration of the EBNA1-bound DNA, suggesting that it can bind the EBNA1-DNA complex.

I also examined the stimulatory effect of USP7 on DNA binding by EBNA1395-641 by incubating a fixed amount of EBNA1395-641 (sufficient to bind a small fraction of the DNA probe on its own) with increasing amounts of USP7 prior to the addition of the DNA binding site Figure 2-3B, left panel). EMSAs performed in this way showed that USP7 had a dose-dependent effect on the DNA binding activity of EBNA1395-641. The possibility that USP7 itself had some ability to bind the DNA probe was tested by titrating USP7 with the DNA in the absence of any EBNA1, but USP7 alone did not shift the DNA probe even at very high concentrations of USP7 (Figure 2-3B, right panel lanes 8-12). Similarly, the USP7-NTD on its own did not bind the DNA-probe (Figure 2-3B, right panel lanes 1-7).

The experiments in Figure 2-3A indicated that USP7 can bind the EBNA1-DNA complex resulting in a supershift, while the titration performed with lesser amounts of USP7 in Figure 2-3B did not show a supershift. To investigate this discrepancy, I preformed EBNA1-DNA
Figure 2-3. Analyses of the USP7 effect on DNA interactions of EBNA\textsubscript{395-641}. (A) EMSAs showing titrations of EBNA\textsubscript{395-641} with a fixed amount of DNA recognition site in the presence or absence of 10 pmols of USP7 (left panel) or USP7-NTD (right panel). (B) EMSAs performed with a fixed amount of EBNA\textsubscript{395-641} and DNA and the indicated amounts of USP7 (left panel). Titrations of USP7 and the USP7-NTD with DNA in the absence of EBNA1 are shown in the right panel. (C) Complexes of EBNA\textsubscript{395-641} and DNA were preformed then incubated with the indicated increasing amounts of USP7. Complexes formed as in lanes 1, 2 and 7 were then incubated with anti-EBNA1 antibody (R4) prior to polyacrylamide gel electrophoresis.
complexes (using EBNA1\textsubscript{395-641} as above) then added increasing amount of USP7 (Figure 2-3C). EMSAs confirmed that USP7 was able to supershift the EBNA1\textsubscript{395-641}-DNA complex but only at higher concentrations of USP7 (compare lanes 6 and 7 to lanes 2-5). To confirm that the supershifted band contained EBNA1, complexes formed as in lanes 2 and 7 were incubated with an EBNA1-specific antibody prior to electrophoresis. In both cases the antibody supershifted the bands to the gel wells, whereas no effect of the antibody was seen on the migration of the DNA probe in the absence of EBNA1 (Figure 2-3C, lanes 8-10). The results indicate that USP7 can form a ternary complex with DNA-bound EBNA1 under some conditions.

### 2.3.2 Effects of USP7 silencing on EBNA1-DNA interactions in vivo

During initial EBV infection, EBNA1 assembles on its recognition sites in oriP and remains stably bound to these sites in all types of latently infected cell lines. Therefore it was not possible to determine the effects of USP7 on EBNA1 assembly on oriP using latently infected cells. Instead, I assessed the effect of USP7 on the initial association of EBNA1 with oriP by treating EBV-negative nasopharyngeal carcinoma cells (CNE2Z) with siRNA against USP7 or GFP (negative control) and then transfecting these cells with an oriP plasmid expressing EBNA1 or an EBNA1 mutant (Δ395-450) that is specifically defective in binding USP7 (Holowaty et al., 2003b) and a plasmid lacking EBNA1 binding sites (pLacZ) as control for nonspecific DNA binding. Chromatin immunoprecipitation (ChIP) assays were then performed using EBNA1-specific antibodies to assess the degree of EBNA1 association with the oriP FR and DS elements and lacZ (negative control) as compared to nonspecific rabbit IgG. EBNA1 was readily detected on both the DS and FR elements after siGFP treatment but the association with both elements was greatly decreased by USP7 silencing (Figure 2-4A, middle panels). As expected, there was little association of EBNA1 with lacZ and this was unaffected by USP7 silencing (right panel). Consistent with the in vitro results, Δ395-450 bound less efficiently to both the DS and FR elements than did wildtype EBNA1, despite being expressed at equivalent levels as EBNA1 (see Figure 2-4A left panel). Moreover, unlike wildtype EBNA1, the interaction of Δ395-450 with the FR and DS elements was not affected by USP7 silencing. Therefore I conclude that USP7 can stimulate the assembly of EBNA1 on oriP elements in vivo.
Figure 2-4. Effects of USP7 silencing on EBNA1-DNA interactions in vivo. (A) CNE2Z cells were treated with siRNA against USP7 or GFP then co-transfected with pLacZ and with an oriP plasmid expressing EBNA1 or Δ395-450 as indicated or empty oriP plasmid (oriP). Equal amounts of cell lysates were analyzed for protein expression by Western blotting (left panel) and ChIP assays were performed with EBNA1 and nonspecific antibodies for the DS and FR elements of oriP and for the lacZ gene. Results are shown after normalization to nonspecific IgG and input DNA. (B) D98/Raji cells were transfected with siRNA against USP7 or GFP then ChIP assays were performed with EBNA1 antibodies and nonspecific antibodies (IgG) and a primer set near region III. Changes with P values less than 0.01 (**) and less than 0.05 (*) relative to siGFP samples are indicated.
In addition to binding the oriP elements, EBNA1 can interact in a more transient manner with a third region of the EBV genome (referred to as region III), consisting of two lower affinity EBNA1 recognition sites within the BamHI-Q fragment, and this interaction can negatively regulate the Qp promoter used for EBNA1 expression in some types of EBV latency (Nonkwelo et al., 1996; Rawlins et al., 1985a; Yoshioka et al., 2008). Due to the transient nature of the EBNA1 interaction with region III, I asked whether USP7 might promote the EBNA1-region III interaction in latently infected cells. D98/Raji cells were used for these experiments since these EBV-infected cells are more transfectable than the Raji cells from which they were derived. D98/Raji cells were transfected with siRNA against USP7 or GFP then ChIP experiments were performed using EBNA1-specific antibody and primer sets for region III. While I did not achieve complete silencing of USP7 in these experiments (Figure 2-4B, left panel), its down-regulation was consistently found to decrease the association of EBNA1 with region III (Figure 2-4B, right panel), indicating that USP7 can also modulate EBNA1-DNA interactions in the context of an EBV infection.

2.3.3 USP7 is recruited to EBV oriP

The above in vitro analyses raised the possibility that EBNA1 may recruit USP7 to oriP in EBV-infected cells. To test this possibility ChIP experiments were conducted in EBV-positive B-lymphocytes (Raji cells). Antibodies against EBNA1 or USP7 were used to immunoprecipitate these proteins from sheared Raji DNA and compared to non-specific rabbit IgG as a negative control. Immunoprecipitates were analyzed by quantitative real-time PCR using primers specific for the DS and FR regions in oriP and for the promoter region of the BZLF gene, located 40 kb away from oriP. EBNA1 is known to be constitutively bound to the FR and DS elements (Hsieh et al., 1993; Ritzi et al., 2003) and, consistent with this, was readily detected on both the FR and DS DNA fragments (with better recovery of the DS element as has been previously observed;(Deng et al., 2005; Lin et al., 2008; Schepers et al., 2001b) but was not detected on the BZLF1 fragment (Figure 2-5A). The USP7 antibody consistently isolated more FR DNA fragment than either the DS or BZLF1 fragments (Figure 2-5A). Recovery of the FR region (but not the DS region) was significantly higher than that of the BZLF1 region with a
Figure 2-5. Chromatin IP assays for USP7, GMPS and Ub-H2B in EBV genomes. (A) ChIP experiments were performed in Raji cells using antibodies against EBNA1 (left panel), USP7 (middle panel), GMPS (right panel) and nonspecific rabbit IgG as a negative control. Recovered DNA fragments were quantified by real-time PCR using primer sets for the oriP DS and FR regions or the BZLF1 promoter region. (B) D98/Raji cells were treated with siRNA against USP7 or GFP (negative control), then ChIP experiments were performed as in A using antibodies against GMPS (right panel). Down-regulation of USP7 by siUSP7 treatment was confirmed by Western blotting, while GMPS levels were unaffected by this treatment (left panel). (C) D98/Raji cells were treated with siRNA against USP7 or GFP and ChIP assays were performed using antibodies against histone H2B and monoubiquitinated histone H2B (Ub-H2B) and primer sets for the indicated region of the EBV genome (LMP= LMP1 promoter region). Relative ratios of Ub-H2B to total H2B were determined for each treatment and the average fold increase in Ub-H2B after siUSP7 treatment (as compared to siGFP treatment) from multiple experiments is shown.
p-value of 0.0004. The results indicate that USP7 is preferentially recruited to the FR and is consistent with the higher enrichment of EBNA1 at the FR.

2.3.4 USP7 forms a complex with GMP synthetase that deubiquitinates histone H2B

USP7 is known to regulate p53 levels but this would not seem to explain why it is recruited to oriP. However, others in the Frappier lab have previously used affinity chromatography and glycerol sedimentation analysis to identify and confirm the interaction between USP7 and GMP synthetase (GMPS). A previous study reported that Drosophila USP7 formed a complex with GMPS in Drosophila embryos and that this complex deubiquitinated histone H2B, thereby contributing to polycomb-mediated silencing (van der Knaap et al., 2005). Human USP7 was also found to deubiquitinate histone H2B and this activity was also stimulated by human GMPS, consistent with the results from the Drosophila study.

2.3.5 Formation of a DNA-EBNA1-USP7-GMPS quaternary complex

I next investigated the relevance of the USP7-GMPS interaction for EBNA1, in particular whether GMPS could form part of the USP7-EBNA1-DNA complex. I examined this in two ways: First, I tested possible interactions between DNA-bound EBNA1395-641 with GMPS with and without USP7 by EMSAs (Figure 2-6). The binding of EBNA1395-641 to the DNA probe was assessed on its own or after incubation of the same amount of EBNA1 with USP7 or GMPS and the migration of the DNA complexes was assessed. As observed above, USP7 shifted the EBNA1-DNA complex to a slower migrating form indicative of a ternary complex (Figure 2-6, compare lanes 2 and 3). On the other hand, the same amount of GMPS did not alter the mobility of the EBNA1-DNA complexes (Figure 2-6, compare lanes 2 and 4). This was expected since there is no evidence of a direct interaction between EBNA1 and GMPS. However, when equal amounts of USP7, GMPS and EBNA1 were combined (the same amounts as when tested individually), and then added to the DNA, these complexes shifted to a position higher than that of the USP7-EBNA1-DNA ternary complex as shown in lanes 5 and 6 of Figure 2-6 (compare to lane 3). Identical results were obtained when GMPS was added to preformed USP7-EBNA complexes, followed by addition to DNA (Figure 2-6, lane 6). However, neither GMPS, USP7 nor GMPS+USP7 interacted with the DNA in the absence of EBNA1 (Figure 2-6, lanes 8-10).
Figure 2-6. GMPS can form a quaternary complex with USP7, EBNA1 and DNA. The indicated combinations of EBNA1<sub>395-641</sub>, USP7 and GMPS were preincubated then combined with the DNA containing the EBNA1 recognition site and EMSAs were performed as in Figure 2-3C. Excess amounts of USP7 alone or USP7 and GMPS were used relative to EBNA1<sub>395-641</sub>. In lane 6 the USP7-EBNA complex was preformed prior to the addition of GMPS then DNA. The positions of complexes formed by EBNA1 alone, EBNA1+USP7 and EBNA1+USP7+GMPS are indicated by arrowheads 1, 2 and 3 respectively. DNA incubated with the same amounts of GMPS, USP7 or GMPS+USP7 but in the absence of EBNA1 are also shown (lanes 8–10).
The results suggest that USP7 mediates an interaction between GMPS and the EBNA1-DNA complex resulting in the formation of a quaternary complex.

The possible association between USP7-GMPS complexes and EBNA1 was also examined in vivo by determining if GMPS localized with EBNA1 and USP7 on EBV chromatin. ChIP experiments performed on Raji cells showed that like USP7, GMPS was preferentially detected at the FR element of oriP over the DS element or the BZLF1 region (Figure 2-5A, right panel). This is consistent with the recruitment of the USP7-GMPS complex to the FR through EBNA1.

It was next investigated whether recruitment of GMPS to the FR was dependent on USP7, as suggested by the EMSA experiments. These experiments required down-regulation of USP7 by siRNA treatment and could not be performed in Raji cells due to their low transfection efficiency. Instead, the more readily transfectable D98/Raji fusion cells were used, which retain the EBV genomes from Raji cells (Glaser and Nonoyama, 1974). USP7 was confirmed to be down-regulated in these cells following treatment with siRNA against USP7 but not siRNA against GFP (negative control), while GMPS levels were not affected (Figure 2-5B, left panel). ChIP analysis of GMPS from these cells showed that, as in Raji cells, GMPS was preferentially localized to the FR region, and that down-regulation of USP7 resulted in decreased levels of GMPS at the FR (P value 0.01 relative to FR-siGFP samples; Figure 2-5B, right panel).

If the USP7-GMPS complex functions to deubiquitinate histone H2B, then the loss of this complex from the FR would be expected to increase the level of Ub-H2B in this region. This possibility was investigated by performing ChIP experiments with and without USP7 silencing, using an antibody that recognizes only the ubiquitinated form of H2B (Minsky et al., 2008). To control for possible differences in the amounts of histones at each region, the same experiment was performed with antibody against total histone H2B and then levels of Ub-H2B were expressed as a ratio of this value. In Figure 2-5C (left panel) the change in the fraction of Ub-H2B after USP7 silencing is shown from multiple experiments (in relation to siGFP treatment). While considerable variability was observed on the level of Ub-H2B at the BZLF1 region, it was consistently observed that USP7 silencing resulted in increased levels of Ub-H2B at the FR
and had little effect on Ub-H2B levels at the DS. These results support the model that USP7 is needed for recruitment of GMPS to the FR and subsequent deubiquitination of histone H2B.

Since EBNA1 binding to the FR is known to activate transcription from the LMP1 and Cp promoters (Gahn and Sugden, 1995; Sugden and Warren, 1989) I examined the possibility that the recruitment of the USP7-GMPS complex to the FR might also affect H2B ubiquitination at these promoters. To this end, ChIP was performed on D98/Raji cells before and after silencing USP7, using antibodies against Ub-H2B and total H2B. The recovery of the LMP1 and Cp promoter regions was quantified for each treatment and the change in the fraction of Ub-H2B after USP7 silencing was determined. Silencing of USP7 consistently resulted in increased Ub-H2B at both the LMP1 and Cp promoters, with the strongest effect on the Cp promoter, whereas H2B ubiquitination at the oriLyt region of EBV (negative control) was not affected by USP7 silencing (Figure 2-5C, right panel). These results suggest that the USP7-GMPS complex not only affects H2B ubiquitination at the FR but also at promoters controlled by the FR.

2.3.6 USP7 contributes to transcriptional activation by EBNA1

The above observations suggest that EBNA1-mediated recruitment of the GMPS-USP7 complex to the FR may contribute to transcriptional activation by this element through alteration of Ub-H2B at the FR and/or promoters under FR control. To test this possibility, I treated EBV-negative CNE2Z cells with siRNA against USP7 or GFP then co-transfected them with a reporter plasmid in which expression of chloramphenicol acetyl transferase (CAT) is under FR control and with a plasmid expressing either EBNA1, the EBNA1 Δ395-450 mutant that is unable to bind USP7 or no EBNA1 (oriP plasmid). CAT assays were then performed on each sample to assess the degree of transcriptional activation (Figure 2-7). As expected, strong transcriptional activation was seen after siGFP treatment in the presence of EBNA1 but not in its absence and, as previously reported (Holowaty et al., 2003b), Δ395-450 had slightly reduced transcriptional activity. USP7 silencing caused a significant decrease in transcriptional activation by EBNA1 (P value 0.004) but did not significantly affect transactivation by Δ395-450. These results support the model that recruitment of the USP7-GMPS complex by EBNA1 contributes to EBNA1-mediated transcriptional activation.
Figure 2-7. Effect of USP7 silencing on EBNA1-mediated transcriptional activation. CNE2Z cells were treated with siRNA against USP7 or GFP then were co-transfected with an FR-CAT reporter plasmid and an oriP plasmid expressing EBNA1, Δ395-450 or no EBNA1 (oriP). CAT assays were then performed on equal amounts of cell lysates and the percent of acetylated substrate was determined as a measure of transcriptional activation. Changes with P values less than 0.01 (**) and less than 0.05 (*) relative to siGFP samples are indicated.
2.4 DISCUSSION

EBNA1 forms a stable complex with host cell USP7 and this interaction can promote cell survival, at least in part through interfering with p53 stabilization by USP7 and through disrupting PML nuclear bodies (Holowaty et al., 2003a; Holowaty et al., 2003b; Saridakis et al., 2005; Sivachandran et al., 2008). Here I provide the first evidence that the EBNA1-USP7 interaction also contributes to EBNA1 functions at EBV oriP. This study stemmed from the unexpected observation that USP7 greatly stimulated the DNA binding activity of EBNA1 in vitro and could form a ternary complex with DNA-bound EBNA1. EBNA1 appears to be constitutively bound to oriP elements in latent EBV infections in proliferating cells (Hsieh et al., 1993; Ritzi et al., 2003) and, in these cases, the functional relevance of these observations for oriP-related functions most likely lies in the ability of USP7 to form a ternary complex with DNA-bound EBNA1, as verified at the FR element in EBV-infected cells. In keeping with this hypothesis, I found that USP7 within this complex can mediate an interaction with GMPS which promotes deubiquitination of histone H2B and that USP7 contributes to EBNA1-mediated transcriptional activation. However, I have also shown that USP7 can stimulate the assembly of EBNA1 on oriP elements in transfected plasmids, suggesting that USP7 might play a role in the initial association of EBNA1 with these elements upon initial EBV infection, and/or during the switch from the EBV latency form in nonproliferating cells, in which EBNA1 is not expressed (referred to as the latency program (Thorley-Lawson and Gross, 2004)), to latency forms in proliferating cells in which EBNA1 is expressed and bound to oriP. In addition, I have shown that USP7 can stimulate EBNA1 binding to region III in the EBV genome which, under some circumstances, negatively regulates EBNA1 expression (Nonkwelo et al., 1996; Yoshioka et al., 2008), raising the possibility of a role for USP7 in EBNA1 autoregulation from the Qp promoter.

It has been previously shown that EBNA1 residues 441-450 bind to the USP7-N terminal domain (USP7-NTD) (Holowaty et al., 2003a; Saridakis et al., 2005). The ternary complex formed between USP7 and DNA-bound EBNA1 also appears to require the interaction of the USP7-NTD with the EBNA1 441-450 region for the following two reasons: first, the USP7-NTD was sufficient to supershift the EBNA1-DNA complex; and second, USP7 did not supershift the complex formed by DNA and EBNA1_{452-641}, which lacks the USP7 binding site but retains full DNA binding activity.
However, it is curious that I observed partial but not complete stimulation of EBNA1 DNA binding by the USP7-NTD. The ability of all USP7 stable domains to bind EBNA1 has been previously assessed by examining the retention of partially proteolyzed USP7 on an EBNA1 affinity column and only the USP7-NTD was found to bind EBNA1 (Holowaty et al., 2003a). However, this does not eliminate the possibility that other regions of USP7 might have weak affinities for EBNA1. My in vitro data are consistent with a model in which the USP7-NTD binds EBNA1 residues 441-450 to bring USP7 to EBNA1, enabling subsequent weaker or less specific interactions of other regions of USP7 with the EBNA1 DNA binding or C-terminal regions (452-641). This might explain why the DNA binding activity of EBNA1$_{452-641}$ was weakly stimulated by USP7. Another possible interpretation of the in vitro data is that the interaction of the USP7-NTD with EBNA1 is stabilized by the rest of USP7 due to effects on the structure of the USP7-NTD. However, I do not think this is likely because the USP7-NTD is a TRAF domain that is stably folded in the absence of the rest of USP7 (Hu et al., 2006; Saridakis et al., 2005).

While stoichiometric amounts of USP7 were sufficient to stimulate the DNA binding activity of EBNA1, only at higher USP7 concentrations was USP7 observed to be stably associated with the EBNA1-DNA complex in vitro. This indicates that the affinity of USP7 for free EBNA1 is higher than for DNA-bound EBNA1 and that a higher effective concentration of EBNA1 or USP7 may be necessary to drive the interaction of these proteins on DNA. This conclusion is also supported by the observation that USP7 is preferentially associated with EBNA1 on the FR element over EBNA1 on the DS element of oriP. The FR element is bound by 20 EBNA1 dimers as compared to 4 EBNA1 dimers at the DS element and, in both cases, the dimers within the element interact with each other to form a larger EBNA1 complex (Frappier and O'Donnell, 1991a; Goldsmith et al., 1993). As a result, the effective concentration of EBNA1 at the FR is higher than at the DS and this may drive recruitment of USP7.

An increasing number of human cellular protein binding targets of USP7 have been identified, including p53, Mdm2, FOXO, March 7 and PTEN, all of which can be deubiquitinated by USP7 (Cummins et al., 2004; Li et al., 2004; Li et al., 2002; Nathan et al., 2008; Song et al., 2008a; van der Horst et al., 2006). Proteomic profiling of USP7 protein
interactions in the Frappier laboratory has identified GMPS as another USP7 binding partner. The interaction of USP7 with GMPS is unique in that it appears to affect the activity of USP7 for specific substrates, as opposed to being a substrate itself. This is supported by the fact that GMPS levels are not altered when USP7 is silenced (as shown in Figure 2-5B).

The finding that human USP7 forms a stable complex with GMPS and this complex is important for efficient histone H2B deubiquitination fits well with the observations of van der Knaap et al (van der Knaap et al., 2005), where Drosophila USP7 was found to co-purify with GMPS and deubiquitinate histone H2B in vivo.

ChIP assays consistently showed higher recruitment of USP7 and GMPS to the oriP FR over the DS and the BZLF1 promoter region, although some degree of interaction of USP7 and GMPS was also detected at the DS and BZLF1 regions as compared to the IgG negative control. This may indicate that these proteins are widespread on chromatin, where they could regulate multiple processes that are affected by H2B ubiquitination (Weake and Workman, 2008). Although H2B monoubiquitination has been reported to be associated with increased transcription through effects on both initiation and elongation (Fleming et al., 2008; Minsky et al., 2008; Tanny et al., 2007; Zhu et al., 2005), in some instances H2B monoubiquitination appears to inhibit transcription (Minsky and Oren, 2004; Shema et al., 2008; Wyce et al., 2007; Zhao et al., 2008). Therefore the contribution of H2B monoubiquitination to gene expression is complicated and possible contributions to other processes such as DNA replication are largely unexplored. USP7 silencing was observed to increase H2B ubiquitination at the FR as well as at LMP1 and Cp promoters and to decrease transcriptional activation from the FR element, suggesting that H2B ubiquitination is inhibitory to transcription controlled by the FR. This is consistent with previous observations that the EBNA1 mutant that fails to bind USP7 has decreased transcriptional activation function (Holowaty et al., 2003b).

