Epigenetic Crossroads of the Epstein-Barr Virus B-cell Relationship

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Abstract

Epstein-Barr virus (EBV) is a gamma-herpesvirus that establishes lifelong infection in the majority of people worldwide. EBV uses epigenetic reprogramming to switch between multiple latency states in order to colonize the memory B-cell compartment and to then periodically undergo lytic reactivation upon plasma cell differentiation. This review focuses on recent advances in the understanding of epigenetic mechanisms that EBV uses to control its lifecycle and to subvert the growth and survival pathways that underly EBV-driven B-cell differentiation versus B-cell growth transformation, a hallmark of the first human tumor virus. These include the formation of viral super-enhancers that drive expression of key host dependency factors, evasion of tumor suppressor responses, prevention of plasmablast differentiation, and regulation of the B-cell lytic switch.

Keywords

Introduction

Human herpesviruses persistently infect immunocompetent hosts through a biphasic lifecycle that involves latent infection of a long-lived reservoir cell and occasional lytic reactivation. Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that crosses the tonsillar epithelium, infects B-cells and manipulates growth, survival and differentiation pathways to reach the memory B-cell compartment, the reservoir for lifelong infection [1].

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Recent advances continue to shed light on how EBV manipulates epigenetic pathways to reprogram host and viral programs in each of these states.

Whereas the lytic cycle promotes virus production and cell-to-cell spread, latency is critical for infected- cell proliferation and survival [2]. Upon B-cell infection, a brief pre-latent state occurs, where lytic transcripts are produced, including those encoding the immediate early transcription factors Zta and Rta, viral BCL2 homologs BHRF1 and BALF1, and the viral IL-10 homolog BCRF1 [3–7]. EBV-encoded miRNAs promote cell cycle and suppress apoptosis [8]. Latency IIb follows, with Epstein Barr nuclear antigens (EBNA) 1, 2 and LP, vBCL2 and non-coding RNAs (ncRNA) expressed (Figure 1). This program drives B-cell hyper-proliferation [4] and transitions to latency III, comprised of six EBNA, two latent membrane protein (LMP) and ncRNAs. *In vitro*, latency III transforms B-cells into immortalized lymphoblastoid cell lines (LCL). *In vivo*, EBV instead switches to latency II in immunocompetent hosts upon germinal center entry, where EBNA1, LMP1/2A and ncRNA are expressed. Upon memory cell differentiation, EBV restricts expression to EBNA1 and ncRNAs by the latency I program [2,9,10]. This progression is thought to result from the accumulation of epigenetic silencing marks on viral promoters [11,12] (Figure 1). Reactivation from latency requires de-repression of immediate early genes *BZLF1* and *BRLF1*.

Here, we review recent advances in the understanding of epigenetic contribution to EBV latency states, lytic reactivation, EBV super-enhancers, silencing of tumor suppressors *BCL2L11* and *PRDM1*, and in EBNA3-mediated suppression of LMP1/2A-driven plasmablast differentiation. Space limitations preclude discussion of equally interesting epigenetic mechanisms in EBV-infected epithelial cells [13].

Promoter methylation control latency states

In pre-latency as viral DNA becomes chromatinized, the viral W promoter (Wp) drives EBNA expression [14–16]. Zta preferentially activates early gene promoters with methylated 5'-cytosine residues (5mC) termed meZREs, not widely distributed at this timepoint, preventing progeny virus production at this early timepoint [9,17,18]. Progressive EBV genome compaction and methylation silences leaky lytic transcription [6,14,15,17], while increasing EBNA2 and LP expression activate the C promoter (Cp) to induce EBNA1 and 3 proteins. EBNA2 and host RBP-Jκ activate the unmethylated LMP promoter (LMPp) to induce LMP1 and LMP2A expression, triggering latency III (Figure 2A)[2].

Chromatin conformation has key roles in maintenance of EBV latency [19]. The looping factor CTCF binds to multiple EBV genome sites and is post-translationally regulated by the host enzyme Poly(ADPribose) polymerase (PARP1), which colocalize at specific EBV genomic sites. PARP1 stabilizes CTCF binding to maintain type III latency chromatin configurations [20]. PARP1 and CTCF suppress the *BZLF1* promoter [21]. Curiously, knockdown of PARP1, but not CTCF promotes viral reactivation, suggesting that PARP1 may have additional cellular cofactors that act redundantly with or independently of CTCF in lytic switch regulation.

