JVI Journals.ASM.org

Screening of the Human Kinome Identifies MSK1/2-CREB1 as an Essential Pathway Mediating Kaposi's Sarcoma-Associated Herpesvirus Lytic Replication during Primary Infection

Fan Cheng,[a] Tanvee Vinod Sawant,[a] Ke Lan,[b] Chun Lu,[c] Jae U. Jung,[a] Shou-Jiang Gao[a]

Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, California, USA[a]; Unit of Tumor Virology, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, People's Republic of China[b]; Department of Microbiology and Immunology, Nanjing Medical University, Nanjing, People's Republic of China[c]

ABSTRACT

Viruses often hijack cellular pathways to facilitate infection and replication. Kaposi's sarcoma-associated herpesvirus (KSHV) is an oncogenic gammaherpesvirus etiologically associated with Kaposi's sarcoma, a vascular tumor of endothelial cells. Despite intensive studies, cellular pathways mediating KSHV infection and replication are still not well defined. Using an antibody array approach, we examined cellular proteins phosphorylated during primary KSHV infection of primary human umbilical vein endothelial cells. Enrichment analysis identified integrin/mitogen-activated protein kinase (integrin/MAPK), insulin/epidermal growth factor receptor (insulin/EGFR), and JAK/STAT as the activated networks during primary KSHV infection. The transcriptional factor CREB1 (cyclic AMP [cAMP]-responsive element-binding protein 1) had the strongest increase in phosphorylation. While knockdown of CREB1 had no effect on KSHV entry and trafficking, it drastically reduced the expression of lytic transcripts and proteins and the production of infectious virions. Chemical activation of CREB1 significantly enhanced viral lytic replication. In contrast, CREB1 neither influenced the expression of the latent gene LANA nor affected KSHV infectivity. Mechanistically, CREB1 was not activated through the classic cAMP/protein kinase A (cAMP/PKA) pathway or via the AKT, MK2, and RSK pathways. Rather, CREB1 was activated by the mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2). Consequently, chemical inhibition or knockdown of MSKs significantly inhibited the KSHV lytic replication program; however, it had a minimal effect on LANA expression and KSHV infectivity. Together, these results identify the MSK1/2-CREB1 proteins as novel essential effectors of KSHV lytic replication during primary infection. The differential effect of the MSK1/2-CREB1 pathway on the expression of viral latent and lytic genes might control the robustness of viral lytic replication, and therefore the KSHV replication program, during primary infection.

IMPORTANCE

Kaposi's sarcoma-associated herpesvirus (KSHV) is a human tumor virus associated with several cancers. Through genome-wide kinase screening, we found that KSHV activates the MSK1/2-CREB1 pathway during primary infection and that it depends on this pathway for viral lytic replication. Inhibition of this pathway blocks KSHV lytic replication. These results illustrate a mechanism by which KSHV hijacks a cellular pathway for its replication, and they identify a potential therapeutic target.

Viruses depend on cell signaling pathways for successful infection and replication. Identification of pathways hijacked by viruses not only reveals the mechanisms of infection and replication of these viruses but also provides novel therapeutic targets. Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus etiologically associated with Kaposi's sarcoma (KS), a vascular tumor of endothelial cells commonly found in AIDS patients, and with two B-cell lymphoproliferative diseases, namely, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (1–3). The early phase of primary KSHV infection is a highly regulated multistep event consisting of virion attachment and binding, membrane fusion, internalization, intracellular trafficking, and early viral gene expression (4). KSHV infection induces phosphorylation of cellular proteins, leading to the activation of signal transduction pathways. A number of these pathways regulate KSHV entry, trafficking, and viral gene expression (4). Binding of KSHV glycoproteins to cellular receptors activates focal adhesion kinase (FAK), Src, phosphatidylinositol-3-kinases (PI3Ks), and mitogen-activated protein kinases (MAPKs), including MEK/extracellular signal-regulated kinase (MEK/ERK), p38, and Jun N-terminal kinase (JNK), facilitating KSHV internalization and trafficking (5–10). This process depends on rearrangements of the actin and microtubule cytoskeletons and on factors, such as Rho-GTPases and diaphanous-2 (Dia-2), that regulate their dynamics (8, 11). KSHV entry and trafficking rely on the dynamics of the ubiquitin/proteasome system and on activation of the E3 ligase c-Cbl to maintain the endosomal activities and cellular signaling (12, 13).

Successful KSHV infection requires the coordinated expres-

Received 28 April 2015 Accepted 21 June 2015

Accepted manuscript posted online 24 June 2015

Citation Cheng F, Sawant TV, Lan K, Lu C, Jung JU, Gao S-J. 2015. Screening of the human kinome identifies MSK1/2-CREB1 as an essential pathway mediating Kaposi's sarcoma-associated herpesvirus lytic replication during primary infection. J Virol 89:9262–9280. doi:10.1128/JVI.01098-15.

Editor: R. M. Longnecker

Address correspondence to Shou-Jiang Gao, shoujiag@usc.edu.

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

doi:10.1128/JVI.01098-15

sion of viral genes. Whether KSHV enters into latency or undergoes lytic replication depends on the extent of expression of viral lytic genes. Several signaling pathways, including ERK, p38, and JNK pathways, promote the expression of viral lytic genes, while the NF-κB pathway promotes the expression of viral latent genes (9, 14, 15). In permissive primary human umbilical vein endothelial cells (HUVEC), in which the ERK, p38, and JNK pathways are highly activated upon KSHV infection, the virus undergoes robust productive lytic replication before entering into latency (9, 16, 17). In contrast, in nonpermissive primary human dermal microvascular endothelial cells (DMVEC) and human foreskin fibroblasts (HFFs), KSHV enters a default latency program with minimal viral lytic activity, which parallels the hyperactivation of the NF-κB pathway (15, 18–20). The ERK, p38, and JNK pathways promote lytic replication by activating AP-1 complexes to induce the expression of RTA (Orf50), the master transactivator of KSHV lytic replication, and other viral lytic genes (9, 21). On the other hand, the NF-κB pathway inhibits viral lytic replication by suppressing the AP-1 complexes (22).

Despite these observations, whether KSHV hijacks any other cellular pathways to promote infection and replication during primary infection has not been examined systematically. The present study aimed to identify additional essential signaling pathways and kinases that regulate primary KSHV infection. To this end, we performed an antibody-based kinome screening assay. This assay uses 334 phospho-site-specific antibodies against 287 different phosphorylation sites in ~200 protein kinases, phosphatases, and other cell signaling proteins that regulate cell proliferation, stress, and apoptosis. Our screen identified 29 phospho-sites on 24 kinases or signaling molecules whose phosphorylation levels were significantly altered at early time points (<15 min) of primary KSHV infection. These hits included protein kinase B (AKT1) and c-Jun, whose activation and roles in primary KSHV infection were previously reported (9, 15). Other hits, such as protein kinase C-δ (PKCδ), were previously implicated in KSHV reactivation (23), though the role of PKCδ in primary KSHV infection has not been explored.

The top hit, CREB1 (cyclic AMP [cAMP]-responsive element-binding protein 1), is a member of a subfamily of bZIP transcription factors that includes ATF1 and CREM. CREB1, through binding to a conserved DNA sequence called the cAMP-responsive element (CRE), acts downstream of a wide variety of signals in cells to mediate many functions, including immune function, memory, metabolism, and cell survival (24–28). CREB1 has been implicated in regulating viral gene expression of a number of viruses, including hepatitis B virus (HBV) (29), herpes simplex virus 1 (HSV-1) (30), human cytomegalovirus (HCMV) (31), and human T-cell leukemia virus (HTLV) (32, 33). A previous study showed that activation of the cAMP-dependent protein kinase A (cAMP/PKA) pathway, the classic CREB1 upstream pathway, is sufficient to reactivate KSHV from latency (34). However, it has been reported that KSHV infection of DMVEC is nonproductive, with minimal lytic activity (19), despite CREB1 activation via the cAMP/PKA pathway (35). Interestingly, KSHV infection of lymphatic endothelial cells (LEC) is also nonproductive (36), though it activates CREB1. This results in the induction of miR-132, which targets p300 to downregulate the proinflammatory cytokines interleukin-6 (IL-6), IL-1β, and beta interferon, thus relieving their suppressive effects on KSHV infection (37). However, IL-6 and IL-1β can activate the MAPK pathways and promote KSHV lytic replication (38). Therefore, the role of CREB1 in KSHV lytic replication remains controversial and has not been examined in primary KSHV infection.

The activity of CREB1 is regulated by various extracellular stimuli, including growth factors, cytokines, and stress responses. PKA, which is activated by cAMP, was the first kinase shown to phosphorylate CREB1, at Ser133 (39). PKA phosphorylation of CREB1 at Ser133 promotes the recruitment of the coactivator proteins CBP and p300 (39, 40). Several other kinases, such as calmodulin kinases (CaMKs) and AKT, downstream of the Ca^{2+} signaling and PI3K pathways, respectively, were subsequently identified to phosphorylate this site (24). The ERK and p38 MAPKs also induce CREB1 phosphorylation. However, in this case, CREB1 is not the direct phosphorylation target of these MAPKs; rather, CREB1 is phosphorylated by one of the downstream kinases, including the ~90-kDa ribosome S6 kinases (RSKs), the mitogen- and stress-activated kinases (MSK1 and MSK2), or MAPK-activated protein kinase 2 (MK2; also called MAPKAP-K2) (41). MSK-mediated CREB1 phosphorylation has been implicated in innate immunity, learning, memory, and addiction (25, 42, 43). A recent study showed that MSK-mediated CREB1 activation plays an important role in HCMV gene expression in lipopolysaccharide (LPS)-induced HCMV reactivation (44).