The increased detection of USP7 and GMPS at the FR element and their effects on Ub-H2B levels in this region suggests that EBNA1 can employ the USP7-GMPS complex for its own purposes, at least in part by decreasing the level of Ub-H2B. In addition to functioning in transcriptional activation, the EBNA1-bound FR element mediates the segregation of the EBV
episomes in mitosis (Frappier, 2004; Krysan et al., 1989; Lin et al., 2008; Reisman and Sugden, 1986), may enhance DNA replication from the DS (Harrison et al., 1994; Reisman et al., 1985a) and causes an impediment to replication fork progression (Dhar and Schildkraut, 1991; Ermakova et al., 1996; Gahn and Schildkraut, 1989). It is conceivable that any of these processes could be affected by the state of H2B ubiquitination, since EBV genomes in latent infection are known to exist as nucleosomal arrays (Shaw et al., 1979). It has been previously shown that EBNA1Δ395-450 that does not bind USP7 has increased DNA replication activity (Holowaty et al., 2003b), suggesting that H2B monoubiquitination could promote DNA replication although other interpretations are also possible.

Histone modifications at oriP are just beginning to be examined and so far these studies have been focused on histone H3 acetylation and methylation of the oriP DS region. Acetylated histone H3 is generally enriched at the DS but a decrease was observed in late G1 that appears to account for the delayed replication of EBV genomes (Zhou et al., 2005; Zhou et al., 2009). Histone H3 dimethyl K4 was also enriched at the DS region while H3 methyl K9 was decreased at this region (Day et al., 2007; Zhou et al., 2005). The findings herein indicate that monoubiquitination of H2B is another histone modification that is modulated at oriP and that this modification is affected by EBNA1. EBNA1 binding to USP7 serves to alter cellular processes in order to facilitate cell survival. Here I have shown that USP7 interaction is not limited to soluble EBNA1 but also occurs with EBNA1 bound to EBV episomes where it could regulate the plasmid maintenance and transcriptional functions of EBNA1 in EBV latent infection.
THE USP7-EBNA1 INTERACTION ALTERS THE CELLULAR ENVIRONMENT AND PROMOTES EBV-HOST CELL TRANSFORMATION

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And


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I performed all experiments in this chapter
3.1 INTRODUCTION

In cells latently infected with EBV, replication and maintenance of the viral genome requires the latent origin of replication, oriP, and the EBNA1 protein. The oriP is comprised of two functional elements, the dyad symmetry (DS) and the family of repeats (FR), which contain four and twenty EBNA1 binding sites respectively (Rawlins et al., 1985b; Reisman et al., 1985b). Replication of EBV episomes or oriP-containing plasmids requires EBNA1 binding to the DS (Gahn and Schildkraut, 1989). EBNA1 binding to the FR is required for the mitotic segregation of oriP-containing plasmids and transactivation of several latency genes (Krysan et al., 1989; Reisman and Sugden, 1986). Evidence suggests that, in addition to carrying out functions at the oriP, EBNA1 may also directly contribute to host-cell immortalization by EBV. For instance, EBNA1 is expressed in all EBV-associated tumors and in many tumors it is the only EBV protein expressed (Rickinson, 2001). Additionally, inhibition of EBNA1 function through dominant negative effects in Burkitt’s lymphoma cells decreases survival of these cells (Kennedy et al., 2003). However, there has been a lack of molecular mechanisms that would explain the role of EBNA1 in EBV-mediated host cell immortalization. Since EBNA1 does not exhibit any enzymatic activity, it is believed that it employs cellular proteins to mediate its functions.

To date several EBNA1-interacting cellular proteins have been identified, including but not limited to members of the nucleosome assembly protein NAP1, TAF-1α, TAF-1β, the bromodomain protein Brd4 and the ubiquitin specific protease 7 (USP7) (Holowaty et al., 2003b; Lin et al., 2008; Shire et al., 1999; Shire et al., 2006; Van Scoy et al., 2000; Wang and Frappier, 2009; Wang et al., 1997). Most of these interacting partners contribute to EBNA1 replication, segregation and transactivation functions at the oriP that rely on EBNA1 DNA binding. Since EBNA1 forms stable interactions with these proteins, it is conceivable that EBNA1 can interfere with the functions of these proteins by sequestering them from their normal binding partners or usurping these proteins to cause aberrant regulation of their physiological targets or other proteins that are normally not regulated by these proteins. In doing so, EBNA1 could alter the cellular environment to be more conducive to viral persistence and oncogenic transformation.
The possibility that EBNA1 can alter the functions of cellular proteins seemed particularly intriguing when several studies showed involvement of USP7 in the regulation of a key tumor suppressor protein, p53. A study by Li et al first showed that USP7 can bind and deubiquitinate p53, causing the stabilization of p53 (Li et al., 2002). Stabilization of p53 by USP7 can subsequently lead to p53-mediated growth arrest and apoptosis (13). These findings indicated that ubiquitination of p53 is a dynamic process and that USP7 can regulate p53-mediated effects by rescuing p53 marked for ubiquitin-dependent degradation. Subsequently the role of USP7 in p53 regulation appeared to be more complex than was originally thought. USP7 was not only found to stabilize p53, but also Mdm2, which is one of the ubiquitin ligases of p53 (Cummins et al., 2004; Cummins and Vogelstein, 2004; Li et al., 2004). While complete ablation of USP7 caused the expected decrease in Mdm2 levels, it was accompanied by a concomitant increase in p53 levels. This rise in p53 levels was attributed to lower levels of Mdm2, which is too unstable in the absence of USP7 to mark p53 for degradation.

Alteration of p53 function by EBNA1 would be a possible mechanism by which EBNA1 could contribute to EBV-mediated host cell survival. Biochemical and structural studies conducted previously in our lab have been fruitful in furthering support for this possibility. EBNA1 was found to bind the N-terminal domain of USP7 (USP7-NTD), which is the same domain bound by p53 (Holowaty et al., 2003a). However EBNA1 has 10-fold higher affinity than p53 for the USP7-NTD (Holowaty et al., 2003a). Additionally, X-ray crystallography and NMR studies have shown that EBNA1 and p53 bind the same pocket in the USP7-NTD (Saridakis et al., 2005; Sheng et al., 2006). These structural studies also revealed that EBNA1 makes more extensive contacts than p53 with the USP7-NTD and this likely explains the higher affinity of EBNA1 for USP7. These observations suggest that EBNA1 may affect the cellular levels and function of p53 by sequestering USP7. In support of this proposal, it was shown that EBNA1 can attenuate p53-mediated apoptosis (Saridakis et al., 2005). This effect of EBNA1 was shown to be USP7-dependent as a deletion mutant of EBNA1 (Δ395 – 450) that lacks its USP7 binding sequence did not interfere with p53-mediated apoptosis (Saridakis et al., 2005).

Furthermore, work by Frappier and colleagues has shown that EBNA1 disrupts promyelocytic leukemia (PML) nuclear bodies (PML-NBs, also called ND10s) in
nasopharyngeal carcinoma cells by inducing the degradation of the PML protein (Sivachandran et al., 2008). Disruption of PML-NBs by EBNA1 required USP7 expression and the EBNA1-USP7 interaction, indicating that this interaction can modulate cellular events in addition to p53 levels. This development has been exciting given the functions of PML and PML-NBs, which include p53 activation, apoptosis, senescence, DNA damage repair and innate antiviral responses (Salomoni et al., 2008). Due to these important roles, loss of PML-NBs is associated with cancer development or progression in a variety of solid tumours, and disruption of PML function through fusion to RARα plays a causative role in promyelocytic leukemia (Gambacorta et al., 1996; Gurrieri et al., 2004; Koken et al., 1995; Reineke and Kao, 2009).

In this chapter I will discuss the impact of the EBNA1-USP7 interaction on the cellular environment, particularly the functions of p53 and PML. I will also discuss how these events may contribute to EBV-mediated immortalization and transformation of host cells.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and transfections

Osteosarcoma cells, U2OS, were grown in H21 medium supplemented with 10% fetal bovine serum (FBS) and EBV-negative CNE2 and CNE2E cells were grown in α-MEM supplemented with 10% FBS. For experiments in Figure 3-1, U2OS cells in 60 mm dishes were transfected with 2 μg of pc3oriP plasmid expressing either EBNA1, Δ395–450, or no EBNA1 by using Lipofectamine 2000 (Invitrogen). 5 hr later, cells were moved to 15 cm dishes and grown in the presence of 0.4 mg/ml G418 to select for cells containing the pc3oriP plasmids for 2 weeks, before Western analysis.

Transfection of CNE2 cells to generate CNE2E cells stably expressing EBNA1 was previously described (Sivachandran et al., 2008). For RNA interference experiments, CNE2E cells in 6cm dishes were transfected with 50 pmol of siRNA against GFP (GCAAGCUGACCCUGAAGUUCAU) or against EBNA1 (GGAGGUUCCAACCCGAAAUTT) using 2μL of Lipofectamine 2000 (Invitrogen). siRNA transfections were repeated twice at 24 hours intervals for a total of 3 rounds of siRNA transfections over 72 hours. For HeLa experiments in Figure 3, cells grown in 10 cm dishes
were transfected with 8 µg of either pc3OriP (empty vector) or pc3OriPE plasmid expressing EBNA1, using 16 µL of Lipofectamine 2000.

3.2.2 Effect of EBNA1 on p53 levels in U2OS cells

To study effects of EBNA1 on p53 levels in U2OS cells after 2 weeks of G418 selection, cells were either harvested or UV irradiated in a Stratagene 1800 ultraviolet crosslinker at 50 × 100 µJ/cm² and harvested 4 or 8 hr post-UV treatment. Cells were lysed and 30 µg of total protein was subjected to Western analysis as discussed below.

3.2.3 Effect of EBNA1 on p53 in CNE2 and HeLa cells

To study the effect of EBNA1 on p53 levels, 80 µg of CNE2 and CNE2E cells before and after siRNA treatment for EBNA1 were subjected to western blotting. For p53 stabilization, p53 acetylation and p21 expression some cells were treated with 10 µg/mL of etoposide as indicated in the figure legends before harvesting and western analysis. p53 acetylation was also assayed in HeLa cells as follows. HeLa cells were transfected with 4 µg pc3OriP or pc3OriPE. EBNA1 expression in HeLa cells was confirmed in approximately 80% of the cells by IF prior to etoposide treatment 48 hours post-transfection. 100 µg of cell lysate was Western blotted.

3.2.4 Western blotting

Cells were lysed in 9 M urea, 5 mM Tris.Cl pH 6.8, sonicated briefly and subjected to centrifugation for 1 minute at 15,000 rpm in a microcentrifuge. 30 µg to 100 µg of total protein as indicated was subjected to SDS-PAGE and transferred to PVDF membrane (Amersham). Membranes were blocked in blocking buffer (5% milk in PBS (137 mM NaCl, 2.7 mM KCl, 0.01 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4)), then incubated with primary antibodies diluted in blocking buffer. For acetyl-p53 blots, primary antibody was diluted in 5% BSA, 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20. Primary antibodies used were DO-1 from Santa Cruz and pAB180 (Banks et al., 1986) for p53, Ab-1 for Actin (Calbiochem), R4 serum for EBNA1 (Holowaty et al., 2003b), antibody 2525 for acetyl-p53 K382 (Cell Signaling Technologies) and antibody 187 for p21 (Santa Cruz, sc-817). After primary antibody incubation, membranes were washed in PBS with 0.1% Tween 20 (PBS-T) then incubated with the secondary antibodies goat anti mouse-HRP (Santa Cruz, SC-2055) or goat anti-rabbit-HRP (Santa Cruz, SC-2004).
Following washes in PBS-T, blots were developed using chemiluminescence ECL reagent (Perkin Elmer). For experiments in Figure 3-1, blots were developed by using the ECL plus system (Amersham), and enhanced chemifluorescence was quantified by using a Typhoon 9400 scanner (Amersham) and ImageQuant 5.0 software. p53 levels were determined in relationship to the actin loading control.

3.2.5 Apoptosis assay

CNE2 and CNE2E cells were treated with 10 µg/mL of etoposide as stated above. 48 hours later, cells were processed for TUNEL staining using the APO-BrdU TUNEL Assay Kit (Invitrogen, MP2321’0) 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.

3.2.6 FACS analysis

CNE2 and CNE2E cells (before and after siRNA treatment) seeded at 60% confluence in 10 cm dishes were treated with UV (50x10^2 µJ/cm^2) 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 FACScalibur (Becton Dickinson, USA) and cell cycle analysis was performed using Modfit LT 3.1 (Verity Software House).

3.2.7 Cell viability assay

Cell viability was measured using a 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 the time of harvesting. 24 hours later, cells were treated with etoposide (10 µg/mL) or UV (50x10^2 µJ/cm^2). 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.

3.3 RESULTS

3.3.1 EBNA1 inhibits p53 stabilization by USP7
The *in vitro* observations discussed above suggest that EBNA1 may lower p53 levels by disrupting the p53-USP7 interaction in vivo. To test this possibility, I transfected U2OS cells with an *oriP* plasmid expressing EBNA1, Δ395–450, or no EBNA1 and grew the cells under selection for the plasmid for 2 weeks. At that point, EBNA1 and Δ395–450 were found to be expressed in about 60% of the cells (data not shown). p53 expression in these cells was analyzed by Western blotting before and after UV irradiation, which initiates the DNA damage response and p53 stabilization (Figure 3-1). Although the levels of p53 in the cells expressing and not expressing EBNA1 varied prior to UV treatment (i.e. p53 levels in EBNA1-expressing cells were either equivalent to or lower than those lacking EBNA1 expression), the degree to which p53 levels increased upon UV induction was consistently less in EBNA1-expressing cells than in cells expressing Δ395–450 or not expressing EBNA1 (Figure 3-1A). Figure 3-1B shows p53 levels after UV treatment from multiple experiments. Therefore, EBNA1 can interfere with the stabilization of p53 by USP7 in response to UV irradiation in this experimental condition.

While the results above suggested that EBNA1 can interfere with p53 stabilization and confirmed that this effect was most likely through EBNA1 interaction with USP7, I wanted to confirm this effect of EBNA1 expression in a cell background relevant to latent EBV infection. To address this issue, I switched my studies to CNE2 cells derived from an NPC-tumor, which was EBV-positive originally, although these cells have lost the EBV genome in cell culture and thus do not express any EBV genes. This has been technically advantageous, because one can transfect an EBNA1 expression construct and select for EBNA1 expressing cells using antibiotics. This gives rise to a population of cells stably expressing relatively low levels of EBNA1 that can be used to study the effects of EBNA1 in an EBV-relevant cell background in the absence of other viral proteins, which may otherwise be confounding. From here on, I will be drawing comparisons between the parental cell line, CNE2, which does not express any EBNA1 and the engineered CNE2E cell line expressing EBNA1.

I first looked at p53 levels in CNE2 and CNE2E cells under steady state conditions. p53 protein levels were considerably lower in EBNA1 expressing CNE2 cells, compared to the parental CNE2 cells (Figure 3-2A). I further wanted to confirm that the lower p53 levels were due to EBNA1 expression. To this end I transfected CNE2E cells with siRNA against EBNA1
Figure 3-1. EBNA1 expression affects p53 stabilization through USP7 binding in U2OS cells. (A) U2OS cells expressing EBNA1, Δ395–450, or no EBNA1 were lysed before (0), 4 hr after, or 8 hr after UV irradiation and analyzed for p53 and actin levels by Western blotting. (B) Combined data from three experiments showing relative levels of p53 after UV induction in U2OS cells expressing EBNA1, Δ395–450, or no EBNA1. SD is indicated by the error bars.
or control siRNA against GFP. siRNA treatment decreased EBNA1 protein levels several-fold, though residual EBNA1 protein was still detectable by Western blot (Figure 3-2B top panel). Nevertheless, p53 protein levels were partly restored in EBNA1 siRNA-treated cells, confirming that EBNA1 expression is directly responsible for the reduced steady state p53 levels (Figure 3-2B). Since Mdm2 is also a target of USP7 and regulates p53 stability, I also looked at Mdm2 levels. Mdm2 levels were also diminished in EBNA1 expressing cells and restored after silencing of EBNA1 expression using siRNA (Figure 3-2A and B, second panel from bottom). Nevertheless, even though Mdm2 is important to mark p53 for degradation, the net effect of EBNA1 appears to be lower levels of p53. This is not unusual since residual Mdm2 may be sufficient to polyubiquitinate p53 and since Mdm2 is not the only ubiquitin ligase for p53 (Dornan et al., 2004; Leng et al., 2003).

A critical attribute of p53 function is stabilization and activation of p53 after induction of DNA damage. USP7 plays a key role in the stabilization of p53 after genotoxic stress (Brooks and Gu, 2006; Shan et al., 2008). Therefore, I next wanted to determine whether EBNA1 can hinder stabilization of p53 after DNA damage induction. As a source of DNA damage, I used etoposide, a topoisomerase inhibitor that leads to DNA double-strand breaks (van Maanen et al., 1988). I examined p53 levels in CNE2 and CNE2E cells before and after etoposide treatment. Etoposide treatment stabilized p53 in both CNE2 and CNE2E cells, but the stabilization of p53 was severely compromised in CNE2E cells (Figure 3-3A, compare lanes 2 and 4).

### 3.3.2 EBNA1 inhibits p53 activation

The requirements for an optimal p53 response to the induction of DNA damage are two-fold: in addition to being stabilized by USP7, p53 undergoes several post-translational modifications that not only ensure p53 stability is sustained but also are important to activate p53 as a transcription factor. One such modification is p53 acetylation, which has been shown to be indispensable for p53 activation and its function as a transcription factor (Tang et al., 2008). Given that EBNA1 disrupts PML-NBs (Sivachandran et al., 2008) and that PML-NBs are critical sites for p53 acetylation (Pearson et al., 2000), one of the questions I wanted to address was whether EBNA1 expression can also hinder p53 acetylation. To this end, I used an antibody
Figure 3-2. Effect of EBNA1 on p53 levels in NPC CNE2 cells. (A) Extracts of CNE2 parent cells or EBNA1 expressing CNE2E cells were analyzed by western blotting for EBNA1, p53 and actin. (B) CNE2E cells were transfected with siRNA for EBNA1 or GFP. Lysates from transfected cells were subjected to western blotting as in A.
Figure 3-3. EBNA1 inhibits p53 stabilization and activation after DNA damage. (A and B) CNE2 and CNE2E cells were treated with etoposide (+) or left untreated (−) and equal amounts of total cell lysates were analyzed by SDS-PAGE and Western blotting for total p53 (A) and p53 acetylated on K382 (B). Actin loading controls are also shown. (C) 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 analyzed by Western blotting for EBNA1, total p53, p53 acetylated on K382 and actin.
specific for acetylated p53 and compared p53 acetylation levels in CNE2 and CNE2E cells before and after induction of DNA damage by etoposide. p53 is efficiently acetylated at lysine 382 after etoposide treatment of CNE2 cells (Figure 3-3B, compare lanes 1 and 2). On the other hand, p53 acetylation is completely lost in CNE2E cells, even after etoposide treatment (Figure 3B lane 4). It has been previously shown that EBNA1 has no effect on PML in HeLa cells and PML-NBs remain intact even after EBNA1 overexpression (Sivachandran et al., 2008). Thus HeLa cells offered an elegant control to confirm whether EBNA1 expression inhibits p53 acetylation by disrupting PML-NBs. Interestingly, after etoposide treatment, p53 was acetylated in EBNA1 expressing HeLa cells to levels comparable to those in control HeLa cells with no EBNA1 (Figure 3-3C, compare lanes 2 and 4). This result further supports the idea that EBNA1 inhibits p53 activation by disrupting PML-NBs.

3.3.3 EBNA1 impairs p53 functions in p21 activation and apoptosis

The results above suggest that EBNA1 inhibits the two critical aspects of an optimal p53 response, p53 stabilization and p53 activation. I next wanted to determine whether these events have a measurable effect on the function of p53. p53 exerts its effects as a tumor suppressor primarily by functioning as a transcription activator (Riley et al., 2008). One of the most widely studied p53 targets is the p21 gene. p21 is an inhibitor of the CDK2-Cyclin E complex and thus arrests cell cycle progression in S phase (Abbas and Dutta, 2009). p21 protein levels are kept to a minimum under steady state, but p53 activates transcription at the p21 promoter in response to DNA damage (el-Deiry et al., 1993). In order to assess the effects of EBNA1 on p53 function, I first looked at levels of p21 protein in CNE2 and CNE2E cells using Western analysis before and after etoposide treatment. In CNE2 cells, p21 protein levels, which were undetectable before etoposide treatment, increased noticeably after etoposide treatment. In CNE2E cells, expression of p21 after etoposide treatment was considerably diminished, even though they had higher basal expression of p21 before etoposide treatment (Figure 3-4A).

The p53 response to DNA damage can also result in programmed cell death or apoptosis, in addition to cell cycle arrest. Therefore I also wanted to determine whether EBNA1 interferes with cell death by apoptosis. I compared the percentages of CNE2 and CNE2E cells undergoing apoptosis after 48 hours of etoposide treatment, using a TUNEL assay. The
Figure 3-4. EBNA1 alters p53 function in NPC cells. (A) CNE2 and CNE2E cells were treated with etoposide (+) or left untreated (−) and equal amounts of total cell lysates were analyzed by SDS-PAGE and Western blotting for p21 and actin. (B) CNE2 and CNE2E cells were treated with etoposide then analyzed 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) and 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.
percentage of TUNEL-positive cells after etoposide treatment was decreased two-fold in the presence of EBNA1 in CNE2E cells (Figure 3-4B).

3.3.4 EBNA1 increases cell survival after DNA damage

I also compared the viability of CNE2 and CNE2E cells after etoposide and UV treatment and found that EBNA1-expressing CNE2E cells survive better than the parental CNE2 cells, particularly after etoposide treatment (Figure 3-4C). This is consistent with the observation mentioned above that EBNA1 expression makes cells resistant to apoptosis.

3.3.5 EBNA1 interferes with DNA repair

In addition to p53 activation, PML-NBs are also important for inducing the repair of DNA damage after genotoxic stress (Dellaire and Bazett-Jones, 2007). Since EBNA1 disrupts PML-NBs and p53 function, which are both important for the optimal detection of damaged DNA and the execution of the DNA damage response, I asked whether EBNA1 can also impair DNA repair mechanisms. I examined the effect of EBNA1 expression on DNA repair by comparing FACS profiles of CNE2 and CNE2E cells before and after the induction of DNA damage using either UV or etoposide. Unrepaired DNA damage manifests itself as the accumulation of cells in S phase, while cells that can repair DNA damage pass through S-phase and arrest in either G2/M or G1 depending on the checkpoint that is activated (Biard, 2007; Boe et al., 2006; Callegari and Kelly, 2007). Therefore, the silencing of PML and other proteins involved in DNA repair mechanisms increases the percentage of cells in S phase (Biard, 2007; Boe et al., 2006). Accordingly, I consistently observed that CNE2E cells had a higher fraction of cells in S phase compared to CNE2 cells after DNA damage induction (Figure 3-5, compare panel ii with v and panel iii with vi), even though the cell cycle profiles of the two cell lines are similar when left untreated (Figure 3-5, compare panel i with iv). From multiple experiments I determined that the percentage of CNE2 cells in S phase after UV or etoposide treatment was 55.8 ± 2.5 and 55.4 ± 1.5 respectively. On the other hand, 66.5±2.2 and 91.4±0.2 percent of CNE2E cells were accumulated in S phase after UV or etoposide treatment respectively. The differences in cell cycle profiles with and without EBNA1 were statistically significant with a p value of less than 0.001. I further confirmed that this effect was due to EBNA1 expression, as knockdown of EBNA1 in CNE2E cells using siRNA reduced the percentage of cells
Figure 3-5. Effects of EBNA1 on DNA repair. 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 analyzed for DNA content by FACS. The percentage of cells in each cell cycle stage was determined using Modfit and is shown for each sample.
accumulating in S phase after DNA damage compared to control cells transfected with siRNA against GFP (Figure 3-5, compare panel viii with xi and panel ix with xii). Together these data show that EBNA1 impairs the DNA repair ability of cells and this effect is consistent with disruption of PML-NBs.

