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Christian Münz *Editor*

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One Herpes Virus: Many Diseases

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Editor

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Contents

Part I
EBV Latency

EBNA1

Lori Frappier

Abstract Epstein–Barr nuclear antigen 1 (EBNA1) plays multiple important roles in EBV latent infection and has also been shown to impact EBV lytic infection. EBNA1 is required for the stable persistence of the EBV genomes in latent infection and activates the expression of other EBV latency genes through interactions with specific DNA sequences in the viral episomes. EBNA1 also interacts with several cellular proteins to modulate the activities of multiple cellular pathways important for viral persistence and cell survival. These cellular effects are also implicated in oncogenesis, suggesting a direct role of EBNA1 in the development of EBV-associated tumors.

Contents

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1 Introduction

Epstein–Barr nuclear antigen 1 (EBNA1) is expressed in all forms of EBV latency in proliferating cells and was the first reported EBV latency protein (Reedman and Klein 1973). EBNA1 has been extensively studied and shown to have multiple important roles in EBV infection. These include contributions to both the replication and mitotic segregation of EBV episomes that lead to stable persistence of EBV episomes in latent infection. EBNA1 also activates the transcription of other EBV latency genes important for cell immortalization. These functions require EBNA1 binding to specific DNA elements in the EBV latent origin of DNA replication (*oriP*). In recent years, it has become apparent that EBNA1 functions are not limited to its roles on EBV episomes but rather that EBNA1 also alters the cellular environment in multiple ways that contribute to cell survival and proliferation and viral persistence. EBNA1 lacks enzymatic activities but is able to affect many processes due to interactions with a variety of cellular proteins. This chapter reviews the multiple functions and mechanisms of action of EBNA1.

2 EBNA1 Functions at EBV Genomes

2.1 DNA Replication

The origin of latent DNA replication, termed *oriP* (for plasmid origin), was identified by screening EBV DNA fragments for the ability to enable the replication and stable maintenance of plasmids in human cells that were latently infected with EBV (Yates et al. 1984). Subsequent studies showed that the only viral protein required for the replication of *oriP* plasmids was EBNA1 (Yates et al. 1985). Both EBV episomes and *oriP* plasmids were found to replicate once per cell cycle, mimicking cellular replication and providing a good model system for human DNA replication (Yates and Guan 1991; Sternas et al. 1990). Note that *oriP* is not the only origin of replication for EBV episomes, as replication forks have also been found to initiate from a poorly defined region outside of *oriP* that appears to be independent of EBNA1 (Little and Schildkraut 1995; Norio et al. 2000; Ott et al. 2011).

O*riP* contains two functional elements: the dyad symmetry (DS) element and the family of repeats (FR) (Reisman et al. 1985) (Fig. 1). The DS contains four EBNA1 recognition sites, two of which are located within a 65-bp DS sequence (Reisman et al. 1985; Rawlins et al. 1985). The DS element is the origin of

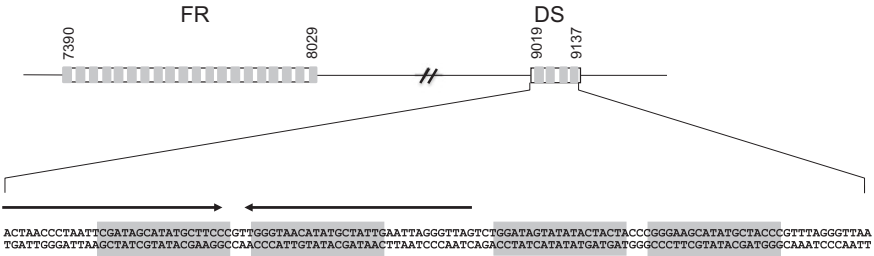

Fig. 1 Schematic representation of *oriP*. Organization of the *oriP* DS and FR elements showing genome nucleotide coordinates and EBNA1 binding sites (*gray boxes*). For the DS element, the positions of the four EBNA1 binding sites and 65-bp dyad symmetry sequence (*arrows*) are indicated

replication within *oriP* (Gahn and Schildkraut 1989) and has been shown to be both essential and sufficient for plasmid replication in the presence of EBNA1 (Wysokenski and Yates 1989; Harrison et al. 1994; Yates et al. 2000). Efficient replication from the DS element requires all four EBNA1 binding sites; however, a low level of DNA replication can be achieved with only two adjacent EBNA1 sites, provided that the 3-bp spacing between these sites is maintained (Koons et al. 2001; Harrison et al. 1994; Yates et al. 2000; Atanasiu et al. 2006; Bashaw and Yates 2001; Lindner et al. 2008). The FR element consists of 20 tandem copies of a 30-bp sequence, each of which contains an EBNA1 binding site (Rawlins et al. 1985; Reisman et al. 1985). The primary function of the FR element is in the mitotic segregation and transcriptional activation functions of EBNA1 as discussed below, although in some cell lines the FR also appears to be required for replication from the DS (Hodin et al. 2013). In addition, when bound by EBNA1, the FR can affect DNA replication by inhibiting the passage of replication forks, forming a major pause site (Gahn and Schildkraut 1989; Dhar and Schildkraut 1991; Norio and Schildkraut 2001, 2004; Ermakova et al. 1996).