In the current study, we have shown that activation of CREB1 is essential for a productive KSHV lytic replication program but has no effect on KSHV infectivity or the expression of the latent gene LANA (Orf73) during primary infection of HUVEC. Interestingly, we found that CREB1 activation is independent of the cAMP/PKA pathway but depends on MSKs. Chemical inhibition or short hairpin RNA (shRNA)-mediated knockdown of MSKs inhibits the KSHV lytic replication program but has no effect on KSHV infectivity and the expression of the latent gene LANA. These results identify the MSK1/2-CREB1 proteins as novel critical effectors of ERK and p38 that are essential for KSHV lytic replication and show that the MSK1/2-CREB1 pathway differentially regulates the expression of viral latent and lytic genes to control the robustness of viral lytic replication, and therefore determine the KSHV replication program, during primary infection.

MATERIALS AND METHODS

Antibodies and reagents. A monoclonal antibody of isotype IgG2a (clone 6A) specific to the KSHV small capsid protein (Orf65) was used to stain KSHV particles (16). A rat anti-LANA monoclonal antibody was purchased from Abcam (Cambridge, MA). An anti-KSHV K-bZIP (Orf-K8) antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). A monoclonal antibody was used to detect RTA (45). The primary antibodies against phospho-CREB1 (Ser133), CREB1, PKA, phospho-MSK1 (Thr581), and MSK1 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to phospho-PKA (Ser338), phospho-MSK2 (Thr568), and MSK2 were purchased from Abcam. An antibody to β-tubulin was purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 568-conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-rat IgG, horseradish peroxidase (HRP)-conjugated goat anti-mouse, and HRP-conjugated goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology.

Kinase inhibitors and antagonists were obtained from the following sources: LY294002 and forskolin (FSK) were from VWR International, LLC (Radnor, PA), and SB747651A and PF3644022 were from Tocris (Minneapolis, MN).

Cell culture. Primary HUVEC were cultured in VascuLife VEGF complete medium (Lifeline Cell Technology, Frederick, MD). Recombinant

KSHV BAC16-infected iSLK (iSLK-BAC16) cells were maintained in the presence of 1 μg/ml puromycin, 250 μg/ml G418 (Sigma-Aldrich), and 1,200 μg/ml hygromycin B (46). Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin solution (Genesee Scientific, San Diego, CA).

Virus preparation. A volume of concentrated virus was prepared from iSLK-BAC16 cells as previously described (46). Briefly, iSLK-BAC16 cells were induced with both doxycycline (1 μg/ml) and sodium butyrate (1 mM) in the medium described above, but without hygromycin, puromycin, and G418. Four days later, the supernatant was collected, centrifuged at 5,000 × *g* for 10 min, and then filtered (0.45-μm pore size) to eliminate cell debris. Virus particles were pelleted by ultracentrifugation (100,000 × *g* for 1 h with a 20% sucrose cushion at 4°C), using an SW32 Ti rotor (Beckman Coulter Inc., Brea, CA). The final pellet was dissolved in culture medium overnight and used for infection after titration.

Virus titration. A fresh virus preparation or culture supernatant was titrated by infecting HUVEC as previously described (16, 47). Briefly, the virus preparation or supernatant was subjected to 2-fold serial dilution and used to infect HUVEC at 2×10^4 cells/well in a 24-well plate, at 20 μl/well, in the presence of Polybrene. Three to six infection repeats were carried out for each sample. Four hours following infection, the medium was replaced with fresh medium. The plates were examined with an inverted fluorescence microscope at 48 h postinfection to determine the number of cells expressing green fluorescent protein (GFP) in each well. Infectious units (IUs) were calculated based on the number of GFP-positive cells generated with a 1-ml sample.

Virus infection. We conducted primary infection of HUVEC to examine factors that might regulate KSHV infection and replication. Fresh virus preparations with a titer of 2×10^6 IUs were used in the experiments. HUVEC were infected with KSHV as previously described (16, 47). For all experiments, cells were infected at a multiplicity of infection (MOI) of 2 IUs per cell unless specified otherwise. To prepare a replication-defective virus, KSHV was exposed to a UV source for 15 min, which reduced virus infectivity from 70 to 80% to less than 1%. To determine the effects of kinase inhibitors or agonists on KSHV infection, HUVEC grown to confluence in 36-mm dishes were first serum starved for 4 h and then treated with kinase inhibitors or agonists (LY294002, SB747651A, PF3644022, or FSK) for 1 h at 37°C prior to KSHV infection. Cells were then infected with KSHV in the presence of the inhibitors or agonists. Cells were collected at different time points, as specified, and used to examine the expression of viral transcripts and proteins. To examine the production of infectious virions, supernatants were collected at day 4 postinfection and subjected to titration as described in the previous section.

Kinex antibody microarray and data analysis. HUVEC that were serum starved for 4 h were mock infected or infected with KSHV for 5, 10, and 15 min. Cells were rinsed with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.2), and cell lysates were collected following the manufacturer's instructions (Kinexus, Vancouver, British Columbia, Canada). Pooled KSHV-infected and mock-infected samples were examined for phosphorylated proteins by using Kinex KAM-1.2FN 800 Ab antibody microarrays (Kinexus). Briefly, 50 μg of cell lysate from each sample was covalently labeled with a proprietary fluorescent dye (Kinexus). Free dye molecules were then removed by gel filtration at the completion of labeling reactions. After blocking of nonspecific binding sites, an incubation chamber was mounted onto the microarray to permit the loading of the KSHV-infected and mock-infected samples side by side on the same chip and to prevent mixing of the samples. Following sample incubation, unbound proteins were washed away. Each array produced a pair of 16-bit images, which were captured with a Perkin-Elmer ScanArray Reader laser array scanner (Waltham, MA). Signal quantification was performed with ImaGene 8.0 from BioDiscovery (El Segundo, CA), using the predetermined settings for spot segmentation and background correction.

The array signal in each sample was corrected to the array background and $\log_2$ transformed, and a Z score was calculated using the following formula: $Z = (X - \mu)/\sigma$, where Z is the Z score, X is the value of the sample, μ is the population mean, and σ is the standard deviation. Thus, the Z score (or standard score) is a statistical measurement of a sample value's relationship to the mean. It indicates how many standard deviations a sample value is from the mean. The Z ratio was further calculated by taking the difference between the average observed protein Z scores for the mock-infected and KSHV-infected groups and dividing it by the standard deviation of all the differences for that particular comparison. Kinases or signaling molecules were considered to be hits if the calculated Z ratio was ≥ 1 or ≤ -1 and the variation between adjacent duplicate spots of the same antibody (% error range) was less than 30%. All the hits, together with previously published kinases and signaling molecules activated during primary KSHV infection, were analyzed for the enrichment of networks/pathways by Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA).

Immunofluorescence assay (IFA). For the detection of CREB1 activation following primary KSHV infection, HUVEC infected with KSHV for specified times were fixed in 4% paraformaldehyde for 10 min. Following three washes with PBS, the cells were incubated with a rabbit anti-phospho-CREB1 monoclonal antibody (Cell Signaling Technology) at a 1:800 dilution for 60 min. The cells were then washed three times with PBS, followed by incubation with an Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibody for 60 min. The cells were again washed with PBS three times and then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were visualized with a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments, Inc., Melville, NY).

The KSHV LANA protein was detected as previously described (48), with minor modifications. At 48 h postinfection, KSHV-infected cells were fixed in methanol for 10 min. Following three washes with PBS, the cells were incubated with a rat anti-LANA monoclonal antibody (Abcam) at a 1:500 dilution for 60 min. The cells were then washed three times with PBS, followed by incubation with an Alexa Fluor 568-conjugated goat anti-rat IgG secondary antibody for 60 min. The cells were again washed with PBS three times and then stained with DAPI.

The method for examining virus entry and trafficking was described previously (12). Briefly, following shRNA knockdown of CREB1 or MSKs or treatment with an MSK inhibitor, the cells were inoculated with virus and incubated for 6 h in the presence of the inhibitors, fixed in 2% paraformaldehyde, and processed for immunostaining of KSHV particles by use of an anti-Orf65 antibody. The numbers of virus particles docked at the perinuclear regions were calculated.

Western blot analysis. Cells infected with KSHV were collected for analysis at the specified times. Total protein preparations were separated in sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and detected with antibodies. Specific signals were revealed with chemiluminescence substrates and recorded using a UVP MultiSpectral imaging system (UVP LLC, Upland, CA).