3.4 DISCUSSION

The functions of EBNA1 in EBV latent infection fall into at least two broad categories. First, EBNA1 mediates processes on EBV genome sequences, particularly at the oriP (Rickinson, 2001). Secondly, evidence also suggests that EBNA1 contributes to oncogenic transformation of EBV-infected cells. However, until recently, there has been a dearth of molecular mechanisms that explain the latter role of EBNA1. My observations discussed above, along with other work emerging from the Frappier laboratory, is beginning to fill some of this void. Here I have shown that, through its interaction with USP7, EBNA1 interferes with the normal regulation of the tumor suppressor p53 by USP7. Additionally, I also explored the consequences of EBNA1-mediated disruption of PML-NBs (Sivachandran et al., 2008). Given the findings that USP7 and its interaction with EBNA1 is required for this effect (Sivachandran et al., 2008), and in light of observations that implicate USP7 in PML regulation (discussed further in this thesis), it is highly likely that EBNA1 also disrupts PML-NBs through its interaction with USP7.

EBNA1 expression led to destabilization of p53 in U2OS and this effect required EBNA1 interaction with USP7. This observation is consistent with the substantial biochemical and structural studies showing that EBNA1 binds the same pocket in the USP7-NTD as p53, but with higher affinity and can compete with p53 for USP7 binding (Holowaty et al., 2003a; Saridakis et al., 2005; Sheng et al., 2006). Taken together, these observations imply that, by binding USP7, EBNA1 disrupts the p53-USP7 interaction and interferes with the stabilization of p53 by USP7.

My observations in U2OS cells, however informative, were not in a cell background relevant to EBV latent infection. To support the idea that these effects of EBNA1 on p53 were relevant to latent EBV infection, I employed the CNE2 cell line. CNE2 cells were derived from an EBV-positive nasopharyngeal carcinoma, but have lost the EBV genome in cell culture (Sun
et al., 1992). CNE2E cells were described previously (Sivachandran et al., 2008) and were engineered to stably express EBNA1 at levels comparable to those in cells natively infected with EBV. Comparison of CNE2 and CNE2E cells thus allows one to study the effects of EBNA1 in isolation in an EBV-relevant cell background. Stable EBNA1 expression in CNE2E cells led to marked reduction in the steady state levels of p53 when compared to the parental CNE2 cells, though the extent of this effect was more striking than in U2OS cells. This is likely because only about 60% of U2OS cells expressed EBNA1 at the time of harvesting, while close to 100% of CNE2 cells stably expressed EBNA1. Knockdown of EBNA1 using siRNA rescued p53 levels, confirming that the reduction in p53 was a reversible event, solely due to EBNA1 expression and not an artifact of the stable transfection process. Additionally, stabilization of p53 in response to DNA damage was also attenuated in CNE2E cells, consistent with the results from U2OS cells. This is a striking effect of EBNA1 expression since, after DNA damage induction, conditions optimally favour p53 interaction with and stabilization by USP7, as opposed to USP7 interactions with Mdm2 and MdmX, the negative regulators of p53 (Meulmeester et al., 2005b).

CNE2E cells were also deficient in activation of p53 by acetylation, compared to CNE2 cells. Even though basal levels of p53 are lower in CNE2E cells compared to CNE2 cells, the level of p53 acetylation was disproportionately lower in EBNA1 expressing CNE2E cells. This effect is in line with previous observations that PML-NBs are important for p53 acetylation by CBP (Pearson et al., 2000) and that EBNA1 disrupts PML-NBs (Sivachandran et al., 2008).

Diminished stabilization and activation of p53 in CNE2E cells would suggest that EBNA1 interferes with the p53 response after stress. The type and extent of genotoxic stress, determine the type of p53 response and thus the fate of the cell (Riley et al., 2008). DNA damage may either result in cell cycle arrest, primarily mediated by transcriptional activation of the p21 gene by p53, or result in expression of another set of genes responsible for programmed cell death (apoptosis) (Riley et al., 2008). In agreement with the poor stability and activation of p53 in the presence of EBNA1, both p21 expression and p53-mediated apoptosis after DNA damage were compromised in EBNA1 expressing cells. Although p21 levels were slightly higher in EBNA1 expressing cells compared to control cells before the induction of DNA
damage, p53-independent transcription of the p21 gene in the absence of DNA damaging agents has been observed and may be at play here (Gartel et al., 2000).

Though mutations in the p53 gene are among the most common genetic alterations in cancers (Nigro et al., 1989), p53 mutations in nasopharyngeal carcinomas, an EBV-associated cancer, are relatively rare (Chang et al., 2002; Effert et al., 1992; Lo et al., 1992; Spruck et al., 1992). Thus, it is conceivable that alternative mechanisms inactivate p53 function in NPC. Work discussed herein offers one such mechanism. My work showing EBNA1-mediated inactivation of p53 through USP7 parallels work done by others showing that shRNA-mediated knockdown of p53 in CNE2 cells results in decreased p21 expression, increased proliferation and an enhanced ability of these cells to form tumors in vivo (Sun et al., 2009).

Finally, EBNA1 expressing CNE2E cells had an impaired ability to repair DNA, which is consistent with the role of PML-NBs in DNA damage repair (Dellaire and Bazett-Jones, 2007). What is remarkable is that CNE2E cells were resistant to apoptosis and survived just as well as or better than the parental CNE2 cells, despite this impairment. 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 by neutralizing p53 function. EBNA1 interaction with USP7 appears to be important for both processes.
CHAPTER 4

USP7 IS A NEGATIVE REGULATOR OF PML PROTEINS AND PML NUCLEAR BODIES

A version of this paper is submitted for publication as:


Experiments depicted in Figure 4-9 were done by Xueqi Wang. Constructs for USP7 mutants were made by Tin Nguyen. I did all other experiments in this chapter.
4.1 INTRODUCTION

The promyelocytic leukemia protein (PML) is a tumor suppressor, whose aberrant function is associated with acute promyelocytic leukemia (APL). PML was originally identified as a fusion protein which results from a translocation of the PML gene to the RARα locus and characterizes most forms of APLs (de The et al., 1990; Melnick and Licht, 1999). The resulting fusion protein, PML-RARα is generally believed to antagonize the functions of both PML and RARα through a dominant negative effect, though evidence of gain of function also exists (Melnick and Licht, 1999; Salomoni and Pandolfi, 2002; Sternsdorf et al., 2006). In conjunction with other proteins, PML forms discrete multiprotein structures referred to as PML nuclear bodies (PML-NBs), thus named since PML is essential for formation of these bodies (Ishov et al., 1999).

The PML gene is comprised of 9 exons and alternative splicing of its transcript yields at least seven distinct isoforms of PML (PML I – VII) (Jensen et al., 2001; Nisole et al., 2005). While PML VII is cytoplasmic, most isoforms reside in the nucleus and largely localize to PML-NBs. Two other major components of PML-NBs are Sp100 and Daxx (Ishov et al., 1999; Zhong et al., 2000). Additionally, a variety of cellular proteins either transit through or constitutively reside at PML-NBs. The variety of these proteins is mirrored by the range of cellular functions regulated by PML-NBs namely p53 activation, apoptosis, senescence, DNA damage repair and innate antiviral response (Salomoni et al., 2008). In addition to their constituents, the number and size of PML-NBs can also vary with cell type, cell cycle phase and differentiation stage or in response to stress (Dellaire and Bazett-Jones, 2004).

Expression of PML in both cancer and normal tissue is subject to transcriptional, post-transcriptional and post-translational regulation (Reineke and Kao, 2009). Given the importance of PML-NBs in tumor suppression, it is conceivable that PML expression is attenuated or PML-NB function is otherwise inactivated in cancers. Indeed, PML-NB function is commonly lost both in leukaemias and solid tumors. In promyelocytic leukemia, PML function and PML-NB formation is lost due to the dominant negative effects of PML-RARα (Reineke and Kao, 2009). Moreover, PML-expression is abolished or diminished in solid tumors of a variety of histological origins (Gambacorta et al., 1996; Gurrieri et al., 2004; Koken et al., 1995). Interestingly, in many tumors, this is not accompanied by a concomitant change in PML transcript levels when compared to normal
tissues (Gurrieri et al., 2004). Thus the attenuation of PML levels in such tumors occurs at the protein level, possibly through aberrant post-translational modification of PML.

PML is subject to a variety of post-translational modifications, which modulate PML levels both in steady state and in response to stress (Reineke and Kao, 2009). First PML is phosphorylated by casein kinase-2 (CK2), an event that targets PML for degradation via ubiquitin-dependent and proteasome-mediated pathways (Lallemand-Breitenbach and de The, 2006; Scaglioni et al., 2006). However, the ubiquitin ligase responsible for CK2-induced polyubiquitination and degradation of PML is not presently known. Perhaps the most studied modification of PML is the conjugation of small ubiquitin-like modifiers (SUMO) 1, 2 and 3. PML is normally SUMOylated, a modification that is important for several aspects of PML function, not the least of which is PML-NB formation (Salomoni et al., 2008). PML is also extensively SUMOylated in response to arsenic treatment of cells, where SUMOylation primes PML for interaction with a SUMO-dependent ubiquitin ligase RNF4, which ubiquitinates PML and commits it to proteasome-mediated degradation (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Weisshaar et al., 2008). Additionally, at least two more ubiquitin ligases, E6AP and SIAH, are implicated in PML-ubiquitination and degradation (Fanelli et al., 2004; Louria-Hayon et al., 2009). Although post-translational modifications are integral to PML-NB functions and maintenance of PML levels, they are incompletely understood (Reineke and Kao, 2009). In particular, the understanding of processes that govern PML-ubiquitination is still in its infancy. For instance, while there is evidence for PML de-SUMOylation by SENP-1 and SENP-5 (Gong and Yeh, 2006; Ohbayashi et al., 2008), whether deubiquitinating enzymes (DUBs) confer similarly tighter regulation of PML-ubiquitination and degradation remains unknown.

Studies with herpesviral proteins have offered another perspective on the regulation of PML levels and functions. The ICP0 protein of herpes simplex virus (HSV), which is an E3 ubiquitin ligase, has long been known to disrupt PML-NBs and cause the degradation of PML protein (Everett et al., 1998; Everett and Maul, 1994; Maul and Everett, 1994). The RING domain responsible for the E3 ligase activity of ICP0 is important for degradation of PML and disruption of PML-NBs (Everett and Maul, 1994; Maul and Everett, 1994). Although the expression of ICP0 leads to increased polyubiquitination of PML and ICP0 relies on the ubiquitin-proteasome pathway for PML
degradation, it is not clear whether ICP0 directly ubiquitinates PML (Boutell et al., 2003).
Additionally, at least in some cell backgrounds, the ability of ICP0 to cause the degradation of PML and disrupt PML-NBs also correlates with its ability to interact with the ubiquitin-specific protease, USP7 (Parkinson and Everett, 2000). USP7 was originally identified as an interacting partner of ICP0 that partially localized to PML-NBs (Everett et al., 1997b; Meredith et al., 1995; Meredith et al., 1994). Further studies have revealed that USP7 regulates the auto-ubiquitinating activity and thus the stability of ICP0 (Boutell et al., 2005; Canning et al., 2004). USP7 has also been widely studied as a regulator of the p53 tumor suppressor (Cummins et al., 2004; Cummins and Vogelstein, 2004; Li et al., 2004; Li et al., 2003; Li et al., 2002). Deubiquitination of p53, its E3 ubiquitin ligase Mdm2 and the functional regulator MdmX by USP7 offers an elegant mechanism to fine tune p53 levels and p53 activity, parameters that are critical in determining response to genotoxic stress.

Using TAP-tagging and affinity column approaches, an interaction between the Epstein-Barr virus (EBV) protein EBNA1 and USP7 was previously identified (Holowaty et al., 2003b). Unlike ICP0, EBNA1 does not appear to be stabilized by USP7 (Holowaty et al., 2003b). However, the USP7-EBNA1 interaction has important consequences for the host and the virus alike. In keeping with the role of USP7 in the p53 pathway, I have shown that EBNA1 can interfere with the stabilization of p53 by USP7 by effectively competing with p53 for its binding site in the USP7 N-terminal domain (NTD) (Holowaty et al., 2003a; Saridakis et al., 2005). EBNA1 can also recruit USP7 to EBV sequences that regulate viral gene expression and genome persistence where, in complex with GMP synthetase, USP7 can deubiquitinate histone H2B and affect EBNA1-mediated transcriptional activation. In addition, like ICP0, EBNA1 was recently shown to disrupt PML-NBs by inducing the degradation of the PML protein (Sivachandran et al., 2008). This effect of EBNA1 was not seen using an EBNA1 mutant defective in USP7-binding nor was it seen when wildtype EBNA1 was expressed in conjunction with USP7 silencing. The combined results implicate USP7 in the regulation of PML-NBs.

In this study I explored the possibility that USP7 directly regulates PML levels and thus PML-NBs. Contrary to what has been seen with other targets of USP7, I found that USP7 negatively regulates PML levels and PML-NBs. Interestingly, both CK2 and RNF4 were dispensable
for PML-NB disruption by USP7, as was the catalytic activity of USP7. Overall, I describe a novel mode of PML regulation and a novel function for USP7, independent of its catalytic activity.

4.2 MATERIALS AND METHODS

4.2.1 Plasmids

To generate the pCANmycUSP7 plasmid, USP7 cDNA was PCR amplified from the pET3a-USP7 plasmid (a gift from Roger Everett). The amplified fragment was ligated into HindIII and XbaI sites of pCANmyc plasmid, with a pcDNA3.1 (Invitrogen) backbone. pCANmycC223S plasmid was generated by QuickChange mutagenesis of pCANmycUSP7 using the following primers: 5’CAG GGA GCG ACT TCT TAC ATG AAC AGC CTG3’ and 5’CAG GCT GTT CAT GTA AGA AGT CGC TCC CTG3’. USP7 NTD and USP7 CTD fragments were generated by PCR-amplification of the sequences encoding these domains from pCANmycUSP7 using the primers

5’CGCCGCAAGCTTCCGAAAAAAAAAAACGCAAAGTGATGAACCACCAGCAGCAGC 3’
and 5’ CCGGGATCCTCCTTCTTGAATCCCCACGCAACTCC 3’ for the NTD and

5’CGCCGCAAGCTTCCGAAAAAAAAAAACGCAAAGTGGAAGCCCATCTCTATATGCAA G 3’ and 5’GCGGGATCCTCAGTTATGGATTTTAATGGCC 3’ for the CTD.

The sequence coding for the SV40 T antigen nuclear localization signal was included in the 5’ primers to generate an in-frame NLS at the N-terminus of each domain. Amplified fragments were ligated into pCMVmyc (Wang and Frappier, 2009) between HindIII and BamHI sites. The plasmid expressing HA-ubiquitin was kindly provided by Dr. Ronald T. Hay (University of Dundee).

4.2.2 Cell lines and transfections

CNE2 cells were derived from an EBV-positive nasopharyngeal carcinoma in which the EBV genomes were lost from the cells during growth in culture (see CNE2Z in (Sun et al., 1992)). CNE2 and H1299 cells (a gift from Linda Penn) were grown in α-MEM media supplemented with 10% fetal bovine serum. Where indicated, cells in 6cm dishes were transfected with 50 pmoles of siRNA against GFP (GCAAGCUGACCCUGAAGUUCAU), USP7 (CCCCAAAATTATTCCGCAGAAGACG), Mdm2 (CCGGAUUCGAUGCGUCACCUG), CK2 or RNF4 using up to 2 μl of Lipofectamine 2000 (Invitrogen). STEALTH siRNA for CK2 and RNF4 were
purchased from Invitrogen, while E6AP siRNA mix was purchased from Santa Cruz. siRNA transfections were repeated twice at 24 hours intervals for a total of 3 rounds of siRNA transfections over 72 hours, unless otherwise stated. 24 hours after the last transfection, cells were either harvested for western blotting, subjected to immunofluorescence microscopy or moved to larger vessels for further transfections. For overexpression of USP7 or USP7 mutants, CNE2 cells were transfected with the pertinent plasmids as indicated in the figure legends.

4.2.3 Immunofluorescence microscopy

Cells grown and transfected on coverslips were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 0.01 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) for 30 minutes at room temperature and permeabilized with 1% Triton X-100 in PBS for 5 minutes at room temperature. For experiments in Figure 4-3, cytosolic and nucleoplasmic proteins were extracted from cells by incubating in 0.5% Triton X-100 on ice for 4 minutes prior to fixing. Samples were then blocked with 4% BSA in PBS, followed by incubation with primary antibodies against USP7 (Holowaty et al., 2003b), PML (PG-M3; Santa Cruz) or myc (Santa Cruz). Samples were subsequently incubated with the secondary antibodies goat anti-rabbit Alexafluor 488 (Invitrogen, A11008) and goat anti-mouse Alexa 555 (Invitrogen, A21424). Coverslips were counterstained with DAPI and mounted on slides using ProLong Gold antifade (Invitrogen). Samples were analyzed on a Leica inverted fluorescence microscope using a 40x oil immersion objective and images were taken using the OpenLAB (ver.X.0) software.

4.2.4 Western Blotting

Cells were lysed in 9 M urea, 5 mM Tris.Cl pH 6.8, sonicated briefly and subjected to centrifugation for 1 minute at 15,000 rpm in a microcentrifuge. 30 µg of total protein was subjected to SDS-PAGE and transferred to PVDF membrane (Amersham). Membranes were blocked with 5% milk in PBS, then incubated with primary antibodies against full length USP7, PML (Bethyl, A301-167A), Sp100 (Santa Cruz, sc-25568), hDaxx (C-20; Santa Cruz, SC-7000,), CK2α (Abcam , ab10466-50), c-Myc (A-14; Santa Cruz, SC-789), β-Actin (Ab-1; Calbiochem, CP01), SUMO-1 (Zymed), Mdm2 (Santa Cruz, SC-965), E6AP EE-7 (Santa Cruz, SC100612), HA (12CA5 monoclonal antibody, kindly provided by Dr Alan Cochrane; (Field et al., 1988)) or RNF4 (K7979
Membranes were washed in PBS with 0.1% Tween 20 then incubated with the secondary antibodies goat anti mouse-HRP (Santa Cruz, SC-2055), goat anti-rabbit (Santa Cruz, SC-2004) or donkey anti-goat-HRP (Santa Cruz, SC-2020). Following washes in PBS with 0.1% Tween, blots were developed using chemiluminescence ECL reagent (Perkin Elmer).

4.2.5 PML ubiquitination Assay

After siRNA as indicated above, CNE2 cells were moved to 15 cm dishes and transfected with 5 µg of plasmid expressing HA-tagged ubiquitin. 48 hours post transfection, cells were treated with 10 µM MG132 (Sigma) for 10 hours. Cells were then harvested and pellets were frozen at -70°C. Cell pellets were thawed at 37°C for 5 minutes and lysed by resuspending in 200 µL of SDS lysis buffer (62.5 mM Tris.Cl pH 6.8, 2% SDS, 10% glycerol, protease inhibitor cocktail (Sigma, P8340),1mM N-ethyl maleimide) and boiling for 5 minutes. Samples were then briefly sonicated followed by centrifugation at 15,000 rpm in a microcentrifuge. Lysates were then diluted 5-fold in IP buffer (50 mM Tris.Cl pH 8.0, 150 mM NaCl, 1% NP-40) and precleared with protein A/G sepharose beads (Santa Cruz, SC-2003) and 2 µg normal rabbit IgG (Santa Cruz, SC-2345). Cleared lysates were incubated overnight with PML antibody (Bethyl, A301-167A). After extensive washing in IP buffer, immunoprecipitates were eluted by resuspending the beads in loading buffer (60 mM Tris.Cl pH 6.8, 1%SDS, 100 mM DTT, 5% glycerol). Eluted complexes were then subjected to western blot analysis.

4.2.6 Immunoprecipitation

Immunoprecipitations were performed using nuclear extracts of CNE2 cells. To this end, asynchronous CNE2 cells were resuspended in hypotonic buffer (10 mM Hepes pH7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail P8340 (Sigma)), Dounce homogenized with 10 strokes of pestle B and incubated at 4°C for 30 min. Nuclei were pelleted using centrifugation at 5,000 rpm in a microcentrifuge and washed once in hypotonic buffer. Nuclei were lysed by resuspending in RIPA buffer (50 mM Tris.Cl pH 8.0, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 1 mM PMSF and protease inhibitor cocktail (P8340, Sigma)) and incubating at 4°C for 15 minutes. Nuclear lysates were then clarified by centrifugation at 15,000 rpm at 4°C for
10 minutes and cleared with protein A/G sepharose beads (Santa Cruz, SC-2003). 1.5 mg of total protein was incubated overnight at 4°C with 2 μg normal rabbit IgG (Santa Cruz, SC-2345) and 2 μg of USP7 (Bethyl, A300-033A) or PML (Bethyl) antibodies coupled to ExactaCruz resin (SantaCruz) according to manufacturer’s instructions. After extensive washing in IP buffer, immunoprecipitates were eluted by resuspending the beads in loading buffer. Eluted complexes were then subjected to western blot analysis.

4.2.7 Cells expressing single PML isoforms

pLKO.shPML1 expressing anti-PMLs shRNA (Everett et al., 2006) and pLNGY-PML.I, II, IV, V and VI expressing EYFP-tagged shPML1-resistant PML isoforms were kindly provided by Dr. Roger Everett and are described in detail elsewhere (D. Cuchet, A. Sykes, A. Nicolas, A. Orr, H. Sirma and R.D. Everett, in preparation). These plasmids were used to generate lentivirus in 293 cells as previously described (Everett et al., 2008). Culture supernatants containing the lentiviruses were filtered through a 0.45 μm filter prior to use. One ml of shRNA-lentiviral supernatant was then added to 1 × 10^5 CNE2 cells with polybrene (Sigma) at a final concentration of 8 μg/μl, removed after 24h and replaced with medium containing 2 μg/ml puromycin. After 72 hours of selection, the puromycin-containing medium was replaced with fresh medium lacking puromycin. Cells were then cloned by serial dilution in 96 well plates and checked for PML expression by immunofluorescence microscopy using antibody against all PML isoforms (PG-M3; Santa Cruz). A clone with no detectable PML-NBs (designated as CNE2Z-shPML) was further confirmed by Western blotting to lack PML expression and was used to generate cell lines expressing single PML isoforms as follows: 1 × 10^4 CNE2Z-shPML cells were incubated with 250 μl culture supernatant containing lentivirus encoding a shRNA-resistant PML isoform and cells containing the second lentivirus were selected for in 1 mg/ml G418 (GIBCO) for 7 days. Fluorescence microscopy was performed for the EYFP tag to confirm the presence of NBs containing EYFP-PML. Western blots were also performed on 80 μg of total cell extract using anti-PML antibody to confirm the expression of the PML isoform.

