Deregulation of the cell cycle machinery by Epstein–Barr virus nuclear antigen 3C

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Review
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Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus associated with a large number of lymphoid and epithelial malignancies. As a successful pathogen it has co-evolved with its human host for millions of years. EBV has the unique ability to establish life-long latent infection in primary human B lymphocytes. During latent infection, a small subset of viral proteins is expressed. These proteins are essential for maintenance of the EBV genome as well as the deregulation of various signaling pathways that facilitate the proliferation and survival of the infected cells. Epstein–Barr nuclear antigen (EBNA)3C is one of the latent proteins shown to be essential for transformation of primary human B lymphocytes *in vitro*. EBNA3C primarily functions as a transcriptional regulator by interacting with a number of well known cellular and viral transcriptional factors. We have recently identified several binding partners for EBNA3C including proteins that regulate cell cycle and chromatin remodeling. We are actively engaged in discerning the biological significance of these interactions. This review summarizes our current understanding of how EBNA3C usurps cellular pathways that promote B-cell transformation.

Epstein–Barr virus (EBV) or human herpesvirus (HHV)-4 is a ubiquitous HHV that belongs to the genus *Lymphocryptovirus* (LCV) of the γ -herpesvirus subfamily. It is closely related to other LCV found in non-human primates including chimpanzees and rhesus monkeys [1]. The majority of the adult population worldwide is infected with EBV and the virus can persist in a latent state in B lymphocytes for the lifetime of the infected host [2]. Primary infection by EBV occurs in early childhood and is generally subclinical. However, primary infection during adolescence or adulthood typically manifests as infectious mononucleosis, a self-limiting lymphoproliferative disorder [3]. EBV is also associated with lymphomas of B, T and natural killer (NK) origin such as endemic Burkitt's lymphoma (BL), immunoblastic lymphomas of immunocompromised patients, Hodgkin's lymphoma, a proportion of peripheral T-cell lymphomas and nasal T/NK lymphomas [4]. An increasing array of nonlymphoid malignancies are also being attributed to EBV infection such as undifferentiated nasopharyngeal carcinomas, a fraction of gastric cancers and smooth muscle tumors in AIDS or transplant patients [4].

EBV was discovered in 1964 by Tony Epstein and Yvonne Barr who identified herpesvirus-like particles from cells cultured from a BL patient

[5]. The EBV genome (B95-8 strain) was the first HHV genome to be sequenced [6]. The genome consists of a linear, dsDNA that is approximately 180 kb pairs long and has reiterated 0.5 kb terminal repeats at the ends of the genome. A large tandemly repeated DNA sequence of approximately 3 kb in size within the genome called internal repeat-1 divides the genome into long and short unique regions. Importantly, the internal repeat-1 contains the Wp promoter that drives latent Epstein–Barr nuclear antigen (EBNA) gene expression on initial entry of EBV into primary B lymphocytes [7,8]. The genome encodes approximately 85 genes and the genes expressed during the lytic phase have homologs in other HHVs, but genes expressed during latency are unique to EBV.

EBV latency

Latency is the hallmark of all herpesviruses allowing them to persist for the lifetime of their respective hosts. During latency, a limited number of transcripts are expressed from the viral genome. EBV transforms primary B cells *in vitro* into continuously proliferating lymphoblastoid cell lines (LCLs) provided that immune T cells are eliminated [9]. These LCLs serve as an invaluable tool for investigating EBV latency/transformation. The LCLs express B-cell activation markers and thus phenotypically resemble

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activated B lymphocytes [10]. In LCLs, the viral genome is maintained in multiple episomal copies [11].

EBV displays distinct patterns of latency as determined by the infected host cell and these patterns are defined by the specificity of the latent gene expression [12]. One of the best-characterized latency programs referred to as latency III (also called the growth program due to its association with autonomous B-cell proliferation) is found in LCLs. Among EBV-associated malignancies, latency III is found in immunoblastic B lymphomas in post-transplant and AIDS patients [13]. These lymphomas represent the *in vivo* counterparts of the transformed LCLs and most likely result when EBV-infected B cells escape a virus-specific cytotoxic T lymphocyte (CTL) response. In healthy immune individuals, the CTL response to latently-infected cells is mainly directed against immunodominant epitopes within the EBNA3 family proteins [14]. *In vivo*, latency III is seen during primary exposure of B cells to EBV resulting in a population of cells that express the full spectrum of latent genes including a family of six nuclear proteins called EBNAs (1–6), three latent membrane proteins (LMPs), two abundant noncoding, nonpolyadenylated RNAs (EBV-encoded small nonpolyadenylated RNAs [EBERs]) and a family of rightward transcripts derived from the BamHI-A region of the EBV genome (Bam HI-A region rightward transcript [BART]). Strikingly, EBERs and BARTs are abundantly present in cell lines representing different latency types and in all EBV-associated diseases. Despite their ubiquitous expression and potential to encode several open reading frames, the regulation and biological function of BARTs are still unclear. The relative position of these latent genes on the EBV genome is shown in FIGURE 1. The major functions associated with EBV latency genes are shown in TABLE 1. Genetic studies have identified