RT-qPCR. The expression levels of viral genes were analyzed by reverse transcription–quantitative real-time PCR (RT-qPCR) using previously described procedures (17). Briefly, total RNAs from KSHV-infected HUVEC were prepared with Tri reagent as recommended by the manufacturer (Sigma). The RNA was treated with RNase-free DNase (Thermo Fisher Scientific, Inc., Waltham, WA) and reverse transcribed to obtain the first-strand cDNA by use of the Maxima reverse transcriptase system (Thermo Fisher Scientific, Inc.). For each sample, a control without reverse transcriptase was conducted in parallel. qPCR was then performed with the cDNA, using the gene-specific PCR primers described in Table 1. α-Tubulin was used as the internal control. Each sample was assayed in three repeats.

shRNA knockdown. shRNA plasmids were constructed by inserting annealed oligonucleotides containing the shRNA sequences specific for the target genes into the EcoRI and AgeI sites downstream of the U6 promoter in the pLKO.1 vector (Sigma-Aldrich). Recombinant lentivi-

TABLE 1 Sequences of primers used for RT-qPCR

Gene	Primer sequence (5′-3′)	
	Forward	Reverse
CREB1	TTAACCATGACCAATGCAGCA	TGGTATGTTTGTACGTCTCCAGA
α-Tubulin	AGATCATTGACCTCGTGTTGGA	ACCAGTTCCCCCACCAAAG
Orf50 (RTA)	CACAAAAATGGCGCAAGATGA	TGGTAGAGTTGGGCCTTCAGTT
Orf-K8 (K-bZIP)	CATGCTGATGCGAATGTGC	AGCTTCAACATGGTGGGAGTG
Orf65	ATATGTCGCAGGCCGAATAC	CCACCCATCCTCCTCAGATA
Orf73 (LANA)	CCAGGAAGTCCCACAGTGTT	AGACACAGGATGGGATGGAG

ruses carrying shRNAs were produced by cotransfecting 293T cells with a mixture of plasmid DNAs consisting of pMD-G (vesicular stomatitis virus glycoprotein [VSV-G] envelope), pCMV-ψR8.91 (Gag/Pol/Rev), and the pLKO/1-shRNA vectors (Aldevron, Fargo, ND), using the Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Virus-containing culture supernatants were collected 2 days after transfection and concentrated.

Knockdown with shRNAs was performed as previously described (49). Briefly, HUVEC were infected with shRNA-carrying lentiviruses in the presence of 10 μg/ml Polybrene (Sigma-Aldrich). Stable cell lines were selected by culturing the cells with 1.0 μg/ml puromycin (Calbiochem, La Jolla, CA) for 5 days. Western blotting or RT-qPCR was used to determine the effect of shRNA knockdown. The CREB1 shRNA knockdown sequences were GCCTGCAAACATTAACCATGA for shRNA1 and ACGGTGCCAACTCCAATTTAC for shRNA2; the MSK shRNA knockdown sequences were as follows: AGCAACCTTCCACGCCTTTAA (MSK1) and GCCACCTTCATGGCATTCAAC (MSK2) for shMixed1 and ACCTATGACTTGTTTGGAAAT (MSK1) and GCAGGGTGTATCCGAGGAAGC (MSK2) for shMixed2.

RESULTS

Kinex antibody array assay identifies signaling molecules activated at early time points during primary KSHV infection. We conducted an antibody-based screen using a Kinex antibody microarray to identify kinases and signaling molecules that had significant changes in phosphorylation levels at early time points during primary KSHV infection. KSHV infection of HUVEC is productive, resulting in an active viral lytic transcriptional program and the production of infectious virions (16, 17). Serum-starved HUVEC were mock infected or infected with KSHV for 5, 10, and 15 min. We selected three time points in order to capture the phosphorylation and dephosphorylation events that occurred immediately following KSHV infection. Cell lysates collected at

TABLE 2 Kinex antibody microarray analysis results

Target protein	Phospho-site	Full target protein name	Z ratio
CREB1	S129 + S133	cAMP response element-binding protein 1	3.39
JAK2	Y1007 + Y1008	Janus protein tyrosine kinase 2	2.74
JAK1	Y1034	Janus protein tyrosine kinase 1	2.64
IRS1	Y612	Insulin receptor substrate 1	2.41
PKCδ	T507	Protein serine kinase C delta	2.41
IκBε	S22	NF-κB inhibitor epsilon	2.22
PKBα (AKT1)	S473	Protein serine kinase B alpha	2.19
IRS1	S639	Insulin receptor substrate 1	1.83
Integrin-β1	S785	Integrin beta 1 (fibronectin receptor beta subunit, CD29 antigen)	1.77
eIF4B	S422	Eukaryotic translation initiation factor 4B	1.74
IR (INSR)	Y999	Insulin receptor	1.71
c-Jun	S243	Jun proto-oncogene-encoded AP1 transcription factor	1.61
Hsp27	S82	27-kDa heat shock protein beta 1 (HspB1)	1.49
PKBα (AKT1)	S473	Protein serine kinase B alpha	1.49
EGFR	Y1092	Epidermal growth factor receptor tyrosine kinase	1.48
c-Kit	Y721	Kit/Steel factor receptor tyrosine kinase	1.43
IκBα	Y42	Inhibitor of NF-κB alpha (MAD3)	1.41
eIF2α	S52	Eukaryotic translation initiation factor 2 alpha	1.41
c-Jun	T91	Jun proto-oncogene-encoded AP1 transcription factor	1.39
ZAP70/Syk	Y319/Y352	Zeta-chain (TCR)-associated protein tyrosine kinase, 70-kDa/spleen protein tyrosine kinase	1.38
PKBα (AKT1)	T308	Protein serine kinase B alpha	1.37
EFNB2	Y316	EPH-related receptor tyrosine kinase ligand 5	1.23
eIF2α	S52	Eukaryotic translation initiation factor 2 alpha	1.22
c-Kit	Y936	Kit/Steel factor receptor tyrosine kinase	1.21
c-Jun	S63	Jun proto-oncogene-encoded AP1 transcription factor	1.15
FRS2	Y348	Fibroblast growth factor receptor substrate 2	−1.07
GAP-43	S41	Growth-associated protein 43 (neuromodulin)	−1.11
PKCγ	T655	Protein serine kinase C gamma	−1.22
STAT5A	S780	Signal transducer and activator of transcription 5A	−1.31
p53	S392	Tumor suppressor protein p53 (antigen NY-CO-13)	−2.04