The DNA replication activity of EBNA1 requires its DNA binding domain as well as additional sequences in the N-terminal half of EBNA1 (Fig. 2) (Yates and Camiolo 1988; Van Scoy et al. 2000; Kim et al. 1997; Kirchmaier and Sugden

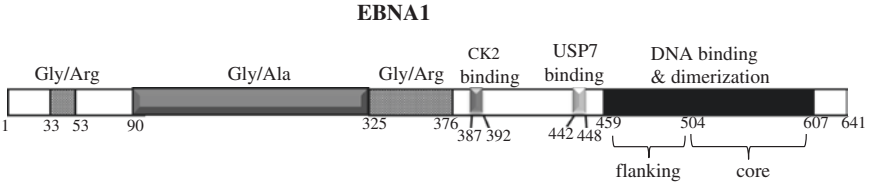

Fig. 2 Organization of the EBNA1 protein. Positions of some of the key elements of EBNA1 are indicating, including the two Gly–Arg-rich regions, variable Gly–Ala repeat, USP7 and CK2 binding sites, and DNA binding and dimerization domain (*black*), along with flanking and core subcomponents of the DNA binding and dimerization domain. Amino acid numbers are indicated *below*

1997; Mackey and Sugden 1999; Shire et al. 1999; Ceccarelli and Frappier 2000; Wu et al. 2002). Single localized deletions or mutations that disrupt EBNA1 DNA replication function have not been identified; rather, this function appears to involve redundant contributions of at least two EBNA1 regions (amino acids 8–67 and 325–376; Fig. 2). These N-terminal sequences can tether EBNA1 to cellular chromosomes (see Sect. 2.2), and the finding that they can be functionally replaced by a nucleosome interacting sequence suggests that chromosome tethering contributes to the replication function of EBNA1 (Hodin et al. 2013). In addition, deletion of EBNA1 amino acids 61–83 or 395–450, or point mutation of G81 or G425 within these regions, has been found to increase replication efficiency (Holowaty et al. 2003c; Wu et al. 2002; Deng et al. 2005). These point mutations disrupt EBNA1 binding to tankyrase, suggesting that tankyrase negatively regulates replication by EBNA1, possibly through the poly-ADP ribosylation of EBNA1 (Deng et al. 2005). In addition, the EBNA1 395–450 region is responsible for binding and recruiting to *oriP* the host ubiquitin-specific protease 7 (USP7), suggesting that USP7 may negatively regulate replication (Holowaty et al. 2003c). This contention was further supported by a study showing that the latent origin binding proteins of other gamma-herpesviruses also recruit USP7 to their origins and that disruption of this interaction increases DNA replication (Jager et al. 2012). Therefore, negative regulation of replication by USP7 appears to be a conserved feature of gamma-herpesvirus replication.

EBNA1 is bound to the *oriP* elements throughout the cell cycle, indicating that EBNA1 binding to the DS is not sufficient to activate DNA replication (Hsieh et al. 1993; Niller et al. 1995; Ritzi et al. 2003). While EBNA1 is the only EBV protein involved in latent-phase DNA replication, it lacks any enzymatic activities, including DNA helicase and origin melting activities present in the origin binding proteins of some viruses (Frappier and O'Donnell 1991b). Therefore, EBV depends heavily on host cellular proteins to replicate its episomes. Several studies have shown that the cellular origin recognition complex (ORC) and minichromosome maintenance (MCM) complex are associated with the DS element of *oriP*, implicating them in the initiation and licensing of EBV DNA replication (Schepers et al. 2001; Chaudhuri et al. 2001; Dhar et al. 2001). A functional role for ORC in *oriP* plasmid replication was shown by the failure of these plasmids to stably replicate in a cell line containing a hypomorphic *ORC2* mutation (Dhar et al. 2001). EBV replication was also found to be inhibited by geminin, a protein that inhibits rereplication from cellular origins by interacting with Cdt1 (Dhar et al. 2001). This suggests that Cdt1 loads the MCM complexes on EBV origins, as it does on cellular origins.

EBNA1 has been shown to be important for ORC recruitment to the DS (Schepers et al. 2001; Dhar et al. 2001; Julien et al. 2004). In addition, EBNA1 was found to interact with Cdc6 and this interaction increased ORC recruitment to the DS in vitro (Moriyama et al. 2012). Interestingly, ORC is not recruited by EBNA1 bound to the FR element, suggesting that the DS DNA sequence or arrangement of the EBNA1 binding sites are important for ORC recruitment (Schepers et al. 2001; Chaudhuri et al. 2001; Dhar et al. 2001; Moriyama et al.

2012). ORC recruitment by EBNA1 was initially reported to involve EBNA1N-terminal sequences including the Gly–Arg-rich regions, and in vitro studies suggested that this interaction was mediated by RNA molecules (Norseen et al. 2008; Moriyama et al. 2012). However, a second study found that, in the presence of Cdc6, EBNA1 could recruit ORC to the DS in an RNA-independent manner (Moriyama et al. 2012). In addition, it was recently reported that the EBNA1 DNA binding domain is sufficient for ORC recruitment to the DS (Hodin et al. 2013). EBNA1 may also facilitate the recruitment of telomere repeat binding factor 2 (TRF2) to site in the DS (Deng et al. 2002, 2003; Moriyama et al. 2012). TRF2 then appears to contribute to ORC recruitment to the DS in conjunction with EBNA1 (Julien et al. 2004; Atanasiu et al. 2006) and also affects the timing of replication in S phase through recruitment of additional proteins (Zhou et al. 2009, 2010).

EBNA1 has also been found to recruit template activating factor Iβ (TAF-Iβ also called SET) to both the DS and FR elements, through a direct interaction with the 325–376 Gly–Arg-rich region of EBNA1 (Holowaty et al. 2003b; Wang and Frappier 2009). TAF-Iβ appears to negatively regulate replication from *oriP* as TAF-Iβ depletion was found to increase *oriP* plasmid replication, while TAF-Iβ overexpression inhibited it (Wang and Frappier 2009). Since TAF-Iβ is a nucleosome-associated protein that can recruit either histone acetylases or deacetylases (Seo et al. 2001; Shikama et al. 2000), TAF-Iβ may negatively regulate replication from *oriP* by affecting the chromatin structure of the origin.

2.2 Mitotic Segregation

In latency, EBV episomes are present at low copy numbers that are stably maintained in proliferating cells. This stable maintenance requires a mechanism to ensure even partitioning of the episomes to the daughter cells during cell division. The mitotic segregation or partitioning of the EBV episomes requires two viral components, EBNA1 and the *oriP* FR element (Lupton and Levine 1985; Krysan et al. 1989; Lee et al. 1999). EBNA1 and the FR can also confer stability on a variety of constructs when combined with heterologous origin sequences (Krysan et al. 1989; Kapoor et al. 2001; Simpson et al. 1996). EBNA1 binding to its multiple recognition sites in the FR is crucial for its segregation function, as is the central Gly–Arg-rich region of EBNA1 (325–376) (Shire et al. 1999).

