JVI Journals.ASM.org

Cellular Differentiation Regulator BLIMP1 Induces Epstein-Barr Virus Lytic Reactivation in Epithelial and B Cells by Activating Transcription from both the R and Z Promoters

Jessica A. Reusch,[a] Dhananjay M. Nawandar,[a] Kenneth L. Wright,[b] Shannon C. Kenney,[a,c] Janet E. Mertz[a]

McArdle Laboratory for Cancer Research[a] and Department of Medicine,[c] University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA; Department of Immunology, Moffitt Cancer Center, Tampa, Florida, USA[b]

ABSTRACT

Epstein-Barr virus (EBV) maintains a lifelong latent infection within a subset of its host's memory B cells, while lytic EBV replication takes place in plasma cells and differentiated epithelial cells. Therefore, cellular transcription factors, such as BLIMP1, that are key mediators of differentiation likely contribute to the EBV latent-to-lytic switch. Previous reports showed that ectopic BLIMP1 expression induces reactivation in some EBV-positive (EBV^+) B-cell lines and transcription from Zp, with all Z^+ cells in oral hairy leukoplakia being $BLIMP1^+$. Here, we examined BLIMP1's role in inducing EBV lytic gene expression in numerous EBV^+ epithelial and B-cell lines and activating transcription from Rp. BLIMP1 addition was sufficient to induce reactivation in latently infected epithelial cells derived from gastric cancers, nasopharyngeal carcinomas, and normal oral keratinocytes (NOK) as well as some, but not all B-cell lines. BLIMP1 strongly induced transcription from Rp as well as Zp, with there being three or more synergistically acting BLIMP1-responsive elements (BRE) within Rp. BLIMP1's DNA-binding domain was required for reactivation, but BLIMP1 did not directly bind the nucleotide (nt) −660 Rp BRE. siRNA knockdown of BLIMP1 inhibited 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced lytic reactivation in NOK-Akata cells, cells that can be reactivated by R, but not Z. Thus, we conclude that BLIMP1 expression is both necessary and sufficient to induce EBV lytic replication in many (possibly all) EBV^+ epithelial-cell types, but in only a subset of EBV^+ B-cell types; it does so, at least in part, by strongly activating expression of both EBV immediately early genes, *BZLF1* and *BRLF1*.

IMPORTANCE

This study is the first one to show that the cellular transcription factor BLIMP1, a key player in both epithelial and B-cell differentiation, induces reactivation of the oncogenic herpesvirus Epstein-Barr virus (EBV) out of latency into lytic replication in a variety of cancerous epithelial cell types as well as in some, but not all, B-cell types that contain this virus in a dormant state. The mechanism by which BLIMP1 does so involves strongly turning on expression of both of the immediate early genes of the virus, probably by directly acting upon the promoters as part of protein complexes or indirectly by altering the expression or activities of some cellular transcription factors and signaling pathways. The fact that EBV^+ cancers usually contain mostly undifferentiated cells may be due in part to these cells dying from lytic EBV infection when they differentiate and express wild-type BLIMP1.

Epstein-Barr virus (EBV) is a human gammaherpesvirus that infects over 90% of the world's population. Like all herpesviruses, it has both latent and lytic phases to its life cycle. Initial infection is generally mild and asymptomatic when the virus is acquired before adolescence. If infected after adolescence, the host may develop infectious mononucleosis (IM) (1, 2). After the primary lytic infection, EBV establishes a lifelong latent infection in a subset of its host's memory B cells, where its genome is maintained as an episome (3). Latent EBV infection has been linked to several epithelial and B-cell malignancies, including Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC), some gastric cancers, and posttransplant lymphoproliferative disease (4–12).

EBV-positive [EBV^+] cells can reactivate into lytic replication, leading to the production and shedding of infectious virus. EBV lytic replication is initiated by activation of transcription from one or both of its immediate early (IE) promoters, Zp and Rp, leading to production of its two IE proteins, Z (also called Zta, ZEBRA, or EB1, the product of the *BZLF1* gene) and R (also called Rta or EB2, the product of the *BRLF1* gene), respectively (13). In most EBV^+ cell lines, synthesis of Z is sufficient to initiate reactivation (14–17). R can frequently initiate reactivation as well, given that R and Z are transcription factors that can usually activate each other's promoters (18–24). However, R, not Z, is necessary in some cases such as telomerase-immortalized normal oral keratinocytes (NOK) that have been infected with the Akata strain of EBV (25). Together, R and Z induce expression of the viral early (E) genes, including BMRF1, which encodes the viral DNA polymerase processivity factor (also known as early antigen diffuse [EAD]) (26–29). Thus, regulation of Zp and Rp serves as the gatekeeper to the EBV latent-to-lytic switch in a cell-type-dependent manner.

Received 26 September 2014 **Accepted** 13 November 2014

Accepted manuscript posted online 19 November 2014

Citation Reusch JA, Nawandar DM, Wright KL, Kenney SC, Mertz JE. 2015. Cellular differentiation regulator BLIMP1 induces Epstein-Barr virus lytic reactivation in epithelial and B cells by activating transcription from both the R and Z promoters. J Virol 89:1731–1743. doi:10.1128/JVI.02781-14.

Editor: L. M. Hutt-Fletcher

Address correspondence to Janet E. Mertz, mertz@oncology.wisc.edu.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.02781-14

Zp has been intensively studied, with there being numerous well-mapped positive and negative regulatory domains (reviewed in references 30 to 33). Unfortunately, much less is known to date about regulation of Rp (reviewed in reference 33) (see Fig. 4A below). Among Rp's known *cis*-acting regulatory elements are binding sites for the cellular factors NF1, YY1, EGR-1, and ZEB1/2. Sp1/Sp3 binding sites have also been identified within Rp; they are necessary for both constitutive promoter activity and auto-activation by R (34). Rp is also activated by Z via three Z-responsive elements (ZRE) and further activated by Z and R in combination (35).

Spontaneous lytic reactivation of EBV is normally initiated during the differentiation of B cells into plasma cells (PC); it is also observed in the more differentiated layers of the oral epithelium, indicating that the environment of a differentiated cell plays a critical role in this process (36, 37). However, the identities of the several cellular factors that contribute to initiating the EBV latent-lytic switch during differentiation and the precise mechanisms by which they do so have yet to be determined. To elucidate the connection between cellular differentiation and lytic EBV reactivation, we have been investigating whether cellular factors known to play key roles in epithelial and/or B-cell differentiation can induce EBV reactivation. Previously, we (38) along with others (39) showed that XBP-1 is one such factor, inducing lytic EBV replication by activating transcription from Zp. Here, we examined the mechanisms of EBV reactivation by another such factor, B-lymphocyte-induced maturation protein 1 (BLIMP1, also known as PRDI-BF1 and PRDM1).

BLIMP1 is necessary for differentiation of B cells into plasma cells (40, 41). It is also a key player in epithelial cell differentiation (42, 43). The 789-amino-acid BLIMP1 protein contains five zinc finger domains, the first two of which are critical for the ability of the protein to bind directly to DNA via sequences resembling the BLIMP1 consensus site, 5′-(A/C)AG(T/C)GAAAG(T/C)(G/T)-3′ (see Fig. 5A below) (44, 45). BLIMP1 transcriptionally silences a myriad of genes during plasma cell differentiation, including those encoding the transcription factors PAX5, Oct-2, and ID3, as well as genes involved in cell cycle progression such as p53 and c-*Myc* (46–49). BLIMP1 represses its target genes through a variety of mechanisms, including recruiting Groucho family proteins, histone deacetylase 1 and 2 (HDAC1/2), and histone methyltransferase G9a (50–54).

Given that the PAX5 and Oct-2 proteins inhibit Z activity (55, 56) and that PAX5 represses synthesis of XBP-1 in lymphoid cells (57), one might expect that addition of BLIMP1 would induce EBV lytic gene expression in some EBV$^+$ B-cell lines under some conditions; this hypothesis has been validated (58). Other investigators reported that while expression of EBV Z protein was restricted to BLIMP1-positive epithelial cells in oral hairy leukoplakia (OHL) tissue samples, this relationship did not hold in IM tonsillar B cells, suggesting that BLIMP1 may be necessary for induction of EBV lytic gene expression during differentiation of epithelial but not B cells (59).

Here, we show that expression of BLIMP1 is sufficient to induce EBV lytic gene expression in EBV$^+$ epithelial cell lines derived from gastric cancers, NPCs, and NOK. We demonstrate that BLIMP1 is also necessary for differentiation-induced lytic reactivation of EBV in at least some epithelial cell types. We further show that BLIMP1 can induce transcriptional activation of Rp over 300-fold as well as Zp at 20- to 50-fold in some epithelial cell lines, and we identify several regions within Rp that act synergistically to enable this high-level activation by BLIMP1. Thus, we conclude that BLIMP1 is a key player in EBV lytic reactivation in epithelial cells; BLIMP1 does so, at least in part, by inducing expression of the *BRLF1* as well as the *BZLF1* gene.

MATERIALS AND METHODS

Cell lines. The gastric carcinoma cell line AGS (obtained from the American Type Culture Collection [ATCC]) and the EBV-infected clone derived from it, AGS-Akata (a gift from Lindsey Hutt-Fletcher), were maintained in F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 100 U/ml penicillin and 100 μg/ml streptomycin (Pen-Strep; Life Technologies). The NPC cell line HONE1 (a gift from Ron Glaser), the EBV-infected clone derived from it, HONE-Akata (a gift from Lawrence Young), the EBV-infected NPC line, CNE2-Akata (a gift from K. W. Lo via Diane Hayward), the naturally derived EBV-infected gastric carcinoma cell line SNU-719 (60), and all EBV$^+$ B-cell lines were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS and Pen-Strep. Though originally derived from NPCs, the HONE1 and CNE2 cell lines may have become contaminated with HeLa cells, resulting in somatic hybrids (61). NOK (a gift from Karl Munger via Paul Lambert) is a telomerase-immortalized normal oral epithelial keratinocyte cell line; NOK-Akata cells were derived from it by infection with the Akata strain of EBV (25). They were maintained in keratinocyte serum-free medium (K-SFM) supplemented with 0.2 ng/ml epidermal growth factor and 25 μg/ml bovine pituitary extract (Life Technologies). Akata EBV-infected epithelial cells were maintained in medium also supplemented with G418 at 400 μg/ml for AGS-Akata and HONE-Akata and 50 μg/ml for NOK-Akata. The B-cell lines Sal and Oku (gifts from Alan Rickinson via David Vereide), MutuI (a gift from Alan Rickinson), and KemI and KemIII (gifts from Jeffrey Sample) are derived from EBV$^+$ BLs. Sal and Oku cells maintain a Wp-restricted latency (62, 63), MutuI and KemI cells maintain a type I latency, and KemIII cells maintain a type III latency. Raji cells were obtained from the ATCC. Human embryonic kidney 293T cells (obtained from the ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS and Pen-Strep. All cells were maintained at 37°C in a 5% CO_2 atmosphere.

Plasmids. Plasmid DNAs were purified using Qiagen Plasmid Maxi Kits as described by the manufacturer. Plasmid pcDNA-FLAG-PRDI-BF1 (pcDNA3-BLIMP1) expresses an amino-terminal FLAG-tagged BLIMP1 (64). Plasmids pc-PRDI-BF1-ΔPR (pcDNA3-ΔPR), pc-PRDI-BF1-ΔPRO (pcDNA3-ΔPro), and pc-PRDI-BF1-ΔZn (pcDNA3-ΔZn) express the C-terminal FLAG-tagged BLIMP1 mutant variants described in Ghosh et al. (65); their structures are schematically shown in Fig. 5A below. Plasmid pSG5-Z [a gift from Diane Hayward [66]) expresses the EBV IE protein, Z. Plasmid pGL3-CIITAp3 contains the firefly luciferase-coding region under the control of the promoter of the cellular *CIITA* gene which is known to be strongly repressed by BLIMP1 (65, 67). Plasmid pCpGL-Zp-668 (25) contains the region of nucleotides (nt) −668 through +15 of EBV Zp relative to the transcription initiation site cloned between the SpeI and BglII restriction sites of pCpGL, a CpG-free vector, driving expression of the firefly luciferase-coding region (a gift from Michael Rehli [68]). Plasmid pCpGL-Rp-673 is a luciferase reporter construct that contains the region of nt −673 to +38 of Rp relative to the transcription initiation site cloned between the SpeI and BglII restriction sites of pCpGL.