4.3 RESULTS

4.3.1 USP7 negatively regulates PML-NBs
USP7 is known to be partially associated with PML-NBs, but the functional significance of this association is unclear. Since it has previously been shown that disruption of PML-NBs by EBV protein EBNA1 in nasopharyngeal carcinoma cells requires USP7, I investigated the potential role of USP7 in regulating the formation or destruction of PML-NBs. To this end, I treated the EBV-negative nasopharyngeal carcinoma cell line, CNE2, with control siRNA for GFP (siGFP) or siRNA for USP7 (siUSP7). USP7 siRNA treatment generally down-regulated USP7 levels in all cells efficiently and evenly as determined by immunofluorescence (IF) microscopy (data not shown). In one such experiment, however, this treatment led to a decrease in USP7 protein levels in most but not all cells (Figure 4-1A). This allowed for a direct comparison of PML-NBs in silenced and non-silenced cells in the same culture and in the same field of view. In both silenced and non-silenced cells, PML mostly showed characteristic punctate staining with some diffuse nuclear staining (Figure 4-1A). In cells in which USP7 was silenced, PML staining appeared much stronger than in cells in which USP7 expression was still detectable. The average number of PML-NBs per cell was found to increase from 13 ± 0.1 in control cells to 18 ± 0.8 in USP7-silenced cells. The distribution of the number of PML-NBs per cell revealed that USP7-silenced cells had a large increase in the percentage of cells with more than 19 PML-NBs and a decrease in the number of cells with less than 15 PML-NBs (Figure 4-1A, right panel).

I further examined the role of USP7 in regulating PML-NBs, by overexpressing myc-tagged USP7 in CNE2 cells. While the number of PML-NBs greatly varied from cell to cell, overexpression of USP7 led to a reduction in the number of PML-NBs (Figure 4-1B, top panel). In multiple experiments the average number of PML-NBs decreased from 13 ± 0.12 in untransfected cells to 9 ± 0.9 in cells overexpressing USP7. More importantly, for USP7 overexpressing cells, there was a dramatic increase in the percentage of cells with fewer than 10 PML-NBs compared to untransfected cells (Figure 4-1B, top panel). Together the results show that USP7 has a destabilizing effect on PML-NBs.

4.3.2 USP7 catalytic activity is dispensable for PML-NB disruption

The destabilizing effect of USP7 on PML-NBs was counterintuitive because the deubiquitinase activity of USPs generally results in the stabilization of target proteins. This prompted me to ask whether the catalytic activity of USP7 is required for its effect on PML-NBs.
Figure 4-1. USP7 regulates PML-NB levels. (A) CNE2 cells were treated with siRNA against USP7 (siUSP7) or GFP (siGFP) then cells were fixed and stained for PML and endogenous USP7. The number of PML-NBs was counted for a minimum of 50 cells for each sample in three independent experiments. Histogram on the right represents the average distribution of number of PML-NBs in siGFP (white bars) and siUSP7 (black bars) samples, where error bars represent standard deviation from the three independent experiments. Images shown are for siUSP7 samples in which green stained cells serves as an internal control for cells in which USP7 expression has not been silenced. (B) CNE2 cells were transiently transfected with 2µg of a plasmid expressing WT USP7 or USP7 mutants as indicated or with the empty plasmid (Mock). 24 hours post-transfection, cells were fixed and stained using PML (red) and c-Myc (green) antibodies. Effect of USP7 overexpression on the number of PML-NBs was quantified as in A, except at least 100 cells were counted for each sample.
To this end, CNE2 cells were transfected with a construct expressing full length USP7 with a point mutation in cysteine 223 (C223S) known to be critical for ubiquitin cleavage (Li et al., 2002) (Figure 4-2). Interestingly, expression of C223S reduced the number of PML-NBs to a greater degree than WT USP7 (Figure 4-1B, 2nd row). The average number of PML-NBs in C223S-positive cells was reduced to 7 ± 1.2 as compared to 13 ± 0.12 in control cells, with a substantially larger percentage of cells containing fewer than 10 PML-NBs than for control cells or even for cells expressing WT USP7. These results indicate that USP7 regulates PML-NBs through a mechanism that is independent of its catalytic activity.

I next examined which USP7 domain(s) was responsible for PML-NB disruption. It has previously been shown that both the N-terminal (amino acids 1-205; NTD) and C-terminal (amino acids 560-1102; CTD) regions of USP7 form stable structural domains that mediate protein interactions (Holowaty et al., 2003a) (see Figure 4-2). Therefore I expressed these domains individually in CNE2 cells (fused to a nuclear localization sequence to ensure nuclear uptake) and examined PML-NB numbers and morphology. Expression of either the NTD or CTD dramatically decreased the number of PML-NBs, comparable in magnitude to C223S (Figure 4-1B, bottom two panels). I also noticed that, in addition to reducing the number of PML-NBs, USP7 overexpression also altered their morphology. While there were fewer PML-NBs in cells expressing USP7, C223S, NTD and CTD, the bodies that remained in these cells also tended to be larger and stained brighter for PML than those in untransfected cells (as shown in Figure 4-1B). I also expressed the USP7 catalytic domain (208-560) on its own in the CNE2 cells and saw no obvious changes in the size or number of the PML NBs relative to untransfected cells (Figure 4-1B, bottom row). Taken together these results indicate that USP7 negatively regulates PML-NBs, through two independent domains and that its catalytic activity is dispensable for this effect.

4.3.3 USP7 N- and C-terminal domains localize to PML-NBs

In most cells USP7 is found throughout the nucleus, often forming discrete foci, some of which associate with PML-NBs. However how USP7 associates with PML-NBs is not known. To better understand how the USP7 NTD and CTD affect PML-NBs, I asked whether, like WT USP7, they associated with PML-NBs. IF microscopy analysis of ectopically expressed USP7 or USP7 mutants yielded bright pan nuclear staining, making it difficult to determine if the USP7 proteins
formed PML-associated foci. To circumvent this problem, I analyzed the localization of USP7 and USP7 mutants in triton-extracted CNE2 cells soon after transfection when PML-NBs are largely intact. Triton extraction resulted in loss of most of the nucleosolic staining, allowing the detection of USP7 foci. I found ectopically expressed myc-tagged full length USP7 formed foci that colocalized with PML-NBs (Figure 4-3, top panel) in a manner similar to endogenous USP7. Additionally, both the USP7 NTD and the CTD formed nuclear foci, some of which were closely associated with PML-NBs (Figure 4-3, middle and bottom panels). This suggests that these USP7 domains disrupt PML-NBs through interactions with them.

4.3.4 USP7 regulates PML protein levels

PML-NBs can be disrupted due to loss of PML proteins or due to failure of the PML proteins to interact to form a NB. To determine which of these mechanisms is utilized by USP7, I examined the levels of the PML proteins before and after silencing USP7 in CNE2 cells. Treatment of CNE2Z cells with USP7 siRNA efficiently decreased USP7 levels in a dose-dependent manner, compared to treatment with siRNA for GFP (Figure 4-4A, top panel). In SDS-PAGE, PML proteins migrate as a ladder of multiple isoforms representing products of alternatively spliced transcripts and their post-translational modified versions (Jensen et al., 2001). Compared to the siGFP control, USP7 silencing led to an increase in most or all isoforms of PML (Figure 4-4A, middle panel). This increase in PML levels was directly proportional to the extent of USP7 silencing (Figure 4-4A, compare lanes 1 and 2). In addition, an increase in the levels of an isoform migrating near the 100 kDa marker was more prominent than for other isoforms, although the identity of this isoform has not been determined. USP7 silencing appeared to specifically affect PML proteins, as opposed to other PML-NB components, as the levels of the Sp100 and hDaxx proteins, known to be highly associated with PML-NBs, were largely unaffected by USP7 silencing (Figure 4-4B).

USP7 can alter levels of the p53 tumor suppressor (Cummins et al., 2004; Cummins and Vogelstein, 2004; Li et al., 2004; Li et al., 2002) and p53 can activate PML transcription (Chan et al., 1997; de Stanchina et al., 2004). To ensure that the increase in PML levels after USP7 silencing was not due to changes in the stability of p53, I repeated USP7 silencing experiments in p53-negative H1299 cells. Silencing of USP7 caused an increase in PML levels even in the absence of p53 (Figure 4-4C), indicating that the ability of USP7 to modulate PML levels is independent of p53.
Figure 4-2. The USP7 proteins used in this study. Pertinent regions of USP7 including the catalytic domain, p53 binding domain and the point mutation (C233S) that abrogates catalytic activity are indicated relative to amino acids numbers (shown below).
Figure 4-3. Localization of USP7 mutants in Triton-X 100-treated cells. CNE2 cells were transfected with USP7 or USP7 mutants as in Figure 4-1, except cells were harvested 18 hours post-transfection and were Triton extracted, prior to fixing and staining with PML and c-Myc antibodies. Arrows identify USP7 foci that localize to PML-NBs.
Figure 4-4. USP7 regulates PML protein levels and physically interacts with PML. (A-C) CNE2 cells (A and B) or H1299 cells (C) were subjected to 2 rounds (+) or 3 rounds (++) of transfections with siRNA (siUSP7) against USP7 or GFP (siGFP). Equal amounts of total cell lysates were analyzed by western blotting using the antibodies indicated. (D) CNE2 cells were treated with siRNA against USP7 or GFP then were transfected with a plasmid expressing HA-tagged ubiquitin. PML ubiquitylation was then analyzed by immunoprecipitating endogenous PML and western blotting with HA antibody. (E and F) Endogenous PML (E) or USP7 (F) was immunoprecipitated from CNE2 nuclear extracts using anti-PML or anti-USP7 antibody then western blotted using the reciprocal antibody as indicated. Normal rabbit IgG was used as a negative control (IgG) for non-specific immunoprecipitation. ‘Input’ represents 5% of the nuclear lysate used for immunoprecipitation.
Since PML protein degradation is mediated by polyubiquitination, I asked whether USP7 affects the degree of PML ubiquitination. To this end, CNE2 cells were transfected with a construct expressing HA-tagged ubiquitin along with siRNA against GFP or USP7. Transfected cells were also treated with the proteasome inhibitor MG132 to allow polyubiquitinated proteins to accumulate. Immunoprecipitation of endogenous PML recovered comparable amounts of PML from both siGFP and siUSP7 treated samples (Figure 4-4D, left panel) but, when the same samples were probed for HA, the level of PML containing HA-ubiquitin was seen to be greatly decreased after USP7 silencing (Figure 4-4D, right panel). Therefore USP7 is required for optimal polyubiquitination of PML.

### 4.3.5 USP7 physically interacts with PML

USP7 has been known to associate with PML-NBs since its discovery as an ICP0-interacting protein (Meredith et al., 1995) but whether or not this involves an interaction with PML proteins is unknown. Therefore I tested whether USP7 and PML proteins physically associate. To this end, I immunoprecipitated endogenous PML from nuclear extracts using antibody that recognizes all PML isoforms. Blotting the immunoprecipitates for USP7 revealed that a fraction of the endogenous USP7 interacts with the PML proteins (Figure 4-4E).

I also conducted the reciprocal immunoprecipitation experiment in which an antibody against USP7 was used to pull down USP7 (Figure 4-4F), as indicated by two bands characteristic of USP7 (Antrobus and Boutell, 2008). Western blots for total PML revealed that one PML isoform preferentially coimmunoprecipitated with USP7 (Figure 4-4F). Interestingly, this isoform migrates at the same position as the isoform which is preferentially stabilized after USP7 silencing (see Figure 4-4A). These data suggest that USP7 physically interacts with PML-NBs through PML proteins and has a preference for one PML isoform.

### 4.3.6 Casein kinase 2 is dispensable for USP7 induced PML-NB disruption

Scaglioni and colleagues (Scaglioni et al., 2006) showed that casein kinase 2 (CK2) phosphorylates PML proteins on serine 517, which primes them for polyubiquitination and proteasome-mediated degradation. Therefore, I wondered if the USP7-mediated degradation of PML required PML phosphorylation by CK2. I tested this possibility by assaying USP7-mediated
PML-NB disruption in CNE2 cells deprived of CK2 activity in the following two ways. First, I silenced CK2α using siRNA and then overexpressed either USP7 or C223S in the siRNA treated cells. CK2α expression was efficiently decreased by siRNA treatment and did not affect USP7 levels, as shown in Figure 4-4A (compare lanes 3 and 4). Consistent with previous reports of a role for CK2 in PML turnover (Scaglioni et al., 2006), CK2 silencing resulted in increased levels of PML-NBs in general. However depletion of CK2 had no noticeable effect on the ability of USP7 or C223S to disrupt PML-NBs (Figure 4-5B).

To rule out the possibility that residual CK2α remaining after silencing was sufficient to bring about USP7-induced PML-NB disruption, I assessed the effect of USP7 and C223S on PML-NBs in CNE2 cells treated with emodin, an inhibitor of CK2 catalytic activity. As reported previously (Scaglioni et al., 2006), emodin treatment on its own increased PML staining. However inhibition of CK2 activity by emodin did not hinder disruption of PML-NBs by either USP7 or C223S (Figure 4-5C). These observations indicate that USP7 negatively regulates PML-NBs in a manner independent of CK2 phosphorylation of PML.

4.3.7 USP7 regulation of PML-NBs is independent of E6AP and Mdm2

Since USP7 is important for PML ubiquitination, it is possible that USP7 recruits a ubiquitin ligase to PML-NBs, which polyubiquitinates PML proteins and commits them to degradation. Recently, E6AP has been identified as a ubiquitin ligase for PML proteins (Louria-Hayon et al., 2009). I wanted to determine whether E6AP is important for USP7-mediated regulation of PML. To this end, I treated CNE2 cells with siRNA for GFP and siRNA for E6AP before overexpressing USP7 or C223S. siRNA treatment silenced E6AP expression (Figure 4-6A, compare lanes 2 and 3), but this did not prevent the USP7- or C233S- induced degradation of PML-NBs (Figure 4-6B). Additionally, I did not find silencing of USP7 to affect levels of E6AP (Figure 4-6A, compare lanes 1 and 2). It is possible that USP7 functions through an E3 ubiquitin ligase that is not yet known to polyubiquitinate PML. USP7 interacts with at least two human ubiquitin ligases, Mdm2 and MARCH7 (Li et al., 2004; Nathan et al., 2008). Indeed, Mdm2 has been shown to interact with PML, but its role in ubiquitinating PML and regulating its stability has not been explored. I wanted to test whether Mdm2 could regulate PML levels and whether it had any role to play in
Figure 4-5. Lack of a requirement for CK2α for USP7-induced PML-NB degradation. (A) CNE2 cells were transfected with siRNAs against RNF4, USP7, CK2α or GFP and protein levels were assessed by western blotting using the antibodies indicated. (B) 24 hours after the last round of siRNA transfections, CNE2 cells silenced for CK2α expression were transfected with a plasmid expressing myc-tagged USP7 or my-tagged C223S (as in Fig 1B). 24 hours later cells were fixed and stained with anti-PML and anti-c-Myc antibodies. (C) CNE2 cells were treated with 50 μg/mL emodin 24 hours prior to transfection with USP7 or C223S expression plasmids. 6 hours post transfection, cells were given a second 24 hr emodin treatment, followed by fixation and immunofluorescence microscopy as in ‘B’
Figure 4-6. Lack of roles for Mdm2 and E6AP in USP7 induced PML regulation. (A) CNE2 cells were transfected with siRNAs against GFP, USP7 or E6AP. 24 hours after the last round of transfections, cells were harvested and lysed. 50 µg of total protein was subjected to SDS-PAGE and immunoblotting to assess the levels of protein levels were assessed by western blotting using the antibodies indicated. (B) 24 hours after the last round of siRNA transfections, CNE2 cells silenced for E6AP expression were transfected with a plasmid expressing myc-tagged USP7 or myc-tagged C223S (as in Figure 4-5B). 24 hours later cells were fixed and stained with anti-PML and anti-c-Myc antibodies. (C) CNE2 cell were transfected with 3 rounds of siRNA for GFP and Mdm2. Proteins were visualized as in A, using the antibodies indicated. Position of the 75kDa isoform of Mdm2 unaffected by siRNA treatment is marked by an asterisk. (D) CNE2 cells silenced for Mdm2 expression were transfected with a plasmid expressing myc-tagged C223S and subjected to immunofluorescence microscopy as in B.
USP7-mediated degradation of PML. I transfected CNE2 cells with siRNA for GFP (control) and siRNA for Mdm2. siRNA treatment silenced only the 90 kDa isoform of Mdm2, while there was little change in the levels of the 75 kDa isoform (Figure 4-6B). Nevertheless, this treatment did not have any effect on the levels of PML (Figure 4-6C). Accordingly, Mdm2 silencing did not prevent USP7 induced disruption of PML-NBs. The role of MARCH7 in PML regulation could not be tested due to insufficient silencing (data not shown). These data suggest that USP7 effect on PML is independent of E6AP and at least one isoform of Mdm2, though a role for Mdm2 is not completely ruled out.

4.3.8 USP7 is important for arsenic-induced PML degradation

Arsenic is known to induce PML SUMOylation, enlargement of PML-NBs, recruitment of proteasomes to PML-NBs and eventual degradation of PML (Miller et al., 2002). Since arsenic also causes degradation of PML-RARα, this property of arsenic is exploited as a treatment for acute promyelocytic leukemia (Miller et al., 2002). I wanted to determine whether USP7 is also important for arsenic-induced degradation of PML. To this end, CNE2 cells were transfected with siRNA against GFP or USP7 in quadruplicates and two of each sample were treated with arsenic trioxide while the other two were left untreated (Figure 4-7A). As expected, silencing of USP7 resulted in PML stabilization in both replicates in the absence of arsenic trioxide (Figure 4-7A, compare lanes 1 and 3 with lanes 2 and 4). In siGFP samples, treatment with arsenic trioxide led to degradation of PML as expected (Figure 4-7A, compare lanes 1 and 3 with 5 and 7). Darker exposures revealed the presence of high molecular weight forms of PML in arsenic treated samples, indicative of PML modified with SUMO and/or ubiquitin. Arsenic treatment of siUSP7 did not reduce PML levels to the same degree as in siGFP control cells (Figure 4-7A, compare lanes 5 and 7 with lanes 6 and 8) and resulted in a disproportionate accumulation of higher molecular weight forms of PML. This suggests that, even though PML is being post-translationally modified after arsenic treatment, its degradation is impeded in the absence of USP7.

The PML high molecular weight forms were further analyzed for the presence of SUMO by immunoprecipitation of total PML from each sample and blotting for endogenous SUMO1 (Figure 4-7B). This confirmed that the slower migrating PML forms evident after USP7 silencing and arsenic treatment (lane 4) were SUMOylated. Note that these SUMO1-modified forms are not
Figure 4-7. USP7 is important for arsenic-induced PML degradation. (A) CNE2 cells were treated with siRNA for USP7 (U) and siRNA for GFP (G) in quadruplicates. 24 hours post-transfection, two samples for each siRNA set were treated with 1 µM As₂O₃ for 6 hours then equal amounts of cell extracts were analyzed by western blotting using the antibodies indicated (B) CNE2 cells were treated with siRNA and As₂O₃ as in ‘A’. PML was immunoprecipitated from untreated and treated cells and SUMOylated PML was analyzed western blotting using anti-SUMO1 antibody.
detectible in the control siGFP sample, likely because they are degraded readily in the presence of USP7. These results indicate that USP7 is dispensable for arsenic-induced SUMOylation of PML but is important for subsequent degradation of these modified isoforms.

4.3.9 USP7 regulation of PML-NBs is independent of RNF4

The mechanism by which PML-NBs are degraded after arsenic treatment was recently discovered to be dependent on polyubiquitination by RNF4 (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Weisshaar et al., 2008), establishing RNF4 as an ubiquitin ligase for PML. Arsenic treatment promotes RNF4 association with PML-NBs, polyubiquitination of PML by RNF4 and degradation of PML. Since USP7 also negatively regulates PML, I wondered whether USP7 worked through RNF4. To test the requirement of RNF4 for USP7-mediated regulation of PML-NBs, I treated cells with siRNA against RNF4 prior to overexpressing USP7 or C223S. While siRNF4 treatment significantly decreased RNF4 levels (Figure 4-5A, compare lanes 1 and 3), it did not noticeably affect the ability of USP7 or C223S to disrupt PML-NBs (Figure 4-8). In addition, USP7 silencing did not affect RNF4 levels (Figure 4-5A, compare lanes 2 and 3) nor did it affect the ability of RNF4 to associate with PML after treatment with arsenic (Figure 4-8B). These findings suggest that USP7 induces degradation of PML by a mechanism that is independent of RNF4-mediated PML degradation.

4.3.10 USP7 regulates individual PML isoforms

Silencing of USP7 led to an increase in the levels of all isoforms of endogenous PML, although one PML form was preferentially affected. Since PML-NBs are comprised of a mix of PML isoforms, I was interested to determine whether PML regulation by USP7 required a specific PML isoform. To this end, endogenous PML was first silenced in CNE2 cells by stably expressing shRNA against all PML isoforms from a lentivirus as previously described by Everett et al (Everett et al., 2006). Expression of shRNA led to depletion of all PML isoforms beyond the limit of detection in CNE2 cells (Figure 4-9A, compare first two lanes). YFP-tagged PML isoforms resistant to the initial shRNA were then individually and stably expressed in the PML-depleted cells using a second lentivirus. PML isoforms I, II, IV, V and VI were expressed to varying but detectible degrees, while expression of PML III could not be detected and thus was not included in further analysis (Figure 4-9A and data not shown). In addition, others in the lab have confirmed by
Figure 4-8. Lack of a role of RNF4 for USP7-induced PML-NB degradation. (A) CNE2 cells were transfected with siRNAs against RNF4 or GFP as in Figure 4. 24 hours after the last round of siRNA transfections, CNE2 cells silenced for RNF4 expression were transfected with a plasmid expressing myc-tagged USP7 or my-tagged C223S (as in Fig 1B). 24 hours later cells were fixed and stained with anti-PML and anti-c-Myc antibodies. (B) CNE2 Cells were treated with siRNA against USP7 or GFP in duplicate. 24 hours post transfection, samples were either left untreated or treated with As$_2$O$_3$ for 8 hours. Following arsenic treatment, samples were fixed and processed for IF microscopy using anti-RNF4 and anti-PML antibodies and counter-stained with DAPI.
Figure 4-9. **USP7 regulates individual PML isoforms.** (A) Endogenous PML expression was silenced in CNE2 cells then silenced cells were engineered to stably express single YFP-tagged PML isoforms I, II, IV, V and VI. PML content of these cell lines was analyzed, relative to parental CNE2 cells, by western blotting equal amount of whole cell lysates and probing with PML antibody recognizing all PML isoforms. The position of each unmodified recombinant PML protein is indicated by an asterisk and the position of molecular weight markers are indicated on the left. A blot for actin is also shown as a loading control. (B) Cells described in A were transfected with siRNA against USP7 (U) or GFP (G) then equal amount of cell lysate were analyzed by western blotting using the indicated antibodies.
fluorescence microscopy that the individual recombinant PML proteins form nuclear bodies that closely resemble those formed by endogenous PML. Each of these cell lines was treated with siRNA against USP7 or GFP and effects on the levels of the individual PML proteins were examined by Western blotting. As shown in Figure 4-8B, USP7 silencing led to increased levels of PML isoforms I and IV, with the largest effect on PML I, while minimal to no effect of USP7 silencing was observed on PML II, V and VI. These data confirm that USP7 can regulate PML proteins even when expressed from a heterologous promoter, and show that USP7 has a preference for some PML isoforms.