EBNA1 functions in segregation by tethering the EBV episomes to the cellular mitotic chromosomes. EBNA1, EBV episomes, and *oriP*-containing constructs have all been found to associate with mitotic chromosomes (Harris et al. 1985; Delecluse et al. 1993; Simpson et al. 1996; Grogan et al. 1983; Petti et al. 1990), and the association of *oriP* plasmids with mitotic chromosomes was shown to depend on the EBNA1-chromosome interaction (Kanda et al. 2001; Kapoor et al. 2005). In addition, EBNA1 mutants that are nuclear but defective in mitotic chromosome attachment fail to partition *oriP* plasmids (Hung et al. 2001; Shire

et al. 1999; Wu et al. 2000). EBNA1 and EBV episomes are not localized to particular regions of mitotic chromosomes, but rather are widely distributed over the chromosomes, leading to the initial suggestion that EBNA1 and EBV episomes interact randomly with chromosomes (Harris et al. 1985). However, subsequent studies have indicated that initial pairing of EBV episomes on sister chromatids may ensure their equal distribution to the daughter cells and that this pairing may stem from the catenation of the newly replicated EBV plasmids (Delecluse et al. 1993; Kanda et al. 2007; Dheekollu et al. 2007; Nanbo et al. 2007). In addition, the FR element has been found to direct EBV genomes to chromatin regions with histone modifications typical of active chromatin (Deutsch et al. 2010).

Studies with EBNA1 deletion mutants showed that the central Gly–Arg-rich region of EBNA1 (amino acids 325–376) was critical for chromosome attachment and that N-terminal sequences (8–67) also contribute to this interaction (Wu et al. 2000, 2002; Shire et al. 1999, 2006; Marechal et al. 1999; Hung et al. 2001; Kanda et al. 2013). Interestingly, fusion proteins in which these EBNA1 regions have been replaced by other chromosome binding sequences are also able to support *oriP* plasmid maintenance (Hung et al. 2001; Sears et al. 2003). Both the central Gly–Arg repeat of EBNA1 and sequences spanning the smaller Gly–Arg-rich N-terminal sequence (amino acids 33–53) can cause proteins to associate with mitotic chromosomes when fused to them (Hung et al. 2001; Marechal et al. 1999; Sears et al. 2004). However, deletion of the N-terminal Gly–Arg sequence within EBNA1 does not affect EBNA1's ability to maintain *oriP* plasmids or to associate with mitotic chromosomes, indicating that it is the central Gly–Arg-rich region that is normally used by EBNA1 for chromosome interactions and segregation (Nayyar et al. 2009; Wu et al. 2002). This region contains a repeated GGRGRGGS sequence that is phosphorylated on the serines and methylated by PRMT1 or PRMT5 on the arginine residues (Laine and Frappier 1995; Shire et al. 2006).

The segregation of viral genomes by attachment to cellular chromosomes is not unique to EBV but is a strategy also used by Kaposi sarcoma associated herpesvirus (KSHV) and papillomavirus. In each case, the viral origin binding (LANA for KSHV and E2 for papillomavirus) tethers the viral plasmid to the cellular chromosome through interactions with one or more cellular proteins (You 2010; Krithivas et al. 2002; Barbera et al. 2006; Parish et al. 2006). For EBNA1, interactions with the cellular protein, EBP2, appear to be important for metaphase chromosome attachment and segregation function (Wu et al. 2000; Shire et al. 1999; Kapoor and Frappier 2003, 2005). EBP2 is largely nucleolar in interphase but redistributes to the chromosomes in mitosis (Wu et al. 2000). The EBNA1 325–376 region critical for chromosome attachment mediates EBP2 binding, and there is a close correspondence between the effect of EBNA1 mutations on EBP2 and metaphase chromosome interactions (Shire et al. 1999; Wu et al. 2000, 2002; Shire et al. 2006; Nayyar et al. 2009). In addition, EBP2 depletion in various cell lines, including the EBV-positive C666-1 nasopharyngeal carcinoma (NPC) cells, resulted in redistribution of EBNA1 from the metaphase chromosomes to the soluble cell fraction and a corresponding release of *oriP* plasmids from the chromosomes (Kapoor et al. 2005). EBP2 was also found to enable EBNA1 to segregate

plasmids in budding yeast by facilitating EBNA1 attachment to the yeast mitotic chromosomes (Kapoor et al. 2001; Kapoor and Frappier 2003).

Detailed studies on the timing of chromosome association in human cells showed that EBNA1 associates with the chromosomes earlier in mitosis than EBP2 and that EBNA1 and EBP2 only associate on the chromosomes in metaphase to telophase (Nayyar et al. 2009). This suggests that EBNA1 initially contacts the chromosomes by an EBP2-independent mechanism and that subsequent interactions with EBP2 in mid-to-late mitosis might be important to maintain EBNA1 on chromosomes. The initial chromosome contact could involve direct DNA binding or interactions with chromosome-associated RNA molecules, since the 325–376 and N-terminal arginine-rich regions have been found to have some capacity to interact with DNA and RNA in vitro, and drugs that bind G-quadruplex RNA have been reported to decrease the mitotic chromosome association of EBNA1 (Sears et al. 2004; Norseen et al. 2008, 2009; Snudden et al. 1994). In addition, FRET analysis identified an interaction between EBNA1 and EBP2 in the nucleoplasm and nucleolus in interphase suggesting additional roles for this interaction, including the possibility that the EBNA1-EBP2 interaction in interphase is important for EBNA1-chromosome interactions in mitosis (Jourdan et al. 2012). This possibility is reminiscent of findings for bovine papillomavirus segregation, in which an interphase interaction between the viral E2 protein and host ChlR1 protein is required in order for E2 to associate with mitotic chromosomes and segregate papillomavirus genomes (Feeney et al. 2011; Parish et al. 2006).