that out of these 11 latent genes, four are absolutely essential for *in vitro* B-cell transformation namely EBNA2, EBNA3A, EBNA3C and LMP1, with EBNA-leader protein being important but not essential [15–17].

The other restricted forms of EBV latency are type II latency (also called the rescue program) and type I latency, which is the most restricted form. Type II latency is seen in infected B cells derived from the germinal center of lymphoid organs and is also seen in EBV-associated Hodgkin's disease, peripheral T-cell lymphomas and nasopharyngeal carcinoma [18–20]. The infected cells express EBNA1 and the LMP proteins that provide signals allowing infected lymphoblasts to survive and differentiate into memory B cells. In latency I, EBNA1 is the only EBV-encoded protein expressed in addition to the EBERs. It is believed that this is the minimum possible level of EBV gene expression that can support viral genome maintenance in a dividing host cell. This is achieved by silencing the major EBNA and LMP latent promoters through methylation [21]. All three epidemiologically distinct forms of EBV-positive BLs (endemic, sporadic or AIDS associated) and early passage EBV plus BL-derived cell lines display the latency I program [22]. However, recent studies demonstrate that alternate patterns of viral latency also exist in endemic BL, which are characterized by the expression of EBNA1 and EBNA3 proteins in the absence of LMPs and EBNA2 [23]. Besides the apparent advantage that such restricted viral antigen expression aids in immune evasion, it was suggested that tumor evolution selects for forms of EBV latency that can antagonize and thus are compatible with the high levels of proapoptotic c-Myc expression [23]. Most likely, these different forms of latency are not tumor specific but reflect forms of infection that a virus adopts during the course of infection [24].

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Figure 1. Genomic organization of Epstein-Barr virus latent genes. The location of Epstein-Barr virus (EBV) latent genes including the six EBNAs (1, 2, 3A, 3B and 3C and EBNA-leader protein), three LMPs (1, 2A, 2B), highly transcribed nonpolyadenylated RNAs (EBER1 and EBER2) and BamHI region transcribing Bam HI-A region rightward transcripts are shown. The genes are shown along with their multiple exons. All EBNAs are transcribed from either Cp or Wp promoter while LMPs have their own promoter. The EBNA-1 transcript originates from Qp promoter during Latency I. The origin of plasmid replication is shown in pink. EBER: Epstein-Barr virus-encoded small nonpolyadenylated RNA; EBNA: Epstein-Barr nuclear antigen; LMP: Latent membrane protein; TR: Terminal repeat.

Table 1. Epstein–Barr virus-encoded latent gene products and their functions.

Gene	Required for transformation	Latency	Major functions
EBNA1	No	I, II, III	<ul style="list-style-type: none"> • Tethers the viral genome to the host cell chromosome, mediating episomal maintenance [81] • Gly-Ala repeat inhibits the processing of EBNA1 through proteasomes thus evading an immune response [82] • Upregulates the expression of RAG-1 and RAG-2 [83]
EBNA2	Yes	III	<ul style="list-style-type: none"> • Functional homolog of Notch • Interacts with RBP-Jκ and modulates Notch signaling [84] • Essential for B-cell transformation [85] • Induces LMP1 [86] and CD21 expression [87]
EBNA3A	Yes	III	<ul style="list-style-type: none"> • An EBNA2 antagonist and coactivator [88] • Interacts with RBP-Jκ [89]
EBNA3B	No	III	<ul style="list-style-type: none"> • Interacts with RBP-Jκ [89]
EBNA3C	Yes	III	<ul style="list-style-type: none"> • Essential for B-cell transformation [30] • Interacts with RBP-Jκ [47] • Recruits SCF/skp2 ubiquitin ligase to degrade Rb and p27 [70] • Promotes LMP1 expression in the presence of EBNA2 [90] • Interacts with Chk2 [77], c-Myc [71] and cyclin A [74]
EBNA-LP	No	III	<ul style="list-style-type: none"> • Upregulates factors necessary for B-cell growth [91] • Binds p53 and Rb [92]
LMP1	Yes	II, III	<ul style="list-style-type: none"> • Mimics signaling through B-cell activation receptor CD40 [93] • Activates nuclear factor-κB pathway to promote B-cell survival [94] • Upregulates IL-10 [95] • Interacts with TRAFs [96]
LMP2A/2B	No	II, III	<ul style="list-style-type: none"> • Negative modulator of B-cell signaling [97] • Promotes B-cell survival [98]
EBER-1/2	No	I, II, III	<ul style="list-style-type: none"> • Antiapoptotic [99]
BART	No	I, II, III	<ul style="list-style-type: none"> • Not known