4.4 DISCUSSION

USP7 was first discovered as a host cell binding partner of the herpes simplex virus protein ICP0 (Meredith et al., 1994). Its alternative name, HAUSP, which stands for herpesvirus associated ubiquitin specific protease, was further justified with the detection of a stable interaction with another herpesvirus protein EBNA1 (Holowaty et al., 2003b). Since then, USP7 has emerged as a key regulator of the p53 tumor suppressor (Shan et al., 2008) and several other cellular proteins (Nathan et al., 2008; Song et al., 2008a; van der Horst et al., 2006; van der Knaap et al., 2005). USP7 can regulate the stability, function and even the sub-cellular localization of its substrates and in each case, its deubiquitinating activity is required for any form of regulation. Here I showed that USP7 negatively regulates the levels of PML proteins and the formation of PML-NBs, by inducing the polyubiquitination of PML.

A function of USP7 in PML regulation ties in well with its known interactions with herpesvirus proteins ICP0 and EBNA1, both of which associate with and disrupt PML-NBs (Everett et al., 1997b; Meredith et al., 1994; Sivachandran et al., 2008). For ICP0 to disrupt PML-NBs, its interaction with USP7 is important at least in some cell backgrounds (Parkinson and Everett, 2000). The ability of EBNA1 to cause degradation of PML and disrupt PML-NBs is tightly tied to USP7, since PML-disruption by EBNA1 requires USP7 expression and does not occur with an EBNA1 mutant defective in binding USP7. Therefore, a direct role of USP7 in the regulation of PML levels would be consistent with the idea that herpesvirus proteins ICP0 and EBNA1 promote the ability of USP7 to cause degradation of PML proteins and disrupt PML-NBs.
While USP7 has long been known to be associated with PML-NBs, the mechanism of this association has not been investigated. I found that both the N- and C-terminal domains of USP7 were sufficient to mediate this association. These domains were also sufficient to disrupt PML-NBs, suggesting that this disruption involved the association between USP7 and PML-NBs. The USP7 NTD is a TRAF domain that is known to bind p53, Mdm2 and MdmX in addition to the viral EBNA1 protein (Li et al., 2002; Meulmeester et al., 2005a; Saridakis et al., 2005; Sheng et al., 2006). The C-terminal half of USP7 (referred to here as the CTD) also appears to have protein interactions as its main function and has been reported to bind FOXO in addition to the viral ICP0 protein (Holowaty et al., 2003a; van der Horst et al., 2006). It is not clear that any of these known interactions would account for the targeting to and disruption of PML-NBs by these USP7 domains, and it is likely that additional functionally important interactions with these domains remain to be uncovered.

I also asked whether the association of USP7 with PML-NBs involved the direct or indirect interaction of USP7 with PML proteins. In keeping with this hypothesis, USP7 and PML were observed to physically interact in coimmunoprecipitation experiments. Although the PML antibody coimmunoprecipitated only a small amount of endogenous USP7 from nuclear extracts, this is in keeping with previous observations that only a small pool of USP7 forms nuclear foci and that only a fraction of these foci colocalize with PML-NBs. In reciprocal experiments, USP7 antibody coimmunoprecipitated PML and interestingly, only one isoform of PML was recovered at levels detectable by western blotting. This suggests the possibility that USP7 is recruited to PML-NBs via a direct or indirect interaction with a specific PML isoform, where it can then bring about degradation of all PML isoforms. It is not yet clear if the USP7-PML interaction is direct or mediated by another protein. EBNA1 also interacts with a single isoform of PML (Sivachandran et al., 2008) and the size of the isoform that interacts with both EBNA1 and USP7 is consistent with the size of PML-IV (Condemine et al., 2006). This similarity may underscore the importance of USP7 for EBNA1-mediated PML-NB disruption.

Another interesting feature of PML-NBs is their sensitivity to arsenic treatment. Arsenic trioxide treatment leads to extensive SUMOylation, ubiquitination and degradation of PML and thus loss of PML-NBs (Lallemand-Breitenbach et al., 2008; Miller et al., 2002; Tatham et al., 2008;
Weisshaar et al., 2008). I found USP7 was important for this process. PML degradation after arsenic treatment was notably hampered in cells in which USP7 was down-regulated. Additionally, I noted an accumulation of high molecular weight forms of PML in USP7-depleted cells after arsenic treatment. Further examination revealed the slow migrating forms of PML to be SUMOylated. This supports the notion that USP7 does not interfere with SUMOylation of PML after arsenic treatment, but is important for a subsequent process. In light of my previous observation that USP7 is important for optimal ubiquitination of PML under steady state conditions, it is likely that USP7 is also involved in this process after arsenic treatment.

Two key regulators of PML protein levels that have been identified are CK2 and RNF4. While CK2 phosphorylates PML proteins and targets them for ubiquitination and proteasome-dependent degradation under steady state conditions, (Scaglioni et al., 2006), RNF4 is a ubiquitin ligase responsible for PML degradation, particularly after arsenic treatment (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Weisshaar et al., 2008). I found both of these regulatory proteins to be dispensable for USP7-induced degradation of PML-NBs. This suggests that USP7 operates to regulate PML as a component of an as yet unidentified system independent of CK2 and RNF4.

The observation that USP7 could regulate the levels of most, if not all endogenous PML isoforms and their modified variants was intriguing given that I only detected a physical interaction with one isoform of PML. This prompted me to examine whether USP7 relied on a specific isoform to bring about degradation of the others. Using cells expressing single PML isoforms, it was found that PML isoforms I, II and IV were regulated by USP7, independently of each other, while PML V and VI were largely unaffected by USP7 silencing. Since the expression of these individual PML isoforms is not driven by the same promoter as for the endogenous PML proteins, these experiments also show that the effects of USP7 siRNA on PML expression are not due to indirect effects from induction of the interferon response. Experiments in cells with individual PML isoforms indicated that USP7 modulates some isoforms more than others with the biggest effect on recombinant PML I. In the context of a native PML-NB, USP7 appears to differentially affect the PML isoforms, showing the greatest effect on an isoform of a size consistent with PML IV. A PML isoform of this size also preferentially immunoprecipitates with USP7 from cells with wildtype PML NBs. Taken
together, the results suggest that preferences of USP7 for specific PML isoforms underlies its ability to trigger loss of PML NBs and proteins.

The observations that USP7 promotes PML-ubiquitination and negatively regulates PML levels were counter-intuitive, since USP7 generally stabilizes its target proteins by removing their ubiquitin moieties. One explanation of this apparent contradiction would be that USP7 stabilizes a ubiquitin ligase that targets PML for ubiquitin-mediated degradation. There is precedent for this possibility as, under some circumstances, USP7 can negatively regulate p53 levels by stabilizing the Mdm2 E3 ligase (Li et al., 2004; Li et al., 2002). However, for PML regulation, this possibility is unlikely given that the USP7 catalytic activity is not required to disrupt PML-NBs. Indeed, mutants of USP7 devoid of its catalytic activity were able to disrupt and degrade PML-NBs when overexpressed in CNE2 cells to an extent similar to or better than WT USP7. A more likely scenario is that USP7 recruits a negative regulator, for example a ubiquitin ligase, to PML-NBs or that USP7 plays a role in recruitment of PML to the proteasome for degradation. In support of these possibilities, USP7 has been identified as a component of the proteasome (Besche et al., 2009; Bousquet-Dubouch et al., 2009) and has so far been reported to interact with three cellular E3 ubiquitin ligases, Mdm2, MARCH7 and CHFR, in addition to the viral E3 ligase, ICP0 (Li et al., 2004; Meredith et al., 1994; Nathan et al., 2008; Oh et al., 2007). The 90 kDa isoform of Mdm2 does not appear to be involved in PML stability, as Mdm2 silencing of this isoform had no noticeable effect on PML levels. The 90 kDa isoform is the one responsible for the degradation of p53 but, the 75 kDa isoform, which remained after siRNA treatment, is an N-terminal truncation with the C-terminal E3 RING domain intact. Thus, this isoform of Mdm2 could still ubiquitinate and regulate PML. It is unlikely that USP7 works through CHFR, since CHFR protein levels are undetectable in CNE2 cells used in these studies. Additionally, the role of MARCH7 could not be tested, due to poor silencing. Clearly, further studies are required to fully examine the role of USP7-associated E3 ligases in PML regulation.

The ability of USPs to function independently from their catalytic activity is not unique to USP7, as USP18 (also called UBP43) has been shown to negatively regulate interferon signaling through protein interactions that are independent of its isopeptidase activity (Malakhova et al., 2006). Overall my results point to a novel model of PML regulation in which USP7 triggers
degradation of PML by a mechanism that does not require phosphorylation of PML by CK2 or polyubiquitination by RNF4 or E6AP. Unlike all previously reported USP7 functions, its role in PML regulation does not require the catalytic activity of USP7 but rather is a function of the USP7 protein interaction domains. It is becoming increasingly clear from numerous recent publications that USP7 is an important regulator of many cellular processes. This work further emphasizes the diverse roles of USP7 by showing that the cellular functions of USP7 extend beyond its role as a deubiquitinating enzyme.
CHAPTER 5

INSIGHTS INTO SUBSTRATE RECOGNITION BY USP7

Parts of Figures 1, 2 and 4 and Table 1 of this chapter have been published as:


And,


Figures 5, 6 and the data in Table 2 form part of the following paper currently in press:


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The crystal structures of USP7-NTD bound by Mdm2 and MdmX peptides were determined by Vivian Saridakis. Samples for mass spectrometry were prepared by Vicky PKH Nguyen. I performed all other experiments in this section.
5.1 INTRODUCTION

The turnover of many proteins is regulated by the intricate interplay between polyubiquitination by E3 ubiquitin ligases and deubiquitination by ubiquitin-specific proteases (USPs). Human USP7 has recently emerged as a key regulator of the p53 pathway. USP7 was first identified as a protein that bound the ICP0 protein of herpes simplex virus and hence was initially named herpesvirus-associated USP (HAUSP) (Everett et al., 1997b; Meredith et al., 1994). USP7 also binds the Epstein-Barr nuclear antigen-1 (EBNA1) protein from Epstein-Barr virus (EBV), which has several important roles in latent EBV infection (Holowaty et al., 2003b). The targeting of USP7 by more than one virus suggests its importance in controlling key cellular processes important for viral infection.

USP7 has been shown to have multiple roles in regulating the p53 pathway. Initially, USP7 was found to interact specifically with and deubiquitinate p53, resulting in its stabilization (Li et al., 2002). Whereas decreased USP7 expression levels had the expected effect of destabilizing p53, ablation of USP7 expression was found to have the opposite effect, resulting in p53 stabilization (Li et al., 2004). This stabilization of p53 seems to be due to the increased ubiquitination and destabilization of Mdm2, the E3 ligase largely responsible for the ubiquitination of p53 (Cummins et al., 2004; Cummins and Vogelstein, 2004; Li et al., 2004). This implicates Mdm2 as a second direct target of USP7, an assumption supported by co-immunoprecipitation of the two proteins and in vitro deubiquitination of Mdm2 by USP7 (Li et al., 2004). A third connection to the p53 pathway has recently been reported with the identification of MdmX as a USP7 target; MdmX inhibits transcription activation by p53 and is targeted for degradation by Mdm2 (Meulmeester et al., 2005a; Meulmeester et al., 2005b).

The mechanism of target-protein recognition by USP7 is only partly understood. The domains of USP7 responsible for binding p53, EBNA1 and ICP0 have been identified and are distinct from the catalytic domain, whereas the domain(s) that bind Mdm2 and MdmX remain to be determined. EBNA1 and p53 both bind the N-terminal domain (NTD) of USP7, whereas ICP0 binds a domain in the C-terminal half of the protein (Holowaty et al., 2003a; Hu et al., 2002). The structural basis for the EBNA1 interaction with USP7 has been revealed by a 1.7-Å crystal structure of the USP7 NTD bound to an EBNA1 peptide (Saridakis et al., 2005). The USP7 NTD
is a TRAF domain that binds the EBNA1 peptide at a position similar to that reported for other peptide–TRAF domain interactions. However, the sequence of the bound EBNA1 peptide and the USP7 residues contacted are substantially different from previously reported TRAF-domain interactions and result in a high-affinity interaction ($K_d = 0.9 \mu \text{M}$). A four residue motif, $^{444}\text{EGGS}^{447}$, was found to be important for USP7 binding, where E444 and S447 made specific contacts with the USP7-NTD. A glutamate to alanine mutation at residue 444 hampered binding of the EBNA1 peptide to the USP7-NTD, while a serine to alanine at 447 completely disrupted binding. NMR chemical shift experiments have indicated that p53 binds USP7 at the same position as does EBNA1 but makes fewer contacts, probably accounting for the lower affinity of the p53-USP7 interaction ($K_d = 10 \mu \text{M}$) (Saridakis et al., 2005). The p53 fragment bound by USP7 has been localized to the regulatory region (Hu et al., 2002) and the actual sequence recognized by USP7 and the nature of the contacts have also been determined using structural studies (Sheng et al., 2006). Two closely spaced sequences $^{359}\text{PGGS}^{362}$ and $^{364}\text{AHSS}^{367}$ were found to bind the USP7-NTD in the same groove bound by EBNA1, highlighting the importance of the serine in the fourth position.

On the other hand, such detailed insight into the molecular recognition of Mdm2 and MdmX is lacking. Here I outline a number of biochemical approaches I undertook to define the regions on Mdm2 and MdmX important for their interactions with USP7. My findings were subsequently used by others for structural analysis of Mdm2-USP7 and MdmX-USP7 interactions as was previously done for p53 and EBNA1.

5.2 MATERIALS AND METHODS

5.2.1 Purification of Mdm2, MdmX and USP7 proteins

USP7-NTD containing residues 1-205, 54 – 205 and 62 – 205 was expressed from pET15b plasmids in Escherichia coli BL21 pLysS cells and purified as described previously (Holowaty et al., 2003a). Plasmids expressing Mdm2 fragments or MdmX fused to GST were generated by PCR amplification of relevant sequences and insertion between the BamHI and Xho1 sites of the pGEX-4T-2 plasmid (Amersham). Proteins were expressed in BL21 pLysS E. coli after a 3-h induction at 37 °C, purified on glutathione-Sepharose (Amersham) using standard methods and dialyzed against an assay buffer of PBS (10 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 137
mM NaCl, 2.7 mM KCl) with 5% glycerol (v/v), 1 mM benazidine and 0.5 mM PMSF. A GST fusion containing EBNA1 residues 395–450 was produced as previously described (Saridakis et al., 2005).

5.2.2 GST Pull-down assays

For USP7-binding assays, purified full-length USP7 or USP7 NTD (residues 62–205) was incubated with GST alone or a GST fusion protein in assay buffer in a 1:1 molar ratio at 4 °C for 1 h in the presence of 50 µL of glutathione-Sepharose beads (Amersham). The mixture was then transferred to a microcolumn, and after extensive washing with assay buffer, bound proteins were eluted with 20 mM reduced glutathione and detected by SDS-PAGE and Coomassie staining. For pull-down assays with MdmX, fractions were transferred to PVDF membrane after SDS-PAGE. GST-MdmX and USP7-NTD were visualized by immunoblotting using the GST antibody (Santa Cruz) and R2B2 antibody for USP7 (Holowaty et al., 2003b).

5.2.3 Intrinsic tryptophan fluorescence assays

Mdm2 or MdmX peptides were titrated (0 to 120 µM) with wild type USP7-NTD<sub>1–205</sub> (1 µM) in 50 mM Tris pH 7.5, 150 mM NaCl, EDTA, and 1 µM DTT. The change in tryptophan fluorescence was measured and K<sub>d</sub> values for USP7/HAUSP binding were calculated as described previously (Holowaty et al., 2003a).

5.2.4 Gel filtration analysis of protein interactions

Interaction of USP7 NTD<sub>62–205</sub> with GST-MDM2<sub>1–160</sub> and competition with EBNA1 fragment 395–450 was assayed as described previously (Holowaty et al., 2003a) except that 0.04 µmol of each protein was combined in a 300 µL final volume and dialyzed overnight against 20 mM Tris (pH 7.5), 200 mM NaCl, 1 mM DTT, 1 mM PMSF and 1 mM benazidine. Each dialyzed sample (250 µL) was then applied to a Superdex-200 10/30 column (Amersham). Fractions (500 µL) were collected and 50 µL of each pertinent fraction was analyzed by SDS-PAGE and Coomassie blue staining.

5.2.5 Crystallization, data collection and structure determination
USP7 fragment 54–205 (100 mg/mL) was cocrystallized with Mdm2 peptide (141-ELQEEKPSSS-150) or MdmX peptide (395LDLAHSSESQ404) at five-fold molar excess of peptide. Clusters of rods appeared after two rounds of microseeding using EBNA1–USP7 crystals as seeds in crystallization tools (Nextal Biotech). The seeded crystals grew after one week at 4 °C in the dark in solution containing 30% (w/v) PEG 4000, 0.1 M Tris (pH 8.5) and 0.2 M lithium sulfate. X-ray data from frozen crystals of peptide–USP7 complexes were collected on home rotating anode diffractometers. Data were integrated and scaled using HKL2000 (Otwinowski, 1997). A summary of data-collection statistics is presented in Table 2.

The structure was determined using the molecular-replacement component of CNS version 1.1 (Brunger et al., 1998). Electron density visualization and model building were done with O (Jones et al., 1991). Rigid-body and simulated-annealing torsion-angle refinement were normally followed by individual B-factor refinement and performed using CNS 1.1 (Brunger et al., 1998). Several rounds of refinement were combined with model rebuilding in O after inspection of both 2Fo –Fc and Fo –Fc maps. All residues are in the most-favored and additionally allowed regions of the Ramachandran plot. Residues 54–62 and 107–111 are disordered in the final models of all the complexes. In all cases, the bound peptide was located in the Fo –Fc difference electron density map after the first round of refinement using only the protein model. The quality of the resulting peptide electron density was very good, allowing residues 145-EKPSSS-150 of the MDM2 peptide to be built into the model. A summary of refinement statistics is presented in Table 5 – 2. SPOCK and PyMOL were used in the production of the figures (Christopher, 1998; DeLano, 2002).

5.2.6 Generation of EBNA1 expressing Adenovirus

Adenovirus expressing SPA-tagged EBNA1 and E444A/S447A were generated using the ViraPower Adenoviral Gateway Expression Kit (Invitrogen), with some modifications. Sequences encoding the SPA-tag were PCR amplified from PMZS3F (Zeghouf et al., 2004) using the primers 5’ CCGAATTCTAATGGAAAAAGAGAAGATGGAAAAG 3’ AND 5’ CCGCGGCCGCCTACTTGTCATCGTCATCC 3’ and cloned into Not1 and EcoR1 sites of the pENTR4 entry vector (Invitrogen) to generate pENTR4-SPA. Sequences encoding EBNA1 and E444A/S447A were PCR amplified from pc3Orip plasmids using primers 5’
GGATCCGACCACATGTCTGACGAGGGGCCAGG 3’ and 5’
CCGTTACCCTCCTGCCCTCTACC 3’ and ligated into the BamH1 and Kpn1 sites of pENTR-SPA (gift from Dr Jack Greenblatt), in frame with the SPA tag coding sequences. cDNA encoding the tagged protein in the entry vector was then recombined into the pAd/CMV/V5-DEST destination vector (Invitrogen), using the Gateway LR recombinase (Invitrogen) according to the manufacturer’s instructions. Expression clones were then transfected into the 293A packaging cell line to generate crude viral lysate, which was eventually used to amplify the virus in 293A cells.

5.2.7 Affinity purification of FA-tagged proteins

EBNA1 and E444A/S447A encoding sequences were PCR amplified from pc3Orip plasmids and ligated into Xho1 and Not1 sites of pMZS-FA (kindly provided by Dr Jack Greenblatt). Constructs expressing FA-tagged proteins were transfected in 293 cells, using Lipofectamine 2000 (Invitrogen). 48 hours post transfection, cells were harvested and cell pellets (1 g) stored at -80°C. Cell pellets were thawed and lysed in TAP-lysis buffer (50 mM Tris pH8, 200 mM NaCl, 1% NP40) supplemented with protease inhibitors (Sigma, P8340). 30 mg of total protein (at 7 mg/mL) was incubated with 100 µL of IgG resin (Amersham) for 3 hours at 4°C. After extensive washing in TAP wash buffer (10 mM Tris.Cl pH 8.0, 150mM NaCl, 0.1% NP40), complexes were eluted by TEV cleavage using 50 units of TEV protease (Invitrogen) in TEV buffer (10 mM Tris.Cl pH 8.0, 150mM NaCl, 0.1% NP40, 0.1 mM EDTA) overnight at 4°C. Eluted complexes were then incubated with anti-FLAG M2 resin (Sigma) for 3 hours at 4°C. After extensive washing in TAP wash buffer, protein complexes were eluted by resuspending in non-reducing SDS-loading buffer (0.25 M Tris.Cl pH 6.8, 5% glycerol, 2% SDS, 0.1% bromophenol blue) and boiling for 5 minutes. After centrifuging briefly, supernatant was collected and DTT to was added to 1 mM final concentration prior to loading on a 10% denaturing polyacrylamide gel.

5.2.8 Immunoprecipitation

For immunoprecipitation experiments in Figure 5-6, 293 cells were transduced with adenovirus engineered to express EBNA1 and E444A/S447A. 48 hours post-transduction, cells
were harvested and lysed in RIPA buffer (50mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate) containing protease inhibitor cocktail P8340 (Sigma). 1mg of total protein from cleared lysate was immunoprecipitated with 30 µL of anti-FLAG-M2 affinity gel resin (Sigma). After extensive washing in RIPA buffer, immunoprecipitates, along with 5% of input were subjected to SDS-PAGE and immunoblotting with USP7 antibody (Holowaty et al., 2003b).

5.2.9 Mass spectrometry analysis

Dried gel bands were excised and rehydrated, first in 100 mM ammonium bicarbonate and then in 60 mM ammonium bicarbonate/40% acetonitrile, followed by reshrinking in 100% acetonitrile. Proteins in excised gel slices were reduced with 3 mM DTT, followed by washing in 60 mM ammonium bicarbonate/40% acetonitrile and then in 100% acetonitrile. Proteins were then alkylated in 20 mM iodoacetoamide, followed by digestion with trypsin at 12.5ng/µL. Digested proteins were extracted from gel slices first in 50 µL of 100 mM ammonium bicarbonate, then twice in 50 µL of 5% formic acid/50%acetonitrile and pooled. LC/MS analyses were done by the Proteomics core facility at Sunnybrook research institute, Toronto, Ontario. Samples were subject to IonTrap LC/MS analysis using the Agilent 1100 series mass spectrometer. Agilent spectrum Mill software (Re. A.03.03.084SR4) was used to search the NCBI non-redundant human protein data base, with 206981 entries (release date 07-05-2009).