2.3 EBV Transcriptional Activation

Another function of EBNA1 at EBV episomes is in transcriptional activation. EBNA1 can act as a transcriptional activator when bound to the *oriP* FR element, enhancing the expression of reporter genes on FR-containing plasmids in a distance-independent manner (Lupton and Levine 1985; Reisman and Sugden 1986). The EBNA1-bound FR was also shown to activate expression from the viral Cp and LMP promoters, suggesting a role for EBNA1 in inducing the expression of the EBNA and LMP EBV latency genes in latent infection (Sugden and Warren 1989; Gahn and Sugden 1995). The EBNA1 residues required for transcriptional activation have been mapped to the 65–83 N-terminal sequence (Wu et al. 2002; Kennedy and Sugden 2003) as well as to the central Gly–Arg-rich region (residues 325–376) also required for segregation function (Yates and Camiolo 1988; Ceccarelli and Frappier 2000; Wang et al. 1997; Van Scoy et al. 2000). EBNA1 requires both of these regions to activate transcription, as deletion of either one abrogates the transcriptional activation function of EBNA1 (Wu et al. 2002; Ceccarelli and Frappier 2000). A Δ61–83 EBNA1 mutant was found to be fully active for replication and segregation functions, indicating that transcriptional activation is a distinct EBNA1 function (Wu et al. 2002). Similar conclusions

were reached with a Δ65–89 EBNA1 mutant in the context of an infectious EBV, where EBNA1 Δ65–89 was shown to be defective in activating expression of the EBNA genes from the Cp promoter, but still supported stable plasmid replication (Altmann et al. 2006). EBV containing the Δ65–89 EBNA1 was also shown to be severely impaired in the ability to transform cells, indicating the importance of EBNA1-mediated transcriptional activation for EBV infection (Altmann et al. 2006).

Two cysteine residues within the N-terminal transactivation sequence (at positions 79 and 82) have been shown to be important for transactivation activity and to mediate an interaction with zinc (Aras et al. 2009). There is also evidence that the transcriptional activity of EBNA1 is zinc-dependent, suggesting that a zinc-dependent structure formed in the N-terminal transactivation region mediates the activity of this sequence (Aras et al. 2009). The 61–83 region also mediates an interaction with Brd4 (Lin et al. 2008), a cellular bromodomain protein that interacts with acetylated histones to regulate transcription (Wu and Chiang 2007). Within the EBV genome, Brd4 was shown to be preferentially localized to the EBNA1-bound FR enhancer element (Lin et al. 2008). Furthermore, Brd4 depletion inhibited EBNA1-mediated transcriptional activation, suggesting that EBNA1 uses Brd4 to activate transcription (Lin et al. 2008). Interestingly, an interaction between Brd4 and papillomavirus E2 proteins (the functional equivalent to EBNA1) has been shown to be important for transcriptional activation by E2 (Schweiger et al. 2006; McPhillips et al. 2006; Ilves et al. 2006), suggesting that EBNA1 and E2 may use common mechanisms to activate transcription. Whether or not the EBNA1–Brd4 interaction is zinc-dependent remains to be determined.

The EBNA1 325–376 region mediates interactions with several cellular proteins, some of which have been implicated in the transcriptional activity of EBNA1. For example, P32/TAP, which interacts with Arg-rich sequences, has been detected at *oriP* by chromatin immunoprecipitation, and its C-terminal region has some ability to activate a reporter gene when fused to the GAL4 DNA binding domain (Van Scoy et al. 2000; Wang et al. 1997). However, it is not clear whether P32/TAP is important for EBNA1-mediated transcriptional activation. The related nucleosome assembly proteins, NAP1, TAF-Iβ (also called SET), and nucleophosmin, also interact with the EBNA1 325–376 sequence and are known to affect transcription in multiple ways (Holowaty et al. 2003c; Wang and Frappier 2009; Park and Luger 2006; Malik-Soni and Frappier 2012, 2013). A role for NAP1, TAF-Iβ, and nucleophosmin in EBNA1-mediated transcriptional activation is supported by the finding that each protein is recruited to the FR element by EBNA1 and that EBNA1 transactivation activity is decreased upon depleting any of these proteins (Wang and Frappier 2009; Malik-Soni and Frappier 2013). Depletion of nucleophosmin had the biggest effect on EBNA1-mediated transcriptional activation, suggesting, either that the EBNA1-nucleophosmin interaction is the most important for transcription function, or that nucleophosmin is more limiting in the cell than NAP1 or TAF-Iβ (Malik-Soni and Frappier 2013). Note that another histone chaperone protein, nucleolin, was also recently reported to contribute to EBNA1 functions including transactivation, but this appears to be due to

an effect on EBNA1 binding to *oriP* (Chen et al. 2014). As a whole, the data suggest that recruitment of both nucleosome assembly proteins and Brd4 are important for transcriptional activation by EBNA1, reflecting the requirement for the two transcriptional activation sequences.

It is expected that transcriptional activation by EBNA1 will involve changes to histone modifications and this may include ubiquitylation of histone H2B. This is suggested by the finding that EBNA1 binds to a complex of USP7 and GMP synthetase that functions to deubiquitylate H2B and recruits it to the FR (Sarkari et al. 2009). USP7 depletion results in increased levels of monoubiquitylated H2B at the FR and decreased transcriptional activation, suggesting that monoubiquitylation of H2B inhibits EBNA1-mediated transcriptional activation. In keeping with this result, an EBNA1 mutant defective in USP7 binding has decreased ability to activate transcription (Holowaty et al. 2003c).

2.4 Autoregulation

In addition to interactions with the *oriP* FR and DS elements, EBNA1 was found to bind a third region of the EBV genome near the Qp promoter that is used to express EBNA1 in the absence of other EBNAs (Jones et al. 1989; Sample et al. 1992; Nonkwelo et al. 1996). EBNA1 binding to two recognition sites located downstream of Qp was reported to repress EBNA1 expression from Qp (Sample et al. 1992). Since EBNA1 has lower affinity for these sites than either the DS or FR elements, EBNA1 would only bind the Qp sites when its levels are high enough to saturate the FR and DS elements, providing a feedback mechanism to shut off EBNA1 expression when EBNA1 levels are high (Jones et al. 1989; Ambinder et al. 1990). While EBNA1 was initially thought to inhibit expression from Qp by repressing transcription, a more recent study found that EBNA1 acts post- or co-transcriptionally to inhibit the processing of primary transcripts (Yoshioka et al. 2008).