BART: Bam HI-A region rightward transcript; EBER: Epstein–Barr virus-encoded small nonpolyadenylated RNA; EBNA: Epstein–Barr nuclear antigen; LMP: Latent membrane protein; LP: Leader protein; Rb: Retinoblastoma protein; RBP-Jκ: Recombination signal binding protein for Jκ; TRAF: Tumor necrosis factor receptor-associated factor.

The three latency types display different patterns of promoter usage. This alternative usage is considered central to the virus strategy to persist in the host. Whereas LMP genes are transcribed from their own promoters, all six EBNA genes are expressed by splicing of a common primary transcript that initiates from one of the two alternative promoters, Wp or Cp [8,25]. Wp is the initial promoter utilized after infection that preferentially drives the expression of EBNA2 and EBNA-leader protein [26]. Shortly thereafter, there is a promoter switch from Wp to Cp, located just upstream, which results in the expression of all six EBNA genes. The viral transcriptional factor EBNA2 is required for the Wp-to-Cp switch [27]. Latency I, where the transcriptional program is restricted to only EBNA1 gene, involves transcription driven from the Qp promoter that selectively drives EBNA1 transcripts [28]. The other promoters including Cp, Wp and LMP are suppressed. Although some cellular factors important for initial activation of the Wp promoter have been identified, the mechanism of promoter switching and the repression of the Wp promoter remains poorly understood.

EBNA3C

The EBNA3 proteins are nuclear proteins that are encoded by three genes arranged in tandem in the viral genome. They are large hydrophilic, proline-rich, charged proteins that share a similar genomic organization. Each EBNA3 transcript is encoded by a short 5'-exon and a long 3'-exon. There is a limited homology between the three family members specifically at the amino terminus of the molecule suggesting that they may have evolved from the common ancestor by gene duplication [29]. A recombinant molecular genetic approach was utilized to evaluate the individual role of all three EBNA3 genes. Recombinant viruses were generated by introducing a stop codon in the reading frame of each EBNA3 gene. The mutation in the EBNA3A and EBNA3C gene but not in EBNA3B gene affected the ability of the recombinant virus to infect and transform primary B cells demonstrating that EBNA3A and EBNA3C are essential for B-cell transformation *in vitro*, whereas EBNA3B is dispensable [30,31].

EBNA3C (also known as EBNA6) is a 992 amino acid (aa) protein with a molecular weight of approximately 160 kd [32]. EBNA3C

localizes exclusively in the nucleus in discrete punctate bodies or speckles [33]. The localization of EBNA3C in these speckles remain stable throughout the cell cycle as shown by blocking different stages of the cell cycle using various drugs in Hela cells expressing GFP-tagged EBNA3C [34]. EBNA3C contains three functional nuclear localization signals, one at the amino terminal (aa 72–80), one in the middle (aa 412–418) and one at the carboxy terminus (aa 939–945), and the region responsible for EBNA3C's localization to these discrete nuclear bodies has been mapped to aa 733–808 [33]. Immunofluorescence studies revealed that EBNA3C co-localized with survival of motor neuron protein (SMN) [34]. The SMN protein is part of a large complex that plays an important role in small nuclear ribonucleoprotein (snRNP) assembly, pre-mRNA splicing and transcription. Interestingly, EBNA3C has also been shown to interact and co-localize with DP103, another component of the SMN complex. The binding of EBNA3C to DP103 has been mapped to a region that overlaps the SMN-binding region [35]. The functional significance of these interactions is not known but do suggest that EBNA3C may have a role in RNA processing as well.