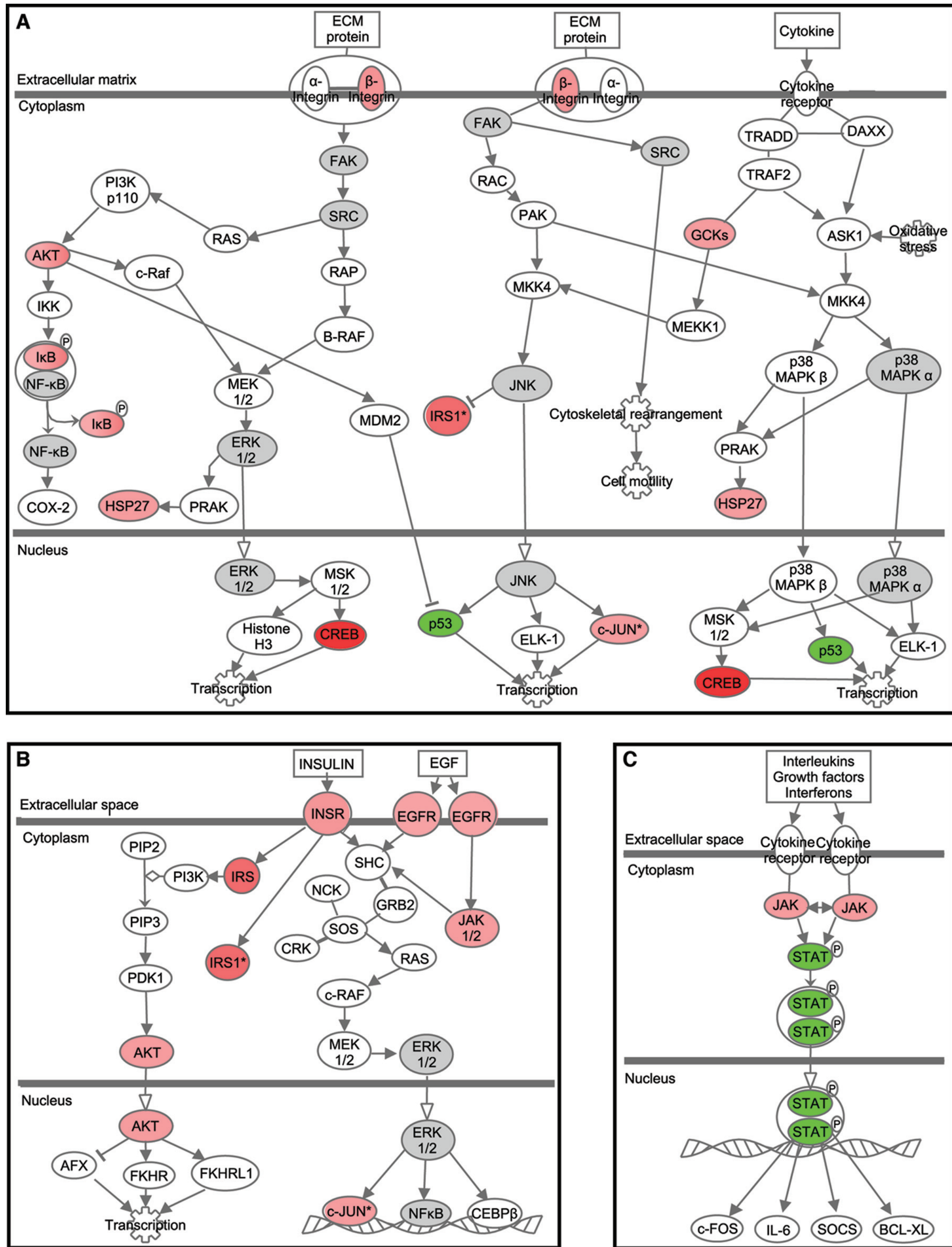

FIG 1 Signaling networks activated during primary KSHV infection as analyzed by Kinex antibody array and IPA. (A to C) Signaling networks activated during primary KSHV infection. Serum-starved HUVEC were mock infected or infected with KSHV for 5, 10, and 15 min. Cell lysates were collected, pooled, and analyzed with a Kinex antibody microarray. The antibody array data shown in Table 2, as well as kinases or signaling molecules that were previously reported to be activated during primary KSHV infection, were used as inputs and analyzed by IPA. The top enriched networks and their associated pathways were the integrin/MAPK network (A), the insulin/EGFR network (B), and the JAK/STAT network (C). Kinases or signaling molecules whose phosphorylation levels were increased by KSHV infection are shown in red, while those with a decreased phosphorylation status are shown in green, with the color intensities correlating with the levels of changes. The kinases or signaling molecules that were previously reported to be activated by KSHV infection are shown in gray, while those that were not known to be regulated by KSHV during primary infection are shown in white. ECM, extracellular matrix.

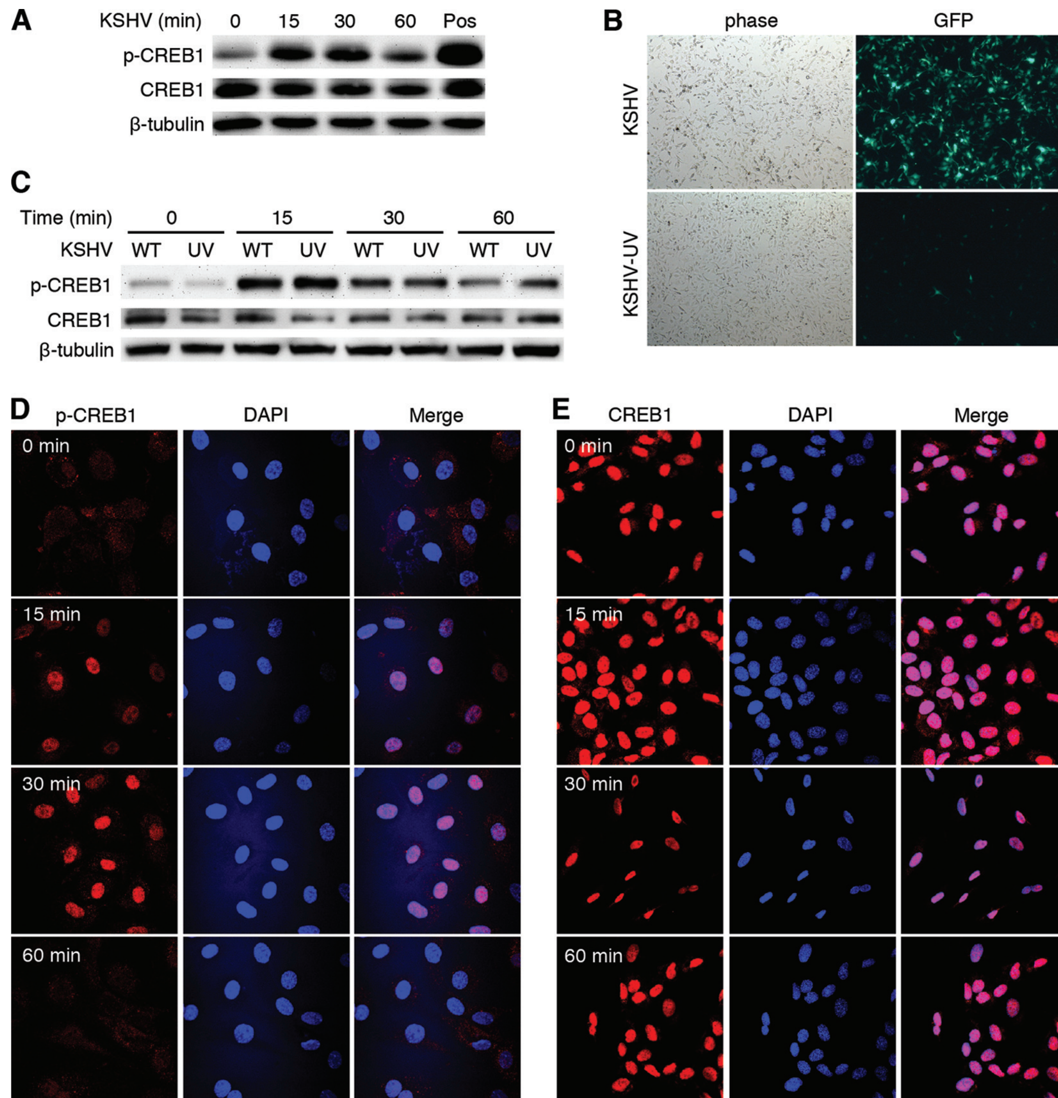

FIG 2 CREB1 is activated during primary KSHV infection. (A) Kinetics of CREB1 activation during primary KSHV infection. HUVEC were infected with KSHV and collected at the indicated times postinfection. Cells subjected to UV treatment for 30 min were used as a positive control (Pos). Whole-cell lysates were collected for Western blot analysis of phospho-CREB1 (S133) (p-CREB1) and total CREB1. An anti-β-tubulin antibody was used to normalize the sample loading. (B) UV-inactivated KSHV has reduced infectivity. UV-inactivated or untreated KSHV was used to infect HUVEC. GFP-positive cells were detected at day 2 postinfection. (C) CREB1 activation is independent of viral gene expression. Untreated KSHV (WT) or UV-inactivated KSHV (UV) was used to infect HUVEC and collected at the indicated times postinfection. Phospho-CREB1 (S133) and total CREB1 were then examined as described for panel A. (D and E) IFA detection of CREB1 activation during primary KSHV infection. HUVEC were infected with KSHV for the indicated times, fixed, and stained for phospho-CREB1 (S133) (D) or total CREB1 (E).

different time points were pooled and examined with the antibody array. We considered a kinase or signaling molecule to be a hit if its Z ratio, which reflected the difference in phosphorylation levels between KSHV-infected and mock-infected cells, was ≥ 1 or ≤ -1 and the variation between adjacent duplicate spots of the same protein was less than 30%. Based on these criteria, we found that a total of 23 unique phospho-sites of 18 proteins were increased, while 5 unique phospho-sites of 5 proteins were decreased, following KSHV infection (Table 2). These hits included PKB/AKT, integrin β1, and c-Jun, whose roles in primary KSHV infection were reported previously (5, 9, 15). The roles of the other hits in primary KSHV infection had not been reported before, though PKCδ was shown to mediate KSHV reactivation (23). These kinases and signaling molecules might potentially mediate different events during primary KSHV infection.

To place the identified hits in their corresponding signaling pathways, we performed IPA. Since the Kinex analysis captured only snapshots of the signaling cascades, we included kinases and signaling molecules that were reported to be activated during primary KSHV infection, such as FAK, SRC, ERK, and p38, in the pool of molecules used for network/pathway analysis. We found that integrin-MAPK, insulin-epidermal growth factor receptor (insulin-EGFR), and Janus kinase-signal transducer and activator of transcription (JAK/STAT) networks/pathways were enriched upon KSHV infection (Fig. 1A to C). The activation of integrin-MAPK pathways during primary KSHV infection has been stud-