3 EBNA1–DNA Interactions

3.1 Interactions with the EBV Genome

EBNA1 specifically recognizes an 18-bp palindromic sequence present in multiple copies in the *oriP* DS and FR elements as well as in the BamHI-Q fragment containing the Qp promoter (Rawlins et al. 1985; Jones et al. 1989; Ambinder et al. 1990, 1991; Frappier and O'Donnell 1991b; Shah et al. 1992). Sequence variation within the multiple copies of this palindrome results in different affinities of EBNA1 for the FR, DS, and BamHI-Q regions and for individual sites within these regions (Ambinder et al. 1990; Summers et al. 1996). EBNA1 has highest

affinity for the FR and DS regions and remains bound to these sites throughout the cell cycle (Hsieh et al. 1993; Niller et al. 1995; Ritzi et al. 2003).

EBNA1 interacts with its recognition sites through its C-terminal domain (amino acids 459 and 607; Fig. 2), which also mediates the dimerization of EBNA1 (Ambinder et al. 1991; Chen et al. 1993; Summers et al. 1996; Shah et al. 1992). EBNA1 forms very stable homodimers both in solution and when bound to its recognition sites (Frappier and O'Donnell 1991b; Ambinder et al. 1991; Shah et al. 1992). The crystal structure of the DNA binding and dimerization domain was determined both in solution and bound to the EBNA1 consensus binding site (Bochkarev et al. 1995, 1996). The structure showed that dimerization was mediated by residues 504–604 (referred to as the core domain), which form an eight-stranded antiparallel β-barrel, comprised of four strands from each monomer and two α-helices per monomer (Fig. 3). This core domain is strikingly similar to the structure of the DNA binding domain of the E2 protein of papillomavirus, despite a complete lack of sequence homology (Edwards et al. 1998; Hegde et al. 1992). Residues 461–503 flank the core domain (flanking domain) and are comprised of an α-helix oriented perpendicular to the DNA and an extended chain that tunnels along the base of the minor groove of the DNA (Fig. 3). Both the helix and the extended chain make sequence-specific DNA contacts. In addition, a direct role of the core domain in DNA recognition was suggested by analogy to the E2 DNA binding domain and later confirmed by mutational analyses (Cruickshank et al. 2000). Combined, the structural and biochemical studies indicate that the core and flanking domains of EBNA1 work together to load EBNA1 on its recognition site, likely through a two-step DNA binding mechanism. In keeping with this model, thermodynamic and kinetic analyses of the EBNA1 DNA binding domain–DNA interaction revealed two DNA association and dissociation events (Oddo et al. 2006). In addition, the ability of EBNA1 to bind its recognition sites, both in vitro and in vivo, was found to be greatly stimulated by USP7 through its interaction

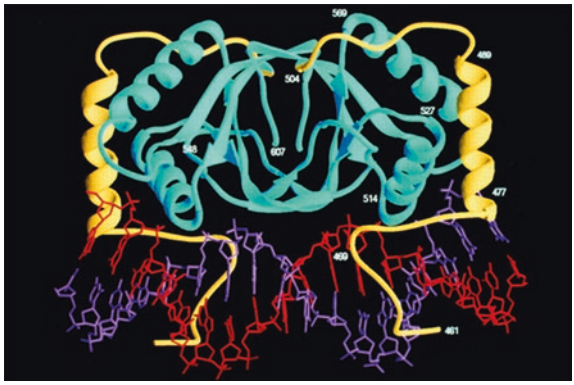

Fig. 3 Crystal structure of the EBNA1 DNA binding and dimerization domain bound to DNA. The core and flanking components of the DNA binding and dimerization domain are shown in *green* and *yellow*, respectively. EBNA1 amino acid numbers are indicated. Reprinted with permission from Bochkarev et al. (1996)

with EBNA1 amino acids close to the flanking domain (442–448; Fig. 2) (Sarkari et al. 2009), suggesting that this USP7 interaction may facilitate the DNA loading of the flanking domain.

The interaction of the EBNA1 DNA binding and dimerization domain with a single recognition site causes the DNA to be smoothly bent and causes localized regions of helical overwinding and underwinding (Bochkarev et al. 1996). The overwinding is caused by the EBNA1 flanking domain residues that traverse along the minor groove (amino acids 463–468) (Bochkarev et al. 1998; Summers et al. 1997) and this results in the increased sensitivity of one T residue within the DS sites to permanganate oxidation (Frappier and O'Donnell 1992; Hearing et al. 1992; Hsieh et al. 1993; Summers et al. 1997). EBNA1 dimers assemble cooperatively on adjacent sites in the DS (Summers et al. 1996; Harrison et al. 1994), and this is predicted to induce additional changes in the DNA structure (such as unwinding), in order to accommodate the closely packed dimers (Bochkarev et al. 1996). The strict requirement for the 3-bp spacing that separates neighboring sites in the DS for origin function suggests that the proper interaction between the EBNA1 dimers bound to these sites is crucial for the initiation of DNA replication, possibly because of the DNA structural changes that it imparts (Bashaw and Yates 2001; Harrison et al. 1994). Interactions of EBNA1 dimers on the multiple sites within the DS and FR elements likely also contribute to the pronounced bending of these elements that have been observed and to the appearance of EBNA1 as a large single complex on each element (Frappier and O'Donnell 1991a; Goldsmith et al. 1993; Bashaw and Yates 2001).