All of the pCpGL-Rp mutant variants described here are derivatives of this "full-length" pCpGL-Rp-673 promoter construct. For 5′ deletion variants of Rp, the number in the plasmid name indicates the endpoint of the deletion in nucleotides upstream of the transcription initiation site. To construct these pCpGL-Rp mutants, the appropriate PCR-amplified region of Rp was inserted between the SpeI and BglII restriction sites of pCpGL. The Rp 2-bp substitution mutants, mt1, -2, -3, -4, and -5, were produced by site-directed mutagenesis of pCpGL-Rp-673 according to

the Stratagene QuikChange site-directed mutagenesis kit protocol using the following primers: mt1, 5′-GACTAGTCAAGCTGCAGGTGACCAC GTAAAGCCAC-3′; mt2, 5′-CTAGTCAAGCTGCACACAACCACGTAA AGCCACAAG-3′; mt3, 5′-CTAGTCAAGCTGCACATGGGCACGTAA AGCCACAAG-3′; mt4, 5′-GTCAAGCTGCACATGACTGCGTAAAGC CACAAGCTTG-3′; and mt5, 5′-GTCAAGCTGCACATGACCAGATAA AGCCACAAGCTTG-3′.

Methylation of pCpGL-Zp-668, pCpGL-Rp-673, pCpGL-mt3, and pCpGL-Rp-655 was performed *in vitro* using the methyltransferase M.SssI (NEB) according to the manufacturer's instructions. Digestion with restriction enzyme HpaII (NEB), which cleaves its recognition sequence only when the DNA is unmethylated, was used to confirm methylation of the DNAs.

Transient-transfection and reporter gene assays. For immunoblot analyses, HONE-Akata, AGS-Akata, NOK-Akata, and SNU-719 were transiently transfected with the indicated DNAs using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. For luciferase assays, transient transfections of AGS, NOK, and 293T cells were carried out in 12-well plates using Lipofectamine 2000, with the medium changed 3 h after addition of the transfection reagent/DNA mixture to minimize cell death. Cells were harvested at 48 h posttransfection and processed with 5× lysis buffer (Promega) according to the manufacturer's instructions. Relative luciferase units were measured in a BD Monolight 3010 Luminometer (BD Biosciences) with Promega luciferase assay reagent. Each condition was performed in triplicate on two or more separate occasions with similar results. Extracts were also subjected to immunoblot analysis (data not shown) to verify that similar BLIMP1 or Z protein levels were present in each sample following cotransfection with the indicated expression plasmids.

Immunoblot analysis. Cells were harvested in SUMO buffer and processed as previously described (69). Proteins in the whole-cell extracts were separated by electrophoresis at 100 V for 90 min in SDS–10% polyacrylamide gels and transferred to nitrocellulose membranes (ISC Biosystem). The membranes were blocked by preincubation for 1 h at 23°C with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA), and 0.1% Tween 20 solution when probed with BLIMP1 antibody and 5% milk–0.1% Tween 20 solution when probed with all of the other antibodies. Membranes were then incubated with the primary antibody in their respective blocking buffer overnight at 4°C. The primary antibodies were β-actin (1:2,000 [A5441; Sigma-Aldrich]), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000 [A00192; Genscript]), α-tubulin (1:5,000 [T5168; Sigma-Aldrich]), BLIMP1 (1:500 [9115S; Cell Signaling]), involucrin (1:3,000 [I9018; Sigma-Aldrich]), Z (BZLF1; 1:500 [sc-53904; Santa Cruz]), and EAD (BMRF1; 1:3,000 [MAB8186; Millipore]). Additionally, we used a rabbit antibody specific to the EBV R protein (1:2,000) generated for us by Pierce Biotechnology from the peptide EDP DEETSQAVKALREMAD which corresponds to R amino acid residues 506 to 524. Membranes were washed and then incubated with the secondary antibody for 1 h at room temperature. Secondary antibodies were horseradish peroxidase (HRP)-conjugated mouse (1:10,000 [31430; ThermoScientific]) or rabbit (1:10,000 [NA934; GE Healthcare]) antibody as appropriate. Blots were developed with Luminata Cresendo Western HRP Substrate (WBLUR0100; Millipore). The band intensities were quantified with ImageJ software and internally normalized to GAPDH.

Infectious virus assay. CNE2-Akata cells (latently infected with a green fluorescent protein [GFP]-expressing EBV) were transfected in 22-mm-diameter wells with either (i) 100 ng of pcDNA3-BLIMP1 plus 400 ng of pcDNA3.1 or (ii) 500 ng of pcDNA3.1 using Lipofectamine 2000. The medium was harvested 4 days later and passed through a 0.8-μm-pore-size filter. Ten microliters of this medium was added to 4×10^5 Raji cells in 15.4-mm-diameter wells. Twenty-four hours later, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; 20 ng/ml) and sodium butyrate (3 mM) were added to the infected Raji cells, and incubation was continued for another 24 h. The number of GFP-positive (GFP^+) Raji cells, representing green Raji units (GRU), was determined by UV microscopy (70).

Chromatin immunoprecipitation (ChIP) assays. HONE-Akata cells were transfected in 10-cm-diameter dishes with 6 μg of pcDNA3.1 or pcDNA3-BLIMP1 as indicated on Fig. 6. Two days later, the cells were cross-linked by incubation in fresh 1% paraformaldehyde for 10 min at room temperature. The cross-linking reaction was quenched by addition of glycine to 125 mM, and the cells were lysed by Dounce homogenization. The lysate was sonicated three times for 30 s to yield DNA fragments approximately 500 bp in size. The DNA-protein complexes were immunoprecipitated by incubation at 4°C overnight with 2 μg of anti-FLAG (F1804; Sigma) or mouse anti-IgG (sc-2025; Santa Cruz) as a control. Immunoprecipitated DNA-protein complexes were sequentially washed with low salt (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), lithium chloride (0.5% sodium deoxycholate, 1 mM EDTA, 250 mM LiCl, 0.5% IGEPAL CA-630 [18896; Sigma], 10 mM Tris-HCl, pH 8.1), and Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) solutions. Protein-DNA cross-linking was reversed by incubation at 65°C overnight. DNA was purified using Qiagen gel extraction kits.

PCR for 30 cycles was performed on the chromatin immunoprecipitated (ChIPed) DNAs to determine the relative presence of specific fragments using GoTaq Flexi DNA polymerase (M8291; Promega). Primers were as follows: Rp +63 to −139, 5′-CTCTTACCTGCGTCTGTTTGT G-3′ and 5′-CTCTCTGCTGCCCACTCATACT-3′ (55); Rp −512 to −770, 5′-ATAAGGCTGCCAATAAGGTGCGTG-3′ and 5′-GGATGTC CAGAGTGCCTACAATGTCT-3′; c-Myc, 5′-CGCCTGCGATGATTTA TACTC-3′ and 5′-CGCCTACCATTTTCTTTTGC-3′ (42); Qp, 5′-GAC CACTGAGGGAGTGTTCCACAG-3′ and 5′-ACACCGTGCGAAAAGA AGCAC-3′; and β-globin, 5′-AGGGCTGGGCATAAAAGTCA-3′ and 5′-GCCTCACCACCAACTTCATC-3′ (25). PCR products were electrophoresed at 100 V for 20 min in a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer (pH 8.0) containing 0.5 μg/ml ethidium bromide and visualized by UV light.

Quantification of the ChIPed DNA was performed by quantitative PCR (qPCR) using iTaq Universal SYBR green supermix (172-5121; Bio-Rad). The DNAs were detected with an ABI Prism 7900 real-time PCR system (Applied Biosystems). Input samples were diluted to 5%, 1%, and 0.2% with distilled H_2O (dH_2O) containing 100 μg/ml sheared salmon sperm DNA (AM9680; Ambion). Primers were as follows: c-Myc, 5′-CG CCTGCGATGATTTATACTC-3′ and 5′-CGCCTACCATTTTCTTTTG C-3′ (42); Rp −40 to −173, 5′-GCATGGGCGGGACAATCGCAATATA A-3′ and 5′-CCAGCCAGATGTTCAGGAACCAAA-3′; Rp −708 to −859, 5′-CAGACATCCAATGACCACTGAGG-3′ and 5′-GAGTCATC GAGGTCAGGGATTTC-3′; and β-globin: 5′-GGCAACCCTAAGGTGA AGGC-3′ and 5′-GGTGAGCCAGGCCATCACTA-3′ (71). A standard curve was calculated from the threshold cycle (C_T) of the input dilution series and used to calculate the relative amount of each specific DNA present in the ChIPed samples. All assays were performed in triplicate.

EMSAs. HONE1 cells in 10-cm-diameter dishes were transfected with 6 μg of pcDNA3-BLIMP1 or pcDNA3.1. Nuclear extracts were harvested at 48 h posttransfection and prepared as previously described (72). Electrophoretic mobility shift assays (EMSAs) were performed using these nuclear extracts essentially as previously described (73) with the addition of 0.5 μM $ZnCl_2$. As a control, the nuclear extracts were incubated with FLAG-specific antibody (F1804; Sigma) at 23°C for 20 min prior to addition of the double-stranded radiolabeled probe DNA and incubation for an additional 20 min at 23°C. Probe DNA sequences were as follows: CIITAp3, 5′-GTCCACAGTAAGGAAGTGAAATTAATTTCAGAG-3′ (64); Rp −673 to −633, 5′-CAAGCTGCACATGACCACGTAAAGCCA CAAGCTTGTGGACC-3′; and Rp −642 to −601, 5′-CTTGTGGACCA ACATGTTCAGGAGATTGACAGGACTACACTG-3′. These mixtures were electrophoresed in a nondenaturing 4% polyacrylamide gel in 0.5×

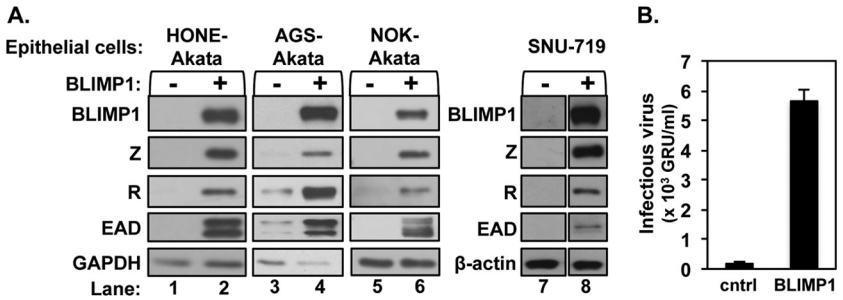

FIG 1 Immunoblots showing BLIMP1 induction of lytic reactivation in EBV-positive epithelial cell lines. (A) EBV$^+$ HONE-Akata, AGS-Akata, and NOK-Akata in 35-mm-diameter wells were transiently transfected as follows: HONE-Akata, (i) 300 ng of pcDNA3-BLIMP1 (+) or pcDNA3.1 (−) plus (ii) 700 ng of SG5 empty control vector; and AGS-Akata and NOK-Akata, 1 μg of pcDNA3.1 or 750 ng of pcDNA3-BLIMP1 plus 250 ng pcDNA3.1. GAPDH served as a loading control. SNU-719 cells in 22-mm-diameter wells were transiently transfected with 500 ng of pcDNA3.1 or 100 ng of pcDNA3-BLIMP1 plus 400 ng pcDNA3.1. Whole-cell extracts were prepared at 48 h posttransfection. The control samples were processed concurrently with their corresponding BLIMP1-containing ones, with an irrelevant intervening lane between 7 and 8 omitted. β-Actin served as a loading control. (B) Raji assay showing that addition of BLIMP1 to CNE2-Akata cells induces the entire lytic cycle of infection, leading to greatly enhanced production of infectious virus within 4 days.

TBE running buffer at 4°C for 2 h at 200 V. Gels were dried and exposed to a phosphor screen.

siRNA knockdown. NOK-Akata cells in a 12-well plate were transfected with 20 pmol of a universal scrambled control small interfering RNA (siRNA) (SR30004; Origene) or a BLIMP1-targeting siRNA (SR300437B; Origene) using Lipofectamine RNAiMAX according to the manufacturer's instructions. Three days later, the cells were treated with TPA (20 ng/ml) for 24 h immediately prior to harvesting and processing for immunoblot analysis.

Lentivirus infection of B cells. A BLIMP1-expressing lentivirus, CD713-BLIMP1, was constructed by insertion of the BLIMP1-coding sequences between the EcoRI and BamHI restriction sites of pCDH-MSCV-MCS-EF1-GFP-puro (CD713B-1; System Biosciences); the BLIMP1-coding DNA was obtained by digestion of pCDNA3-BLIMP1 DNA with EcoRI and BamHI restriction enzymes (NEB) and gel purification.