EBNA3C is a remarkably stable protein expressed at low levels in LCLs. Surprisingly, *in vitro* translated EBNA3C is degraded when incubated with purified 20S proteasome suggesting that in infected EBNA3C cells can somehow escape the proteasome-mediated degradation pathway [36]. It is quite possible that it interacts with or recruits a complex with deubiquitination activity, which allows bypassing of proteasome degradation machinery. Interestingly, another latent protein, EBNA1, has been shown to bind a cellular deubiquitinating enzyme USP7 with high affinity and so regulates the ability of USP7 to deubiquitinate p53 [37].

EBNA3C as a transcriptional regulator

Analysis of the EBNA3C amino acid sequence has revealed features associated with many viral and cellular transcription factors. These features include a region that resembles a basic DNA binding domain adjacent to a potential leucine zipper motif (bZIP) and regions (rich in acidic, proline and glutamine residues) often seen in domains known to be associated with modulation of gene transcription (FIGURE 2). EBNA3C primarily functions as a transcriptional regulator by interacting with various cellular and viral factors. Studies have shown that EBNA3C can act as both an activator as well as a repressor

in transient reporter assays. EBNA3C does not bind directly to DNA, although it has a putative bZIP domain at the amino terminal located between residues 244–291 [38]. The bZIP family of transcription factors including c-Jun and c-Fos contain a highly conserved basic region involved in DNA binding and the leucine zipper (a heptad repeat of leucine residues) required for dimerization [39]. These proteins dimerize through their leucine repeats to form a coiled coil with two flanking α -helices that constitute the basic region that contacts DNA bases. Structural and biochemical analysis of the bZIP domain of EBNA3C demonstrated that this domain is not capable of forming stable homodimers. This unusual feature was attributed to the presence of atypical residues present in the zipper domain [38].

The first experimental evidence implicating EBNA3C as a transcriptional activator was provided by gene transfer experiments that demonstrated the ability of EBNA3C to activate the expression of both cellular and viral genes. In EBNA3C expressing an EBV-negative BL cell line there is an increased expression of the complement receptor CD21 known to function as an EBV receptor [40]. In similar experiments, heterologous expression of EBNA3C was also shown to increase the expression of the LMP1 gene in Raji cells (an EBV-positive BL cell line lacking an EBNA3C open reading frame) arrested early in the G1 phase of the cell division cycle [41]. In transient promoter assays EBNA3C activated transcription from the LMP1 promoter but had no effect on the CD21 promoter [42]. Given the essential contribution of LMP1 to EBV-mediated transformation, the fine tuning of the LMP1 promoter seems to be an important function of EBNA3C. A transactivation domain rich in glutamine/proline residues with similarities to the cellular transcription factor Sp1 has been mapped to aa 724–826 in transient Gal4 directed transcription reporter assays [40]. However, amino acid residues constituting an EBNA3C repression domain (aa 365–545) were found necessary and sufficient for the coactivation of the LMP1 promoter with EBNA2 [43]. It was suggested that coactivation could be due to the squelching of a highly specific inhibitor that is otherwise active at sites of EBNA2 transcription complexes. Interestingly, the same region was also required for binding small ubiquitin-related modifier, SUMO-1 and SUMO-3 [44]. Post-translational modifiers of the SUMO family have emerged as key regulators of protein function with roles in diverse processes including regulation of transcription,

Figure 2. Schematic of Epstein-Barr nuclear antigen 3C protein with the identified domains and known interaction sites for various proteins. Domains for interaction with p300 are present at both the amino (aa 1–207) and the carboxy termini (aa 620–992) of Epstein-Barr nuclear antigen (EBNA)3C. Prothymosin-α specifically interacts with the region between aa residues 366–400. The interaction domain for RBP-Jκ (aa 183–240), the leucine zipper (aa 241–291), acidic domains, proline rich domain and glutamine rich residues (QP region) are indicated. Three NLS are present, at the amino-terminal (aa 72–80), one in the middle (aa 412–418) and one at the carboxy-terminus (aa 939–945). The PLDLS motif responsible for binding with the transcriptional co-repressor CtBP is present at residues 728–732. The regions involved in activation and repression are indicated. The region responsible for binding Cyclin A, SCF/SKP2 ligase complex and c-Myc is situated at the amino terminus between aa residues 130–190. NLS: Nuclear localization signal; RBP-Jκ: Recombination signal binding protein for Jκ; SMN: Survival of motor neuron protein.

chromatin structure and DNA repair [45]. It is quite possible that interaction and modification by SUMO proteins have a role in EBNA3C transcriptional effects and regulation of cell growth in EBV transformed LCLs.