Accumulation of Cp methylation accompanies the shift to latency II, where the Q promoter (Qp) instead drives EBNA1, and a CTCF site insulates Qp from spreading repressive marks [22] (Figure 2A). Cytotoxic T-cell responses exert selective pressure for this antigenic silencing [23], and EBV likely also responds to cellular cues, for example resulting from germinal center entry. Progressive LMPp methylation leads to latency I, reducing immunogenicity [24]. Cellular cues likely contribute to this reprogramming, perhaps resulting from memory cell differentiation, germinal center exit or downmodulation of mitogenic transcription factors [4].

Changes in EBV and host genome methylation patterns in latency III have recently been linked to the host demethylase ten-eleven translocation 2 (TET2), a tumor suppressor that is frequently mutated in hematological cancers [25]. TET2 converts 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5'hmC) to de-repress target gene expression. Whereas TET2 overexpression results in increased latency III protein expression, TET2 knockdown blocks latency III and triggers lytic expression [24,26]. Building on this hypothesis, latency III expression drives B-cell activation, and TET2 expression is higher in activated B-cells than in germinal center (GC) B-cells. Demethylating agents upregulate latency III transcripts in latency I B-cells. Further supporting key methylation roles, EBNA2, RBP-Jκ and EBF1 induce TET2 expression and then co-occupy key LCL genomic sites with TET2 [24,26].

Epigenetic regulation of EBV lytic reactivation

Reactivation requires immediate early gene de-repression. Zta is homologous to bZIP family transcription factors and binds to AP-1-like Z-response elements (ZREs) [27–29]. Alanine-to-serine substitutions homologous to Zta convert host AP-1 into a TF that binds methylated EBV DNA and that activates some EBV early genes [30], underscoring the close relationship between AP-1 and ZTA. Similarly, Rta binds R-response elements (RREs) with the consensus sequence $5'\text{-}GNCCN_9GGNG\text{-}3'$ (where N is any nucleotide). Zta and Rta induce one another as well as EBV early genes [27,31]. As such, Zp and Rp tightly control Zta and Rta expression, respectively [27].

Importantly, repressive H3K27me3 methylation deposited by polycomb-group proteins repress ZREs and RREs. This shielding is important for latency [32], and cellular factors such as MEF2D, ZEB1 and ZEB2 also inhibit early lytic replication by recruiting histone deacetylases to Zp and by negatively regulating cis-acting Zp elements. However, these factors instead enhance lytic induction upon B-cell receptor engagement or TGF-β1 treatment [27].

Zta or Rta overexpression triggers lytic reactivation and virus production [33,34]. However, Zta and Rta lytic-inducing effects are not equal in their binding and activation of methylated promoters [35]. Zta only efficiently binds and activates CpG methylated viral promoters, while Rta only efficiently binds and activates unmethylated and perhaps CpG methylated promoters [27,35]. Adding another layer of depth, 5'hmC modification inhibits promoter binding and activation by ZTA [24,36]. Rta binding is unaffected by 5'hmC, yet 5'hmC enhances Rta-mediated promoter activation [36] (Figure 2B). TET2 knockdown enhances immediate-early expression in latency III cells [26].

Curiously, Zta expression triggers lytic reactivation only in a subpopulation of B-cells, for reasons that are incompletely understood. STAT3, which is activated upon EBV entry, contributes to this phenotype, as cells with elevated STAT3 are refractory to reactivation [37]. Mechanistically, STAT3 regulates the KRAB-zinc finger proteins SZF1 and ZNF557 to silence multiple EBV lytic genes, in part through the enzyme TRIM28, which recruits the histone methyltransferase SETDB1, the DNA methyltransferases 3A/B, the NuRD histone deacetylase complex and the heterochromatin amplification factor HP1 [38]. Physiological EBV reactivation triggers are incompletely understood. Differentiation of latently infected memory B-cells into plasma-cells appears to be a major trigger [39]. Further supporting this hypothesis, treatment of latently infected cells with the immunomodulatory agent lenalidomide stimulates reactivation via enhanced PI3K signaling and activation of the downstream AKT and GSK kinases, a pathway also activated by B-cell receptor signaling in plasma cell differentiation. Lenalidomide effects on lytic induction are suppressed by PI3K inhibition, denoting important PI3K roles in this reactivation pathway [40,41]. Lenalidomide also suppresses expression of Ikaros, a host transcription factor positively regulated by PI3K-dependent Forkhead-box-O1 (FoxO1) [40]. Lenalidomide decreases FoxO1 and Ikaros levels and results in enhanced Zta/Rta expression. Ikaros knockdown suppresses EBV reactivation [42] (Figure 2C).