EBNA1 complexes bound to the DS and FR elements of *oriP* can also interact with each other cause the looping out of the intervening DNA (when interaction occur within an *oriP* molecule) and the linking of multiple *oriP* molecules (when interactions occur between *oriP* molecules) (Frappier and O'Donnell 1991a; Goldsmith et al. 1993; Su et al. 1991; Middleton and Sugden 1992). The DNA looping and linking interactions stabilize EBNA1 binding to the DS and involve homotypic interactions mediated by two different regions of EBNA1; a stable interaction mediated by amino acids 327–377 and a less stable interaction mediated by residues 40–89 (Frappier et al. 1994; Laine and Frappier 1995; Mackey et al. 1995; Mackey and Sugden 1999; Avolio-Hunter and Frappier 1998). The looping/linking interactions of EBNA1 are not restricted to EBNA1 complexes formed on the DS or FR elements but also occur between single EBNA1 dimers bound to distant recognition sites (Goldsmith et al. 1993). The contribution of DNA looping and linking to EBNA1 functions remains unclear but the amino acids required for these interactions overlap with those required for EBNA1 replication, segregation, and transcriptional activation functions (Mackey and Sugden 1999; Wu et al. 2002; Shire et al. 1999).

In vivo EBV genomes are assembled into nucleosomes with a spacing similar to that in cellular chromatin (Shaw et al. 1979; Dyson and Farrell 1985). Since nucleosomes tend to inhibit sequence-specific DNA interactions, the ability of EBNA1 to bind its site in the DS in the context of a nucleosome was examined. Surprisingly, EBNA1 was able to access its recognition sites within the

nucleosome and destabilized the nucleosome structure such that the histones could be displaced from the DNA (Avolio-Hunter et al. 2001). Efficient assembly of EBNA1 on the FR and DS elements was also observed on larger *oriP* templates containing physiologically spaced nucleosomes (Avolio-Hunter and Frappier 2003). The disruption of the DS-nucleosome by EBNA1 required all four recognition sites in the DS and was intrinsic to the DNA binding and dimerization domain of EBNA1 (Avolio-Hunter et al. 2001). The ability of EBNA1 to destabilize nucleosomes might be important for initiating DNA replication, a process known to be sensitive to nucleosome positioning. In addition, the ability of EBNA1 to access its sites within a nucleosome is likely to be important at times when chromatin is established prior to EBNA1 expression, for example, when latently infected resting cells (which do not express EBNA1) switch to proliferating forms of latency in which EBNA1 is expressed.

3.2 Interactions with Cellular DNA Sequences

The fact that EBNA1 can activate transcription, when bound to the EBV FR element, has prompted several studies to determine whether EBNA1 might also interact with specific sequences in cellular DNA to affect cellular gene expression. Chromatin IP (ChIP) experiments performed for EBNA1 from EBV-positive lymphoblastoid cell lines, followed by promoter array analysis, identified several EBNA1-associated DNA fragments, some of which were confirmed to be directly bound by EBNA1 in vitro (Dresang et al. 2009). While this approach identified a new EBNA1 recognition sequence (distinct from those in *oriP*), EBNA1 binding to this sequence did not activate reporter gene expression, so the significance of these EBNA1-cellular DNA interactions is not clear. ChIP combined with deep sequencing was also used to determine EBNA1 binding sites in B cells, identifying many EBNA1-associated sites, several of which were close to transcriptional start sites for cellular genes (Lu et al. 2010). The expression of some of these cellular genes was decreased upon EBNA1 depletion and induced by EBNA1 expression, suggesting that EBNA1 may affect their transcription. Like the previous study, these EBNA1 sites differed from those in *oriP*, but some were similar in sequence to those identified by Dresang et al. (2009). In addition, a cluster of high-affinity EBNA1 binding sites was identified on chromosome 11 between the divergent FAM55D and FAM55B genes, although the expression of these genes was not affected by EBNA1 (Lu et al. 2010). Canaan et al. (2009) conducted microarray experiments to compare cellular transcripts in B cells and 293 cells with and without EBNA1 and identified a small percentage of transcripts that were affected by EBNA1. In addition, EBNA1 was found to ChIP to most of these gene promoters, suggesting that it directly regulated them. However, whether or not EBNA1 bound directly to these promoters or was recruited through protein interactions was not determined.

The transcriptional activation function of EBNA1 on the EBV genome requires EBNA1 binding to multiple tandem recognition sites in the FR (Wysokenski and Yates 1989), and therefore, it seems unlikely that EBNA1 binding to any single recognition site would be sufficient to activate cellular transcription. To increase the probability of identifying functionally relevant EBNA1 interactions with cellular DNA, D'Herouel et al. (2010) used nearest neighbor position weight matrices to identify repeated EBNA1 binding sites in the human genome. The sites they identified had considerable overlap with those found by Dresang et al. (2009). Although the significance of the repeated EBNA1 sites that they identified remains to be determined, it is interesting that they include weak binding sites near the c-Jun and ATF promoters, which were previously shown to be activated by and associated with EBNA1 in NPC cells (O'Neil et al. 2008).

By comparing cell cycle-specific transcripts from EBV-negative B cells with and without EBNA1 expression, Lu et al. (2011) identified survivin (an inhibitor of apoptosis) as an EBNA1 target gene. EBNA1 increased the levels of survivin transcripts and protein and was shown to associate with the survivin promoter. Induction of survivin protein and transcripts required the EBNA1 residues 65–89 containing the N-terminal transcriptional activation sequence (Wu et al. 2002; Kennedy and Sugden 2003), suggesting that EBNA1 was activating the transcription of the survivin gene. However, since activation of the survivin promoter by EBNA1 involves the Sp1 binding sites, EBNA1 may be recruited to the promoter through the Sp1 host protein, as opposed to binding directly to the DNA. Similarly, Owen et al. (2010) found that EBNA1 increased the level of TFIIIC and ATF-2 transcripts and was associated with their promoter regions, consistent with a direct role in transcriptional activation. Finally, EBNA1 was recently reported to induce the expression of cellular let-7a microRNAs (miRNA) in nasopharyngeal and gastric carcinoma cells (Mansouri et al. 2014). EBNA1 increased the level of let-7a primary transcripts in a manner dependent on its N-terminal transactivation sequence, suggesting that EBNA1 directly induces their transcription. However, whether or not this involves a direct interaction of EBNA1 with DNA sequences regulating these primary transcripts remains to be determined.