Full length EBNA3C fused to the DNA-binding domain of yeast transactivator GAL4, repressed reporter gene expression in a BL-derived EBV negative B-cell line [46]. The repression domains of EBNA3C were mapped to a region rich in acidic and proline residues (aa 280–525) and one rich in proline and glutamine residues (aa 580–992). These studies established EBNA3C's dual ability to modulate transcription by acting both as a transactivator or repressor. The mechanisms that mediate this switch as an activator or repressor may include differential modification or the recruitment of different cofactors. One such transcriptional repressor that interacts with EBNA3C is the cellular transcriptional factor recombination signal binding protein for Jκ (RBP-Jκ; also known as CBF-1), a downstream signaling protein in the Notch pathway [47]. The RBP-Jκ binding site is located at the amino terminus of EBNA3C (mapped to amino acids between 183 and 240) and is conserved among all EBNA3 proteins [48]. EBNA3C was shown to repress transcription from the viral Cp promoter in a manner dependent on the RBP-Jκ interaction site in EBNA3C and the RBP-Jκ binding site in Cp [47]. In transient gene

reporter assays EBNA3C–RBP-Jκ interaction repressed EBNA2–RBP-Jκ mediated transcription by preventing RBP-Jκ from associating with DNA. *In vitro* gel retardation assays showed that EBNA3C interfered with the ability of RBP-Jκ to bind to DNA and inclusion of EBNA3C in EBNA2–RBP-Jκ binding reactions led to decreased association of EBNA2 with DNA. Since EBNA2 activates the Cp promoter, it was speculated that the EBNA3 proteins might mediate feedback inhibition of Cp [47].

Another candidate corepressor that was found to interact with EBNA3C is C-terminal binding protein (CtBP) [49]. The interaction was seen both *in vitro* and *in vivo* through a PLDLS motif located in EBNA3C at aa 728–732 [49]. CtBP is a highly conserved cellular phosphoprotein with roles in the regulation of development, proliferation and apoptosis [50]. CtBP was initially identified as a cellular protein that interacts with the C terminus of adenovirus E1A oncoproteins and was found to be essential for the immortalization of primary rodent cells by E1A [51]. Functionally, however, CtBP binding made little contribution to repression by Gal4–EBNA3C and no apparent contribution to repression of Cp by EBNA3C [49]. It is quite probable that other factors like RBP-Jκ, recruited by EBNA3C, are more important for repression activity.

Previous studies from our laboratory implicates EBNA3C's involvement in the regulation of chromatin remodeling by targeting acetylase,

deacetylase or other factors associated with these complexes [52–57]. Regulation of chromatin structure by post-translational modifications of histones is an important mechanism in controlling gene transcription. The addition of acetyl groups to the core histones results in the disassociation of chromatin and nucleosomal structure, thereby rendering the transcriptional regulatory sites accessible to the transcriptional machinery. Transcriptional coactivators, such as p300 and CREB-binding protein, have been shown to exhibit histone acetyltransferase activity, which is shown to enhance transcription activities. The histone acetyltransferase inhibitor trichostatin A was shown to inhibit EBNA3C-mediated repression of Cp in transient transfection assays suggesting the involvement of the histone deacetylation complex in the repression mediated by EBNA3C [52]. Furthermore, EBNA3C was shown to bind histone-modifying deacetylase enzyme (HDAC1) *in vitro* and the residues involved in binding were shown to be similar to that which bind RBP-J κ [52].

These lines of investigations also identified a cellular protein, prothymosin- α (ProT α), which interacts with EBNA3C between aa 366–393 *in vitro* and *in vivo* [53]. ProT α is a small, acidic nuclear protein ubiquitously expressed in most eukaryotic cells [54]. It has been shown to associate with histone H1 and is capable of transforming Rat1 cells in culture inducing foci formation in soft agar assays [55]. In addition to its association with EBNA3C, ProT α was also shown to associate with p300 and histone H1 in EBV-infected B cells [56]. Moreover, it was also shown that EBNA3C complexed with ProT α interacts with HDAC1, HDAC2 and the corepressors mSin3A and NCoR in cell lines expressing EBNA3C [57]. Based on these interaction studies, a model was proposed to explain EBNA3C regulation of transcription and histone acetyltransferase activity associated with p300 and ProT α . According to the model, ProT α interacts with histone H1 resulting in decondensation of the nucleosomes, thus allowing access for transcription and coactivation complexes including p300. This then leads to the acetylation of histone and enhanced transcription. The expression of EBNA3C displaces this complex leading to the downmodulation of the histone acetylation activity.