5.3 RESULTS

5.3.1 Mapping of the Mdm2-USP7 interaction

Previous studies have suggested that USP7 binds Mdm2 (Cummins and Vogelstein, 2004; Li et al., 2004). We examined whether this interaction is direct by pull-down assays using equimolar amounts of purified full-length USP7 and Mdm2 fragments fused to GST. N-terminal fragments of Mdm2 were used as opposed to full length Mdm2 given the poor stability of the full length protein. GST fusions containing Mdm2 residues 1–280, 1–160 or 110 – 220, each caused the retention of USP7 on glutathione resin, which was not seen with GST alone (Figure 5-1A). This indicated that the Mdm2-USP7 interaction is direct and is likely mediated by sequences between residues 110 and 160. To determine whether this interaction occurs through the USP7
NTD, I repeated the pull-down assay with GST-Mdm2 and the USP7 NTD (Figure 5-1B). Analysis was difficult with GST-Mdm2_{1-280} and GST-Mdm2_{110-220} because of degradation products. However, the USP7-NTD was clearly retained on the glutathione resin by Mdm2_{1-160}, to a similar degree as by EBNA1 (Figure 5-1B).

I also examined the interaction of Mdm2_{1-160} with the USP7 NTD by gel-filtration analysis (Figure 5-2). The two proteins showed different migrations when run individually through a gel filtration column (Figure 5-2A, top and middle panels). However, in keeping with the pull-down results, when incubated together, a complex was formed between the two proteins that comigrated on the gel-filtration column (Figure 5-2A, bottom panel). The interaction was disrupted by addition of the USP7-binding fragment of EBNA1 (395–450) to the binding reaction (Figure 5-2B). This fragment formed a complex with the USP7 NTD, indicating that Mdm2 is probably bound to the same USP7 pocket as EBNA1, but with lower affinity.

To identify the Mdm2 peptide bound by USP7, I looked for potential recognition sequences within Mdm2_{1-160} that match the P/AXXS USP7-binding motif of p53 or the EXXS USP7-binding motif of EBNA1 (Saridakis et al., 2005; Sheng et al., 2006). Five such sequences were identified, and a sixth QXXS motif (peptide 2) was also included, as glutamine could theoretically mediate the same contacts with USP7 that glutamate does. Additionally, another PSTS site outside of the 1-160 residues was also included in the analysis since it matched the PxxS motif. Corresponding 10-residue peptides were synthesized with each potential binding sequence at identical positions (residues 4–7 of the peptide; Figure 5-3A). Interactions of these peptides with the USP7 NTD were determined by monitoring the change in intrinsic fluorescence of USP7 at peptide concentrations up to 100 μM. Whereas no binding was detected with peptides 1 (85-LFGVPSFSVK-94), 2 (109-VVVNQESSDS-118) and 3 (126-RCHLEGGSDQ-135), titratable binding was seen with peptides 4 (141-ELQEEKPSSS-150), 5 (152-LVSRPSTSSR-161), 6 (143-QEEKPSSSHEL-152) and 7 (394-YSQPSTSSSI-403) with the \( K_d \)s shown in Table 5-1. Peptides 4 and 6 are almost entirely overlapping; they are shifted by two residues so that potential binding sites EKPS and PSSS are appropriately positioned in each. The finding that the nonoverlapping peptide 5 was also bound by USP7 indicates that there are at
Figure 5-1. Mapping the Mdm2 and USP7 interaction. (A) GST pull-down assays in which Mdm2 fragment 1–280, 1–160 or 110 – 220 fused to GST was incubated with equal amounts of full-length USP7, applied to glutathione resin, washed and eluted with glutathione. GST alone and GST-EBNA1 were used as negative and positive controls respectively. The column load (L), flow-through (FT) and eluates (E) are shown. (B) GST pull-down assays performed as in A except that the USP7 NTD was used in place of full-length USP7. GST-EBNA1 is shown as a positive control.
Figure 5-2. Gel Filtration analyses of Mdm2 and USP7 interaction. (A) Mdm21–160 fragment and USP7-NTD were incubated briefly at 4°C individually (top and middle panels) or in combination (bottom panel) before subjecting to Gel-filtration chromatography. Equal volumes were collected and analyzed by SDS-PAGE and coomassie blue staining. (B) Gel-filtration analysis of complexes formed by incubating equimolar amounts of the USP7 NTD and the Mdm21–160 fragment (top gel), showing comigration of a portion of USP7 with Mdm2 (fractions 22–26) as well as unbound USP7 (fractions 28–34). Middle gel shows the effect of including equimolar amounts of EBNA1 395–450 in the incubation mix, where USP7 dissociates from the Mdm2 complex and comigrates with EBNA1. Bottom gel shows the migration of EBNA1 395–450 alone in the same gel-filtration analysis.
Figure 5.3. Putative USP7 recognitions sites in Mdm2 and MdmX. Sequences of Mdm2 (A) and MdmX (B) showing peptides tested for USP7-NTD binding (underlined) with potential recognition sequences (boxed).
Table 5-1. Affinities of Peptides for USP7-NTD as determined by change in tryptophan fluorescence.

<table>
<thead>
<tr>
<th>Peptides Tested</th>
<th>$K_d$ for USP7-NTD (µM)*</th>
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<tr>
<td><strong>Mdm2</strong></td>
<td></td>
</tr>
<tr>
<td>Peptide 1 (PSFS)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Peptide 2 (QESS)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Peptide 3 (EGGS)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Peptide 4 (EKPS, PSSS)</td>
<td>7.97±0.65</td>
</tr>
<tr>
<td>Peptide 5 (PSTS)</td>
<td>7.53±0.33</td>
</tr>
<tr>
<td>Peptide 6 (EKSP, PSSS)</td>
<td>8.02±0.30</td>
</tr>
<tr>
<td>Peptide 7 (PSTS)</td>
<td>7.0</td>
</tr>
<tr>
<td><strong>MdmX</strong></td>
<td></td>
</tr>
<tr>
<td>Peptide 1 (AQCS)</td>
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</tr>
<tr>
<td>Peptide 2 (PCNS)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Peptide 3 (AHSS)</td>
<td>8.0</td>
</tr>
<tr>
<td>Peptide 4 (AGAS)</td>
<td>&gt;100</td>
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</table>

*Average of three experiments reported where applicable, ± standard deviation.
least two different binding sites for USP7 between Mdm2 residues 141 and 161. Binding was also detected with peptide 7, which is outside of the 1 – 160 N-terminal residues initially tested for USP7-NTD binding in GST-pull down assays. This suggests that Mdm2 could have two separate regions at opposing ends of the protein, which could bind USP7 independently.

5.3.2 Structure of Mdm2-USP7 Complex

Crystal trials were set with the USP7 NTD and each of the Mdm2 peptides 4, 5 and 6. Crystals of the USP7 NTD bound to peptide 4 (acetyl–141-ELQEEKPSSS-150–amide) were obtained, the structure of the complex was determined using molecular replacement and the model was refined to 2.1-Å resolution (Figure 5-4A and B). The structure of the USP7-NTD bound to EBNA1 and p53 peptides is shown in Figure 5-4D and E for comparison. The structure of USP7 bound to Mdm2 145-EKPSSS-150 is virtually identical to that bound to p53 358-EPGGSR-363, with an r.m.s. deviation of 0.12. The r.m.s. deviation between the Cα's of p53 \textsubscript{359–362} and Mdm2 \textsubscript{147-PSSS-150} is also very small, 0.11 Å (Figure 5-4C), although some deviation occurs in the flanking regions. Upon binding USP7, the Mdm2 peptide buries a total of 714 Å\textsuperscript{2} of surface area, slightly more than the p53 peptides, but substantially less than EBNA1. The common interactions with USP7 observed for the p53 peptides were also observed with the Mdm2 peptide (Figure 5-4A and B). There are several distinguishing interactions made by the Mdm2 peptide that were not seen with either p53 peptide. These include both side chain and backbone interactions from Mdm2 Glu145', Lys146' and Ser148' to USP7 Ser168 and Asn169, mediated through several water molecules. X-ray data collection and refinement parameters are listed in Table 5-2.

5.3.3 Characterization of the MdmX-USP7 interaction

Since both p53 and Mdm2 bind the USP7-NTD and given the sequence similarity between Mdm2 and MdmX, I wanted to test whether MdmX also bound the USP7-NTD. For this I conducted GST-pull down assays using full length GST-MdmX and USP7-NTD. GST-Mdm2 and GST only were used as positive and negative controls respectively. GST-MdmX was able to retain USP7-NTD on the resin, albeit to a much lower extent than the GST-Mdm2 positive control (Figure 5-5, compare lane 1 to 3). Only GST showed no binding to USP7-NTD, as expected.
Table 5-2. X-Ray Data Collection and Refinement Parameters.

<table>
<thead>
<tr>
<th>X-Ray Data</th>
<th>USP7:MdmX&lt;sup&gt;AHSS&lt;/sup&gt;</th>
<th>USP7:Mdm2&lt;sup&gt;PSSS&lt;/sup&gt;</th>
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<td>Space Group</td>
<td>P4&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P4&lt;sub&gt;1&lt;/sub&gt;</td>
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<tr>
<td>Resolution (Å)</td>
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<td>2.1</td>
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<tr>
<td>Unit Cell Axes (Å&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>69.8 x 69.8 x 45.5</td>
<td>69.9 x 69.9 x 45.7</td>
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<td>32 471</td>
</tr>
<tr>
<td>Unique Reflections (#)</td>
<td>21 185</td>
<td>13 160</td>
</tr>
<tr>
<td>Intensity (I/σ&lt;sup&gt;2&lt;/sup&gt;&lt;I&gt;)</td>
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<td>4.3 (2.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.1)</td>
<td>87.0 (83.9)</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt;</td>
<td>0.055 (0.249)</td>
<td>0.112 (0.543)</td>
</tr>
</tbody>
</table>

Refinement

| R<sub>work</sub>           | 0.202                     | 0.207                     |
| R<sub>free</sub>           | 0.228                     | 0.250                     |
| Protein Atoms (#)         | 1161                      | 1134                      |
| Water Molecules (#)       | 107                       | 137                       |
| rmsd bonds (Å)            | 0.005                     | 0.006                     |
| rmsd angles (°)           | 1.4                       | 1.39                      |
| rmsd dihedrals (°)        | 25.2                      | 24.9                      |
| rmsd improper (°)         | 0.86                      | 0.852                     |
| thermal factors (Å<sup>2</sup>) | 18.4                    | 21.6                      |

Ramachandran Plot

| Most Favoured | 0.888                   | 0.866                     |
| Additionally Allowed | 0.104                   | 0.118                     |

Numbers in brackets refer to the highest resolution shell, 1.81 Å to 1.78 Å for the HdmX<sup>AHSS</sup> data and 2.18 to 2.10 Å for the Hdm2<sup>PSSS</sup> data. *R<sub>sym</sub> = Σ |I - <I>| / ΣI where I is the observed intensity and <I> is the average intensity from multiple observations of symmetry-related reflections.
Figure 5-4. Crystal Structure of USP7-NTD bound to Mdm2, p53 and EBNA1 peptides. (A) Transparent surface diagrams of Mdm2-USP7. (B) Detailed interactions between USP7 (light gray) and MDM2 (dark gray). (C) Overlay of MDM2 (blue) and p53 359-PGGS-362 (red) peptides from USP7 complex structures. (D and E) Transparent surface diagrams of EBNA1-USP7 (D) and p53-USP7 (E).
Figure 5-5. Mapping the MdmX-USP7 interaction. GST pull-down assays in which MdmX fused to GST was incubated with equal amounts of USP7-NTD, applied to glutathione resin, washed and eluted with glutathione. GST alone and GST- Mdm21-160 were used as negative and positive controls respectively. Fractions were subjected to SDS-PAGE and proteins were visualized by western blotting using antibodies as indicated.
(Figure 5-5 lane 2). This suggested that MdmX can directly interact with USP7 and that USP7-NTD is sufficient for this interaction.

Knowing the preference of USP7-NTD for a P/AxxS motif, I next analyzed the sequence of MdmX to identify such motifs as putative USP7 binding sequences. Four such motifs (highlighted in Figure 5-3B) were identified in MdmX and 10-mer peptides containing these sequences were synthesized to test binding to USP7-NTD in a tryptophan fluorescence assay. Peptide 1 (with motif 8ACQS 11) and peptide 3 (containing motif 398 AHSS 401) showed the strongest binding to USP7-NTD (estimated Kd ~8\(\mu\)M), while others showed negligible binding (Kd >100\(\mu\)M) (Table 5 – 2). Accordingly, peptides 1 and 3 were used further for co-crystallography with USP7-NTD.

5.3.4 Structure of MdmX peptide bound to USP7-NTD

To reveal the molecular mechanism of the USP7-Hdmx interaction, USP7-NTD and the MdmX peptide (acetyl-395LDLAHSSESQ404-amide) were co-crystallized and the structure was determined using molecular replacement (Figure 5-6A). The model was refined to 1.8 Å resolution with R and Rfree values of 0.202 and 0.228 respectively. The overall structure of the NTD of USP7 formed an eight-stranded anti-parallel beta sandwich similar to the TRAF-C domain of tumor necrosis factor-receptor associated factors. The MdmX AHSS peptide binds to the USP7-NTD by forming a fifth antiparallel \(\beta\)-strand on the sheet formed by strands \(\beta2, \beta3, \beta4\) and \(\beta7\), adjacent to strand \(\beta7\). The MdmX AHSS peptide makes the following contacts with strand \(\beta7\), in addition to the H-bonding contacts made between the backbones of strand \(\beta7\) and the peptide involved in extension of the \(\beta\)-sheet. MdmX Ala398 is stacked between the side chains of USP7 Trp165 and Phe169 and forms hydrophobic interactions with both residues. The side chain of MdmX His399 forms a H-bond with the side chain of USP7 Ser168. The side chain OH of MdmX Ser401 makes important contacts with the NTD of USP7 by forming H-bonds with the side chain and main chain of Asp164. The carbonyl of MdmX Ser401 also forms a H-bond with the side chain of USP7 Arg104 (Figure 5-6B).

The structure of the USP7-NTD complexed with MdmXAHSS is very similar to previously reported USP7-NTD structures with peptides from EBNA1, p53 and Mdm2 with an
Figure 5-6: Crystal Structure of the USP7-NTD-MdmX AHSS Complex. (A) The crystal structure of the USP7-NTD:MdmXAHSS complex is shown with the USP7-NTD in the yellow ribbon configuration and the MdmXAHSS peptide in the green stick configuration. (B) Detailed interactions between USP7-NTD (yellow) and MdmXAHSS (green). (C) Structural superimposition of p53AHSS (pink) and MdmXAHSS (green) peptides with Ala and Ser residues labeled.
average root mean square deviation of 0.15 Å² between C'' atoms. There is a p53 peptide (p53AHSS, 359PGGARAHSS367) with an identical peptide-binding motif (AHSS). The structure and binding properties of p53AHSS to the USP-NTD have already been established (Sheng et al., 2006). A more-detailed comparison between p53 AHSS and MdmX AHSS indicated that the backbones of the two peptides are completely superimposable within the AHSS region with a root mean square deviation of 0.09 Å² (Figure 5-6C).

5.3.5 Relevance of USP7-NTD binding motif in vivo

Data thus far suggest that the serine in the P/A/ExxS motif is critical for binding USP7-NTD, while the glutamate in EBNA1 peptide also contributes to binding (Saridakis et al., 2005). However, all mutational analyses to determine the importance of individual residues in USP7 binding were conducted in vitro and in the context of short peptides. For EBNA1 Glu444 was shown to be important for EBNA1-binding to USP7, while mutation of Ser447 to alanine completely abrogates binding of EBNA1 peptides to USP7-NTD (Saridakis et al., 2005). I wanted to determine the importance of these residues in cells and in the context of full length proteins and for this I employed two approaches. First, I transfected 293 cells with constructs expressing either FA-tagged EBNA1 or a double point mutant of EBNA1, termed E444A/S447A, in which Glu444 and Ser447 were mutated to alanine. The FA tag contains a triple-Flag tag and a protein A sequence separated by a TEV protease cleavage site (Figure 5-7A, top). This allows for tandem affinity purification of complexes containing the tagged protein on an IgG column, using the affinity for Protein A, followed by elution by TEV cleavage and purification on an anti-FLAG column. Using this method, I purified EBNA1- and E444A/S447A-containing complexes from 293 cells. Purified protein complexes were resolved by SDS-PAGE and visualized by silver staining. Remarkably, the profiles of proteins co-purified with EBNA1 and E444A/S447A were virtually identical with one exception (Figure 5-7A, bottom). WT EBNA1 lane contained a protein migrating around 135 kDa, the expected size of USP7, which was absent in the E444A/S447A sample. The ID of this band was confirmed to be USP7 by LC/MS mass spectrometry (MS). 20 distinct peptides were identified for USP7 and covered 20% of the amino acid sequence. Interestingly, MS analysis of the corresponding area in the
Figure 5-7. Importance of the USP7-binding motif in vivo. (A) FA-tagged proteins were overexpressed in 293 cells and either sequentially isolated on IgG and FLAG columns. Protein complexes were resolved by SDS-PAGE and visualized by silver staining. The position of the band and gel area excised for mass spectrometry is indicated with an asterisk and the expected band for EBNA1 is indicated with an arrow. (B) Lysates of transfected cells were subjected to SDS-PAGE and immunoblotting to assess expression of FA-tagged protein. (C) SPA-tagged proteins were overexpressed in 293 cells using an adenovirus based vector, immunoprecipitated using the FLAG antibody and immunoblotted with the same. Input represents 5% of the cell lysate used for immunoprecipitation.
E444A/S447A lane did not identify any USP7 specific peptides. This was not due to poor recovery or expression of E444A/S447A compared to EBNA1 (Figure 5-7B). I also tested binding of SPA-tagged EBNA1, E444A/S447A and β-gal (negative control) to USP7 in a single step immunoprecipitation assay. The SPA tag is similar to the FA tag, but contains a FLAG tag and a calmodulin binding peptide, separated by a TEV cleavage site (Zeghouf et al., 2004). Lysates from cells transduced with adenovirus expressing the SPA-tagged proteins were subjected to immunoprecipitation using the FLAG antibody, followed by immunoblotting for USP7. As expected, USP7 was found to readily interact with EBNA1, while no binding was detected with the β-gal negative control (Figure 5-7C). E444A/S447A showed minimal to no binding to USP7, highlighting the importance of the mutated residues. Together, these results confirm not only that E444 and S447 are important for binding of full length EBNA1 to USP7, but also that their mutation will exclusively disrupt USP7 binding, while other interactions remain intact.

5.4 DISCUSSION

Here I have offered further insight into substrate recognition by USP7-NTD. I have shown that both Mdm2 and MdmX interact directly and independently with USP7 and that USP7-NTD is sufficient for this interaction. I have further delineated the sequences on Mdm2 and MdmX important for interaction with USP7-NTD. These findings have led to subsequent structural studies that have revealed the molecular basis of substrate recognition by USP7-NTD (Sheng et al., 2006). Though MdmX appeared to bind to the USP7-NTD less avidly than Mdm2, this is likely due to poor stability of full length MdmX (as illustrated by the various degradation products) compared to the Mdm2 truncation.

While four of the seven Mdm2 peptides tested and two of the four MdmX peptides tested, bound USP7-NTD, not all peptides with a P/E/AxxS motif that were tested bound USP7-NTD. In peptides that bind, a preference for PSTS, PSSS and AHSS motifs is observed, highlighting the importance of ST, SS and AH as the middle residues in the motif. Additionally, the residues spanning the 4-mer sequence might also play a part, though no consistent trends were observed in the sequences analyzed.
It is worthy of note that like p53, two closely spaced USP7 recognition sites were found in Mdm2 (PSSS in peptides 4 and 6 and PSTS in peptide 5) (Sheng et al., 2006). This redundancy might ensure binding to USP7 in the event one of the sites is mutated. Additionally, closely spaced binding sequences might allow USP7 to bind different sites to cleave ubiquitin from different positions. In p53, multiple lysines between residues 370 and 386 are known to be ubiquitinated (Rodriguez et al., 2000) and this region is immediately downstream of the USP7 binding region. Though ubiquitination sites are not mapped for Mdm2, there are several lysines in close proximity to the USP7-binding sites.

Comparison of the affinities of Mdm2 and MdmX peptides for USP7 to that of p53 and EBNA1 reveals an interesting trend. USP7-binding EBNA1 peptide showed a $K_d$ of around 0.85μM (Holowaty et al., 2003a), which is about 10-fold less than any of the other peptides tested for USP7-NTD binding. This explains why EBNA1 can displace Mdm2 (as shown here) and p53 (Holowaty et al., 2003a) from USP7-NTD in gel filtration assays and can indeed disrupt p53 stabilization by USP7 in vivo (Saridakis et al., 2005). Mdm2 and MdmX peptides showed slightly higher affinity ($K_d$ ~8 μM) than p53 peptides (~10 μM) (Holowaty et al., 2003a; Sheng et al., 2006) for USP7-NTD. Although these small differences between short peptides might not translate exactly to full length proteins, they are consistent with in vivo observations on how these proteins interact with USP7. Under steady state conditions Mdm2 and MdmX are predominantly bound by and stabilized by USP7 (Meulmeester et al., 2005a). Only after post-translational modifications of Mdm2 and MdmX is their interaction with USP7 disrupted, leaving USP7 free to interact with and stabilize p53 (Meulmeester et al., 2005a).

Finally, I have offered the first line of evidence supporting the importance of the E/A/PxxS USP7 binding motif in vivo. It was remarkable to see that mutation of Glu444 and Ser447 in EBNA1 specifically abrogates binding to USP7, while other interactions remain intact. However, it remains to be seen whether a single mutation of Ser447 is sufficient to knock out USP7 binding in cells, as was observed in vitro with a short EBNA1 peptide (Saridakis et al., 2005). While EBNA1 is not yet known to be a substrate of USP7 activity, its mode of interaction is similar to that of other USP7 binding partners. It would be equally interesting to know whether mutation of corresponding residues in p53, Mdm2 and MdmX would yield results
similar to those obtained with EBNA1. Indeed, selective disruption of binding of negative regulators of p53 to USP7 may have potential for therapeutic application.