Two lentiviruses expressing short hairpin RNAs (shRNAs) against BLIMP1 mRNA that had been constructed in pLKO.1-TRC were originally purchased from Thermo Scientific; they can now be obtained from Sigma-Aldrich (shRNA product SHCLNG_NM_001198; The RNAi Consortium [TRC] shRNAs TRCN0000013612 [BLIMP1 no. 1] and TRCN0000013610 [BLIMP1 no. 2]). Two nontargeting shRNAs, cntrl 1 (1864; Addgene) (74) and cntrl 2 (SHC002; Sigma), were used as controls.

293T cells in 10-cm-diameter dishes were cotransfected with (i) 4 μg of the indicated lentivirus vector, (ii) 1.4 μg of pCMV-dR8.2 dvpr (8455; Addgene), and (iii) 0.6 μg of vesicular stomatitis virus G protein (VSV-G) (12259; Addgene). The medium containing the virus was harvested at 48 h and again at 72 h posttransfection; at both times, the virus was passed through a 0.8-μm-pore-size filter and shortly thereafter added to the B cells being infected with it. KemI, Sal, and Oku cells were incubated with 1 μg/ml puromycin beginning at 72 h after infection to select for lentivirus-positive cells. MutuI and KemIII cells showed a robust initial infection and required no further selection for our short-term experiments.

RESULTS

BLIMP1 induces lytic replication in EBV$^+$ epithelial cells. BLIMP1 is an important factor in epithelial as well as B-cell differentiation. Whether BLIMP1 expression is sufficient to induce EBV lytic reactivation in epithelial cells has not been previously investigated. To answer this question, we examined the effect of addition of BLIMP1 to HONE-Akata, AGS-Akata, NOK-Akata, and SNU-719 cells on EBV lytic gene expression. HONE-Akata and AGS-Akata cells were derived from an NPC and gastric carcinoma cell line, respectively, that had been infected with the Akata strain of EBV. BLIMP1 expression in these cells induced high-level synthesis of the lytic viral proteins Z, R, and EAD (Fig. 1, lane 2 versus lane 1 and lane 4 versus lane 3, respectively).

NOK-Akata cells were derived from a telomerase-immortalized normal oral keratinocyte cell line that had been infected with the Akata strain of EBV (25). Given the origin of the cells, they represent a more physiologic model for EBV infection than cells derived from tumors. Once again, addition of BLIMP1 strongly induced EBV lytic gene expression (Fig. 1, lane 6 versus lane 5). We also examined the effect of BLIMP1 addition to SNU-719, a cell line derived from a gastric carcinoma that maintains its original latent EBV. We observed strong induction of Z, R, and EAD synthesis (Fig. 1A, lane 8 versus lane 7) here as well.

To determine whether BLIMP1-induced reactivation proceeded all the way to production of infectious virus, we harvested the culture medium from CNE2-Akata cells transfected 4 days earlier with pcDNA3-BLIMP1 or its empty vector as a control. CNE2-Akata is another EBV$^+$ epithelial cell line, this one derived from an NPC that had been latently infected with a GFP-marked Akata strain of EBV. After filtration to remove cells, the relative amount of infectious EBV present in the medium was determined by superinfection of Raji cells; the BLIMP1-expressing cells produced approximately 40-fold more virus than the control cells (Fig. 1B).

Thus, five distinct EBV$^+$ epithelial cell lines derived from a variety of different, physiologically relevant cell types similarly responded to BLIMP1 addition. Therefore, we conclude that BLIMP1 expression is, indeed, sufficient to induce EBV lytic reactivation in many (possibly all) EBV$^+$ epithelial cell lines.

BLIMP1 stimulates EBV lytic reactivation in B cells in a cell-line-dependent manner. Given that BLIMP1 addition was reported to induce EBV lytic gene expression in Akata BL and several lymphoblastoid cell lines (LCLs) (58), we next asked whether BLIMP1 does so in all EBV$^+$ B-cell lines. To answer this question, we infected a variety of EBV$^+$ BL-derived lines with a BLIMP1-expressing lentivirus, CD713-BLIMP1. We first examined two Wp-restricted latency lines, Sal and Oku, in which the EBV latency proteins EBNA1, -3A, -3B, -3C, and -LP are expressed. As expected, addition of BLIMP1 strongly induced Z, R, and EAD expression (Fig. 2, lane 2 versus lane 1 and lane 6 versus lane 5, respectively).

Next, we examined the effect of BLIMP1 addition in a type III

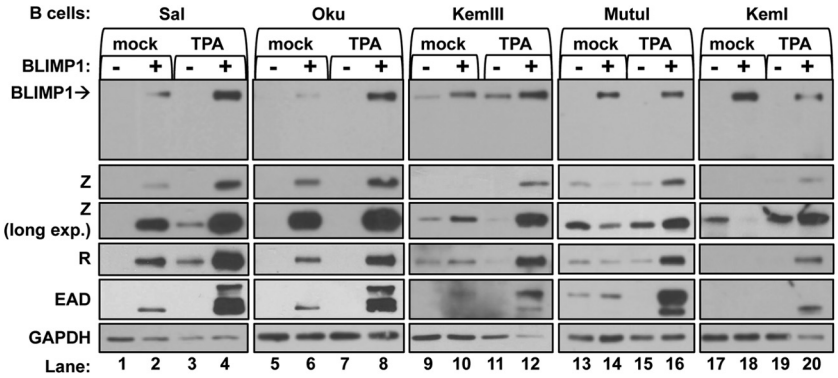

FIG 2 Immunoblots showing BLIMP1 induction of lytic reactivation in EBV-positive B cells. EBV$^+$ BL cells were infected with the lentivirus CD713-BLIMP1 (+) or CD713 (−), its empty vector, as a control. CD713-positive KemI, Sal, and Oku cells were selected by incubation with puromycin (1 μg/ml) for 2 weeks and then incubated with TPA (20 ng/ml) or dimethyl sulfoxide(1 μl/ml; mock) as a control for 48 h immediately prior to preparation of whole-cell extracts. Given high-level lentivirus infection, MutuI and KemIII cells were simply incubated for 4 days postinfection without puromycin selection prior to incubation with TPA or dimethyl sulfoxide for 48 h and harvesting. GAPDH served as a loading control. long exp., longer exposures of the same blots.

latency line, KemIII, in which all nine EBV latency proteins are expressed. KemIII differs from the other cell lines examined here in that it constitutively expresses some BLIMP1 (Fig. 2, lane 9). Nevertheless, it was still somewhat responsive, although less so than Sal and Oku, with smaller increases in expression of Z, R, and EAD starting from a higher background level (Fig. 2, lane 10 versus lane 9).

We also examined the effect of BLIMP1 addition in two type I latency cell lines, MutuI and KemI, in which EBNA1 is the only EBV protein expressed. Unexpectedly, addition of BLIMP1 was not sufficient to induce reactivation; rather, it led to decreased Z protein levels (Fig. 2, lane 14 versus lane 13 and lane 18 versus lane 17). Thus, we conclude that BLIMP1 addition induces reactivation in some, but not all, EBV$^+$ B-cell lines.

TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) is a well-known chemical inducer of EBV reactivation in some, but not all, EBV$^+$ cell lines; it does so by activating the protein kinase C (PKC) pathway (75–77). The five B-cell lines examined here are among ones where incubation with TPA for 2 days has little, if any, effect on EBV lytic gene expression (Fig. 2, lane 3 versus lane 1, lane 7 versus lane 5, lane 11 versus lane 9, lane 15 versus lane 13, and lane 19 versus lane 17). Nevertheless, when BLIMP1 addition was combined with TPA incubation, we observed strong synergistic reactivation in all five of these B-cell lines (Fig. 2, lanes 4, 8, 12, 16, and 20 versus lanes 2 and 3, lanes 6 and 7, lanes 10 and 11, lanes 14 and 15, and lanes 18 and 19, respectively). Thus, we conclude that the cellular environment can affect whether BLIMP1 expression is sufficient to induce EBV lytic reactivation in B cells.

BLIMP1 strongly activates both Zp and Rp. BLIMP1 was previously shown to induce transcription from Zp in a reporter assay (59); whether it affected Rp was not determined. Given that R, but not Z, induces reactivation in NOK-Akata cells (25), we hypothesized that BLIMP1 also induces transcription from Rp. To test this possibility, we cotransfected EBV-negative (EBV$^-$) AGS and NOK cells with a plasmid containing the firefly luciferase gene driven by the Z promoter (pCpGL-Zp-668), R promoter (pCpGL-Rp-673), or their empty vector, pCpGL, together with pcDNA3-BLIMP1 or its empty vector, pcDNA3.1. As expected, BLIMP1 activated transcription from Zp; however, it activated transcription from Rp even more, i.e., approximately 340-fold (Fig. 3A and B). Similar results were observed in EBV$^-$ HONE1 cells (data not shown).

DNA methylation status plays a major role in regulation of expression of EBV genes, with EBV genomes being highly methylated in EBV$^+$ cancers (25, 78–80). Therefore, we next asked whether BLIMP1 induces transcriptional activation from methylated as well as unmethylated Zp and Rp. To answer this question, we *in vitro* methylated or mock treated our pCpGL-Zp-668 and pCpGL-Rp-673 luciferase reporter DNAs prior to cotransfection into AGS cells along with the BLIMP1 expression plasmid. Methylation had no effect on BLIMP1-induced activation of transcription from Zp, while it reduced activation of transcription from Rp approximately 40%, with activation of Rp still severalfold higher than activation of Zp (Fig. 3C). Furthermore, BLIMP1 induced EBV lytic gene expression in cell lines regardless of whether the EBV genome was methylated (Fig. 2, BL-derived lines) or unmethylated (Fig. 1, lane 6, NOK-Akata) (25, 79, 80).

Transcriptional activation is uncharacteristic of BLIMP1, given that it usually functions as a repressor (46, 48, 50, 53, 81, 82). Thus, for a control, we examined likewise the effect of BLIMP1 addition on transcription from the promoter of the cellular gene *CIITA*. CIITA is a transcriptional coactivator that regulates major histocompatibility complex class II (MHC-II) gene expression; its CIITAp3 promoter is known to be strongly repressed by BLIMP1 (65, 67, 83). 293T cells were used in this experiment because the CIITAp3 promoter exhibits high basal activity in these cells, and, therefore, its repression by BLIMP1 can be readily observed. As expected, cotransfection of 293T cells with pcDNA3-BLIMP1 decreased expression from a CIITAp3-driven luciferase reporter by 90% (Fig. 3D).

Thus, we conclude that BLIMP1 strongly induces transcription from both Zp and Rp; it does so regardless of whether these viral promoters are methylated.

BLIMP1-induced activation of Rp involves several synergistically acting elements. To begin to understand how BLIMP1 expression induces high-level transcriptional activation of Rp, we asked what regions of Rp are needed for it to occur. Unfortunately, our current knowledge regarding how Rp is regulated is far from complete (Fig. 4A). Thus, we first looked for BLIMP1 consensus binding sites, 5′-(A/C)AG(T/C)GAAAG(T/C)(G/T)-3′, in Rp; none