The specific mechanism by which Ikaros suppresses EBV lytic reactivation is currently incompletely understood [40,42]. Adding to the importance of the role of PI3K in lytic reactivation, overexpression of B-lymphocyte-induced maturation protein 1 (Blimp1), a cellular master regulator of plasma cell differentiation, induces lytic reactivation [43]. Blimp1 expression is partly controlled by PI3K activation, as knockout of PTEN, a cellular lipid phosphatase that negatively regulates PI3K, resulted in enhanced Blimp1 expression [44] (Figure 2C). Since lenalidomide induces degradation of IRF4, the major B-cell transcription activator of Blimp1, further studies are needed to assess specific elements of PI3K pathway and additional downstream targets that regulate EBV reactivation, but it stands as an important avenue of investigation.

Primary Effusion Lymphoma (PEL) are typically co-infected with EBV and Kaposi Sarcoma-Associated Herpesvirus (KSHV). Interestingly, EBV co-infection enhances KSHV persistence and tumorigenesis in a humanized mouse model [45]. In this model, co-infection results in a plasma-cell like state reminiscent of PEL, with enhanced EBV lytic gene expression. Coinfected B-cells express Zta, without detectable late gene expression. KSHV may therefore utilize EBV early lytic gene expression to drive cell proliferation and survival [45]. Abortive lytic EBV expression was detected in human PEL tumor samples. Open questions include whether a lack of methylated 5'-cytosine meZREs at PEL early gene promoters underlie the abortive lytic pattern, how KSHV contributes to EBV lytic gene expression, and how EBV lytic genes contribute to transformation.

EBNA and LMP1-activated NF-κB Form Viral Super-Enhancers

Super-enhancers (SE) are particularly strong enhancers that are major determinants of cell identity and malignant states [46,47]. Compared with typical enhancers, SE have significantly larger size, transcription factor density and content and are particularly

sensitive to perturbation, such as by BET-bromodomain inhibitors [46]. LCL chromatin immunoprecipitation with deep sequencing (ChIP-Seq) analysis identified SE at host genomic sites [48]. Using LCL EBNA and LMP1-activated NF-κB ChIP-seq datasets [49], two distinct types of viral SE were observed. 888 SE sites are EBNA2 occupied and termed EBNA2 SE, whereas 187 SE are co-occupied by EBNA2, LP, 3A and 3C and five NF-κB subunits and termed EBV SE [48].

To assign EBV SE-promoter linkages, LCL ChIP-seq and Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET) datasets [50] were integrated [51]. ChIA-PET defines 3-dimensional genome organization positioned by specific transcription factors, such as CTCF. EBV SE target 544 promoters at an average distance of 438 kilobases (kb), including the host dependency factors *CFLAR, IRF4, IRF2* and *MYC* [51,52]. EBV further upregulates key targets by typical enhancers, including by EBNA2- and RBP-Jκ-bound enhancers [51,53]. RBP-Jκ is important for *IRF4* induction by EBNA2 [54], and EBNA2 likewise activates typical enhancers upstream and downstream of *MYC* [55]. EBNA3C associates with and stabilizes IRF4 [56].

EBV SE often target multiple host genes, and key genes are targeted by multiple EBV SE. For instance, EBV SE 525 and 428 kb upstream each target *MYC*. While the importance of most EBV SE remain to be established experimentally, deletion of either SE significantly diminishes *MYC* expression, LCL growth and survival [48]. It will be interesting to determine how EBV upregulates *MYC* in latency IIb in the absence of LMP1, or in latency II where LMPs but not EBNAs 2 and 3 are expressed.