EBNA3C & the cell cycle

A general overview of the cell cycle is presented here. The readers are directed to more comprehensive review articles [58–61]. The cell cycle is a

complex series of ordered events involving several proteins that direct the cell to duplicate the genome. The mammalian cell cycle is divided into four distinct phases, referred to as G1, S, G2 and M phase. G1 and G2 phases represent gaps in the cell cycle that occur between DNA synthesis and mitosis. Four main families of proteins, including the cyclin-dependent kinases (CDKs), the cyclins, pRB and CDK inhibitors (CDKIs), regulate the transition from one phase of the cell cycle. CDKs are a family of serine/threonine kinases that are expressed throughout the cell cycle but are activated at specific points [62]. Their activation depends on the temporal synthesis and binding to the regulatory subunits called cyclins whose levels oscillate during the cell cycle. Different cyclins work cooperatively with different CDKs at different phases of the cell cycle. CDK4 and CDK6 are involved in the early G1 phase, whereas CDK2 is required to complete the G1 phase and initiate the S phase. Both CDK4 and CDK6 form active complexes with the type D cyclins (cyclins D1, D2 and D3). CDK2 is sequentially activated by the type E cyclins during the G1/S transition stage while type A cyclins play a role during the S phase. The CDK2–cyclin A complex appears during the late S phase and plays a role in the progression of DNA replication. Active CDKs subsequently phosphorylate key growth-regulating proteins, such as the retinoblastoma tumor-suppressor protein (pRb), resulting in its dissociation with the E2F family of transcription factors [63]. The active E2F then transactivates several promoters including for genes that are involved in DNA synthesis and cyclins (E and A) necessary for S phase entry. The kinase activity of cyclin/CDK complexes in turn is negatively regulated by CDKIs, which belong to one of the two families based on their structural relationship [64]. The first family includes the Ink4a proteins (inhibitors of CDK4), which specifically inhibit the catalytic subunits CDK4 and CDK6. These include p16INK4a, p15INK4b, p18INK4c and p19INK4d. The second family members include Cip/Kip proteins, which regulate the activities of cyclin D-, cyclin E- and cyclin A-dependent kinases and include p21Cip1, p27Kip1 and p57Kip2.

To ensure the accurate replication of a genome, cells are equipped with the checkpoints that are set at various stages of the cell cycle. These checkpoints respond to any cellular damage by arresting the cell cycle to provide time for repair. During DNA damage, there is stabilization and accumulation of p53

[65]. p53 is an extensively studied tumor suppressor protein [66]. The active p53 transactivates CDKI p21, which binds and inactivates the cyclin E–CDK2 complex. Inhibition of CDK2 prevents phosphorylation of retinoblastoma protein (Rb) and subsequently the entry of the cell into the S phase. DNA damage also results in the activation of Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR) kinases, which in turn phosphorylate and activate the downstream kinases Chk2 and Chk1, respectively [58]. The activated form of Chk2 phosphorylates and inactivates Cdc25C, an inhibitory phosphatase that activates CDKs by removing inhibitory phosphate residues. The phosphorylated Cdc25C binds to 14-3-3 protein that sequesters Cdc25C in the cytoplasm preventing the activation of the CDK1–cyclin B1 complex. This results in cell cycle arrest at the G2/M checkpoint.

Viruses in general have yielded many insights into cell cycle control because they encode oncoproteins that modulate cell cycle progression and apoptosis. Oncogenes encoded by transforming viruses, such as large T antigen by simian virus 40, or E6/E7 by human papillomavirus, deregulate cell cycle progression by overriding growth suppressive functions of tumor suppressor proteins like p53 and Rb [67]. EBNA3C has been shown to function as an oncoprotein that can cooperate with activated (Ha⁺) ras to transform rat embryonic fibroblasts, with efficiency comparable to human papilloma viral protein E7 [68]. It was also found that EBNA3C could rescue the suppression of this transformation by CDKI and p16/INK4a [68]. It is noteworthy that viral oncoproteins E1A and E7 antagonize Rb function and are resistant to suppression by p16. This suggested that EBNA3C interacts directly or indirectly with the Rb. A weak *in vitro* interaction was initially seen between glutathione S-transferase-Rb and EBNA3C in pull-down assays. However, no reproducible association was seen in *in vivo* experiments [68]. The molecular mechanism by which EBNA3C regulates the Rb pathway is not fully elucidated. However, Knight and coworkers showed that EBNA3C can form a stable complex with Rb in cells when the proteasome machinery is inhibited [69]. This study also showed that EBNA3C could influence the stability of Rb by recruiting a cellular ubiquitin ligase Skp1–Cul1–F-box complex (SCF–Skp2) [70]. The region of EBNA3C responsible for binding SCF/Skp2 complex was mapped to amino acid residues 130–190. Surprisingly, the same region was shown to