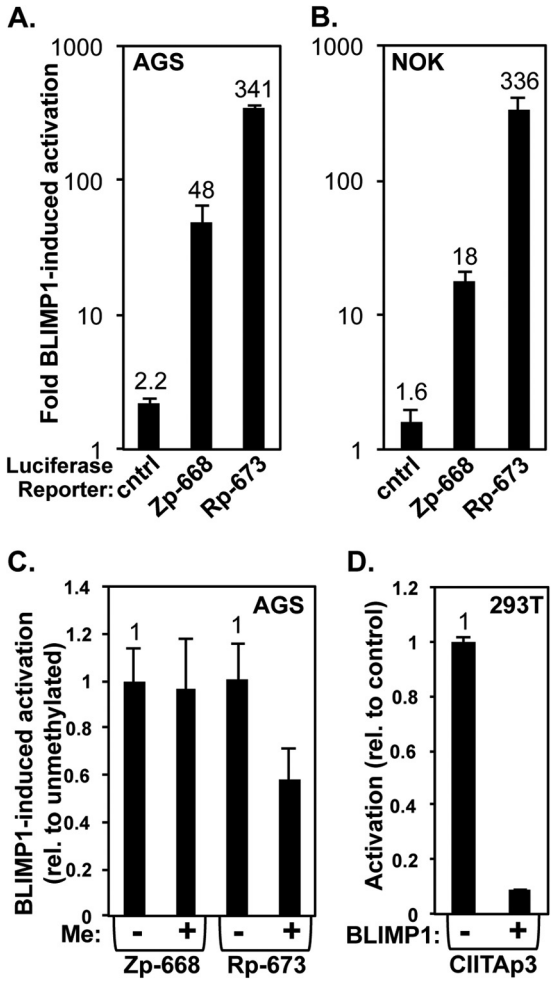

FIG 3 Luciferase reporter assays showing that BLIMP1 strongly induces transcriptional activation from both Zp and Rp. (A) EBV$^-$ AGS cells in 22-mm-diameter wells were cotransfected with (i) 50 ng pcDNA3-BLIMP1 or pcDNA3.1 and with (ii) 450 ng of the CpG-free luciferase reporter (pCpGL) containing no promoter (cntrl), Zp-668, or Rp-673. Fold activation is presented on a $\log_{10}$ scale normalized to each promoter's activity in the absence of BLIMP1. (B) EBV$^-$ NOK cells in 22-mm-diameter wells were cotransfected with (i) 200 ng pcDNA3-BLIMP1 or pcDNA3.1 and with (ii) 300 ng of pCpGL-Zp-668, pCpGL-Rp-673, or pCpGL. Fold activation is presented on a $\log_{10}$ scale. (C) BLIMP1-induced transcriptional activation from methylated Zp and Rp DNA relative to activation from the unmethylated versions of these same DNAs. AGS cells in 22-mm-diameter wells were cotransfected with (i) 50 ng of mock-treated (−) or methylated (+) pCpGL-Zp-668 or pCpGL-Rp-673, (ii) 50 ng of pcDNA3-BLIMP1 or pcDNA3.1, and (iii) 400 ng of pSG5. (D) 293T cells in 22-mm-diameter wells were cotransfected with (i) 10 ng of pcDNA-BLIMP1 (+) or pcDNA3.1 (−) and with (ii) 490 ng of pGL3-CIITAp3 or its empty vector, pGL3-Basic. Cells were harvested at 48 h posttransfection. Data were normalized to each promoter's activity when cells were cotransfected with pcDNA3.1. Data are representative means of assays performed in triplicate on two or more occasions. Error bars indicate standard deviations within one representative set of assays performed in triplicate.

were identified. Next, we constructed a series of 5′-truncated variants of our full-length pCpGL-Rp-673 reporter and compared their ability to be activated by BLIMP1 in AGS cells. Over multiple replicates of this experiment, we observed that while Rp-664 was activated by BLIMP1 similarly to Rp-673, there was a 5- to 11-fold decrease in activation of Rp-655 (Fig. 4B). Further deletion up to nt −267 did not result in additional significant changes in activation by BLIMP1. However, deletion to nt −106 led to a further decrease of 4- to 6-fold, with BLIMP1-induced transcriptional activation of Rp-106 relative to its empty vector control still being a substantial 6- to 10-fold. Thus, we conclude that Rp contains three or more elements through which BLIMP1 induces activation: one maps between nt −664 and −655, a second maps between nt −267 and −106, and a third maps between nt −106 and +38; these elements act synergistically, with each one contributing 5-fold or more for a total activation of over 300-fold.

BLIMP1 activates Rp in part via a nt −660 region element. To identify the exact bases involved in BLIMP1-induced activation via the region of nt −664 to −655 of Rp, we constructed a series of variants of pCpGL-Rp-673 containing 2-bp substitution mutations throughout this region (Fig. 4C) and assayed them for activation by BLIMP1 as described above. All five Rp mutants exhibited some decrease in BLIMP1-induced activation (Fig. 4D). Activation of Rp-mt3 was almost as reduced as that of the deletion mutant Rp-655 which lacks this entire 10-bp region, while the basal transcriptional activities of these mutants were unaffected (Fig. 4D and E).

As an additional control to ensure that this 5-fold loss of Rp activation by BLIMP1 was not due to these mutations inadvertently impairing more generally the ability of Rp to function, we also tested the ability of Z protein to activate the two most impaired of these Rp mutants. Given that Z strongly activates methylated Rp but not unmethylated Rp (25, 78, 80), we *in vitro* methylated pCpGL-Rp-mt3, pCpGL-Rp-655, and full-length wild-type (WT) pCpGL-Rp-673 DNA prior to their cotransfection along with a Z expression plasmid; both of the Rp mutants were normally activated by Z (Fig. 4F). Therefore, we conclude that a sequence element that includes nt −660 and/or −659 is a significant contributor to high-level BLIMP1-induced activation of Rp.

We asked whether the sequence surrounding this nt −660 BLIMP1-responsive element (BRE) might also be present within the BLIMP1-responsive regions of nt −267 to −106 or nt −106 to +38 of Rp; it was not. This finding indicates that BLIMP1 likely induces Rp through several mechanisms.

PR and Zn finger domains of BLIMP1 are necessary for Rp activation. BLIMP1 contains five major domains: two acidic regions located at the N and C termini, a PRDI-BF1-RIZ1 (PR) domain, a proline-rich (Pro) domain, and a Zn finger DNA-binding (Zn) domain. The Pro domain contributes to recruiting cofactors to BLIMP1 complexes, while the Zn finger domain is required for its ability to directly bind DNA (44, 51). To try to gain further insights into the mechanism by which BLIMP1 induces EBV reactivation, we examined the activities of variants of BLIMP1 with deletions in these domains (depicted in Fig. 5A). First, we compared their abilities to induce EBV lytic gene expression after transfection into HONE-Akata cells. While BLIMP1 ΔPro induced expression of Z, R, and EAD to levels similar to those observed with wild-type BLIMP1, ΔPR and ΔZn BLIMP1s failed to induce synthesis of any of these three EBV lytic proteins (Fig. 5B, lanes 2 and 4 versus lanes 3 and 5). Therefore, we conclude that both the PR and Zn finger domains are necessary for BLIMP1-induced reactivation of EBV in epithelial cells.

To determine whether the failure of these mutant proteins to induce reactivation was due, in part, to a defect in Rp activation, we cotransfected AGS cells with pCpGL-Rp-673 and the BLIMP1 expression plasmids. As expected, both ΔPR and ΔZn BLIMP1s were also unable to induce high-level activation of Rp-673

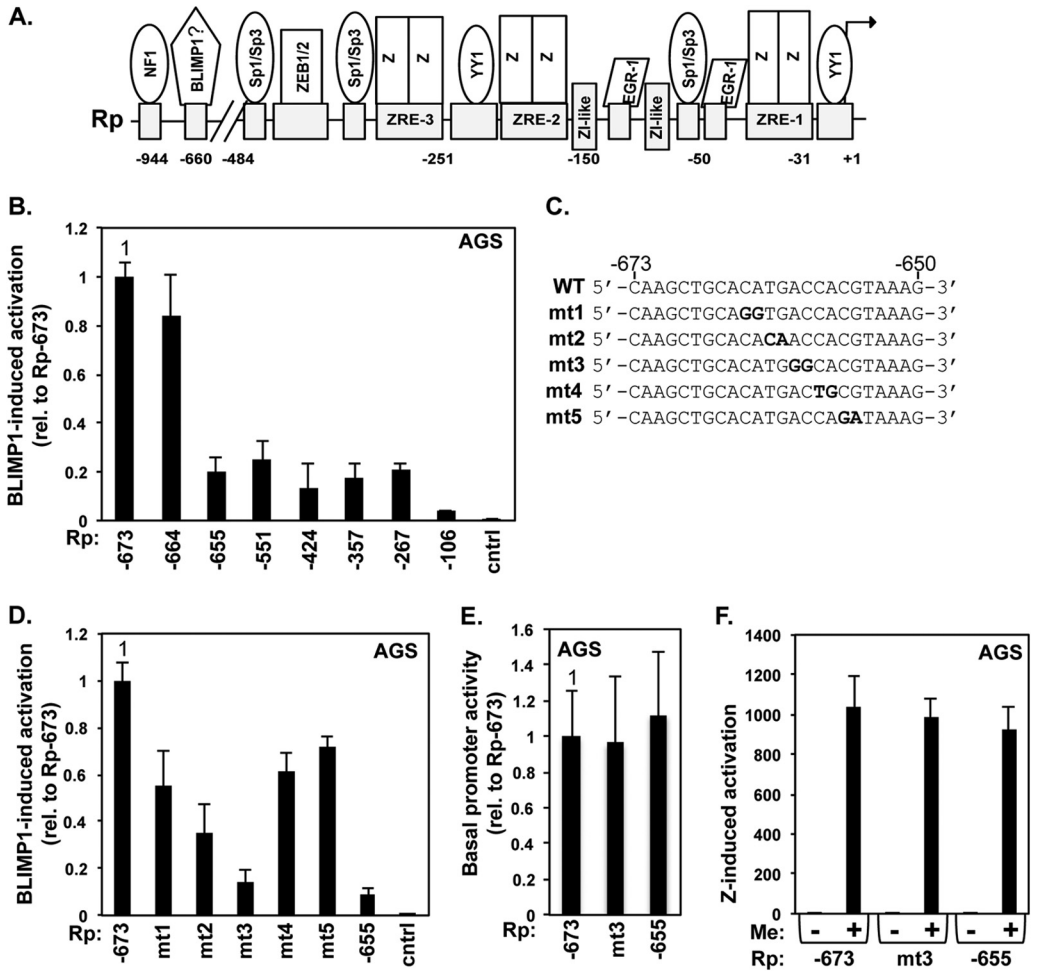

FIG 4 High-level BLIMP1-induced transcriptional activation of Rp involves three or more elements that act synergistically, including one requiring nt −660 and/or −659. (A) Schematic indicating factors known to be involved in regulating Rp expression and the *cis*-acting elements through which they act. The figure is not drawn to scale. (B) Luciferase reporter assays performed with deleted variants of Rp. AGS cells in 22-mm-diameter wells were cotransfected with (i) 50 ng of pcDNA3-BLIMP1 or pcDNA3.1 and with (ii) 450 ng of a variant of pCpGL-Rp-673 containing the indicated 5′ deletion endpoint of Rp. Data were normalized both to each promoter's activity when the cells were cotransfected with pcDNA3.1 and to the relative activity observed with Rp-673. (C) Sequences of the Rp region of nt −673 to −650 of the wild-type Rp-673 and 2-bp substitution mutant variants of it studied here, with the mutations indicated in bold font. (D) Luciferase reporter assays performed with the 2-bp substitution mutants shown in panel C. AGS cells in 22-mm-diameter wells were cotransfected with (i) 50 ng pcDNA3-BLIMP1 or pcDNA3.1 and (ii) 450 ng pCpGL-Rp-673, pCpGL-Rp-655, or one of the 2-bp mutant variants of pCpGL-Rp-673. Data were normalized both to each promoter's activity when cells were cotransfected with pcDNA3.1 and to the activity of the WT Rp-673. (E) Basal transcriptional activity of some of the Rp mutants. The data were obtained from the luciferase assays shown in panel D performed with pcDNA3.1, with normalization to the level observed with Rp-673. (F) Luciferase reporter assays showing that the Rp mutants are not defective in activation by Z. AGS cells in 22-mm-diameter wells were cotransfected with (i) 50 ng of mock-treated (−) or methylated (+) pCpGL-Rp-673, pCpGL-Rp-mt3, or pCpGL-Rp-655 and with (ii) 5 ng of pSG5-Z plus 445 ng of pSG5 or 450 ng of pSG5. Data were normalized to each promoter's activity in the absence of pSG5-Z. All data are representative means of assays performed in triplicate on two or more occasions; error bars indicate standard deviations within one representative set of assays performed in triplicate.

(Fig. 5C). Thus, we conclude that the mechanism through which the PR and Zn finger domains of BLIMP1 are necessary for induction of EBV lytic reactivation in epithelial cells likely involves, at least in part, their roles in inducing *BRLF1* gene expression.

BLIMP1 associates with Rp *in vivo*. Given that BLIMP1 requires its Zn finger domain both to activate Rp and to induce EBV lytic reactivation, we hypothesized that BLIMP1 may bind Rp even though we failed to identify a BLIMP1 consensus sequence within Rp. To test this possibility, we performed ChIP assays for BLIMP1 binding to Rp. HONE-Akata cells were transfected with pcDNA3-BLIMP1 or pcDNA3.1. Forty-eight hours later, the cells were harvested and processed for ChIP. The ChIPed DNA was subjected to PCR analysis for the presence of Rp, with c-Myc as a positive control and EBV Qp and cellular β-globin as negative controls. Consistent with our hypothesis, BLIMP1 associated *in vivo* with both the promoter-proximal and -distal regions of Rp that we showed above contain BREs (Fig. 6A and B).