Presumably any of the above direct interactions of EBNA1 with specific DNA sites would be mediated by the EBNA1 DNA binding domain. However, there have also been reports of less specific interactions of EBNA1 with DNA or chromatin through its Gly–Arg-rich regions, which resemble AT hooks (Sears et al. 2004; Coppotelli et al. 2013). In addition, EBNA1 has been reported to decondense heterochromatin through its Gly–Arg-rich sequences (Coppotelli et al. 2013). However, it is unclear whether this effect involves the association of the Gly–Arg sequences with DNA, chromatin-associated proteins (including the nucleosome assembly proteins known to bind to them), or another mechanism.

4 Cellular Effects of EBNA1

In addition to the roles of EBNA1 at the EBV genome, numerous reports suggest that EBNA1 directly contributes to cell proliferation and survival typical of latent EBV infection. The first implications came from the observations that EBNA1 is the only EBV protein expressed in all EBV-positive tumors and latency types in proliferating cells and is sometimes the only EBV protein expressed. EBNA1 was subsequently shown to be important for efficient B-cell immortalization by EBV (Hume et al. 2003; Altmann et al. 2006) and for the continued proliferation of some EBV-positive tumor cells (Kennedy et al. 2003; Hong et al. 2006; Yin and Flemington 2006). EBNA1 expression in various EBV-negative cancer cells has also been found to increase tumorigenicity (Sheu et al. 1996; Cheng et al. 2010; Kube et al. 1999; Kaul et al. 2007). In addition, EBNA1 expression in the B-cell compartment of a transgenic mouse has been reported to be sufficient to induce B-cell lymphomas (Wilson et al. 1996; Tsimbouri et al. 2002). However, these results were not reiterated in a second independent transgenic mouse study, suggesting that secondary events might contribute to the development of EBNA1-induced lymphomas (Kang et al. 2005, 2008). Nonetheless, the body of evidence indicates that EBNA1 contributes to oncogenesis, likely due to multiple effects on cellular proteins as discussed below and summarized in Fig. 4.

4.1 USP7 Interaction

Proteomics methods identified several cellular proteins that are bound by EBNA1, including an interaction with the cellular ubiquitin-specific protease USP7 [also called HAUSP (Holowaty et al. 2003c; Malik-Soni and Frappier 2012)]. USP7

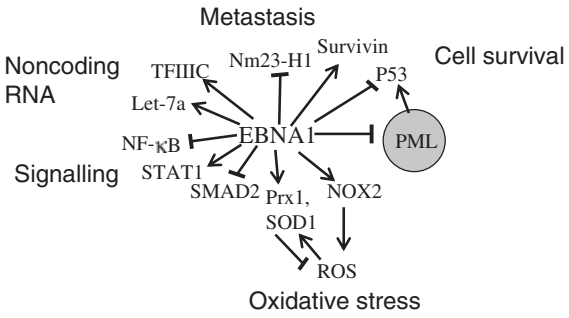

Fig. 4 Summary of EBNA1 cellular effects. The cellular proteins whose functions or levels are affected by EBNA1 are shown, where *arrows* represent positive regulation and blunted *lines* represent negative regulation. Associated cellular processes are also indicated

was originally discovered as a binding partner of the ICP0 protein from herpes simplex virus type 1 and has since been shown to be targeted by proteins from several different herpesviruses (Everett et al. 1997; Salsman et al. 2012; Lee et al. 2011; Jager et al. 2012). USP7 has been reported to bind and regulate several cellular proteins including p53 and Mdm2 (an E3 ubiquitin ligase for p53), which USP7 stabilizes by removing the polyubiquitin chains that normally signal degradation (Li et al. 2002, 2004; Cummins et al. 2004; Nicholson and Suresh Kumar 2011; Frappier and Verrijzer 2011). EBNA1, p53, and Mdm2 compete for the same binding pocket in the N-terminal TRAF domain of USP7; however, EBNA1 was found to outcompete p53 or Mdm2 due to its higher affinity for USP7 (Holowaty et al. 2003a; Saridakis et al. 2005; Sheng et al. 2006). The EBNA1 region just N-terminal to the DNA binding domain was identified as the USP7 binding site, and a subsequent crystal structure of this EBNA1 peptide bound to the USP7 TRAF domain showed that EBNA1 amino acids 442–448 contact USP7 (Fig. 2) (Holowaty et al. 2003a; Saridakis et al. 2005; Sheng et al. 2006; Hu et al. 2006).

In theory, EBNA1 could destabilize either p53 or Mdm2 by blocking their interaction with USP7, resulting in opposite effects on p53 levels. In vivo EBNA1 has not been reported to lower Mdm2 levels, but has been confirmed to lower p53 levels at least in some cell backgrounds. For example, expression of EBNA1 but not a USP7-binding mutant of EBNA1 in U2OS cells was shown to reduce the accumulation of p53 in response to DNA damage and subsequent apoptosis (Saridakis et al. 2005). Similarly, EBNA1 expression in CNE2 NPC cells decreased the accumulation of p53 in response to DNA damage (Sivachandran et al. 2008), and the presence of EBNA1 or EBV in AGS or SCM1 gastric carcinoma cells decreased the steady-state levels of p53 (Sivachandran et al. 2012a; Cheng et al. 2010). This suggests that EBNA1 could promote cell survival by modulating p53 in EBV-infected epithelial cells.

4.2 Effects on PML Nuclear Bodies

Promyelocytic leukemia (PML) nuclear bodies (also called ND10s) are nuclear foci for which PML tumor suppressor proteins form the structural basis. PML bodies are important for several cellular processes, including apoptosis, DNA repair, senescence, and p53 activation by acetylation (Salomoni et al. 2008; Bernardi and Pandolfi 2007; Takahashi et al. 2004; Guo et al. 2000; Wang et al. 1998; Pearson et al. 2000), and their loss has been associated with the development and/or progression of several tumors (Gurrieri et al. 2004; Salomoni et al. 2008). In addition, PML nuclear bodies suppress lytic viral infection as part of the innate antiviral response (Geoffroy and Chelbi-Alix 2011; Everett and Chelbi-Alix 2007; Reichelt et al. 2011). To counter this defense, many viruses encode proteins that disrupt PML nuclear bodies either by interfering with PML protein interactions need to form the bodies or by inducing the degradation of the PML proteins (Everett 2001).