How do EBV SE target promoters by long-range interactions? Conditional EBNA2 or 3 allele studies suggest that EBV nuclear antigens may be DNA looping factors. Conditional inactivation of either EBNA2, 3A or 3C significantly reduces EBV SE looping to *MYC* [48]. EBNA2 recruits the SWI/SNF remodeler BRG1 ATPase to *MYC*-targeting enhancers, and BRG1 is necessary for their interaction with the *MYC* promoter [55]. EBNA2 drives cooperative and combinatorial binding of the EBF1 and RBP-Jκ, which then co-occupy newly induced binding sites with EBNA2 [57]. EBNA3 can instead repress or prevent the establishment of chromatin hubs through modulating enhancer-promoter loop formation [58]. Noncoding RNAs Pol II-transcribed from EBV SE also have roles in EBV SE looping to *MYC* [59].

How EBNA-LP contributes to EBV SE remains to be understood. Recombinant EBV deficient for EBNA-LP transforms B cells from adult donors at reduced efficiency as compared with wildtype EBV and could not fully transform umbilical cord B cells, which died by two weeks post-infection, suggesting epigenetic cell states define EBNA-LP roles [60]. Effects on EBNA2, RBP-Jκ and EBF1 recruitment to viral genome latency promoters were observed. EBNA-LP can also coactivate with the host histone acetyltransferase EP300 [61], an effect that remains to be investigated in the context of EBV SE.

Positive feedback loops enforce expression of key SE targets, such as host microRNA miR-155HG, which is induced by multiple EBV SE and by EBV-SE target IRF4 [51,53]. Similarly, IRF4 and EBNA3C downmodulate *BCL6* [62]. ChIP-seq analysis of SE

landscapes in pre-latency, latency IIb, II and I phases would undoubtedly reveal interesting shared and unique targets.

EBNA3A, 3C, IRF4 and BATF Epigenetic Regulation of Tumor Suppressor *BCL2L11*

EBV induces IRF4 and BATF [52], which are key regulators of B-cell differentiation and activation. Genome-wide Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) screens identified BATF and IRF4 as key EBV-induced host dependency factors necessary for LCL, but not EBV+ Burkitt cell survival [52]. CRISPR screens similarly implicated IRF4 as a key PEL dependency factor [63]. The IRF4 DNA motif is enriched at LCL human genome sites occupied by EBNA3C or EBNA3A [64–66], suggesting that EBV may often use IRF4 to target B-cell sites.

BATF and IRF4 bind cooperatively to DNA sites with an AP1-interferon composite element (AICE). BATF and IRF4 are each critical for EBV repression of the tumor suppressor *BCL2L11*, which encodes the pro-apoptosis factor BIM [52]. Elegant studies identified EBNA-3A, 3C and polycomb as necessary for *BCL2L11* suppression [55,67]. How these factors silence *BCL2L11* is incompletely understood. Since they co-occupy a B-cell genomic site upstream of the BIM promoter [52], we hypothesize that an IRF4/BATF complex anchors EBNA3A, 3C and polycomb complexes that epigenetically silence *BCL2L11.*

LCL *BCL2L11* knockout (KO) was insufficient to rescue subsequent IRF4 CRISPR KO [52], suggesting additional important LCL IRF4 roles. IRF4 or BATF KO downmodulated expression of c-Myc and its target genes. Since IRF4, BATF and EBNA3A/C co-occupy multiple sites within the EBV SE that loop to *MYC,* they may have roles in recruiting EBNA3s to these sites and/or SE looping.

EBNA3A, 3C, IRF4 and BATF Epigenetic Regulation of *PRDM1*

IRF4 upregulates *PRDM1*, which encodes the master plasma cell differentiation regulator Blimp1. Since Blimp1 is expressed at low levels in LCLs, how then does EBV block Blimp1 upregulation? EBV null for EBNA3A/3C induces Blimp1 and plasmablast differentiation. EBNA3A/C are necessary to suppress plasmablast differentiation within the first 12 days post-infection, but not thereafter, suggesting that they drive epigenetic commitment to the B-blast fate [68]. Curiously, although Blimp1 and plasma cell differentiation trigger lytic reactivation, lytic antigens are not observed in this model. Further studies are required to identify host and/or viral factors that maintain EBV latency in this setting. Infection of humanized mice with EBNA3A- or EBNA3C-null EBV did not induce plasmablast differentiation, indicating that EBNA3A and 3C have functional redundant roles in maintaining B-lymphoblast state [69].