While most of the observations here were made in cell-free systems, they are consistent with the interactions of these proteins *in vivo*. More importantly, this work forms the basis of structural analyses leading to the understanding of molecular recognition of substrates by USP7 and thus furthers our understanding of the intricate relationship of these proteins in the p53 pathway.
CHAPTER 6

THESIS SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS
6.1 THESIS SUMMARY

Study of infectious agents and their interaction with the host is an exciting approach to answer biological questions. One not only learns about the biology of the pathogen but also potentially uncovers hidden aspects of that host. Historically, study of infectious agents has made seminal contributions to our understanding of cellular and molecule processes. The discovery of p53 and pRB tumour suppressor proteins are two prominent examples (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Ludlow et al., 1989; Whyte et al., 1988). My work documented in thesis continues with this trend. The interaction of the Epstein-Barr virus protein, EBNA1, with the host protein USP7 has important consequences for the host and the virus alike. I have shown that the USP7-EBNA1 interaction is important for viral persistence, as it promotes the association of EBNA1 with the viral origin of latent replication, which is required for replication of the viral genome, partitioning of the viral genome and transcriptional activation of viral genes important for latent infection. On the other hand, the EBNA1-USP7 interaction is not a favourable one for the host cell. EBNA1 usurps the customary functions of USP7, such as regulation of p53, to promote host cell transformation and immortalization. Fittingly, these studies led me to discover a new function of USP7 in the regulation of the PML protein, required for the formation of multiprotein nuclear structures known as PML-NBs. Finally, I have provided the biochemical groundwork for determining the basis for molecular recognition of substrates by USP7. Coupled with structural analysis of protein interactions of USP7, these studies could be instrumental in therapeutic interventions.

6.2 GENERAL DISCUSSION
6.2.1 USP7 and EBNA1 functions at the oriP

The investigation of the effect of USP7-interaction on EBNA1 DNA-binding stemmed from the observation that the USP7-binding sequences and the DNA-binding and dimerization domain of EBNA1 are in close proximity. Given the size of USP7, I postulated that USP7 binding might inhibit EBNA1 DNA binding. Surprisingly, I discovered that the interaction with USP7 increased the association of EBNA1 with its target DNA in vitro and was important for this association in vivo. The DNA-binding domain is absolutely required for functions of EBNA1 in persistence of latent viral infection, though not much is known about its regulation. Although
sequences other than the DNA-binding domain of EBNA1 have been shown to be important for regulating EBNA1 functions at the oriP, details of how these sequences confer such regulation have not been readily forthcoming. This is beginning to change with the discovery of several host proteins that interact with EBNA1, many of which bind through sequences important for the known functions of EBNA1 (Holowaty et al., 2003b). These interacting partners would likely regulate the functions of EBNA1 in diverse manners. For instance, members of the nucleosome assembly family of proteins promote transcriptional activation by EBNA1, as does the bromodomain protein Brd4 (Lin et al., 2008; Wang and Frappier, 2009). The role of these proteins in transcriptional regulation (viral transcription in the case of Brd4) is relatively well known and they likely facilitate EBNA1 functions in transcription and replication in manners already known for regulatory elements in the host cell. On the other hand, there is novelty in how USP7 contributes to EBNA1 function at the oriP. The ability of USP7 to promote the association of EBNA1 with its target DNA sequences is unprecedented, since this ability is completely independent of deubiquitination by USP7 and no other EBNA1-interacting protein is known to do the same. Moreover, the role of USP7 in EBNA1 functions at oriP is two-fold. USP7 and GMPS are recruited as a H2B deubiquitinating complex that confers epigenetic regulation on the processes at the oriP. Though this resembles one of the native functions of USP7 in eukaryotic cells, it is not clear whether it has the same consequence for viral transcription (or replication) as on the host processes. Deubiquitination of histone H2B by the USP7-GMPS complex negatively regulates transcription and is associated with gene silencing (van der Knaap et al., 2005). Conversely, USP7 was required for efficient activity of EBNA1 in transient reporter assays, though this may be because USP7 is also required for efficient DNA binding by EBNA1. Thus the exact effect of deubiquitination of H2B by USP7 on EBNA1 transactivation at the oriP in vivo is unclear.

Histone modification at the oriP (or in the EBV genome) is just beginning to be explored, and my findings also suggest a role of H2B monoubiquitination in epigenetic regulation of EBV gene expression. Others have thus far studied acetylation and methylation of histones at the oriP. Acetylated histone H3 is enriched at the DS but there is a noticeable decrease in late G1, thought to account for delayed replication of EBV episomes and oriP-containing plasmids (Zhou
et al., 2005; Zhou et al., 2009). OriP is also enriched for dimethylated H3K4 and trimethylated H3K9 (Chau and Lieberman, 2004; Day et al., 2007; Zhou et al., 2005). Although H3K4 methylation is associated with increased transcription and is often preceded by H2B ubiquitination, it is unclear what its role is at the DS. What is also not known is how these methylations at the FR are related to H2B ubiquitylation at the FR. Although EBNA1 activates transcription from the FR, and H2B deubiquitination correlates with decreased transcription, recruitment of the USP7-GMPS complex might be a mechanism to fine tune the level of transcription activation from the FR. It remains to be seen how different histone modifications at the oriP are integrated into a coherent epigenetic regulatory program.

Furthermore, although the effect of USP7 interaction on stimulating EBNA1 DNA-binding is striking, it is unclear how this stimulation is biochemically achieved. One explanation for the in vitro observation may be that USP7 increases the solubility of EBNA1, which would enhance the availability of EBNA1. EBNA1 is highly basic and requires relatively high salt concentrations for solubility (Frappier and O'Donnell, 1991b) and NAP1 and TAF-1β are known to promote EBNA1 solubility (Wang and Frappier, 2009). Though it is possible USP7 can promote EBNA1 solubility, it is unlikely to be the sole reason for the effect on DNA-binding by EBNA1. The truncation mutant of EBNA1, 395 – 640, does not have such stringent solubility requirements and the effect of USP7 on its DNA binding activity was far more pronounced than on full length EBNA1. It is possible that USP7 allosterically regulates the DNA binding activity of EBNA1. No information regarding the structure of EBNA1, other than the DNA-binding domain, is available and structure prediction tools have also been futile. One could however look for changes in the conformation of the EBNA1 DNA-binding domain or other regions of EBNA1 after USP7 binding, which might explain the stimulated DNA binding.

6.2.3 Stimulation of DNA-binding by USP7 is not unique to EBNA1

The ability of USP7 to promote EBNA1-DNA interaction prompted me to investigate the role of USP7 in DNA binding by p53 (see appendix). An important determinant of DNA binding by the core domain of p53 is the auto-regulation of this activity by the C-terminal regulatory domain of p53. The C-terminal domain is heavily modified post-translationally and these modifications affect the ability of p53 to bind DNA (Appella and Anderson, 2001; Brooks
I found that binding to USP7 is yet another means of regulating this property of p53. It is interesting to see that the ability of USP7 to promote DNA-protein interactions is not limited to EBNA1 but also extends to p53, specifically given that p53 and EBNA1 are two very different DNA-binding proteins. However, there are both similarities and subtle differences in how USP7 contributes to the DNA-binding activity of the two proteins. The N-terminal domain of USP7 has been shown to be sufficient for binding both p53 and EBNA1. While USP7-NTD can stimulate DNA-binding by EBNA1, it had no effect on p53 DNA-binding. Additionally, in both cases, the data suggest that regions other than the USP-NTD may contribute to DNA-binding by EBNA1 and p53. EBNA1 can bind DNA in complex with USP7 and can recruit USP7 to EBNA1-binding sequences in the EBV genome. USP7 in turn contributes to functions of EBNA1 at the EBV origin of replication that are important for latent EBV infection. Such a role of USP7 in p53-DNA interactions is not supported by the in vitro observations and has yet to be tested in cells. Clearly, further studies are warranted, ideally in p53-positive primary cells, to decipher a potentially novel mode of p53 regulation by USP7.

Additionally, an important overarching idea regarding the function of USP7 has emerged from studying the role of USP7 in p53 and EBNA1 DNA-binding. It is that USP7 functions may not be limited to its catalytic activity. This idea was further reinforced when I studied the role of USP7 in regulation of PML proteins and PML-NBs.

6.2.4 Alteration of the cellular Environment by EBNA1

The role of USP7 in EBV latent infection is not restricted to EBNA1 DNA-binding and its functions at the oriP. USP7 is intimately tied to regulation of proteins in the p53 pathway and misregulation of this pathway can make the cell highly susceptible to malignant transformation. Although several EBV latent proteins play a role in carcinogenesis (Young and Rickinson, 2004), none had been known to interfere with p53 function. This is at odds with proteins of other DNA tumor viruses, which have been known to target the p53 tumor suppressor. Prominent examples include the E6 protein of papillomavirus (Scheffner et al., 1990; Werness et al., 1990), E1b from adenovirus (Sarnow et al., 1982) and the large T antigen of SV40 (Bargonetti et al., 1992; Farmer et al., 1992; Lane and Crawford, 1979; Linzer and Levine, 1979). Prior work had shown EBNA1 can compete with p53 for USP7-binding in vitro
I have shown that this competition translates to in vivo effects, where EBNA1 can prevent stabilization of p53 by USP7. EBNA1 expression also results in diminished acetylation of p53, a modification which is required for optimal p53 activity as a transcription factor. This is in accordance with the ability of EBNA1 to disrupt PML-NBs, which serve as sites for p53 acetylation by CBP (Pearson et al., 2000). Thus in EBNA1 expressing cells p53-induced apoptosis and expression of p21 is severely compromised.

It is unclear whether p53 function is compromised in EBNA1 expressing cells solely due to suboptimal stabilization of p53 by USP7 or due to disruption of PML-NBs, which are important for p53 activation and apoptosis (Pearson et al., 2000; Takahashi et al., 2004). The data suggest a model (Figure 6-1) in which EBNA1 employs a two-pronged approach to target p53. First EBNA1 disrupts p53-USP7 interaction, thus inhibiting p53 stabilization. Secondly EBNA1 cripples p53 function by disrupting PML-NBs and thus hindering p53 activation. This yields unstable and inactive p53, which fails to function optimally as a transcription factor and as a tumor suppressor.

PML-NBs are also important for DNA damage repair (Boe et al., 2006). In line with this function of PML-NBs, I have shown that EBNA1 expression also impairs the ability of cells to repair DNA damage. Despite this impairment, cells are resistant to programmed cell death, making it likelier for oncogenic events to accumulate. This dichotomy underpins the contribution of EBNA1 to EBV-mediated carcinogenesis, at least in cells of the nasopharynx.

**6.2.5 Model for EBNA1’s role in host cell immortalization and transformation**

Studies, such as those involving transgenic mice, to test whether EBNA1 promotes tumor formation have yielded conflicting results (Kang et al., 2005; Kang et al., 2008; Wilson et al., 1996). Nevertheless, analysis of molecular events instigated by EBNA1 expression in various cell lines and xenografts consistently validate the notion that EBNA1 does contribute to cell survival, cell proliferation and thus tumor formation (Kaul et al., 2007; Kube et al., 1999; Sheu et al., 1996). These studies, coupled with my work, suggest that, while EBNA1 may not be sufficient for the transformation process, it can definitely alter the cellular environment to make transformation more likely should additional tumorigenic events occur. Hence, like other
Figure 6-1. Model for EBNA1-mediated alteration of p53 function in nasopharyngeal carcinoma. (A) In the absence of EBNA1, p53 regulation is intact such that p53 can be readily stabilized by USP7 and activated by acetylation (among other modifications) through PML-NBs in response to stress. Stabilized and active p53 can now initiate a gene expression program to either induce growth arrest or apoptosis, depending on the type and degree of stress. (B) In EBV-positive EBNA1 expressing cells, USP7 is tightly bound by EBNA1 and thus fails to efficiently stabilize p53. On the other hand, EBNA1 also disrupts PML-NBs and impedes acetylation of p53. This renders p53 unstable and inactive and unable to fulfill its function as a tumor suppressing transcription factor.
latent genes, EBNA1 is a contributing factor in EBV-associated malignancies. This is also consistent with the observations that, while EBV-associated tumors may be clonal expansions of EBV-infected cells, the oncogenic potential of EBV works cooperatively with environmental factors, genetics and dietary habits in malignant transformation.

6.2.6 USP7 as a negative regulator of PML

The observation that EBNA1 disrupts PML-NBs (Sivachandran et al., 2008) intrigued me further as it hinted at a possible new function for USP7. When USP7 was discovered as an ICP0-interacting protein, it was found associated with PML-NBS (Everett et al., 1999b; Meredith et al., 1995; Meredith et al., 1994). However, the biological significance of this association has been elusive. Additionally, like EBNA1, ICP0 expression also leads to degradation of PML proteins and PML-NBs and at least in some cell backgrounds, this ability correlated with ICP0 interaction with USP7 (Parkinson and Everett, 2000). The ability of EBNA1 to disrupt PML-NBs correlates more tightly with its ability to interact with USP7, because as Δ395 – 450, a mutant of EBNA1 lacking USP7-binding, fails to disrupt PML-NBs (Sivachandran et al., 2008). These studies implied that USP7 directly regulates PML. Through a series of overexpression and RNAi experiments, I have established that, indeed, USP7 is a negative regulator of PML proteins and PML-NBs. More importantly, I found the catalytic activity of USP7 to be dispensable for this regulation. Generally, deubiquitination by USP7 stabilizes its substrate, and so it is fitting that the catalytic activity is not required for negative regulation of PML proteins by USP7. Another DUB, USP18, can also function independently of its catalytic activity in regulating interferon response (Malakhova et al., 2006). Thus this ability is not unique to USP7. These observations add depth to the functions of these DUBs and suggest that domains other than the catalytic core might be more than interfaces for substrate recognition. In a multiprotein complex, via their protein interaction domains, DUBs may link up other enzymes with their substrates. Given that USP7 is important for optimal ubiquitination of PML, it is conceivable that USP7 couples PML with a ubiquitin ligase. Surprisingly, the contradictory activities of ubiquitination and deubiquitination work cooperatively to fine tune the process of protein ubiquitination and degradation, and USP7 interacts with the cellular ubiquitin ligases Mdm2, MARCH7 and Chfr (Cummins et al., 2004; Li et al., 2004; Nathan et al., 2008; Oh et al.,
While I have not looked at whether MARCH7 regulates PML levels, my preliminary observations do not suggest Mdm2 regulates PML levels. This is consistent with observations by several groups, who have shown that PML and Mdm2 interact but did not find this interaction to impact PML stability (Bernardi et al., 2004; Wei et al., 2003; Zhu et al., 2003). Similarly, I did not find Chfr to regulate PML levels either. I also found USP7 to work independently of the known ubiquitin ligases of PML, namely RNF4 and E6AP.

USP7 also worked independently of Ck2, which phosphorylates PML and primes it for polyubiquitination and degradation. The ubiquitin ligase responsible for Ck2 induced ubiquitination of PML is not known, and since it works downstream of Ck2, USP7 could still work through this ubiquitin ligase even in the absence of Ck2. Additionally, given the diversity of ubiquitin ligases, it is also possible that USP7 works through a completely different protein. Lastly, it is interesting that silencing of USP7 has such a dramatic effect on PML proteins and PML-NBs given only a small fraction of USP7 associates with PML-NBs. However, there is also some PML soluble in the nucleus and not associated with PML-NBs, and so USP7 does not necessarily only target PML proteins at PML-NBs. Also, my studies do not rule out the possibility that USP7 may serve other functions at PML-NBs, which may or may not involve its catalytic activity. Indeed PML-NBs do not serve as mere storage hubs of proteins but sites where protein function is coordinated dynamically (Dellaire and Bazett-Jones, 2004).

6.2.7 Insights into substrate recognition by USP7-NTD

As part of a collaborative effort, I have helped gain insight into substrate recognition by USP7 through its N-terminal TRAF domain. USP7-NTD recognized proteins through a P/A/ExxS motif, although the E at the first position has so far only been noticed in EBNA1. It should be noted that this is not a consensus for USP7-NTD binding. The P/A/ExxS motif rather represents a preferred sequence that is likely to bind USP7-NTD. Since not all P/A/ExxS motifs bind USP7-NTD, the importance of the middle two residues or residues outside the motif cannot be overlooked, yet no consistent patterns have thus far been observed for these sequences. However, this should not detract from the biological significance of these findings or their potential for therapeutic applications. Although USP7 stabilizes p53 when it is overexpressed, its complete ablation through RNAi or genetic knockout has the same effect and promotes tumor
suppression by p53. In the latter situation, this is because Mdm2, which is the major negative regulator of p53 and marks it for degradation, can no longer be stabilized by USP7. Thus, theoretically, selectively blocking the interaction of USP7-NTD with Mdm2 should have the same effect, assuming no other region of USP7 interacts with Mdm2. Small-molecule inhibitors of USP7 have been successfully used to stabilize and activate p53 in a tissue culture system (Colland et al., 2009), but these targeted the catalytic activity of USP7. Targeting USP7 catalytic activity may also relieve inactivation of other tumor suppressors, such as FOXO4 and PTEN, and may have an overall beneficial effect as an anti-cancer therapeutic strategy. On the other hand, it could have nonspecific and/or toxic effects, which were not addressed in the aforementioned study. More importantly, I have shown that the catalytic activity of USP7 may only be one facet of its functions. Therefore, targeting protein interactions through domains other than the catalytic core may still be a viable option for pharmaceutical intervention. Structural and biochemical characterization of these interactions could be invaluable in such endeavors.

6.3 FUTURE DIRECTIONS

6.3.1 Promotion of DNA-binding activity by USP7

The finding that USP7 stimulates DNA binding of two different DNA-binding proteins was quite surprising. For p53, this likely happens because USP7 binds the C-terminal domain of p53, which regulates its DNA-binding activity. This can be further confirmed by using a chimeric p53 C-terminal domain which retains USP7-binding but does not confer autoregulation of DNA-binding. Sauer et al have made such chimeric p53 proteins containing C-terminal domains from the other two members of the p53 family, p63 and p73, which have several isoforms (Sauer et al., 2008). Only the basic C-terminal domain of the p73γ isoform exhibited autoregulation of DNA-binding by p53, while other, more neutral, C-terminal domains did not. These C-terminal domains can be substituted for most of the regulatory domain of p53 without disrupting USP7 binding, as USP7-binding sequences are on the extreme N-terminal of the regulatory domain. On the other hand, how USP7 stimulates DNA-binding by EBNA1 is less clear. If USP7 allosterically regulates this activity, one could monitor conformational changes in the DNA-binding domain of EBNA1 after USP7 binding. One approach involves isotopic
labeling of EBNA1 395 – 641 with $^{15}$N and measuring NMR-chemical shifts in the presence and absence of USP7. This approach has been successfully used to study conformational changes in p53 mutants upon DNA binding (Ayed et al., 2001). Another approach may be to conduct crystallographic studies with USP7-NTD bound to EBNA1 395 – 641 and compare the structure of unbound EBNA1 to that of EBNA1 bound by USP7.

### 6.3.2 Epigenetic regulation of Latent Gene expression by USP7

USP7 is important for EBNA1-dependent transcription activation from the FR in reporter assays. However, this may be because USP7 promotes EBNA1 DNA-binding. The effect of histone H2B deubiquitination by USP7 on EBV latent gene expression needs to be examined in a native setting. Histone H2B ubiquitination generally leads to H3K4 methylation, which is associated with increased transcription. Methylated H3K4 is enriched at the oriP and correlates with transcriptional activation from this region (Chau and Lieberman, 2004). Deubiquitination of H2B by the USP7-GMPS complex may offer finer control of these epigenetic events. Chau et al have previously studied the effect of H3 methylation on EBV latent gene expression by measuring the transcript levels of LMP1 and EBNA2 after blocking protein methylation with inhibitors (Chau and Lieberman, 2004). A similar approach can be taken to assess the role of H2B ubiquitination in EBV gene expression. Levels of EBV latent transcripts can be measured using RT-PCR after overexpression of WT USP7 or its C223S mutant or silencing of USP7 to examine the effect of H2B ubiquitination on EBV latent gene expression.

### 6.3.3 Regulation of PML and PML-NBs by USP7

One of the most surprising discoveries during the course of this work has been that USP7 negatively regulates PML proteins and PML-NBs. My observations suggest that USP7 potentiates the interaction between PML and one of its negative regulators, most likely a ubiquitin ligase. However, thus far I have found all known PML negative regulators to be dispensable for this process, although I have not ruled out the involvement of the USP7-interacting ubiquitin ligase MARCH7. This can be readily addressed by using RNAi or overexpression of MARCH7 and measuring the levels of PML. It is also possible that USP7 works through a ubiquitin ligase as yet unidentified. A proteomic approach to profile USP7 targets may help identify such a protein. An RNAi screen, coupled with 2D-PAGE analysis, has
been used to identify potential targets of USP7 (Kessler et al., 2007), but no proteins that could explain the effect on PML could be readily identified. However, this study was biased towards proteins whose levels were regulated by USP7. The ubiquitin ligase that potentially regulates PML may not be subject to such regulation or could have been missed in this study due to inherent limitations of the experimental approach taken. Another approach may be affinity purification of endogenous USP7-containing protein complexes and may help identify the protein(s) that cooperate with USP7 in PML regulation. One way to do this is to couple USP7 to affigel resin, run human cell extracts over this affigel column and elute and identify proteins retained specifically on the column. This approach has previously been used to identify USP7 interactions in HeLa cells. This led to the discovery of the interaction between USP7 and GMPS but, unfortunately, did not identify any more USP7-interacting proteins. This approach can be altered by using purified individual domains of USP7 or expanding to different cell lines, such as CNE2 where we know that USP7 plays an important role in PML regulation. Alternatively, FA- or SPA-tagged USP7 can be ectopically expressed in cells and protein complexes containing USP7 can be purified in a two-step sequential process as was done with EBNA1. Additionally a yeast-two hybrid approach may also be taken for this purpose. This method was previously used successfully to the identify FOXO as a USP7 target (van der Horst et al., 2006). However, in that study, only a C-terminal region of USP7 was used. Therefore, potentially more USP7 interactions can be identified with either full length USP7 or other individual domains of USP7.