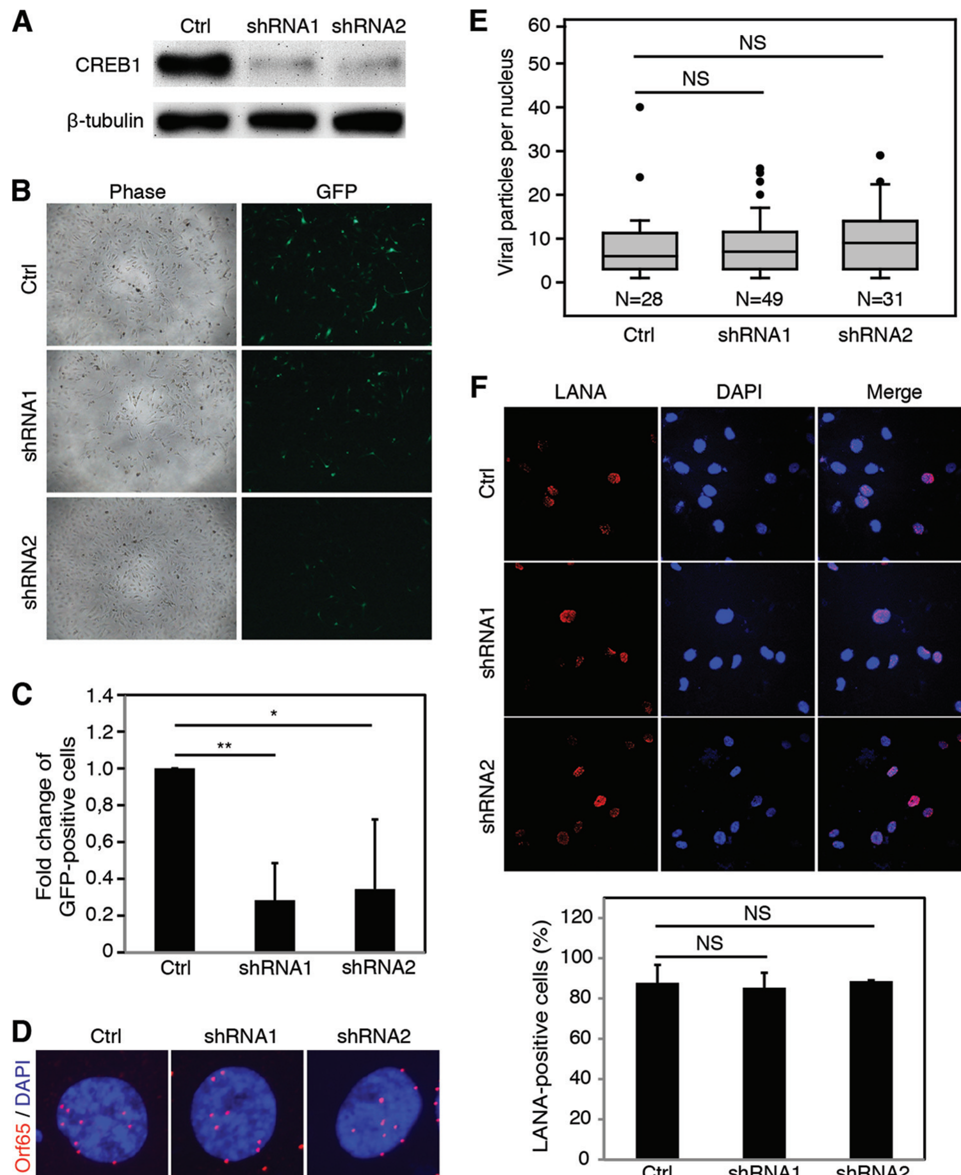

FIG 3 CREB1 is required for the production of infectious virions but not for virus entry, trafficking, and infectivity during primary KSHV infection. (A) shRNA knockdown of CREB1. HUVEC were infected with lentiviruses expressing shRNAs targeting CREB1 (shRNA1 or shRNA2) or expressing control shRNA sequences (Ctrl). Cells were selected with puromycin (1 μg/ml) and analyzed by Western blotting. (B and C) shRNA knockdown of CREB1 inhibits the production of infectious virions. HUVEC with knockdown of CREB1 were infected with KSHV. At 4 h postinfection, cells were extensively washed and replaced with new medium. At day 4 postinfection, the supernatants were collected and used to titrate infectious virions by infecting fresh HUVEC. (B) Representative images showing GFP-positive cells. (C) Similar images were used for quantification. (D) Representative images showing docking of viral particles on the nucleus of a HUVEC with knockdown of CREB1 or that of a control cell. HUVEC with knockdown of CREB1 or control cells were infected with KSHV for 6 h, fixed, and stained for the nucleus (blue) and for KSHV particles (with an antibody to Orf65) (red). (E) shRNA knockdown of CREB1 has no effect on virus entry and trafficking. The total number of KSHV particles successfully docked at each nucleus was quantified and analyzed based on images similar to those in panel D. The results are shown as box-and-whisker plots, with the open boxes representing the 75th and 25th percentiles, the top and bottom short lines representing the 90th and 10th percentiles, respectively, the middle thick lines in the boxes representing the medians, and the open circles outside the 90th and 10th percentiles representing the outliers. N, total number of cells analyzed per sample. (F) shRNA knockdown of CREB1 has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC with knockdown of CREB1 were infected with KSHV, fixed at day 2 postinfection, and stained for LANA. The expression level and pattern of LANA are shown in the top panels, while the number of LANA-positive cells was quantified and is shown in the bottom panel. *, **, and ***, P values of <0.05, <0.01, and <0.001, respectively; NS, not significant.

ied extensively, thus confirming the overall validity of the approach. Nevertheless, a number of MAPKs, such as ERK, p38, and JNK, were not picked up, although their downstream effectors, such as c-Jun, CREB, and HSP27, were in the hit list (Fig. 1A), thus also revealing the limitation of this approach.

The insulin-EGFR pathways were previously shown to be involved in KSHV latency (50). However, their roles in primary KSHV infection had not yet been explored. The JAK/STAT pathways are complex and are involved in interferon or cytokine signaling. Because JAK1 and JAK2 were activated, while STAT5A was

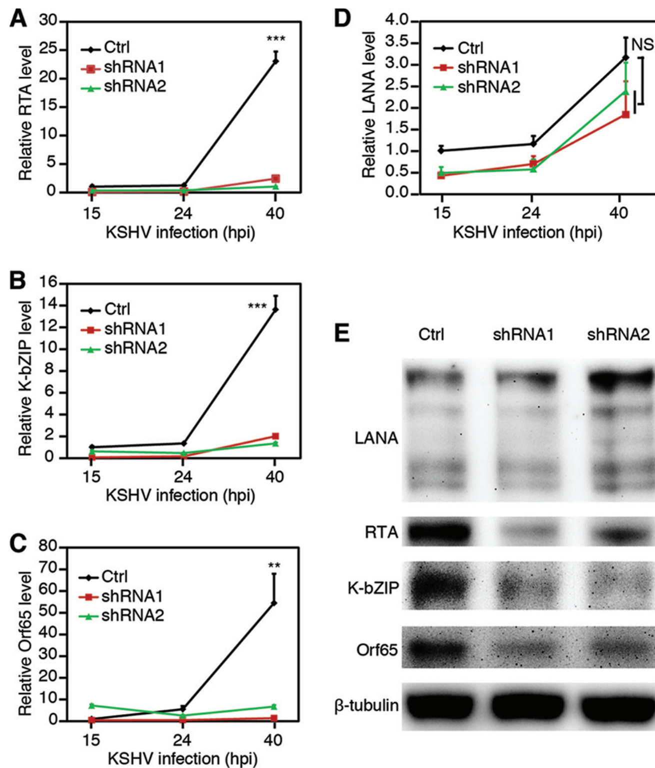

FIG 4 CREB1 is required for the expression of viral lytic but not latent genes during primary KSHV infection. (A to D) shRNA knockdown of CREB1 inhibits mRNA expression of the KSHV RTA (A), K-bZIP (B), and Orf65 (C) lytic genes but has a minimal effect on the latent gene LANA (D). HUVEC with knockdown of CREB1 were infected with KSHV and examined for mRNA expression of KSHV genes by RT-qPCR at 15, 24, and 40 h postinfection. (E) shRNA knockdown of CREB1 inhibits the expression of the KSHV RTA, K-bZIP, and Orf65 lytic proteins but not the latent protein LANA. HUVEC with knockdown of CREB1 were infected with KSHV and analyzed for the expression of viral proteins by Western blot analysis at 48 h postinfection. *, **, and ***, *P* values of <0.05, <0.01, and <0.001, respectively; NS, not significant.

inhibited (Fig. 1C), we were not able to determine the status of the specific pathways that were regulated upon KSHV infection.

The top hit, CREB1, mediates the transcription of downstream genes containing CRE (24). CREB1 is implicated in the integrin-MAPK network and functions as a downstream effector of MAPKs (24). Since the role of CREB1 in KSHV lytic replication is controversial and has not been examined in primary KSHV infection, we decided to further investigate CREB1's role in different stages of primary KSHV infection.

We first confirmed the antibody array data by examining the levels of phospho-Ser133 and total CREB1 in HUVEC following KSHV infection. The level of phospho-S133 CREB1 was increased at 15 and 30 min but had started to decrease at 60 min postinfection, while the total CREB1 level did not show a significant change during this interval (Fig. 2A). The results of immunostaining showed increased levels of phospho-CREB1 at 15 and 30 min and much weaker staining at 60 min postinfection (Fig. 2D), while no change was observed for total CREB1 (Fig. 2E). There was no staining signal with the secondary antibody alone (data not shown). These immunostaining results further confirmed the activation of CREB1 following KSHV infection. Furthermore, UV-inactivated virions activated CREB1 as efficiently as the wild-type (WT) virions did (Fig. 2B and C). These results suggested that KSHV-induced CREB1 phosphorylation was independent of viral gene expression but was mediated through the interactions between KSHV envelope glycoproteins and host cell receptors during the attachment and entry stages of infection.

shRNA knockdown of CREB1 inhibits the KSHV lytic replication program but has no effect on virus entry and trafficking, expression of the latent gene LANA, and KSHV infectivity. To explore the role of CREB1 in primary KSHV infection, we performed shRNA knockdown of CREB1. The Western blot results showed that CREB1 expression was reduced by over 70% in cells transduced with CREB1-specific shRNA-carrying lentiviruses compared to those transduced with the scrambled control-carrying lentivirus (Fig. 3A). CREB1 knockdown cells were infected with KSHV and extensively washed with culture medium at 4 h postinfection to remove any residual input viruses. At day 4 postinfection, the supernatants were collected and titrated. Knockdown of CREB1 reduced virus titers by approximately 70% for both shRNAs (Fig. 3B and C). These results indicated that CREB1 plays an important role in KSHV lytic replication and virion production during primary infection.