To test whether BLIMP1 directly binds Rp DNA, we performed electrophoretic mobility shift assays (EMSAs) using a radiolabeled probe corresponding to the region of Rp encompassing our nt −660 BRE. As negative and positive controls, we used probes corresponding to a nearby region of Rp and the region of the CIITAp3 promoter known to bind BLIMP1 (64), respectively. While BLIMP1 bound well to the positive control, no binding above background was observed with the Rp probe encompassing the nt −660 BRE (Fig. 6C, lanes 7 and 8 versus lanes 3 and 4). These data

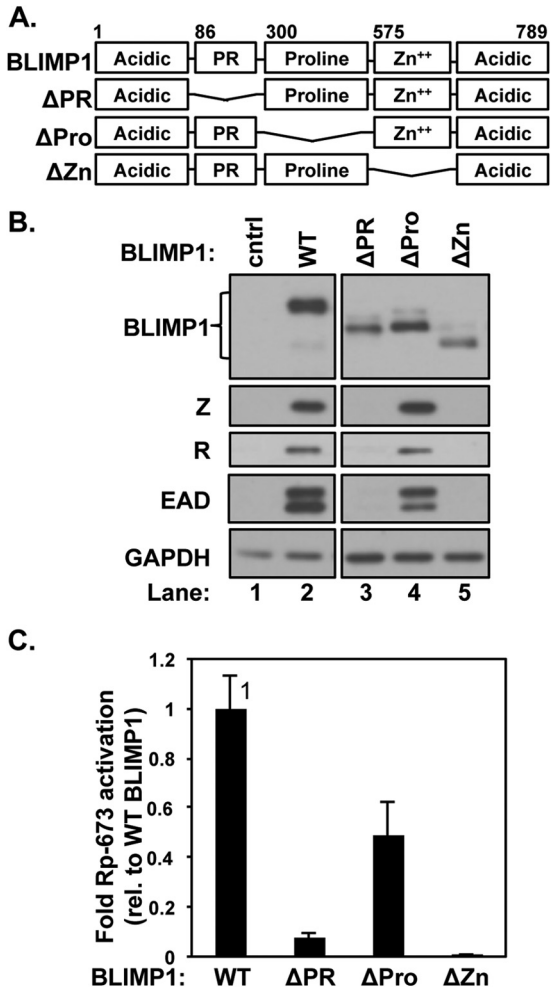

FIG 5 PR and zinc finger domains of BLIMP1 are critical for inducing reactivation. (A) Schematic showing structures of BLIMP1 wild-type and variants with deletions in the PRDI-BF1-RIZ1 (PR), proline-rich (Pro), and Zn finger DNA-binding (Zn) domains. The figure is not drawn to scale. (B) Immunoblot showing relative levels of some EBV lytic proteins following expression of WT BLIMP1 and the BLIMP1 variants depicted in panel A. HONE-Akata cells in 35-mm-diameter wells were transfected with (i) 300 ng of the indicated pcDNA3-BLIMP1 variant or pcDNA3.1 and (ii) 700 ng of pSG5. Whole-cell extracts were prepared 48 h posttransfection. All samples were present in the same immunoblots, with irrelevant lanes omitted. GAPDH served as a loading control. (C) Luciferase assays showing relative abilities of the variant BLIMP1 proteins to activate Rp. AGS cells in 22-mm-diameter wells were cotransfected with (i) 450 ng of pCpGL-Rp-673 and (ii) 50 ng of the indicated pcDNA3-BLIMP1 variant depicted in panel A or pcDNA3.1. Data were normalized to WT BLIMP1-induced activation of Rp-673. Values are representative means of assays performed in triplicate on two separate occasions. Error bars indicate standard deviations within one representative set of assays performed in triplicate.

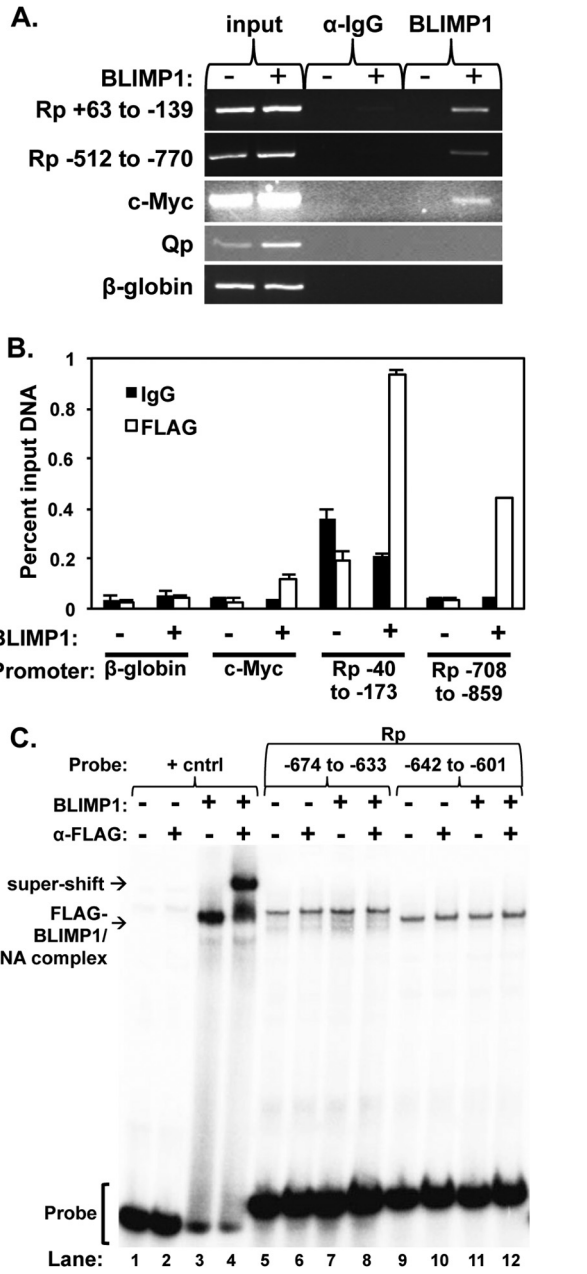

FIG 6 BLIMP1 associates with Rp *in vivo* but does not directly bind the nt −660 BRE *in vitro*. (A) Semiquantitative ChIP assay for BLIMP1 association with Rp. HONE-Akata cells in 10-cm-diameter dishes were transfected with 6 μg of pcDNA3-BLIMP1 (encoding FLAG-tagged BLIMP1) (+) or pcDNA3.1 (−). Cells were processed for ChIP 48 h later and immunoprecipitated with a FLAG-specific or mouse IgG control antibody. ChIPed DNA was subjected to PCR analysis with the indicated primers specific to Rp, with c-Myc as a positive control and EBV Qp and cellular β-globin as negative controls. (B) Quantitative PCR analysis of the ChIPed DNA from the experiment described in panel A. The ChIPed DNA was subjected to quantitative PCR with primers specific to Rp, cellular β-globin, and c-Myc. (C) EMSA to look for direct binding of BLIMP1 to the Rp nt −660 BRE *in vitro*. HONE1 cells in 10-cm-diameter dishes were transfected with 6 μg of pcDNA3-BLIMP1 or pcDNA3.1. Nuclear extracts were prepared 48 h posttransfection and incubated with radiolabeled probes corresponding to the indicated regions of Rp or the cellular promoter, CIITAp3, as a positive control. A FLAG-specific antibody was used to supershift FLAG-BLIMP1:DNA complexes.

indicate that BLIMP1 does not activate Rp by directly binding to the nt −660 BRE; however, it may interact with this region of Rp indirectly as part of a protein complex.

Requirement of BLIMP1 for EBV lytic gene expression. Incubation with TPA induces cellular differentiation in a variety of cell types, including epithelial and B cells (84–88). As expected, treatment of NOK-Akata cells with TPA induced them to differentiate as indicated by an increase in the level of the keratinocyte differentiation factor, involucrin, as well as by activation of expression

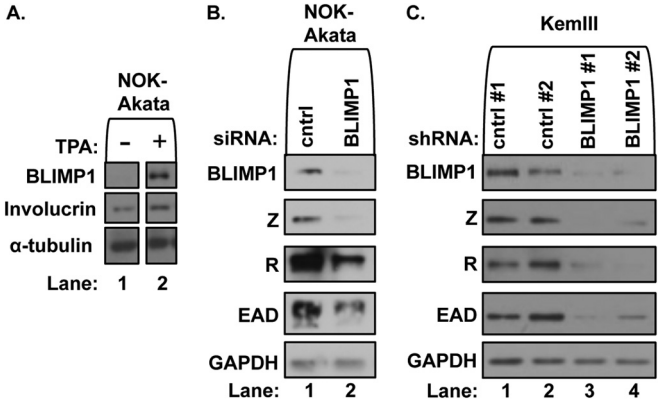

FIG 7 Requirement of BLIMP1 for EBV lytic gene expression in NOK-Akata and KemIII cells. (A) Immunoblot showing effect of TPA treatment on the expression of markers of differentiation in NOK-Akata cells. NOK-Akata cells in 22-mm-diameter wells were treated with TPA (20 ng/ml) (+) or dimethyl sulfoxide (1 μl/ml) (−) as a control for 48 h immediately prior to preparation of whole-cell extracts. The samples were present on the same blots, with an irrelevant intervening lane omitted. α-Tubulin served as a loading control. (B) Immunoblot showing effect of BLIMP1 knockdown in NOK-Akata cells. NOK-Akata cells in 22-mm-diameter wells were transfected with 20 pmol of either a universal scrambled control or a BLIMP1-targeting siRNA. Three days later, the cells were incubated with TPA (20 ng/ml) for an additional 24 h prior to preparation of whole-cell extracts. (C) Immunoblot showing effect of BLIMP1 knockdown in KemIII cells. KemIII cells were infected with a lentivirus expressing the indicated shRNAs. Three days later, the cells were infected with the BLIMP1-expressing lentivirus, CD713-BLIMP1, and incubated for an additional 48 h prior to preparation of whole-cell extracts. GAPDH served as a loading control in the experiments shown in panels B and C.

of endogenous BLIMP1 (Fig. 7A, lane 2 versus lane 1). To determine if BLIMP1 was necessary for the induction of EBV reactivation during epithelial cell differentiation, we transfected NOK-Akata cells with a BLIMP1-targeting siRNA to inhibit BLIMP1 synthesis prior to the addition of TPA. Cells transfected in parallel with a control siRNA expressed a physiological level of BLIMP1 as a consequence of differentiation as well as Z, R, and EAD (Fig. 7B, lane 1). However, the levels of BLIMP1, Z, R, and EAD were reduced relative to those of the control by approximately 90%, 90%, 50%, and 60%, respectively, in the cells that contained the BLIMP1-targeting siRNA (Fig. 7B, lane 2 versus lane 1). Thus, we conclude that the physiological level of BLIMP1 that appears during differentiation of these epithelial cells is probably a necessary contributor to reactivation of EBV.

We also investigated the effect of knocking down BLIMP1 in KemIII cells, which constitutively express some BLIMP1 along with EBV lytic proteins (Fig. 2, lane 9). Being a B-cell line, these cells were infected with a lentivirus expressing a BLIMP1-targeting shRNA (BLIMP1 no. 1 and BLIMP1 no. 2) or a nontargeting shRNA (cntrl 1 or cntrl 2) for 3 days prior to infection with a BLIMP1-expressing lentivirus. Both BLIMP1-targeting shRNAs reduced not only the BLIMP1 level by greater than 80% relative to the control shRNAs but also the levels of Z by greater than 90% and 70%, R by greater than 50% and 90%, and EAD by greater than 80% and nearly 50%, respectively (Fig. 7C, lanes 3 and 4 versus lanes 1 and 2). Thus, we conclude that BLIMP1 is, in at least some cases, also necessary for EBV lytic gene expression in B cells.

DISCUSSION

In this study, we examined the role of BLIMP1, an important regulator of both epithelial and plasma cell differentiation, in inducing EBV out of latency into lytic replication. We showed that BLIMP1 is sufficient to induce EBV lytic gene expression in a variety of EBV$^+$ epithelial cells lines (Fig. 1) as well as in some, but not all, EBV$^+$ B-cell lines (Fig. 2). We confirmed that BLIMP1 induces transcription from Zp and discovered that it also induced transcription from Rp, doing so to a very high level (Fig. 3). The latter transcriptional activation involved, in part, a *cis*-acting element that includes Rp nt −660 and/or −659 (Fig. 4) along with the PR and Zn finger domains of BLIMP1 (Fig. 5). Knockdown of BLIMP1 in both epithelial and B cells led to reduction in expression of EBV lytic genes (Fig. 7). Combining these findings with ones reported previously (58, 59), we conclude that BLIMP1 is a key inducer of EBV lytic reactivation during both epithelial and B-cell differentiation, doing so by inducing expression of both of EBV's immediate early genes, *BZLF1* and *BRLF1*; while not sufficient to induce EBV lytic gene expression in some B-cell types, BLIMP1 expression appears to be sufficient in all of the epithelial cell types examined to date.