EBNA1 was found to induce the loss of PML nuclear bodies in both NPC and gastric carcinoma cells, by inducing the degradation of the PML proteins (Sivachandran et al. 2008, 2012a). Consistent with known PML functions, EBNA1 expression in these cells was also found to decrease DNA repair efficiency, p53 acetylation, and apoptosis in response to DNA damaging agents (Sivachandran et al. 2008, 2012a). The results suggest that, as a result of EBNA1-induced PML loss, cells expressing EBNA1 are more likely to survive with DNA damage, which would be expected to contribute to the development of carcinomas. Importantly, these observations in cell lines appear to hold true in vivo, as a comparison of EBV-positive and EBV-negative gastric carcinoma tumor biopsies showed that PML levels were greatly reduced by the presence of EBV, presumably due to the action of EBNA1 (Sivachandran et al. 2012a).

The mechanism by which EBNA1 induces the degradation of PML proteins involves EBNA1 binding to both USP7 and the host casein kinase 2 (CK2) and recruitment of these proteins to the PML nuclear bodies (Sivachandran et al. 2008, 2010). EBNA1 was found to preferentially interact with PML isoform IV over the other five nuclear PML isoforms, and therefore, EBNA1 may localize to PML nuclear bodies through interactions with PML IV (Sivachandran et al. 2008, 2012b). EBNA1 mutants that fail to bind either USP7 or CK2 can still associate with PML bodies but do not induce their loss (Sivachandran et al. 2008, 2010). Similarly, wild-type EBNA1 does not affect PML nuclear bodies when USP7 or CK2 is depleted. In keeping with these observations, USP7 was subsequently shown to negatively regulate PML proteins (even in the absence of EBV or EBNA1), by a mechanism that is independent of its ubiquitin cleavage activity (Sarkari et al. 2011).

The interaction of EBNA1 with CK2 involves a direct interaction of EBNA1 amino acids 387–394 with the binding pocket in the β-regulatory subunit of CK2 and this interaction requires EBNA1 to be phosphorylated at S393 (Sivachandran et al. 2010; Cao et al. 2014). CK2 was previously identified as a negative regulator of PML and was shown to phosphorylate PML proteins at a particular serine residue that triggers polyubiquitylation and subsequent degradation (Scaglioni et al. 2006, 2008). Through its interaction with CK2, EBNA1 was shown to increase CK2-mediated phosphorylation of PML, which is expected to increase PML polyubiquitylation (Sivachandran et al. 2010). Since CK2 is involved in many cellular processes, it is possible that the interaction of EBNA1 with CK2 also affects additional pathways.

4.3 Modulation of Signaling Pathways

EBNA1 has been reported to affect several signaling pathways. First, EBNA1 expression in three different carcinoma cell lines was found to increase the expression of STAT1 (Wood et al. 2007; Kim and Lee 2007). EBNA1 was subsequently shown to enhance STAT1 phosphorylation and nuclear localization in response to IFNγ (Wood et al. 2007). Second, EBNA1 expression was found to decrease the

expression of TGF-β1-responsive genes suggesting that EBNA1 interferes with TGF-β signaling (Wood et al. 2007). This effect may be due to increased turnover of SMAD2 in the presence of EBNA1, resulting in decreased levels of SMAD complexes needed for TGF-β1-induced transcription (Wood et al. 2007; Flavell et al. 2008). Third, using NF-κB reporter plasmids in carcinoma cell lines, EBNA1 was found to inhibit NF-κB activity and DNA binding (Valentine et al. 2010). Additional experiments showed that the levels, nuclear localization, and phosphorylation of the p65 NF-κB subunit were all reduced in the presence of EBNA1 as was the phosphorylation of the p65 kinase, IKKα/β (Valentine et al. 2010). How EBNA1 elicits any of the above effects is presently unclear as no physical interaction has been detected between EBNA1 and STAT1, SMAD2, p65, or IKKα/β.

4.4 Induction of Oxidative Stress

EBV infection is associated with increased oxidative stress (Lassoued et al. 2008; Cerimele et al. 2005) and this may be at least partly due to EBNA1 expression. Stable or transient EBNA1 expression in B-cell lines was found to increase levels of reactive oxygen species (ROS), DNA damage foci and dysfunctional, uncapped telomeres, and these EBNA1 effects were decreased by ROS scavengers (Gruhne et al. 2009; Kamranvar and Masucci 2011). In addition, EBNA1 was found to increase the expression of the NOX2 NADPH oxidase which might account for the ROS induction (Gruhne et al. 2009). Similarly, a comparison of the nuclear proteome in NPC cells with and without EBNA expression showed that EBNA1 increased the levels of several oxidative stress response proteins including the antioxidants superoxide dismutase 1 and peroxiredoxin 1, known to be induced by ROS (Cao et al. 2011). Further studies confirmed that, in the presence of EBNA1, ROS levels were elevated and that NOX1 and NOX2 transcripts were increased (Cao et al. 2011). Therefore, EBNA1 appears to have multiple effects on the oxidative stress response, although the mechanisms of these effects are not yet known.

4.5 Effects on Noncoding RNA

EBNA1 expression has been reported to increase the transcript and protein levels of the RNA polymerase III transcription factor TFIIIC and, in keeping with this finding, increased the expression of several cellular pol III-transcribed genes (Owen et al. 2010). In the same study, EBNA1 was also found to induce the expression of ATF-2, a pol II transcription factor known to contribute to the expression of EBV EBER noncoding RNAs. The result prompted examination of the effect of EBNA1 on EBER levels and confirmed that EBNA1 induces EBER expression.

The effects of EBNA1 on cellular miRNAs have also been examined, using high-throughput sequencing to compare miRNA levels in two different