KSHV productive infection is regulated at different steps during primary infection, including virus entry, trafficking, and viral gene expression. To identify the step(s) mediated by CREB1, we first examined KSHV entry and trafficking to the cell nucleus. HUVEC with knockdown of CREB1 were infected with KSHV for 6 h. Cells were extensively washed, fixed, and stained for the Orf65 protein to visualize the viral capsids that had successfully trafficked to the perinuclear region. By counting viral capsids docking at the nucleus, we found that similar numbers of virus particles had successfully reached the cell nucleus in both control and CREB1 knockdown cells (Fig. 3D and E). These results indicated that CREB1 was not involved in virus entry and intracellular trafficking.

We further determined if knockdown of CREB1 might affect KSHV infectivity. Because LANA is the major KSHV latent protein that binds to the viral genome to maintain its persistence, detection of the LANA protein, which manifests as a punctate staining pattern, would indicate successful viral infection. HUVEC transduced with lentiviruses carrying CREB1-specific or control shRNAs were infected with KSHV and stained for the LANA protein at day 2 postinfection. Knockdown of CREB1 had no effect on the LANA staining pattern and intensity, the number of punctate dots per cell, and the percentage of LANA-positive cells (Fig. 3F), indicating that CREB1 did not affect LANA protein expression and KSHV infectivity. Taken together, the results show that CREB1 is an essential factor for viral lytic replication and virion production but that it neither regulates viral entry and trafficking nor affects KSHV infectivity during primary infection.

Knockdown of CREB1 inhibits the expression of KSHV lytic but not latent genes. Based on their distinct expression kinetics, KSHV genes are classified into latent and lytic genes. Latent genes, which mainly consist of LANA, vFLIP, vCyclin, and a cluster of microRNAs, promote cell growth and survival and viral latency (51). Lytic genes, consisting of immediate early (IE), early (E), and late (L) genes, are expressed in a coordinated fashion during KSHV lytic replication. The expression of the IE RTA gene is necessary and sufficient for activating KSHV into full lytic replication (51). The early gene Orf-K8 encodes another important regulator of lytic replication, K-bZIP. The late genes, such as Orf65 and

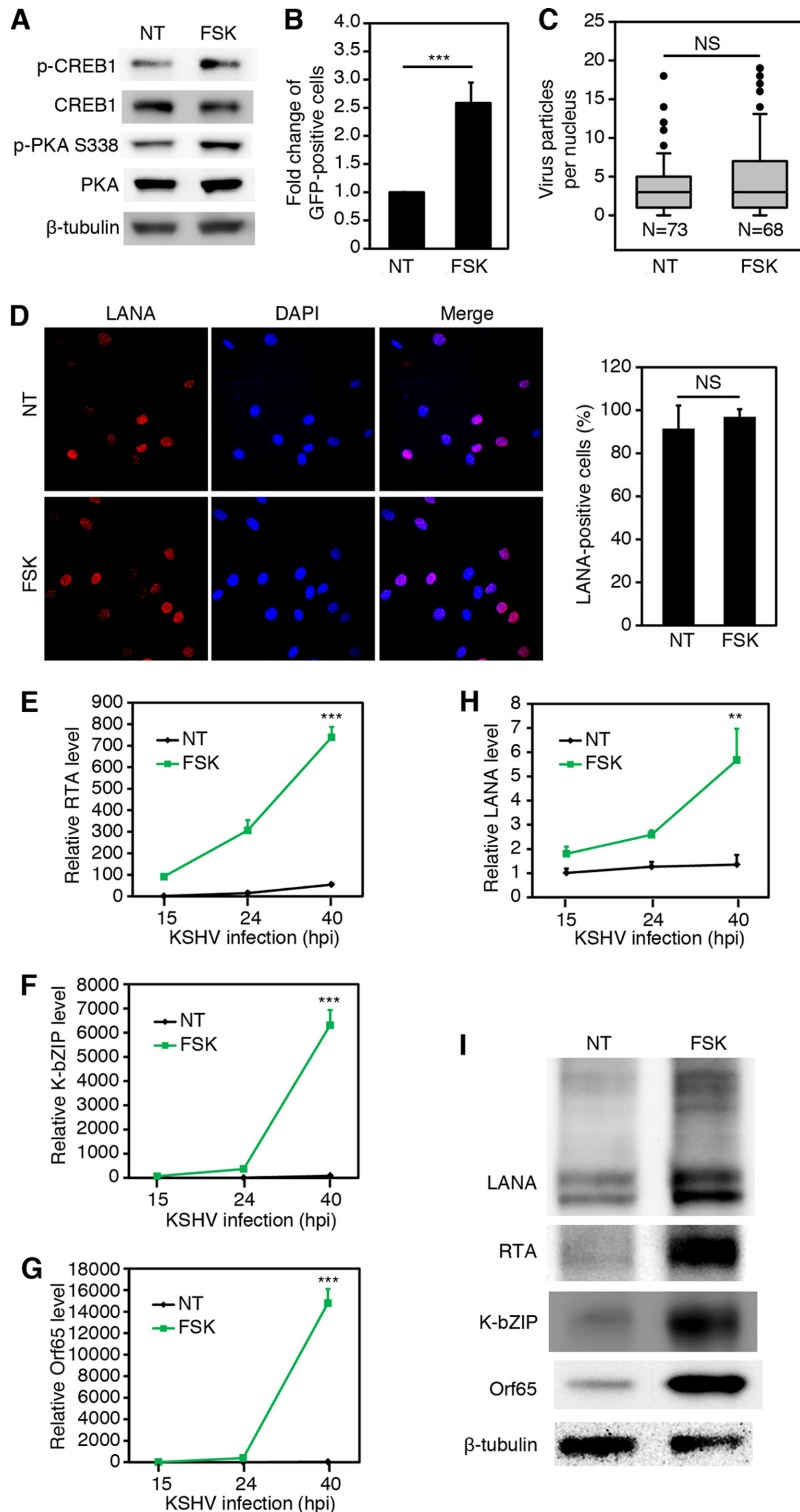

FIG 5 Chemical activation of CREB1 with forskolin (FSK) promotes KSHV lytic replication but has no effect on virus entry, trafficking, and infectivity during primary infection. (A) FSK activates the PKA/CREB1 pathway. HUVEC were treated with FSK for 1 h and then examined for phospho-CREB1 (S133), total CREB1, phospho-PKA (S338), and total PKA. (B) Activation of CREB1 with FSK increases the production of infectious virions. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK. At 4 h postinfection, cells were extensively washed and replaced with new medium containing FSK. At 48 h postinfection, cells were extensively washed and replaced with new medium without FSK. At day 4 postinfection, the supernatants were collected and subjected to titration of infectious virions as described in the legend to Fig. 3B and C. NT, not treated. (C) Activation of CREB1 with FSK has no effect on virus entry and trafficking. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK for 6 h. The number of KSHV particles successfully docked at

Orf39 (glycoprotein M), are mostly composed of genes encoding viral structural proteins and those required for viral lytic replication, and their expression often depends on viral genome replication. During primary KSHV infection of HUVEC, both latent and lytic genes are actively transcribed, with most of them peaking at 48 to 78 h postinfection (17). The results of immunostaining showed that shRNA knockdown of CREB1 did not affect LANA protein expression (Fig. 3F). To test the role of CREB1 in viral lytic gene expression during primary infection, we monitored the kinetics of several representative KSHV lytic genes by RT-qPCR. Knockdown of CREB1 inhibited over 90% of the expression of the RTA, K-bZIP, and Orf65 lytic genes (Fig. 4A to C) but had no significant effect on the expression of the latent gene LANA (Fig. 4D), which was in agreement with the immunostaining results (Fig. 3F). Western blot analysis further confirmed that knockdown of CREB1 decreased over 90% of the expression of the RTA, K-bZIP, and Orf65 viral lytic proteins but had no effect on the expression of the viral latent protein LANA (Fig. 4E). Taken together, these results suggest an essential role of CREB1 in the viral lytic transcriptional program.