BLIMP1-induced EBV reactivation. A previous report showed that BLIMP1 can induce EBV lytic gene expression in Akata BL and several LCLs (58). Here, by looking at epithelial cell lines derived from three different tissue types and B-cell lines expressing three different types of EBV latency, we provided a much broader analysis of the range of EBV$^+$ cell lines in which BLIMP1 can induce EBV reactivation. While our findings confirmed that BLIMP1 addition was sufficient to induce EBV reactivation in some B-cell lines, we discovered others in which it failed to do so (Fig. 2). On the other hand, BLIMP1 induced reactivation in all five of the EBV$^+$ epithelial cell lines we examined (Fig. 1). Consistent with our findings, Buettner et al. (59) observed that all Z$^+$ cells within an OHL lesion are also BLIMP1$^+$, while only one-third to two-thirds of Z$^+$ B cells from tonsils of IM patients contain detectable BLIMP1. Thus, we conclude that BLIMP1 expression leads to induction of EBV lytic gene expression in many (possibly all) EBV$^+$ epithelial cell types but not all EBV$^+$ B-cell types.

Incubation with TPA greatly increased the responsiveness of EBV$^+$ B cells to BLIMP1, even in two type I latency cell lines, MutuI and KemI, that were otherwise unresponsive to it (Fig. 2). This finding reveals a synergy between BLIMP1 and changes in the cellular environment fostered by TPA. Incubation with TPA also led to higher levels of BLIMP1 in Sal, Oku, and KemIII cells. However, the synergy observed with TPA plus BLIMP1 was not solely due to enhancement in BLIMP1 level since it was also observed in MutuI and KemI cells where TPA had a synergistic effect without increasing BLIMP1 levels.

Incubation with TPA leads to activation of numerous cellular transcription factors through activation of the PKC pathway (76, 77) and stimulates cells to differentiate (84–88). Given that EBV is reactivated in one type I latency BL cell line, Akata, with BLIMP1 alone while two others, MutuI and KemI, require TPA along with BLIMP1, we hypothesize that there exists a balance of cellular transcription factors and pathways necessary for BLIMP1-induced reactivation that is already present (or absent) in Akata cells, with TPA modifying their levels and/or activities in MutuI and KemI cells and altering them somewhat in Sal, Oku, and KemIII cells. For example, one such factor may be the cellular transcription factor Ets-1; it can bind BLIMP1, inhibiting its activity (89, 90), while expression of Ets-1 is inhibited in B cells by incubation with TPA (91, 92).

BLIMP1 versus BLIMP1β. Human BLIMP1 is expressed in two major isoforms, α and β. BLIMP1α is the full-length, fully functioning form described here as BLIMP1. BLIMP1β is a truncated isoform, arising from an alternative promoter, that differs from BLIMP1α by beginning with three unique amino acids, MEK, and continuing with BLIMP1 amino acids 102 to 789 (64). BLIMP1β is functionally impaired, exhibiting approximately half the repressive activity of BLIMP1 on natural target promoters (64). Infection of germinal center B cells with some strains of EBV leads to synthesis of BLIMP1β (93). We did not detect the presence of this isoform in any of our EBV$^+$ B-cell lines (Fig. 2), nor did we detect it in the D4 LCL previously derived by infection of primary blood B cells with the B95.8 strain of EBV (data not shown). Therefore, we conclude that BLIMP1β is not expressed in many EBV$^+$ B-cell lines, and it need not contribute to BLIMP1-induced EBV lytic reactivation.

How does BLIMP1 induce EBV reactivation? Here, we showed that while both Zp and Rp are strongly activated by BLIMP1, Rp is activated even more than Zp in epithelial cells (Fig. 3). Thus, while BLIMP1 activation of Zp is probably the primary mechanism by which it induces EBV reactivation in B cells, given that EBV$^+$ B cells are much more responsive to Z than R, BLIMP1 activation of Rp may be the primary mechanism in epithelial cells, given that they are often more and sometimes exclusively responsive to R (15, 16, 20, 25).

To begin to understand the mechanism by which BLIMP1 induces high-level activation of Rp, we performed a deletional analysis of this promoter, identifying three distinct regions that functioned synergistically (Fig. 4). The most upstream BRE was precisely mapped to include nt −660 and/or −659. The only known transcription factor binding site sequence in this region is an E box (5′-CANNTG-3′) spanning nt −666 to −661. However, this E box does not influence BLIMP1-induced Rp activation, given our finding that pCpGL-Rp-664 was as strongly activated as pCpGL-Rp-673. The more promoter-proximal BREs have yet to be mapped precisely; with luck, the identities of the factors that recognize these BREs may become clear once they are.

We examined by both semiquantitative and quantitative ChIP assays whether BLIMP1 activation of Rp might occur via binding to these BREs, finding that BLIMP1 associates with Rp at or somewhere near (i) the nt −660 BRE and (ii) one or both of the promoter-proximal BREs (Fig. 6A and B). Given the limited resolution of these assays, data from ChIP with sequencing would be needed to determine more precisely where BLIMP1 binds within Rp. ChIP assays also cannot be used to distinguish between directly binding to Rp versus indirectly binding via a protein complex. In the case of the nt −660 BRE, we addressed this question by EMSAs and failed to observe BLIMP1 binding to a probe spanning Rp nt −674 to −633 (Fig. 6C). Thus, we conclude that BLIMP1 directly binds the nt −660 BRE but too weakly or transiently to be detected by EMSAs, or directly binds Rp somewhat near but not at the −660 BRE, or indirectly associates with Rp as part of a protein complex.

In some cases, BLIMP1's proline-rich domain is necessary for transcriptional repression via recruitment of Groucho family proteins and HDACs (51–53). In other cases, repression of a target promoter is independent of the proline-rich domain but partially dependent upon BLIMP1's PR domain (65). Here, we mapped the domains of BLIMP1 critical for activation of Rp and induction of EBV lytic gene expression; one of them was the PR domain

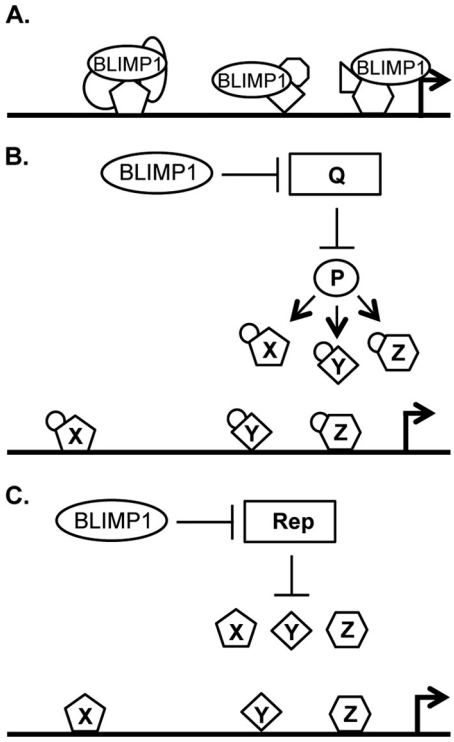

FIG 8 Models for BLIMP1-mediated transcriptional activation of Rp. (A) BLIMP1 may act by indirectly binding to Rp as part of a multiprotein complex(es) that activates transcription from Rp. (B) BLIMP1 may act indirectly through repression of synthesis of a protein(s) (Q) that inhibits the activity or level of a protein(s) (P) that posttranslationally modifies transcriptional regulators of Rp (X, Y, and Z), altering the latter's activities. (C) BLIMP1 may act indirectly through repressing synthesis of a repressor(s) (Rep) of factors (X, Y, and Z) that activate transcription from Rp. Each BRE on Rp could potentially function via one or more of these non-mutually exclusive mechanisms depending upon the cell type and culture conditions.

(Fig. 5). How BLIMP1's PR domain contributes to repression of target genes such as *CIITA* (65) has yet to be determined; the homologous region of the RIZ1 protein is known to serve as a protein-binding interface, allowing for the formation of multiprotein complexes (94). Taken together with our EMSA and ChIP findings (Fig. 6), these data indicate that BLIMP1 may induce Rp activation in part as a component of a protein complex that binds the nt −660 BRE as an activator (Fig. 8A).

The zinc finger DNA-binding domain of BLIMP1 was also found to be essential for induction of EBV lytic gene expression and Rp activation (Fig. 5). Given that BLIMP1 requires only its two most-N-terminal zinc fingers for DNA-binding function (44), we also examined a BLIMP1 mutant variant lacking only these two zinc fingers; it was similarly unable to induce EBV lytic gene expression and Rp activation (data not shown). Thus, the mechanism of EBV reactivation probably involves BLIMP1 binding to both DNA and some transcriptional coregulators.

Although BLIMP1 has been reported to act as a transcriptional repressor, many transcription factors have been shown to act as either activators or repressors, depending upon the promoter context and cellular environment. Given our ChIP data, we hypothesize that BLIMP1 may activate Rp by indirectly binding to it as one component of multiprotein transcription-activating complexes (Fig. 8A). Alternatively, BLIMP1 may act indirectly by repressing

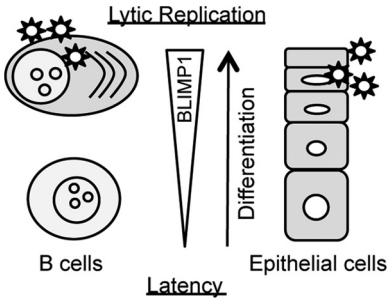

FIG 9 Model for BLIMP1 induction of EBV reactivation during cellular differentiation. As epithelial and B cells differentiate, expression of the gene encoding BLIMP1 is activated, leading to synthesis of BLIMP1 and, consequently, the induction of lytic EBV infection with production of infectious virus in EBV^+ epithelial and some B-cell types.

synthesis of a protein(s) that inhibits the activity or level of another protein(s) that posttranslationally modifies one or more transcriptional regulators of Rp, altering the latter's activities (Fig. 8B). For example, BLIMP1 may inhibit the synthesis of some phosphatases or proteases that target kinases that phosphorylate some coactivators of Rp into their functionally active forms. Another possibility is that BLIMP1 may repress synthesis of a factor(s) that, in turn, represses synthesis of an activator(s) of Rp (Fig. 8C). An example of the latter in B cells might be BLIMP1 repressing expression of PAX5 (48), thereby relieving repression of XBP-1 by PAX5 (57) and thus enabling XBP-1 to activate both Rp and Zp (38, 39). These models are not mutually exclusive. Given that several BREs exist on Rp, they could potentially function via different mechanisms, increasing the possible ways by which Rp can be activated under various growth conditions and cell types.

Requirement for BLIMP1. Lastly, we showed in both epithelial and B cells that knockdown of BLIMP1 led to a reduction in EBV lytic gene expression (Fig. 7). Thus, BLIMP1 is probably a key contributor to induction of EBV lytic reactivation during cellular differentiation given that BLIMP1 expression is activated during the normal physiological process of differentiation in epithelial and B cells (Fig. 9). Combining all of our findings together with those of Buettner et al. (59), we conclude that this activation of BLIMP1 expression may well be necessary as well as sufficient in epithelial cells, but not in B cells, for induction of EBV reactivation.

Conclusion. BLIMP1, a key player in both epithelial and B-cell differentiation, also plays a major role in the induction of EBV lytic gene expression during differentiation of epithelial and B cells. BLIMP1 is sufficient and, at least in some cases, necessary for EBV lytic reactivation in epithelial cells; it does so via inducing expression of both of EBV's immediate early genes, *BZLF1* and *BRLF1*. The fact that EBV^+ cancers usually contain mostly undifferentiated cells may be due in part to these cells dying from lytic EBV infection when they differentiate and express wild-type BLIMP1.

ACKNOWLEDGMENTS

We thank Ron Glaser, Lindsey Hutt-Fletcher, Karl Munger, Alan Rickinson, Jeff Sample, Bill Sugden, David Vereide, and Lawrence Young for cell lines, Diane Hayward, Michael Rehli, and Coral Wille for plasmids, and members of the Johannsen, Kenney, and Mertz laboratories for suggestions and discussions.

This work was supported by U.S. Department of Health and Human Services NIH grants P01CA22443, P30CA14520, R01AI07034, R01DE023939, and T32AI078985 to J.E.M. and S.C.K.