EBNA3A and 3C were found to co-occupy a *PRDM1* locus intronic site[68], but since they do not bind DNA directly, how do they target this site? An overlay of LCL ChIP-seq datasets demonstrates that IRF4 and BATF co-occupy the major EBNA3A/C *PRDM1* locus site

(Figure 3). We hypothesize that LMP1, LMP2A and EBNA2 first induce a BATF/IRF4 complex to target this site, and to then prevent *PRDM1* induction, EBNA3A and C are recruited and drive polycomb proteins to epigenetically silence *PRDM1.*

Transgenic mouse B-cell LMP2A expression induces IRF4 and enhances B-cell survival, but not plasmablast differentiation [70]. By contrast, conditional GC B-cell LMP1/2A co-expression highlyinduces *IRF4* and *PRDM1* [71] and upregulates markers of plasmablast differentiation, including CD38 and CD138 [71]. Taken together with EBNA3 models, it appears that sustained LMP1/2A expression in the absence of EBNA3A or 3C drives plasmablast differentiation, which is fatal in mice that lack T and NK-cells [71] or even perforin [72]. In immunocompetent animals, GC B-cell LMP1/2A-coexpression impedes the GC reaction but does not cause lymphoma [71,72].

These mouse models raise the important question of how latency II GC B-cells differentiate into latency I memory cells rather than plasmablasts. Perhaps epigenetic marks from earlier EBV latency states, coexpression of EBNA1 or viral ncRNAs, LMP1/2A expression levels, and/or T/NK-cell immune pressure play roles in guiding infected GC B-cells towards memory cell states. Conversely, epigenetic mechanisms that drive the latency I state typically observed in EBV+ plasmablastic lymphomas remain to be determined.

EBV suppresses Blimp1 by additional mechanisms, including by *PRDM1* promoter hypermethylation, upregulation of the functionally-impaired Blimp1-beta isoform [73], and by EBV miR-BHRF1–2 [74]. EBV SE target IRF2 also suppresses *PRDM1,* apparently independently of EBNA3A/C, since LCL depletion of IRF2 but not EBNA3A/C de-represses *PRDM1* [52,68]. CRISPR identified the EBV SE target and IRF2 co-repressor IRF2BP2 as important for LCL growth/survival [52], suggesting possible joint roles in *PRDM1* suppression.

Concluding remarks.

Despite recent advances, important epigenetic roles throughout the EBV lifecycle and in EBV oncogenesis remain to be discovered. Humanized mice EBV infection models are rapidly advancing and promise to enable the study of viral and host genome epigenetic changes at key steps of Bcell infection, differentiation and reactivation. For instance, how does EBV alter memory B-cell epigenetic states to maintain latency *in vivo*? Likewise, how epigenetic mechanisms differ in the host/pathogen interactions between Type-I and II Epstein-Barr strains, and for Type II EBV between B and T-lymphocytes, will be interesting to pursue, including in newly developed humanized mouse models. It will be also be interesting to learn how epigenetic mechanisms underlie cell differentiation state-specific roles of LMP2A [75] and EBNA-LP, and how EBV nuclear antigens and host TFs together form viral super-enhancers.