Activation of CREB1 is sufficient to promote KSHV lytic replication during primary infection. Next, we determined if artificial activation of CREB1 was sufficient to promote viral lytic replication during primary infection. Since there is no chemical agonist that can directly activate CREB1, we chose an indirect approach by using the PKA agonist forskolin (FSK). FSK raises the intracellular cAMP level by activating adenylyl cyclase, resulting in the activation of the PKA pathway, the classic upstream pathway of CREB1 (24). Following pretreatment of cells with 10 μM FSK, we infected HUVEC with KSHV in the presence of the agonist. FSK increased the phosphorylation level of PKA at S338 and activated CREB1 (Fig. 5A). Consistent with these results, FSK enhanced the production of infectious virions 2.7-fold (Fig. 5B), indicating that activation of CREB1 alone was sufficient to significantly promote viral lytic replication during primary infection. Similar to knockdown of CREB1, FSK treatment did not affect virus entry and trafficking, had no effect on the expression level and staining pattern of LANA, and conferred a minimal effect on KSHV infectivity (Fig. 5C and D). Consistent with its enhanced effect on the production of infectious virions, FSK significantly enhanced the mRNA expression of the KSHV RTA, K-bZIP, and Orf65 lytic genes, increasing their expression levels 18-, 450-, and 288-fold, respectively, at 40 h postinfection (Fig. 5E to G). Interestingly, FSK also increased the expression of LANA, but only 4.6-fold (Fig. 5H). In agreement with the mRNA results, FSK increased the expression levels of the RTA, K-bZIP, and Orf65 lytic proteins (Fig. 5I). The LANA protein level was also increased by FSK, albeit to a lower level. Taken together, these results show that chemical activation of CREB1 was sufficient to increase viral lytic replication during primary KSHV infection, predominantly by enhancing the expression of viral lytic genes.

Screening of CREB1's direct effectors identifies MSK1 and -2 as upstream CREB1 regulators during primary KSHV infection. Having defined the role of CREB1 in primary KSHV infection, we next attempted to identify the cellular pathway(s) that directly mediated CREB1 activation. The classic CREB1 activation pathway is the cAMP/PKA pathway (24). As a putative protein kinase for CREB1 activation, activation of PKA as a result of an increased second messenger (cAMP) level leads to CREB1 phosphorylation at Ser133 (39). We first measured the change in cAMP level during primary KSHV infection. Using a specific anti-cAMP antibody in an enzyme-linked immunosorbent assay (ELISA), we did not detect any significant change in the cAMP level within the first 1 h of primary KSHV infection (Fig. 6A), during which time CREB1 was activated (Fig. 6B). As expected, FSK drastically raised the intracellular cAMP level (Fig. 6A). Consistent with these results, we did not detect any change of PKA phosphorylation when CREB1 was activated at 15 and 30 min post-KSHV infection (Fig. 6B). Using a specific antibody against the PKA catalytic subunit (PKAc), we did not detect any cytoplasmic-to-nuclear translocation of PKA at these time points (Fig. 6C). Taken together, these results indicated that the cAMP/PKA pathway was not involved in KSHV-induced CREB1 activation during primary infection, although it could actively promote the KSHV lytic transcriptional program if artificially activated (Fig. 5). This finding was contradictory to that of a previous study showing that KSHV activation of CREB1 was mediated by the cAMP/PKA pathway in DMVEC (35), indicating that KSHV activation occurs via distinct signaling pathways in different cell types.

Previous studies revealed that besides PKA, several other protein kinases can directly phosphorylate CREB1. These kinases include AKT, RSKs, MSKs, and MK2. We first checked the activation of these kinases during primary KSHV infection. The Western blot results showed that AKT, MSK1/2, and MK2, but not RSKs, were phosphorylated at the early time points of KSHV infection (Fig. 7A). Similar to the case for CREB1, the increased phosphorylation levels of AKT, MSK1/2, and MK2 peaked at around 15 to 30 min postinfection, suggesting their possible involvement in CREB1 activation. We then used specific inhibitors to investigate the involvement of these kinases in CREB1 activation. LY294002 is an inhibitor of the AKT upstream kinase PI3K, SB747651A is an inhibitor of MSK1/2, and PF3644022 is an inhibitor of MK2. HUVEC were preincubated with the individual inhibitors at 37°C for 1 h, at final concentrations of 12.5 μM, 10 μM, and 10 μM for LY294002, SB747651A, and PF3644022, respectively, and then infected with KSHV in the presence of the inhibitors. Western blot analysis of the infected cells showed that the inhibitor of MSKs, but not that of PI3K or MK2, prevented CREB1 activation (Fig. 7B to D). The inhibitor of MSKs reduced the phosphorylation levels of CREB1 by 77% and 73% at 15 and 30 min postinfection, respectively (Fig. 7B). In contrast, inhibitors of PI3K and MK2 not only did not decrease but in fact increased

the nucleus was analyzed as described in the legend to Fig. 3D and E. (D) Activation of CREB1 with FSK has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK and analyzed for the expression level and pattern of LANA and the number of LANA-positive cells at 48 h postinfection. (E to H) Activation of CREB1 with FSK significantly enhances the mRNA expression of the KSHV RTA (E), K-bZIP (F), and Orf65 (G) lytic genes but has a lesser effect on the latent gene LANA (H). HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK and analyzed for the mRNA expression of viral genes by RT-qPCR at 15, 24, and 40 h postinfection. (I) Activation of CREB1 with FSK increases the expression of the KSHV RTA, K-bZIP, and Orf65 lytic proteins but increases that of the LANA protein to a lesser extent. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK and analyzed for the expression of viral proteins by Western blotting at 48 h postinfection. *, **, and ***, *P* values of <0.05, <0.01, and <0.001, respectively; NS, not significant.

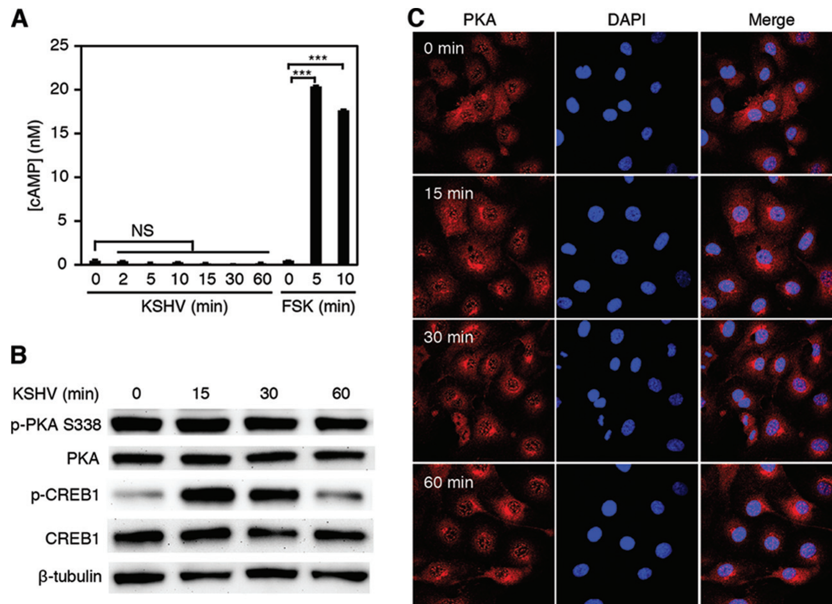

FIG 6 The cAMP/PKA pathway does not mediate CREB1 activation during primary KSHV infection. (A) KSHV does not alter the intracellular cAMP level during primary infection. HUVEC infected with KSHV were used to determine the intracellular cAMP levels at the indicated times postinfection. Cells treated with FSK were used as positive controls. (B and C) PKA is not activated during primary KSHV infection. HUVEC infected with KSHV were examined for phospho-PKA (S338), total PKA, phospho-CREB1 (S133), and total CREB1 by Western blotting (B) or for PKA translocation by immunostaining (C) at the indicated times postinfection. *, **, and ***, P values of <0.05, <0.01, and <0.001, respectively; NS, not significant.

CREB1 activation, even before KSHV infection (0 h). We successfully detected reduced phosphorylation levels of AKT and the MK2 downstream target Hsp27, confirming that the two inhibitors were functional (Fig. 7C and D). Together, these results showed that KSHV-induced activation of CREB1 was mediated by MSKs but not by AKT or MK2.

MSK1 and -2 regulate KSHV productive lytic replication during primary infection, but not virus entry, intracellular trafficking, and KSHV infectivity. To determine the role of MSK1/2 in primary KSHV infection, we infected HUVEC in the presence of the MSK inhibitor SB747651A (10 μM). Inhibition of MSKs decreased the production of infectious virions by approximately 62% (Fig. 8A), indicating an essential role of MSKs in productive KSHV replication during primary infection. On the other hand, inhibition of MSKs did not affect the number of viral particles reaching the perinuclear region, indicating that MSKs did not regulate KSHV entry and intracellular trafficking (Fig. 8B). Furthermore, MSKs did not affect the expression and staining pattern of LANA or KSHV infectivity (Fig. 8C). The results of RT-qPCR analysis showed that inhibition of MSKs reduced the expression of RTA, K-bZIP, and Orf65 transcripts by 64%, 83%, and 63%, respectively (Fig. 8D to F), while no significant change was detected for LANA transcripts (Fig. 8G). The Western blot results confirmed those of RT-qPCR, showing that the levels of RTA, K-bZIP, and Orf65 proteins were reduced 90%, 95%, and 75%, respectively, by the inhibitor, while no change was detected for the LANA protein (Fig. 8H). These results indicate that MSKs play an important role in primary KSHV infection by affecting the viral lytic transcriptional program at the postentry stage.