REFERENCES

1. **Henle G, Henle W, Diehl V.** 1968. Relation of Burkitt's tumor-associated herpes-ytpe virus to infectious mononucleosis. Proc Natl Acad Sci U S A **59:**94–101. http://dx.doi.org/10.1073/pnas.59.1.94.
2. **Cohen JI.** 2000. Epstein-Barr virus infection. N Engl J Med **343:**481–492. http://dx.doi.org/10.1056/NEJM200008173430707.
3. **Lindahl T, Adams A, Bjursell G, Bornkamm GW, Kaschka-Dierich C, Jehn U.** 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J Mol Biol **102:**511–530. http://dx.doi.org/10.1016/0022-2836(76)90331-4.
4. **Epstein MA, Achong BG, Barr YM.** 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet **i:**702–703.
5. **zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle G, Clifford P, Santesson L.** 1970. Epstein–Barr virus in Burkitt's lymphoma and nasopharyngeal carcinoma: EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature **228:**1056–1058. http://dx.doi.org/10.1038/2281056a0.
6. **Desgranges C, Wolf H, De-Thé G, Shanmugaratnam K, Cammoun N, Ellouz R, Klein G, Lennert K, Muñoz N, Zur Hausen H.** 1975. Nasopharyngeal carcinoma. X. Presence of Epstein-Barr genomes in separated epithelial cells of tumours in patients from Singapore, Tunisia and Kenya. Int J Cancer **16:**7–15.
7. **Nalesnik MA, Jaffe R, Starzl TE, Demetris AJ, Porter K, Burnham JA, Makowka L, Ho M, Locker J.** 1988. The pathology of posttransplant lymphoproliferative disorders occurring in the setting of cyclosporine A-prednisone immunosuppression. Am J Pathol **133:**173–192.
8. **Shibata D, Weiss LM.** 1992. Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol **140:**769–774.
9. **Paya CV, Fung JJ, Nalesnik MA, Kieff E, Green M, Gores G, Habermann TM, Wiesner PH, Swinnen JL, Woodle ES, Bromberg JS.** 1999. Epstein-Barr virus-induced posttransplant lymphoproliferative disorders. ASTS/ASTP EBV-PTLD Task Force and The Mayo Clinic Organized International Consensus Development Meeting. Transplantation **68:**1517–1525.
10. **Kieff E, Rickinson AB.** 2007. Epstein-Barr virus and its replication, p 1225–2654. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
11. **Rickinson AB, Kieff E.** 2007. Epstein-Barr virus, p 2655–2700. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
12. **Kimura H, Kawada J, Ito Y.** 2013. Epstein-Barr virus-associated lymphoid malignancies: the expanding spectrum of hematopoietic neoplasms. Nagoya J Med Sci **75:**169–179. http://www.med.nagoya-u.ac.jp/medlib/nagoya_j_med_sci/7534/02_Kimura.pdf.
13. **Feederle R, Kost M, Baumann M, Janz A, Drouet E, Hammerschmidt W, Delecluse H-J.** 2000. The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. EMBO J **19:**3080–3089. http://dx.doi.org/10.1093/emboj/19.12.3080.
14. **Countryman J, Miller G.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc Natl Acad Sci U S A **82:**4085–4089. http://dx.doi.org/10.1073/pnas.82.12.4085.
15. **Takada K, Shimizu N, Sakuma S, Ono Y.** 1986. *trans* Activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. J Virol **57:**1016–1022.
16. **Chevallier-Greco A, Manet E, Chavrier P, Mosnier C, Daillie J, Sergeant A.** 1986. Both Epstein-Barr virus (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J **5:**3243–3249.
17. **Countryman J, Jenson H, Seibl R, Wolf H, Miller G.** 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J Virol **61:**3672–3679.
18. **Flemington E, Speck SH.** 1990. Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1. J Virol **64:**1227–1232.
19. **Sinclair AJ, Brimmell M, Shanahan F, Farrell PJ.** 1991. Pathways of

activation of the Epstein-Barr virus productive cycle. J Virol **65:**2237–2244.
20. **Zalani S, Holley-Guthrie E, Kenney S.** 1996. Epstein-Barr viral latency is disrupted by the immediate-early BRLF1 protein through a cell-specific mechanism. Proc Natl Acad Sci U S A **93:**9194–9199. http://dx.doi.org/10.1073/pnas.93.17.9194.
21. **Ragoczy T, Heston L, Miller G.** 1998. The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J Virol **72:**7978–7984.
22. **Adamson AL, Darr D, Holley-Guthrie E, Johnson RA, Mauser A, Swenson J, Kenney S.** 2000. Epstein-Barr virus immediate early proteins BZLF1 and BRLF1 activate the ATF2 transcription factor by increasing the levels of phosphorylated p38 and c-Jun N-terminal kinases. J Virol **74:**1224–1233. http://dx.doi.org/10.1128/JVI.74.3.1224-1233.2000.
23. **Lin T-Y, Chu Y-Y, Yang Y-C, Hsu S-W, Liu S-T, Chang L-K.** 2014. MCAF1 and Rta-activated BZLF1 transcription in Epstein-Barr virus. PLoS One **9:**e90698. http://dx.doi.org/10.1371/journal.pone.0090698.
24. **Murata T, Narita Y, Sugimoto A, Kawashima D, Kanda T, Tsurumi T.** 2013. Contribution of myocyte enhancer factor 2 family transcription factors to BZLF1 expression in Epstein-Barr virus reactivation from latency. J Virol **87:**10148–10162. http://dx.doi.org/10.1128/JVI.01002-13.
25. **Wille CK, Nawandar DM, Panfil AR, Ko MM, Hagemeier SR, Kenney SC.** 2013. Viral genome methylation differentially affects the ability of BZLF1 versus BRLF1 to activate Epstein-Barr virus lytic gene expression and viral replication. J Virol **87:**935–950. http://dx.doi.org/10.1128/JVI.01790-12.
26. **Hardwick JM, Lieberman PM, Hayward SD.** 1988. A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. J Virol **62:**2274–2284.
27. **Cox MA, Leahy J, Hardwick JM.** 1990. An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators. J Virol **64:**313–321.
28. **Ragoczy T, Miller G.** 1999. Role of the Epstein-Barr virus RTA protein in activation of distinct classes of viral lytic cycle genes. J Virol **73:**9858–9866.
29. **Miller G, El-Guindy A, Countryman J, Ye J, Gradoville L.** 2007. Lytic cycle switches of oncogenic human gammaherpesviruses. Adv Cancer Res **97:**81–109. http://dx.doi.org/10.1016/S0065-230X(06)97004-3.
30. **Speck SH, Chatila T, Flemington E.** 1997. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. Trends Microbiol **5:**399–405. http://dx.doi.org/10.1016/S0966-842X(97)01129-3.
31. **Murata T.** 2014. Regulation of Epstein-Barr virus reactivation from latency. Microbiol Immunol **58:**307–317. http://dx.doi.org/10.1111/1348-0421.12155.
32. **Murata T, Tsurumi T.** 2014. Switching of EBV cycles between latent and lytic states. Rev Med Virol **24:**142–153. http://dx.doi.org/10.1002/rmv.1780.
33. **Kenney SC, Mertz JE.** 2014. Regulation of the latent-lytic switch in Epstein-Barr virus. Semin Cancer Biol **26:**60–68. http://dx.doi.org/10.1016/j.semcancer.2014.01.002.
34. **Ragoczy T, Miller G.** 2001. Autostimulation of the Epstein-Barr virus BRLF1 promoter is mediated through consensus Sp1 and Sp3 binding sites. J Virol **75:**5240–5251. http://dx.doi.org/10.1128/JVI.75.11.5240-5251.2001.
35. **Liu P, Speck SH.** 2003. Synergistic autoactivation of the Epstein-Barr virus immediate-early BRLF1 promoter by Rta and Zta. Virology **310:**199–206. http://dx.doi.org/10.1016/S0042-6822(03)00145-4.
36. **Laichalk LL, Thorley-Lawson DA.** 2005. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. J Virol **79:**1296–1307. http://dx.doi.org/10.1128/JVI.79.2.1296-1307.2005.
37. **Young LS, Lau R, Rowe M, Niedobitek G, Packham G, Shanahan F, Rowe DT, Greenspan D, Greenspan JS, Rickinson AB.** 1991. Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. J Virol **65:**2868–2874.
38. **Bhende PM, Dickerson SJ, Sun X, Feng W-H, Kenney SC.** 2007. X-box-binding protein 1 activates lytic Epstein-Barr virus gene expression in combination with protein kinase D. J Virol **81:**7363–7370. http://dx.doi.org/10.1128/JVI.00154-07.
39. **Sun CC, Thorley-Lawson DA.** 2007. Plasma cell-specific transcription factor XBP-1s binds to and transactivates the Epstein-Barr virus BZLF1 promoter. J Virol **81:**13566–13577. http://dx.doi.org/10.1128/JVI.01055-07.
40. **Turner CA, Jr, Mack DH, Davis MM.** 1994. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. Cell **77:**297–306. http://dx.doi.org/10.1016/0092-8674(94)90321-2.
41. **Shapiro-Shelef M, Lin K-I, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K.** 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. Immunity **19:**607–620. http://dx.doi.org/10.1016/S1074-7613(03)00267-X.
42. **Magnúsdóttir E, Kalachikov S, Mizukoshi K, Savitsky D, Ishida-Yamamoto A, Panteleyev AA, Calame K.** 2007. Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1. Proc Natl Acad Sci U S A **104:**14988–14993. http://dx.doi.org/10.1073/pnas.0707323104.
43. **Chiang M-F, Yang S-Y, Lin I-Y, Hong J-B, Lin S-J, Ying H-Y, Chen C-M, Wu S-Y, Liu F-T, Lin K-I.** 2013. Inducible deletion of the Blimp-1 gene in adult epidermis causes granulocyte-dominated chronic skin inflammation in mice. Proc Natl Acad Sci U S A **110:**6476–6481. http://dx.doi.org/10.1073/pnas.1219462110.
44. **Keller AD, Maniatis T.** 1992. Only two of the five zinc fingers of the eukaryotic transcriptional repressor PRDI-BF1 are required for sequence-specific DNA binding. Mol Cell Biol **12:**1940–1949.
45. **Kuo TC, Calame KL.** 2004. B lymphocyte-induced maturation protein (Blimp)-1, IFN regulatory factor (IRF)-1, and IRF-2 can bind to the same regulatory sites. J Immunol **173:**5556–5563. http://dx.doi.org/10.4049/jimmunol.173.9.5556.
46. **Lin Y, Wong K, Calame K.** 1997. Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. Science **276:**596–599. http://dx.doi.org/10.1126/science.276.5312.596.
47. **Shaffer AL, Lin K-I, Kuo TC, Yu X, Hurt EM, Rosenwald A, Giltnane JM, Yang L, Zhao H, Calame K, Staudt LM.** 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity **17:**51–62. http://dx.doi.org/10.1016/S1074-7613(02)00335-7.
48. **Lin K-I, Angelin-Duclos C, Kuo TC, Calame K.** 2002. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. Mol Cell Biol **22:**4771–4780. http://dx.doi.org/10.1128/MCB.22.13.4771-4780.2002.
49. **Yan J, Jiang J, Lim CA, Wu Q, Ng H-H, Chin K-C.** 2007. BLIMP1 regulates cell growth through repression of p53 transcription. Proc Natl Acad Sci U S A **104:**1841–1846. http://dx.doi.org/10.1073/pnas.0605562104.
50. **Keller AD, Maniatis T.** 1991. Identification and characterization of a novel repressor of beta-interferon gene expression. Genes Dev **5:**868–879. http://dx.doi.org/10.1101/gad.5.5.868.
51. **Ren B, Chee KJ, Kim TH, Maniatis T.** 1999. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. Genes Dev **13:**125–137. http://dx.doi.org/10.1101/gad.13.1.125.
52. **Chen G, Fernandez J, Mische S, Courey AJ.** 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor Groucho in *Drosophila* development. Genes Dev **13:**2218–2230. http://dx.doi.org/10.1101/gad.13.17.2218.
53. **Yu J, Angelin-Duclos C, Greenwood J, Liao J, Calame K.** 2000. Transcriptional repression by Blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. Mol Cell Biol **20:**2592–2603. http://dx.doi.org/10.1128/MCB.20.7.2592-2603.2000.
54. **Gyory I, Wu J, Fejér G, Seto E, Wright KL.** 2004. PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. Nat Immunol **5:**299–308. http://dx.doi.org/10.1038/ni1046.
55. **Robinson AR, Kwek SS, Kenney SC.** 2012. The B-cell specific transcription factor, Oct-2, promotes Epstein-Barr virus latency by inhibiting the viral immediate-early protein, BZLF1. PLoS Pathog **8:**e1002516. http://dx.doi.org/10.1371/journal.ppat.1002516.
56. **Raver RM, Panfil AR, Hagemeier SR, Kenney SC.** 2013. The B-cell-specific transcription factor and master regulator Pax5 promotes Epstein-Barr virus latency by negatively regulating the viral immediate early protein BZLF1. J Virol **87:**8053–8063. http://dx.doi.org/10.1128/JVI.00546-13.
57. **Nera K-P, Kohonen P, Narvi E, Peippo A, Mustonen L, Terho P, Koskela K, Buerstedde J-M, Lassila O.** 2006. Loss of Pax5 promotes plasma cell differentiation. Immunity **24:**283–293. http://dx.doi.org/10.1016/j.immuni.2006.02.003.
58. **Vrzalikova K, Vockerodt M, Leonard S, Bell A, Wei W, Schrader A, Wright KL, Kube D, Rowe M, Woodman CB, Murray PG.** 2011. Down-regulation of BLIMP1α by the EBV oncogene, LMP-1, disrupts the plasma cell differentiation program and prevents viral replication in B cells: implications for the pathogenesis of EBV-associated B-cell lymphomas. Blood **117:**5907–5917. http://dx.doi.org/10.1182/blood-2010-09-307710.