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Highlights

- EBNA2 and TET2-mediated DNA demethylation promotes Latency III in B cells

- EBNA3A/3C and polycomb prevent plasmablast differentiation driven by LMP1/2A

- IRF4, BATF, EBNA3A/3C and polycomb suppress BCL2L11/BIM expression

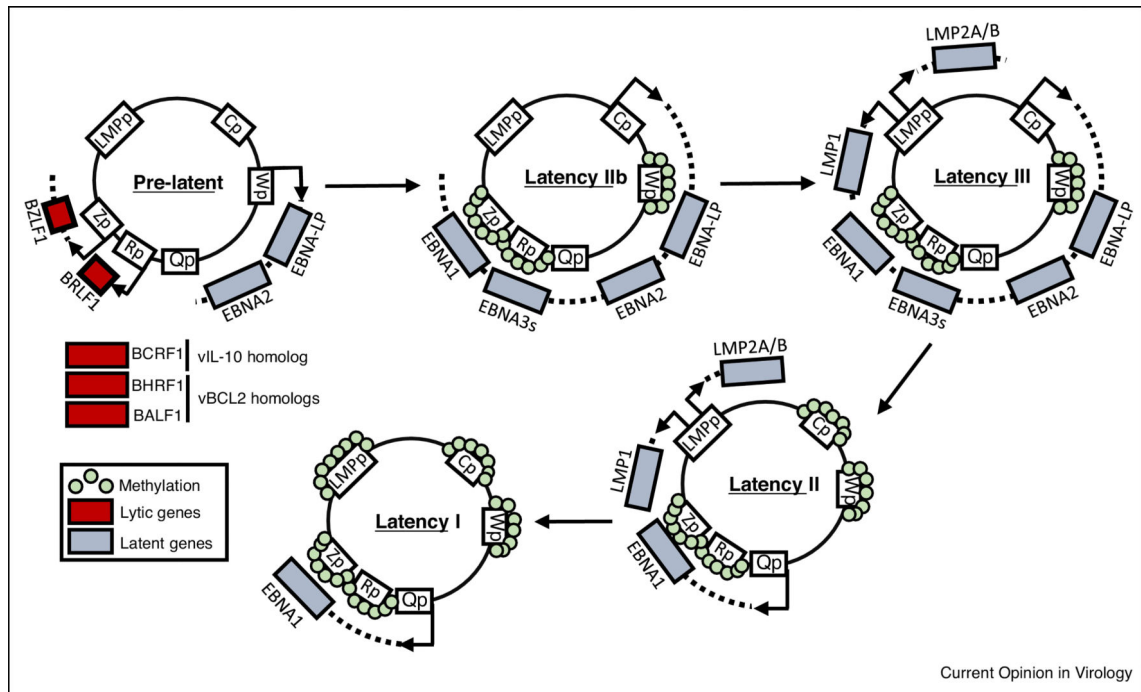- EBV super-enhancers target 544 LCL genes, typically by long-range interactions

Figure 1. Methylation-dependent expressional changes of the EBV genome during latency progression.