strongly associate with the c-Myc oncoprotein [71]. Additionally, the interaction of EBNA3C with c-Myc was mapped to the region of c-Myc that included the highly conserved Skp2-binding domain. Skp2 has been shown to regulate c-Myc stability and also has been shown to function as a coactivator of transcription for c-Myc target genes [72]. EBNA3C was able to stabilize c-Myc and recruit both c-Myc and its cofactor Skp2 to c-Myc-dependent promoters, which result in enhanced c-Myc-dependent transcription. We are presently exploring the possible mechanisms of c-Myc deregulation in EBV-infected cells, which may lead to interesting mechanisms as it does not involve chromosomal translocation or amplification known to be associated with c-Myc.

EBNA3C can also regulate cyclin–CDK complexes. As mentioned above, one of the substrates of cyclin–CDKs complexes include pRb. EBNA3C was shown to bind cyclin A in an *in vitro* pull-down assay and also in an *in vivo* co-immunoprecipitation assay from a B-cell line expressing EBNA3C [73]. There was enhanced cyclin A-associated kinase activity for the substrate histone H1 in cyclin A immune complexes isolated from EBNA3C-expressing U2OS cells [74]. In EBNA3C-expressing BJAB cells there was a decrease in the association of p27 and cyclin A in co-immunoprecipitation experiments compared with the control BJAB cells. This suggested that EBNA3C rescues p27-mediated inhibition of cyclin A/CDK2 kinase activity by decreasing the molecular association between cyclin A and p27 [74]. Targets of EBNA3C linked to the G1/S transition are shown in our model (FIGURE 3).

A recent study demonstrated that EBNA3C can interact with mitochondrial ribosomal protein S18B (MRPS18-2) [75]. The exact function of MRPS18-2 is not known but it was shown to bind the small pocket region of both hypo- and hyperphosphorylated forms of Rb protein, which is a site of interaction with E2F1 [75]. EBNA3C was found to target MRPS18-2 to the nucleus and promote binding between pRb and MRPS18-2 resulting in the disruption of E2F1 with pRb. The authors suggested that EBNA3C may play a major role in the entry of EBV-infected B cells into the S phase by binding to and raising the level of nuclear MRPS18-2 protein, which would inhibit pRb binding to E2F1 competitively and thus lift the block preventing S phase entry.

Other studies demonstrate that EBNA3C not only disrupts the G1/S checkpoint but also the G2/M checkpoint by directly interacting with

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Figure 3. Epstein-Barr nuclear antigen 3C deregulates cell cycle. Epstein-Barr nuclear antigen (EBNA)3C forms a complex with cyclin A and enhances the kinase activity which results in the hyperphosphorylated form of retinoblastoma protein (Rb). Hyperphosphorylation of Rb prevents its interaction with E2F, releasing E2F from an inhibitory constraint and enabling the expression of genes required for entry into the S Phase. EBNA3C also recruits SCF/SKP2 E3-ligase complex and targets Rb and p27 for ubiquitin-mediated degradation, which facilitates G1/S Phase transition.

Chk2, an ATM/ATR signaling effector [76,77]. The cell cycle analysis of BJAB cells expressing EBNA3C treated with the drug nocodazole, which blocks cell cycle at G2/M transition, did not show G2/M checkpoint arrest compared with BJAB cells expressing vector. Furthermore, immunoprecipitation experiments showed that EBNA3C interacts directly with Chk2 and siRNA knockdown of Chk2 expression diminished the ability of EBNA3C to release the nocodazole-induced G2/M block [77].

Carcinogenesis is a multistep process that involves deregulation of cell cycle machinery along with suppression of apoptotic pathways.