59. **Buettner M, Lang A, Tudor CS, Meyer B, Cruchley A, Barros MHM, Farrell PJ, Jäck H-M, Schuh W, Niedobitek G.** 2012. Lytic Epstein-Barr virus infection in epithelial cells but not in B-lymphocytes is dependent on Blimp1. J Gen Virol **93:**1059–1064. http://dx.doi.org/10.1099/vir.0.038661-0.
60. **Park JG, Yang HK, Kim WH, Chung JK, Kang MS, Lee JH, Oh JH, Park HS, Yeo KS, Kang SH, Song SY, Kang YK, Bang YJ, Kim YH, Kim JP.** 1997. Establishment and characterization of human gastric carcinoma cell lines. Int J Cancer **70:**443–449. http://dx.doi.org/10.1002/(SICI)1097-0215(19970207)70:4<443::AID-IJC12>3.0.CO;2-G.
61. **Strong MJ, Baddoo M, Nanbo A, Xu M, Puetter A, Lin Z.** 2014. Comprehensive high-throughput RNA sequencing analysis reveals contamination of multiple nasopharyngeal carcinoma cell lines with HeLa cell genomes. J Virol **88:**10696–10704. http://dx.doi.org/10.1128/JVI.01457-14.
62. **Kelly G, Bell A, Rickinson A.** 2002. Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. Nat Med **8:**1098–1104. http://dx.doi.org/10.1038/nm758.
63. **Kelly GL, Stylianou J, Rasaiyaah J, Wei W, Thomas W, Croom-Carter D, Kohler C, Spang R, Woodman C, Kellam P, Rickinson AB, Bell AI.** 2013. Different patterns of Epstein-Barr virus latency in endemic Burkitt lymphoma (BL) lead to distinct variants within the BL-associated gene expression signature. J Virol **87:**2882–2894. http://dx.doi.org/10.1128/JVI.03003-12.
64. **Györy I, Fejér G, Ghosh N, Seto E, Wright KL.** 2003. Identification of a functionally impaired positive regulatory domain I binding factor 1 transcription repressor in myeloma cell lines. J Immunol **170:**3125–3133. http://dx.doi.org/10.4049/jimmunol.170.6.3125.
65. **Ghosh N, Gyory I, Wright G, Wood J, Wright KL.** 2001. Positive regulatory domain I binding factor 1 silences class II transactivator expression in multiple myeloma cells. J Biol Chem **276:**15264–15268. http://dx.doi.org/10.1074/jbc.M100862200.
66. **Sarisky RT, Gao Z, Lieberman PM, Fixman ED, Hayward GS, Hayward SD.** 1996. A replication function associated with the activation domain of the Epstein-Barr virus Zta transactivator. J Virol **70:**8340–8347.
67. **Ghosh N, Piskurich JF, Wright G, Hassani K, Ting JP-Y, Wright KL.** 1999. A novel element and a TEF-2-like element activate the major histocompatibility complex class II transactivator in B-lymphocytes. J Biol Chem **274:**32342–32350. http://dx.doi.org/10.1074/jbc.274.45.32342.
68. **Klug M, Rehli M.** 2006. Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. Epigenetics **1:**127–130. http://dx.doi.org/10.4161/epi.1.3.3327.
69. **Adamson AL, Kenney S.** 2001. Epstein-Barr virus immediate-early protein BZLF1 is SUMO-1 modified and disrupts promyelocytic leukemia bodies. J Virol **75:**2388–2399. http://dx.doi.org/10.1128/JVI.75.5.2388-2399.2001.
70. **Altmann M, Hammerschmidt W.** 2005. Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. PLoS Biol **3:**e404. http://dx.doi.org/10.1371/journal.pbio.0030404.
71. **Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, Nibbs RJ, Freedman BI, Quinones MP, Bamshad MJ, Murthy KK, Rovin BH, Bradley W, Clark RA, Anderson SA, O'Connell RJ, Agan BK, Ahuja SS, Bologna R, Sen L, Dolan MJ, Ahuja SK.** 2005. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science **307:**1434–1440. http://dx.doi.org/10.1126/science.1101160.
72. **Schreiber E, Matthias P, Muller MM, Schaffner W.** 1989. Rapid detection of octamer binding proteins with "mini-extracts," prepared from a small number of cells. Nucleic Acids Res **17:**6419. http://dx.doi.org/10.1093/nar/17.15.6419.
73. **Kraus RJ, Mirocha SJ, Stephany HM, Puchalski JR, Mertz JE.** 2001. Identification of a novel element involved in regulation of the lytic switch BZLF1 gene promoter of Epstein-Barr virus. J Virol **75:**867–877. http://dx.doi.org/10.1128/JVI.75.2.867-877.2001.
74. **Sarbassov DD, Guertin DA, Ali SM, Sabatini DM.** 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science **307:**1098–1101. http://dx.doi.org/10.1126/science.1106148.
75. **zur Hausen H, Bornkamm GW, Schmidt R, Hecker E.** 1979. Tumor initiators and promoters in the induction of Epstein-Barr virus. Proc Natl Acad Sci U S A **76:**782–785. http://dx.doi.org/10.1073/pnas.76.2.782.
76. **Flemington E, Speck SH.** 1990. Identification of phorbol ester response elements in the promoter of Epstein-Barr virus putative lytic switch gene BZLF1. J Virol **64:**1217–1226.
77. **Davies AH, Grand RJ, Evans FJ, Rickinson AB.** 1991. Induction of Epstein-Barr virus lytic cycle by tumor-promoting and non-tumor-promoting phorbol esters requires active protein kinase C. J Virol **65:**6838–6844.
78. **Bhende PM, Seaman WT, Delecluse H-J, Kenney SC.** 2004. The EBV lytic switch protein, Z, preferentially binds to and activates the methylated viral genome. Nat Genet **36:**1099–1104. http://dx.doi.org/10.1038/ng1424.
79. **Fernandez AF, Rosales C, Lopez-Nieva P, Graña O, Ballestar E, Ropero S, Espada J, Melo SA, Lujambio A, Fraga MF, Pino I, Javierre B, Carmona FJ, Acquadro F, Steenbergen RDM, Snijders PJF, Meijer CJ, Pineau P, Dejean A, Lloveras B, Capella G, Quer J, Buti M, Esteban J-I, Allende H, Rodriguez-Frias F, Castellsague X, Minarovits J, Ponce J, Capello D, Gaidano G, Cigudosa JC, Gomez-Lopez G, Pisano DG, Valencia A, Piris MA, Bosch FX, Cahir-McFarland E, Kieff E, Esteller M.** 2009. The dynamic DNA methylomes of double-stranded DNA viruses associated with human cancer. Genome Res **19:**438–451. http://dx.doi.org/10.1101/gr.083550.108.
80. **Bergbauer M, Kalla M, Schmeinck A, Göbel C, Rothbauer U, Eck S, Benet-Pagès A, Strom TM, Hammerschmidt W.** 2010. CpG-methylation regulates a class of Epstein-Barr virus promoters. PLoS Pathog **6:**e1001114. http://dx.doi.org/10.1371/journal.ppat.1001114.
81. **John SA, Garrett-Sinha LA.** 2009. Blimp1: a conserved transcriptional repressor critical for differentiation of many tissues. Exp Cell Res **315:**1077–1084. http://dx.doi.org/10.1016/j.yexcr.2008.11.015.
82. **Vrzalikova K, Woodman CBJ, Murray PG.** 2012. BLIMP1α, the master regulator of plasma cell differentiation is a tumor suppressor gene in B cell lymphomas. Biomed Pap Med Fac Univ Palacký Olomouc Czech Repub **156:**1–6. http://dx.doi.org/10.5507/bp.2012.003.
83. **Smith MA, Wright G, Wu J, Tailor P, Ozato K, Chen X, Wei S, Piskurich JF, Ting JP-Y, Wright KL.** 2011. Positive regulatory domain I (PRDM1) and IRF8/PU.1 counter-regulate MHC class II transactivator (CIITA) expression during dendritic cell maturation. J Biol Chem **286:**7893–7904. http://dx.doi.org/10.1074/jbc.M110.165431.
84. **Terzaghi M, Klein-Szanto AJ.** 1980. Differentiation of normal and cultured preneoplastic tracheal epithelial cells in rats: importance of epithelial mesenchymal interactions. J Natl Cancer Inst **65:**1039–1048.
85. **Tötterman TH, Nilsson K, Sundström C.** 1980. Phorbol ester-induced differentiation of chronic lymphocytic leukaemia cells. Nature **288:**176–178.
86. **Hawley-Nelson P, Stanley JR, Schmidt J, Gullino M, Yuspa SH.** 1982. The tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate accelerates keratinocyte differentiation and stimulates growth of an unidentified cell type in cultured human epidermis. Exp Cell Res **137:**155–167.
87. **Anisimová E, Prachová K, Roubal J, Vonka V.** 1984. Effects of n-butyrate and phorbol ester (TPA) on induction of Epstein-Barr virus antigens and cell differentiation. Arch Virol **81:**223–237.
88. **Jaken S, Yuspa SH.** 1988. Early signals for keratinocyte differentiation: role of Ca^{2+}-mediated inositol lipid metabolism in normal and neoplastic epidermal cells. Carcinogenesis **9:**1033–1038.
89. **John SA, Clements JL, Russell LM, Garrett-Sinha LA.** 2008. Ets-1 regulates plasma cell differentiation by interfering with the activity of the transcription factor Blimp-1. J Biol Chem **283:**951–962. http://dx.doi.org/10.1074/jbc.M705262200.
90. **John S, Russell L, Chin SS, Luo W, Oshima R, Garrett-Sinha LA.** 2014. Transcription factor Ets1, but not the closely related factor Ets2, inhibits antibody-secreting cell differentiation. Mol Cell Biol **34:**522–532. http://dx.doi.org/10.1128/MCB.00612-13.
91. **Raghunandan R, Frissora FW, Muthusamy N.** 2013. Modulation of Ets-1 expression in B lymphocytes is dependent on the antigen receptor-mediated activation signals and cell cycle status. Scand J Immunol **77:**75–83. http://dx.doi.org/10.1111/sji.12012.
92. **Luo W, Mayeux J, Gutierrez T, Russell L, Getahun A, Müller J, Tedder T, Parnes J, Rickert R, Nitschke L, Cambier J, Satterthwaite AB, Garrett-Sinha LA.** 2014. A balance between B cell receptor and inhibitory receptor signaling controls plasma cell differentiation by maintaining optimal Ets1 levels. J Immunol **193:**909–920. http://dx.doi.org/10.4049/jimmunol.1400666.
93. **Vrzalikova K, Leonard S, Fan Y, Bell A, Vockerodt M, Flodr P, Wright KL, Rowe M, Tao Q, Murray PG.** 2012. Hypomethylation and overexpression of the beta isoform of BLIMP1 is induced by Epstein-Barr virus infection of B cells; potential implications for the pathogenesis of EBV-associated lymphomas. Pathogens **1:**83–101. http://dx.doi.org/10.3390/pathogens1020083.
94. **Huang S, Shao G, Liu L.** 1998. The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. J Biol Chem **273:**15933–15939.