Recent evidence suggests EBV latency begins with a pre-latent stage, in which lytic inducers Zta and Rta, along with the vIL-10 homolog BCRF1 and vBCL2 homologs BHRF1 and BALF1 are expressed and enhance transition into viral latency. Upon accumulation of EBNA2 and EBNA-LP, expression from the viral Cp and LMPp promoters is enhanced, leading to induction of all type III latency genes. Repressive methylation of Cp blocks EBNA2 an 3 expression in latency II, where Qp drives EBNA1 and LMPp drives LMP1/2A co-expression. Once repressive methylation is accrued on LMPp, the quiescent latency I is reached, where only EBNA 1 and viral ncRNAs are expressed.
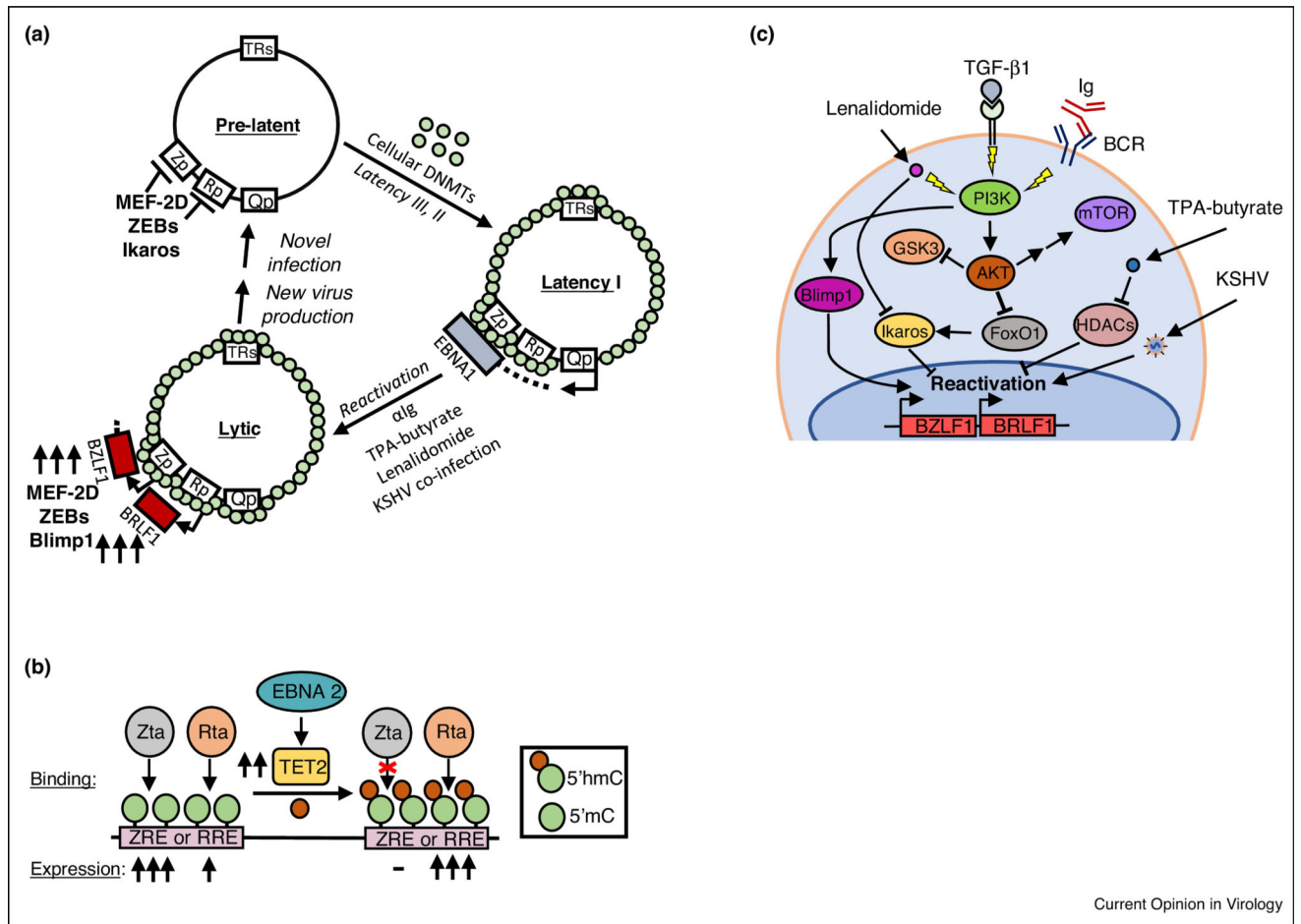
Figure 2. Epigenetic mechanisms affecting the EBV latent-lytic switch.
(A) Schematic diagram depicting repressive methylation (green circles) accrual on the EBV genome by cellular DNA methyltransferases (DMNTs), resulting in latency I. Shown also are selected treatments that can induce lytic gene expression. (B) TET2 conversion of viral genome methylated Z-response element (ZRE) 5'cytosine residues (5mC) into 5' hydroxymethylation (5'hmC) precludes Zta binding and transactivation. 5'hmC does not affect Rta binding, but surprisingly enhances Rta-mediated activation. (C) PI3K pathway activation stimulates EBV lytic reactivation from latency I. Multiple lytic reactivation stimuli activate PI3K to upregulate Blimp1 and downmodulate Ikaros.