6.4 CONCLUSION

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 proteins involved in replication, chromosome attachment and transcription regulation further supports these roles of EBNA1 and suggests these functions are regulated as they are in the host. Yet EBNA1 interactions are not limited to proteins traditionally known to work in these processes and USP7 is one such protein. USP7 has turned out to be a surprising regulator – the first of its kind – of EBNA1 DNA-binding and of epigenetic events important for latent EBV infection. More importantly, the EBNA1-USP7 interaction is instrumental in crippling the functions of two
tumor suppressor proteins, p53 and PML, leaving the cellular environment conducive to transformation and immortalization. During the course of these studies, novel aspects of USP7 functions were also discovered, namely its activity-independent role in PML regulation and promotion of DNA-binding by EBNA1 and p53 (see appendix).

p53 has been one of the most widely-studied proteins in cancer research. It was over 30 years ago when this tumor suppressor was first identified as an interacting partner of the SV40 large T antigen (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). Although a less well known serological approach also identified p53 in transformed cells (DeLeo et al., 1979; Kress et al., 1979; Rotter et al., 1980), the SV40 viral studies remain iconic, and their impact on cancer research arguably unmatched. The historical significance of the work discussed herein may not match that of those seminal studies. Nevertheless, over 30 years later I have managed to find the first direct link between an EBV latent protein and p53. In doing so, I have not only learned more about the biology of EBV but also discovered hidden aspects of that of the host.
APPENDIX

USP7 PROMOTES SEQUENCE-SPECIFIC DNA BINDING BY p53

I performed all experiments discussed in this section.
INTRODUCTION

The tumor suppressor p53, often referred to as the guardian of the genome, functions by integrating signals of cellular stress and controlling cell fate (Vogelstein et al., 2000). Depending on the nature of the stimulus and the extent of cellular stress, p53 orchestrates responses that range from transient cell cycle arrest, allowing for DNA repair and cell survival, to programmed cell death (apoptosis) (Stiewe, 2007). Aberrant function of a tumor suppressor like p53 can lead to unchecked growth and the onset of cancer. It is thus not surprising that inactivation of p53 function through either mutations or interactions with cellular or viral proteins is one of the most common oncogenic events in human cancers (Nigro et al., 1989; Vogelstein et al., 2000; Vousden and Lane, 2007).

p53 fulfills its tumor suppressive function primarily by acting as a transcription factor. p53 binds DNA as a dimer of dimers in a sequence-specific manner to a consensus site comprises two decamer repeats of 5’-PuPuPuC(A/T)(T/A)GPyPyPy-3’ (where Pu is a purine and Py is a pyrimidine) separated by 0 to 13 base pairs (el-Deiry et al., 1992). p53 predominantly activates transcription of target genes, though evidence of transcriptional repression by p53 also exists (Ho and Benchimol, 2003). A growing body of work has also unearthed a cytosolic and transcription independent function of p53 (Green and Kroemer, 2009). In this role, p53 interacts with anti-apoptotic and pro-apoptotic BCL-family proteins and helps bring about permeabilization of the outer mitochondrial membrane. Though cytoplasmic functions of p53 are not strictly dependent on p53 transcription activation, transcriptional regulation by p53 still contributes to the cytosolic functions of p53 since some of the BCL-family members are direct transcriptional targets of p53 (Green and Kroemer, 2009). The importance of DNA binding and transcriptional control by p53 is further highlighted by the observation that many of the p53 mutations found in tumors are clustered in the DNA-binding domain (Soussi and Wiman, 2007) (UMD p53 database 2008_R2; http://p53.free.fr/).

The p53 protein is organized into distinct functional and structural domains. Transcription activation is mediated by the N-terminal transactivation domain (residues 1 – 70). Residues 94 – 292 form the DNA-binding domain, which binds DNA in a sequence-specific manner and is also referred to as the core domain. Further downstream is the oligomerization region (residues 320 –
360) which mediates p53 tetramerization, the functional form of p53 as a transcription factor. The extreme C-terminus of p53 (residues 360–393) forms a lysine- and arginine-rich basic region and possesses non-sequence-specific DNA-binding activity that is independent of the core DNA-binding domain. This region, also known as the regulatory region, was initially thought to negatively regulate the DNA-binding activity of the core domain. This notion was based on the observations that deletion and post-translational modifications of the regulatory region or its interaction with an antibody (PAb 421) directed at a C-terminal epitope lead to an increase in DNA binding by the core domain (Gu and Roeder, 1997; Hupp et al., 1992; Sakaguchi et al., 1998; Takenaka et al., 1995; Wang and Prives, 1995). It was proposed that these modifications of the C-terminal regulatory domain of p53 induce an allosteric conformational change that switches the core domain from a latent form with low affinity for its DNA-binding site to an active form with higher affinity for DNA (Halazonetis et al., 1993; Hupp et al., 1992; Muller-Tiemann et al., 1998). These studies, however, mostly relied on short stretches of naked DNA containing p53-binding sites. The conformational change model was not supported by an NMR-study that showed that full length p53 (latent form) and p53 lacking the C-terminal regulatory domain (active form) were identical in structure (Ayed et al., 2001).

More recent lines of evidence have suggested a positive role for the regulatory region in DNA-binding by the core domain. First, a deletion mutant lacking the C-terminal region (p53Δ30), shows weaker DNA-binding ability than WT p53, when longer molecules of DNA are used (Espinosa and Emerson, 2001). Second, efficient recognition of target sites in circular DNA or stem loop structures requires the C-terminal region of p53 (Gohler et al., 2002; McKinney and Prives, 2002). Third, it was shown that the C-terminal region of p53, through its nonspecific DNA binding activity, helps p53 slide along stretches of DNA (McKinney et al., 2004; Tafvizi et al., 2008). Linear diffusion along DNA allows the p53 core domain to sample sequences and find its target sites. Thus, the p53 C-terminus positively contributes to sequence-specific DNA-binding by the p53 core domain through mechanisms that are not fully understood.

An important regulator of p53 function is the Herpesvirus associated ubiquitin specific protease, HAUSP (or USP7), which deubiquitinates p53 and protects it from proteasome-mediated degradation (Shan et al., 2008). Mapping analysis have shown that the N-terminal domain
(residues 53 – 208) of USP7 are sufficient to bind p53, whereas USP7 binding sequences were mapped within the C-terminal regulatory region (residues 351 – 382) in p53 (Hu et al., 2002; Li et al., 2002). USP7 was originally identified as a binding partner of the ICP0 protein from herpes simplex virus (Meredith et al., 1994) and was shown to interact with another Herpesvirus protein, EBNA1 of Epstein-Barr virus (EBV) (Holowaty et al., 2003b). I have shown that EBNA1 can alter cellular processes, including p53 function, through its interaction with USP7. However, the more traditionally known functions of EBNA1 rely on its DNA binding activity to mediate replication and maintenance of the EBV genome and transactivation of viral genes (Rickinson, 2001). Interestingly, I have shown that USP7 stimulates the DNA binding activity of EBNA1 and is important for transcriptional activation by EBNA1 at the latent origin of EBV replication (Sarkari et al., 2009). Given that USP7 binds the C-terminal domain of p53 (Li et al., 2002) and that this domain regulates DNA binding by the p53 core domain, I asked whether USP7 affects the DNA binding activity of p53 and downstream p53 functions.

In this appendix, I will discuss observations that support a role of USP7 in regulating p53 DNA-binding. This role of USP7 is novel in terms of p53 regulation, since it is independent of deubiquitination of p53 by USP7.

MATERIALS AND METHODS

p53 and USP7 constructs and purification

Constructs expressing p53 mutants and purification of p53 proteins was described previously (Ayed et al., 2001). USP7 constructs and purification were described by Holowaty et al (Holowaty et al., 2003b).

Electrophoretic Mobility Shift Assays (EMSAs)

Labeling of DNA double-stranded probes and EMSAs were performed as previously described (Ayed et al., 2001). Briefly, p53 was incubated with either BSA or USP7 on ice for 5 minutes prior to incubation with 8pmoles of Cy-5 Dye labeled DNA double-stranded probe. Protein-DNA mixes were further incubated at room temperature for 10 minutes in the presence of 1μg salmon sperm competitive DNA and total reaction volume was brought up to 20μL using reaction buffer (20mM Trsi.Cl pH 8.0, 200mM NaCl). Samples were resolved on 5%
polyacrylamide gels at 4°C at 100V. Gels were scanned using a Typhoon 9400 scanner (Amersham) and analyzed using the ImageQuant 5.0 software.

**Western Blotting**

U2OS cells were transfected with either an empty vector or a vector expressing myc-tagged C223S. 24 hours after transfection, cells were either left untreated or treated with 10μg/mL of etoposide for 1, 2 and 4 hours. Cells were then harvested and lysed in 9 M urea, 5 mM Tris.Cl pH 6.8, sonicated briefly and subjected to centrifugation for 1 minute at 15,000 rpm in a microcentrifuge. 50μg of total protein was subjected to SDS-PAGE and transferred to PVDF membrane (Amersham). Membranes were blocked in blocking buffer (5% milk in PBS (137 mM NaCl, 2.7 mM KCl, 0.01 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4)). Primary antibodies used were R2B2 for USP7 (Holowaty et al., 2003b), DO-1 for p53 from Santa Cruz, Ab-1 for Actin (Calbiochem) and antibody 187 for p21 (Santa Cruz, sc-817). After primary antibody incubation, membranes were washed in PBS with 0.1% Tween 20 (PBS-T) then incubated with the secondary antibodies goat anti mouse-HRP (Santa Cruz, SC-2055) or goat anti-rabbit-HRP (Santa Cruz, SC-2004). Following washes in PBS-T, blots were developed using chemiluminescence ECL reagent (Perkin Elmer).

**Chromatin Immunoprecipitation**

U2OS cells were transfected and treated with etoposide as mentioned above. After etoposide treatment, cells were fixed with 1% formaldehyde, lysed in RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.1% Sodium Deoxycholate, 1 mM PMSF) containing protease inhibitor cocktail (Sigma, P8340) and sonicated briefly to shear the DNA. Clarified lysates were precleared with Protein A/G beads (Santa Cruz, SC-2003) and normal mouse IgG (Santa Cruz, SC-2343) prior to immunoprecipitation with p53 DO-1 antibody. Protein cross links were reversed in the immunoprecipitated DNA by incubating at 65°C for 16hrs. DNA was purified using QIAquick Gel Extraction Kit (Qiagen, 28704) and analyzed by quantitative PCR using LightCycler 480 DNA SYBR Green I Master (Roche, 04707516001) and a Rotorgene Q-PCR system (Corbett Research). Primers used for p21 quantification were 5’CTGGACTGGGCACTCTTTGTC3’ and 5’CTCCTACCATCCCTTTCCTC3’.
RESULTS

Effect of USP7 on DNA binding by p53

To assess the effect of USP7 on p53 DNA binding, I conducted electrophoretic mobility shift assays (EMSAs) using a Cy-5 labeled consensus p53 binding sequence as a probe and a version of p53 lacking the transactivation domain, but with the core DNA-binding and the C-terminal regulatory domains intact (p53_{82–393}). Purified p53_{82–393} was incubated with labeled DNA in the presence of USP7 or BSA as a negative control. This version of p53 is termed the latent form since in EMSAs it exhibits poor sequence-specific binding by the core domain, as characterized by lack of distinct shift in the mobility of the DNA probe. Instead, p53_{82–393} forms non-specific heterogeneous complexes with DNA, leading to smearing of DNA-protein complexes as seen in p53+BSA samples (Figure A-1C, lanes 2-6). On the other hand, in the presence of USP7, incubation of p53_{82–393} with the probe readily yielded distinct shifts in mobility of the DNA probe, characteristic of sequence-specific DNA-binding (Figure A-1C, lanes 6-9). I also examined the effect of USP7 on p53 DNA-binding by incubating a fixed amount with increasing amounts of USP7 (Figure A-1D). This showed that USP7 had a dose-dependent stimulatory effect on the DNA-binding ability of p53_{82–393} while the BSA negative control had little to no effect. Both USP7 and BSA showed no binding to the DNA probe under these conditions, even at the highest level of the concentrations used (Figure A-1D, lanes 9 and 10). These results show that USP7 stimulates the DNA-binding activity of p53 and can rescue the autoinhibitory effect of the C-terminal regulatory domain observed in these assays.

USP7 binding is required for the stimulatory effect on p53 DNA-binding

To test whether USP7 binding was responsible for the stimulatory effect on p53 DNA-binding I conducted EMSAs using another version of p53, p53_{82–360} (Figure A-1A). In addition to lacking the N-terminal transactivation domain, this version also lacks the C-terminal regulatory region, which is responsible for USP7 binding and non specific DNA-binding. This version is termed the active form of p53, as it lacks autoinhibition from the C-terminal region. As expected, p53_{82–360} efficiently binds DNA, at concentrations much lower that used for latent
Figure A-1. Effect of USP7 on DNA-binding activity of p53 in vitro. (A and B) Schematic representation of p53 (A) and USP7 (B) proteins used in study. For p53, transactivation, DNA-binding core, tetramerization (tet) and the USP7-binding regulatory domains are indicated. For USP7, p53-binding N-terminal, central catalytic (CAT) and C-terminal domains are indicated. (C) EMSA showing titration of latent p53$_{82-393}$, (60, 120, 240, 480 pmoles) in the presence of BSA negative control (lanes 2 - 5) or presence of USP7 (400pmoles) (lanes 6 – 9). (D) EMSA performed with fixed amount (240pmoles) of p53$_{82-393}$ with 100, 200 and 400 pmoles of USP7 (lanes 3 -5) or BSA (lanes 6 – 8). Incubation of USP7 alone and BSA alone in the absence of p53 are also shown (lanes 9 and 10). (E) EMSA showing titration of active p53$_{82-360}$, in the absence (lanes 2 - 5) or presence of USP7 (lanes 6 – 9). (F and G) EMSAs showing titration of latent p53$_{82-393}$, in presence or absence of USP7-NTD (f) and USP7$_{560-1102}$ or USP7$_{530-870}$ (G). (H) EMSAS shown p53$_{82-393}$ (480pmoles) DNA binding in the presence (lanes 3 and 6) or absence (lanes 2 and 5) of USP7 (400pmoles), with and without a USP7-specific antibody (1 µg), BL851 (Bethyl) (lanes 4-6).
p53, as indicated by distinct shifts it causes in the mobility of the DNA probe (Figure A-1E, lanes 2-5). While the active $p53_{82-360}$ binds better than the latent form, $p53_{82-393}$, its DNA binding was not further stimulated by the presence of USP7 (Figure A-1E, lanes 6-9), suggesting that physical interaction with USP7 is important for the stimulatory effect.

**USP7 C-terminal Sequences Stimulate p53 DNA Binding**

It has been shown that the N-terminal domain of USP7 (USP7-NTD) is sufficient to interact with p53 (Li et al., 2002). I wanted to examine whether the interaction mediated by USP7-NTD was also sufficient to stimulate the DNA-binding activity of p53. To this end, I used EMSAs as above with latent $p53_{82-393}$ in the presence and absence of the USP7-NTD. Surprisingly USP7-NTD did not stimulate sequence-specific DNA-binding by $p53_{82-393}$ as no distinct shifts in the mobility of the labeled probe were observed (Figure A-1F). This suggests that regions other than the NTD in USP7 mediate additional interactions between p53 and USP7, which might account for the stimulatory effect of USP7 on p53 DNA-binding. While USP7-NTD is sufficient to interact with p53, USP7 sequences downstream of the catalytic domain do interact with p53 albeit weakly (Li et al., 2002). Therefore I next tested whether the USP7-CTD (amino acids 560-1102 as shown in Figure A-1A) could account for the effect of USP7 on p53 DNA binding by assaying the DNA binding activity of $p53_{82-393}$ in the presence and absence of this USP7 domain (Figure A1-G). Similar to what I observed with full-length USP7, the USP7-CTD stimulated sequence-specific DNA binding by p53, as compared to the BSA control, suggesting that it is likely responsible for the p53-USP7 interaction that results in increased p53 sequence-specific DNA binding.

**USP7 is not stably associated with the p53-DNA complex**

I have previously shown that USP7 not only stimulates DNA-binding by EBNA1 but also forms a complex with EBNA1 bound to DNA. This led me to question whether p53 can also interact with USP7 while bound to DNA. While EBNA1 bound to USP7 causes a greater shift in EMSAs in the mobility of the probe than EBNA1 alone, this effect was not observed with p53. p53-DNA complexes migrated similarly in the presence or absence of USP7, suggesting USP7 is not part of the shifted complexes (Figure A-1H). To further test this possibility, p53-DNA complexes formed in the presence and absence of USP7 were incubated with a USP7-specific
antibody prior to electrophoresis. The antibody did not have any effect on the mobility of the DNA probe alone or the p53-DNA complex without USP7 (Figure A-1H, lanes 4 and 5). More importantly, the antibody did not alter the mobility of complexes even in the presence of USP7 (Figure A-1H, lane 6), further indicating the absence of USP7 in the shifted complexes. Since mobility in a native gel is dependent on shape, it is possible, however unlikely, that USP7 may be part of the p53-DNA complex, but does not alter it significantly to change its mobility.

**Effect of USP7 on p53-DNA interaction in vivo**

The in vitro results above suggest that USP7 promotes binding of p53 to its target DNA. To examine whether this is true in vivo, I used the following two approaches in U2OS cells, which express wild type p53. I first conducted western blots for p21 as a read out of p53 DNA-binding to and transcription activation at the p21 promoter. p21 protein levels are kept to a minimum under steady state, but p53 activates p53 activates p21 transcription in response to DNA damage. U2OS cells were transfected with either an empty vector or a vector expressing the catalytically dead point mutant of USP7, C223S (Li et al., 2002). C223S binds p53 but does not stabilize it and can indeed work as a dominant negative and destabilize p53 (Li et al., 2002). This was done to ensure that any observed effects of USP7 on p53 DNA-binding were not due to p53 stabilization by USP7. Etoposide treatment of U2OS cells transfected with empty vector led to stabilization of p53, which was accompanied by accumulation of p21 (Figure A-2A, compare lane 1 to 2-4). p53 stabilization was diminished in U2OS cells transfected with C223S, consistent with its dominant negative effects (Figure A-2A, lanes 5-8). However, expression of p21 in these cells increased compared to control cells, and this increase was most dramatic after etoposide treatment (Figure A-2A, top panels, compare lanes 1-4 to 5-8). This observation is consistent with the idea that USP7 promotes p53 binding to and transcription activation at the p21 promoter. To test this possibility more directly I carried out chromatin immunoprecipitation using a p53-specific antibody in C223S overexpressing and vector control U2OS cells before and after etoposide treatment. Immunoprecipitated chromatin was analyzed by quantitative PCR (Q-PCR) using oligos specific for the p21 promoter. This revealed incremental accumulation of p53 at the p21 promoter after etoposide treatment in a
Figure A-2. Ubiquitin-independent regulation of p53 function by USP7. U2OS cells were transfected with an empty vector or vector expressing myc-tagged C223S and treated with etoposide for the indicated times (A and B). (A) Equal amounts of cell lysates were analyzed for protein expression by western blotting using the indicated antibodies where actin is the loading control. (B) Post-transfection and etoposide treatment, samples were cross-linked and ChIP assays were performed with a p53 specific antibody. Immunoprecipitates were analyzed by quantitative PCR using primers for the p21 promoter region and results are shown after normalization to input DNA.
dose-dependent manner. Interestingly, the degree of p53 recruitment to the p21 promoter was virtually identical in C223S overexpressing cells and vector transfected control cells (Figure A-2B), in contrast to the difference in p21 protein levels between these cells.

**DISCUSSION**

The DNA-binding ability of p53 is critical to its function as a transcription factor and thus as a tumor suppressor. The significance of sequence-specific DNA-binding for p53 tumor suppressor function is highlighted by the substantial number of tumor-associated mutations in the core DNA-binding domain (Hainaut and Hollstein, 2000). An important determinant of DNA-binding by the core domain is the autoregulation of this activity by the C-terminal regulatory domain of p53. The C-terminal domain is heavily modified post-translationally and these modifications affect the ability of p53 to bind DNA (Appella and Anderson, 2001; Brooks and Gu, 2003). Here I propose that binding to the ubiquitin specific protease, USP7, is yet another means of regulating this property of p53.

Full length p53, with its core DNA-binding domain and its C-terminal domain intact is referred to as the latent form, since it shows poor sequence-specific DNA-binding in *in vitro* binding assays. This effect is attributed to autoinhibition of sequence-specific DNA-binding by the C-terminal domain. Incubation of latent p53 with USP7 in EMSAs stimulated sequence-specific DNA-binding by latent p53, suggesting that USP7-binding can reverse this autoinhibition. DNA-binding by a C-terminal deletion mutant of p53, p53\textsubscript{8-360}, which lacks the USP7 binding sequences, was not stimulated by USP7 suggesting that binding to USP7 is required for this effect.

The USP7-NTD, which is sufficient to bind p53, had no effect on the DNA-binding activity of p53. While the interaction of p53 with USP7-NTD is important for deubiquitination and stabilization of p53, the results here suggest that perhaps interactions mediated by other regions of USP7 are responsible for the effect on p53 DNA-binding. Though there is evidence for weak binding between p53 and the C-terminal extensions of USP7, the significance of this interaction is not known (Li *et al.*, 2002). I observed that C-terminal sequences of USP7 can stimulate DNA-binding by latent p53.
The effect of USP7 on p53 DNA-binding is tricky to study in cells given that USP7 also stabilizes p53. The C223S point mutation in USP7 helps in getting around this obstacle since the mutant protein interacts with p53 just as well as WT USP7 but does not stabilize it. Overexpression of C223S led to an increase in p21 levels compared to control cells before and after etoposide treatment, while p53 was not stabilized. This is consistent with the idea that USP7 stimulates p53 binding to the p21 promoter to enhance the expression of the p21 gene. Surprisingly, ChIP assays did not support this idea, as p53 recruitment to the p21 promoter was not stimulated in C223S expressing cells. One explanation for this is perhaps that USP7 can upregulate p21 through a p53-independent mechanisms (reviewed in (Abbas and Dutta, 2009)), though there is no evidence of involvement of USP7 in these mechanisms. It is also possible that U2OS cells may not be an ideal system to study this form of regulation of p53 by USP7. It has been proposed that, unlike in primary cells, in established tumor cell lines like U2OS there is little correlation between the extent of promoter occupancy by p53 and the level of transcriptional activity at these promoters and the expression of target genes (Kaeser and Iggo, 2004; Shaked et al., 2008). Nonetheless, the results here support the conclusion that USP7 can promote p53 function in a manner independent of deubiquitination and stabilization.

This investigation was sparked because of my earlier observations that USP7 could stimulate the DNA-binding of the Epstein-Barr virus protein EBNA1. There are similarities and subtle differences in how USP7 may contribute to the DNA-binding activity of the two proteins. The N-terminal domain of USP7 has been shown to be sufficient for binding both p53 and EBNA1. While USP7-NTD can stimulate DNA-binding by EBNA1, it had no effect on p53 DNA-binding. However, in both cases, the data suggest that regions other than USP7-NTD may contribute to DNA-binding by EBNA1 and p53. EBNA1 can bind DNA in complex with USP7 and can recruit USP7 to EBNA1 binding sequences in the EBV genome. USP7, in turn, contributes to functions of EBNA1 at the EBV origin of replication that are important for latent EBV infection. Such a role of USP7 in p53-DNA interactions is not supported by the in vitro observations and has yet to be tested in cells. Clearly further studies are warranted, ideally in p53 positive primary cells, to decipher a potentially novel mode of p53 regulation by USP7.
REFERENCES


Ambinder RF, Shah WA, Rawlins DR, Hayward GS, Hayward SD (1990). Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein-Barr virus DNA. *J Virol* **64**: 2369-79.


Christopher JA. (1998). The Center for Macromolecular Design (Texas A&M University: College Station).


DeLano WL. (2002). DeLano Scientific: San Carlos, California, USA.


Hung SC, Kang MS, Kieff E (2001). Maintenance of Epstein-Barr virus (EBV) oriP-based episomes requires EBV-encoded nuclear antigen-1 chromosome-binding domains, which can be replaced by high-mobility group-I or histone H1. Proc Natl Acad Sci U S A 98: 1865-70.


Maul GG, Everett RD (1994). The nuclear location of PML, a cellular member of the C3HC4 zinc-binding domain protein family, is rearranged during herpes simplex virus infection by the C3HC4 viral protein ICP0. *J Gen Virol* **75** (Pt 6): 1223-33.


Tang J, Qu L, Pang M, Yang X Daxx is reciprocally regulated by Mdm2 and Hausp. Biochem Biophys Res Commun 393: 542-5.