Inhibition of apoptosis is central to tumor development and viruses in general have developed a battery of distinct strategies to modulate this key cellular process. Recent studies showed that latent EBV protects BL cells from apoptosis induced by a variety of cytotoxic agents and implicated the EBNA3 family as responsible for the resistant phenotype [78]. Further investigations exploited recombinant EBVs with deletions of individual EBNA3 genes and evaluated the contribution made by each of the EBNA3 proteins [79]. Analysis of BL cell lines, established following infection by mutant viruses, for their ability to resist apoptosis when exposed to

different cytotoxic drugs revealed that survival was dependent on both EBNA3A and EBNA3C [79]. The study demonstrated that both proteins functionally cooperate to repress the expression of pro-apoptotic Bcl-2-family member, Bcl-2-interacting mediator of cell death (Bim) [79].

Definitive evidence for the importance of EBNA3C in maintaining the growth of LCLs was provided by Maruo *et al.* [80]. The researchers utilized a recombinant EBV that contains EBNA3C under the control of 4-hydroxytamoxifen (4HT)-dependent mutant estrogen receptor to infect primary B cells. Once established, the recombinant LCLs grew like the wild-type LCLs when maintained in the presence of 4-HT. However, upon withdrawal of 4-HT the cells experienced growth arrest, accumulating in sub-G1 phase. The addition of an oriP plasmid expressing EBNA3C was able to rescue the cells from growth arrest. The induction of growth arrest did not affect the

levels of other EBV latent proteins or c-Myc, but decreased the levels of hypophosphorylated Rb molecules and resulted in an accumulation of CDKI p16INK4a via gene transcription [80]. Interestingly, this increase in p16INK4a was associated with an increase in p16INK4a–CDK4 complexes and a decrease in cyclin D2–CDK4 complexes confirming that EBNA3C is not only required for the transformation process of B cells but also for their survival [80].

Conclusion & future perspective

The overriding conclusions that have emerged from the recent studies are that EBNA3C has devised multiple strategies to modulate the pathways that normally regulate the proliferation of B cells. A significant progress has been made towards understanding how EBNA3C contribute towards the transformation process. Our group has elucidated some of these

Executive summary

Epstein–Barr virus: first candidate human tumor virus

- Epstein–Barr virus (EBV) or human herpesvirus (HHV)-4 is a B lymphotropic γ -herpesvirus that infects more than 90% of the adult population worldwide.
- It persists as an episome in a subset of latently-infected memory B cells and is associated with Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and B-cell lymphomas in immunosuppressed people.

EBV latency

- There are at least three patterns of viral latency displayed by different types of tumor involving different patterns of viral promoter usage.
- Latency is characterized by a restricted pattern of viral gene transcription.
- Out of 11 genes that EBV encodes during latency, four are absolutely essential for *in vitro* transformation namely EBNA2, EBNA3A, EBNA3C and LMP1.

Epstein Barr nuclear antigen

- Epstein–Barr nuclear antigen (EBNA)3C is a 992 amino acid long latent protein that localizes exclusively in the nucleus in discrete punctate bodies.
- It is a remarkably stable protein that is expressed in low levels in lymphoblastoid cell lines.

EBNA3C as a transcriptional regulator

- The amino acid sequence of EBNA3C reveals features associated with many viral and cellular transcriptional factors including the basic leucine zipper motif (bZIP).
- EBNA3C can act both as an activator and repressor in transient reporter assays. This dual ability to modulate transcription may include modification or recruitment of different cofactors including RBP-J κ and CtBP.
- EBNA3C also regulates chromatin remodeling by targeting acetylases, deacetylases and other factors associated with these complexes.

EBNA3C deregulates cell cycle

- EBNA3C targets various cell cycle regulators resulting in deregulation of the mammalian cell cycle.
- EBNA3C targets cyclin A complexes *in vivo* and enhances cyclin A-dependent kinase activity by disrupting the CDK inhibitor p27 from kinase complexes.
- EBNA3C also targets the SCF/skp2 E3 ubiquitin ligase complex thereby regulating the activity and stability of cyclin A/CDK2 and pRb complexes.
- EBNA3C stabilizes c-Myc, a critical cell cycle modulator.

Conclusion & future perspective

- A better understanding of pathways modulated by EBNA3C will facilitate the development of targeted therapeutics in the treatment of EBV-related human malignancies.

targeted pathways by identifying the critical players that are involved. However, most of this work is done in artificial systems such as transient or drug selected overexpression systems, *in vitro* translation assays and glutathione *S*-transferase pull-down assays. These experiments often fail to reflect the enormous complexity of *in vivo* biology. Future studies should address these limitations so as to enable us to develop a comprehensive model for the role of EBNA3C in B-cell transformation and for the development of therapeutic agents for treatment of EBV-related diseases.

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