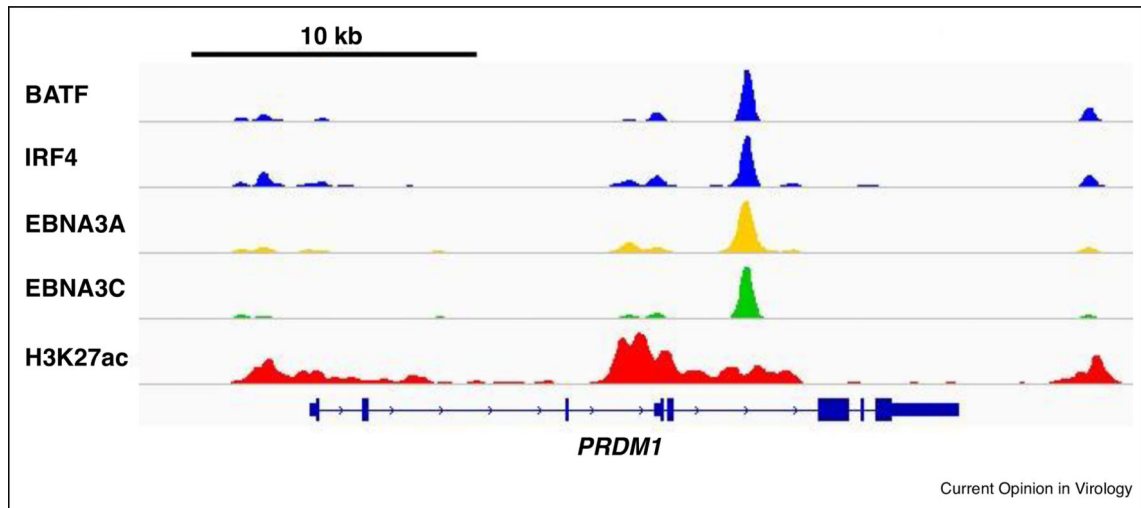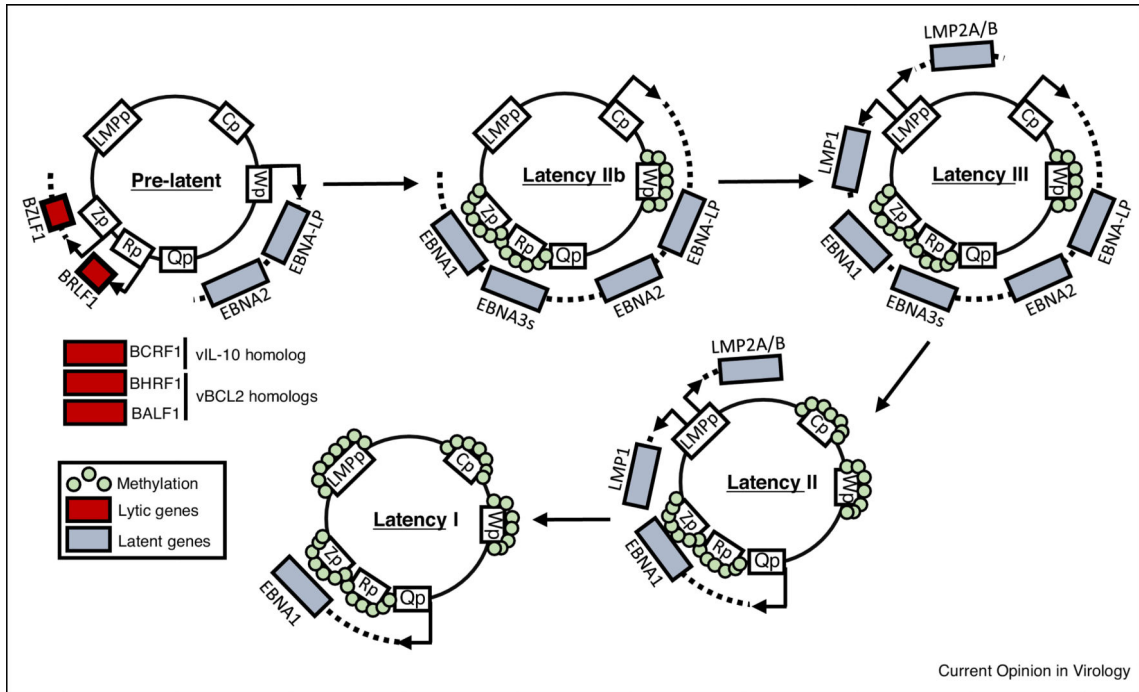
Figure 3. Epigenetic silencing of *PRDM1* in lymphoblastoid B-cells.
Shown are LCL ChIP-seq tracks at the Blimp1-encoding *PRDM1* locus for the host transcription factors BATF and IRF4 (blue), EBV EBNA3A (yellow) and EBNA3C (green) and the histone epigenetic mark histone 3 lysine 27 acetyl (H3K27ac, red). A schematic diagram of the *PRDM1* gene body is shown at bottom.

Overview Figure. Key epigenetic mechanisms regulating EBV and B-cell gene expression.
Upon B-cell infection, the EBV genome rapidly becomes chromatinized to establish latency.
During latency III, EBV superenhancers, comprised of four EBNA and five NF-kB
transcription factor subunts target key host growth and survival genes, including IRF4.
EBNA2-superenhancers. Separately, EBNA3A, 3C and polycomb proteins block expression
of tumor suppressors, including *PRDM1* (which encodes Blimp1) and *BCL2L11* (which
encodes BIM). BATF and IRF4 occupy composite AP1-interferon sites and may anchor
EBNA3/polycomb complexes. As B-cells transit through the germinal center and
differentiate into memory cells, progressive methylation restricts EBV latency gene
expression, whereas the TET2 demethylase promotes latency III. Inducing agents trigger
PI3K activation and de-repression of the master plasma cell differentiation regulator Blimp
to trigger EBV lytic reactivation.