To further confirm the role of MSKs in KSHV lytic replication, we designed shRNAs for both MSK1 and MSK2. Lentiviruses expressing two combinations of shRNAs targeting both MSK1 and MSK2 (shMixed1 and shMixed2) were used to transduce HUVEC. Western blot analysis showed knockdown efficiencies of 75 to 85% for MSK1 and 65 to 75% for MSK2 compared to the efficiencies of the scrambled controls (Fig. 9A). Knockdown of MSKs attenuated CREB1 activation by 60 to 80% (Fig. 9A). Consistent with these results, knockdown of MSKs reduced the production of infectious virions by 57 to 61% during primary KSHV infection (Fig. 9B). Similar to the results observed with the MSK inhibitor, knockdown of MSKs did not affect virus entry and trafficking or KSHV infectivity (Fig. 9C and D). The RT-qPCR results showed that shRNA knockdown of MSKs reduced the expression levels of RTA, K-bZIP, and Orf65 transcripts by 56 to 61%, 43 to 50%, and 32 to 35%, respectively, but had no effect on the expression of LANA transcripts (Fig. 9E). The smaller effect of the MSK shRNAs than that of the MSK inhibitor on the expression of KSHV lytic genes was likely due to the lower efficiencies of the shRNAs. The Western blot results confirmed that MSK shRNAs reduced the expression of the RTA, K-bZIP, and Orf65 proteins but had no effect on the LANA protein (Fig. 9F). Taken together, the results show that MSKs play an important role in regulating viral lytic replication during primary KSHV infection and that such regulation might occur at least partially through phosphorylating and activating the downstream transcription factor CREB1.

DISCUSSION

Deregulation of cell signaling pathways is a common theme in viral infections. Viruses often hijack cellular pathways to achieve successful infection and replication. For example, the MSK-CREB1 pathway has been reported to mediate ERK-induced HCMV reactivation in dendritic cells (44). CREB1 activation promotes HCMV gene expression by binding to the viral major immediate early promoter. Similarly, HSV-1 depends on CREB1 to regulate the expression of an 8.3-kb primary latency-associated transcript during acute and latent infections of peripheral sensory

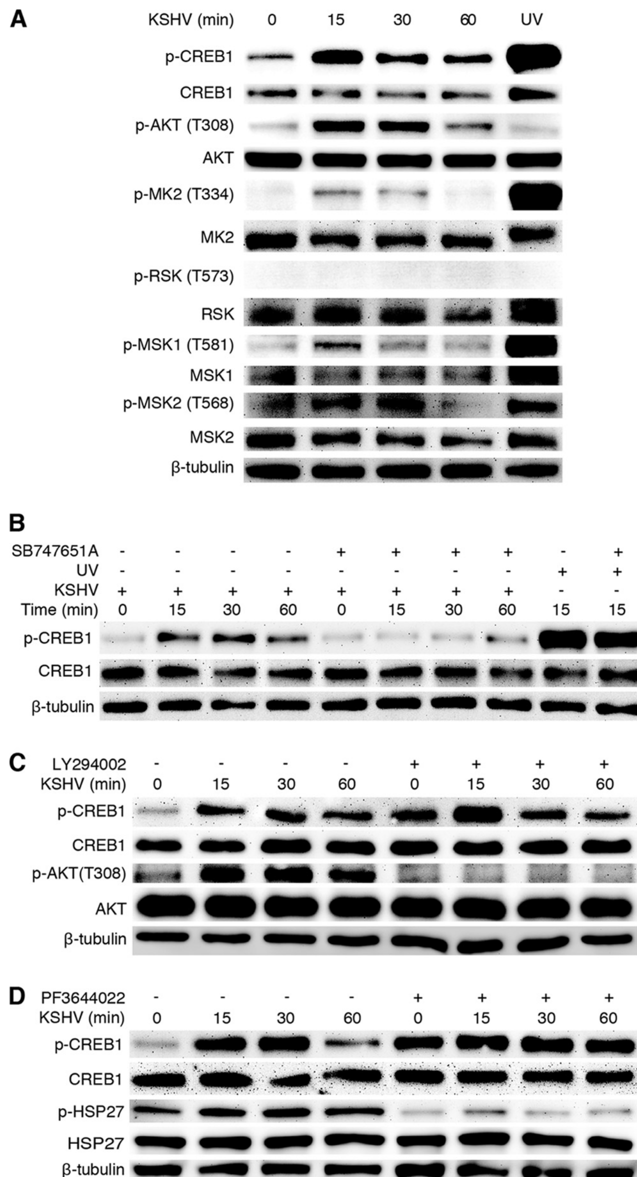

FIG 7 MSK1 and -2, but not AKT, MK2, and RSK, mediate CREB1 activation during primary KSHV infection. (A) Activation of AKT, MK2, and MSK1/2, but not RSK, during primary KSHV infection. HUVEC infected with KSHV were examined for phospho-CREB1 (S133), total CREB1, phospho-AKT (T308), total AKT, phospho-MK2 (T334), total MK2, phospho-RSK (T573), total RSK, phospho-MSK1 (T581), total MSK1, phospho-MSK2 (T568), and total MSK2 by Western blotting at the indicated times postinfection. Cells subjected to UV irradiation for 30 min were used as positive controls for phospho-CREB1, phospho-MK2, phospho-MSK1, and phospho-MSK2. (B to D) MSK proteins, but not AKT and MK2, mediate CREB1 activation during primary KSHV infection. HUVEC pretreated with the MSK inhibitor SB747651A (B), the AKT upstream PI3K inhibitor LY294002 (C), or the MK2 inhibitor PF3644022 (D) were infected with KSHV in the presence of the inhibitors and analyzed for CREB1 activation at the indicated times postinfection. Inhibition of MSKs, PI3K, and MK2 effectively decreased the phosphorylation levels of their downstream targets, CREB1, AKT, and HSP27, respectively.

neurons as well as reactivation from latency (30). KSHV has been shown to activate a number of signaling pathways, such as MAPK and NF-κB pathways, at the early stage of primary infection, and these pathways in return facilitate KSHV infection and gene expression (9, 10, 15, 52).

In this study, we applied an unbiased antibody array approach to identify new kinases or signaling molecules that might play important roles during primary KSHV infection. The Kinex antibody array has a relatively broad coverage of human kinases and signaling molecules. We were interested in kinases and signaling molecules that might be deregulated early during primary KSHV infection, as they might mediate virus attachment, entry, membrane fusion, intracellular trafficking, and viral gene expression. Using stringent criteria, we identified 28 unique phospho-sites from 23 cellular proteins whose phosphorylation levels were significantly altered at the early phases of KSHV infection of HUVEC (Table 2). A number of these hits, including AKT, integrin β1, and c-Jun, were previously reported (5, 9, 15). It would be interesting to confirm the alterations of the newly identified hits and to explore their potential roles in primary KSHV infection.

By performing IPA with the Kinex antibody array results integrated with those reported previously, we identified three enriched networks. All three networks are initiated as a result of ligand-receptor interactions. In this case, they might be due to KSHV binding to its receptors or cytokines/growth factors induced at the early stage of KSHV infection. The first is the integrin/MAPK network (Fig. 1A), parts of which have been reported in several studies (5, 9, 10, 15). Nevertheless, the upstream activators, the downstream mediators, and the interactions among the signaling pathways in this network in the context of primary KSHV infection remain unclear. The current paradigm is that KSHV binding to cellular integrin receptors activates downstream FAK (5), which can lead to the induction of multiple MAPK pathways and the PI3K/AKT pathway (9, 10, 15, 52). Activation of the ERK, JNK, and p38 MAPK pathways promotes KSHV entry and trafficking as well as the viral lytic replication program (9, 52). The AKT pathway mediates activation of the NF-κB pathway, which regulates KSHV latent gene expression (15). While KSHV infection triggers the immediate release of proinflammatory and proangiogenic cytokines, such as IL-6, angiopoietin-2, and IL-8 (10, 53), a number of them, including IL-6, angiopoietin-2, MMP1, MMP2, and MMP9, can also be induced further by the MAPK pathways (10, 54, 55). Importantly, some of these cytokines in turn can activate the ERK, AKT, and NF-κB pathways, thus allowing the establishment of a positive-feedback loop that can further enhance KSHV infection and replication and amplify a proinflammatory and proangiogenic environment during primary KSHV infection.

Another enriched network involved the insulin receptor and EGFR pathways (Fig. 1B). The insulin-like growth factor and insulin receptor signaling pathways have been implicated in latent KSHV infection (50). However, the roles of the insulin receptor and EGFR in primary KSHV infection are still unknown. EGFR is a receptor tyrosine kinase that regulates a number of key cellular processes, including cell proliferation, survival, differentiation, tissue homeostasis, and tumorigenesis. EGFR and ephrin receptor A2 (Eph2A) have been identified as hepatitis C virus (HCV) entry receptors (56). KSHV also uses Eph2A as an entry receptor for infection (57). Binding of glycoproteins gH and gL to EphA2 triggers EphA2 phosphorylation and endocytosis and KSHV entry (57). It would be interesting to explore the role of EGFR in primary KSHV infection.

The JAK/STAT network was enriched in the IPA results (Fig. 1C). In particular, the phosphorylation levels of JAK1 and JAK2 increased >4-fold. A number of proinflammatory cyto-

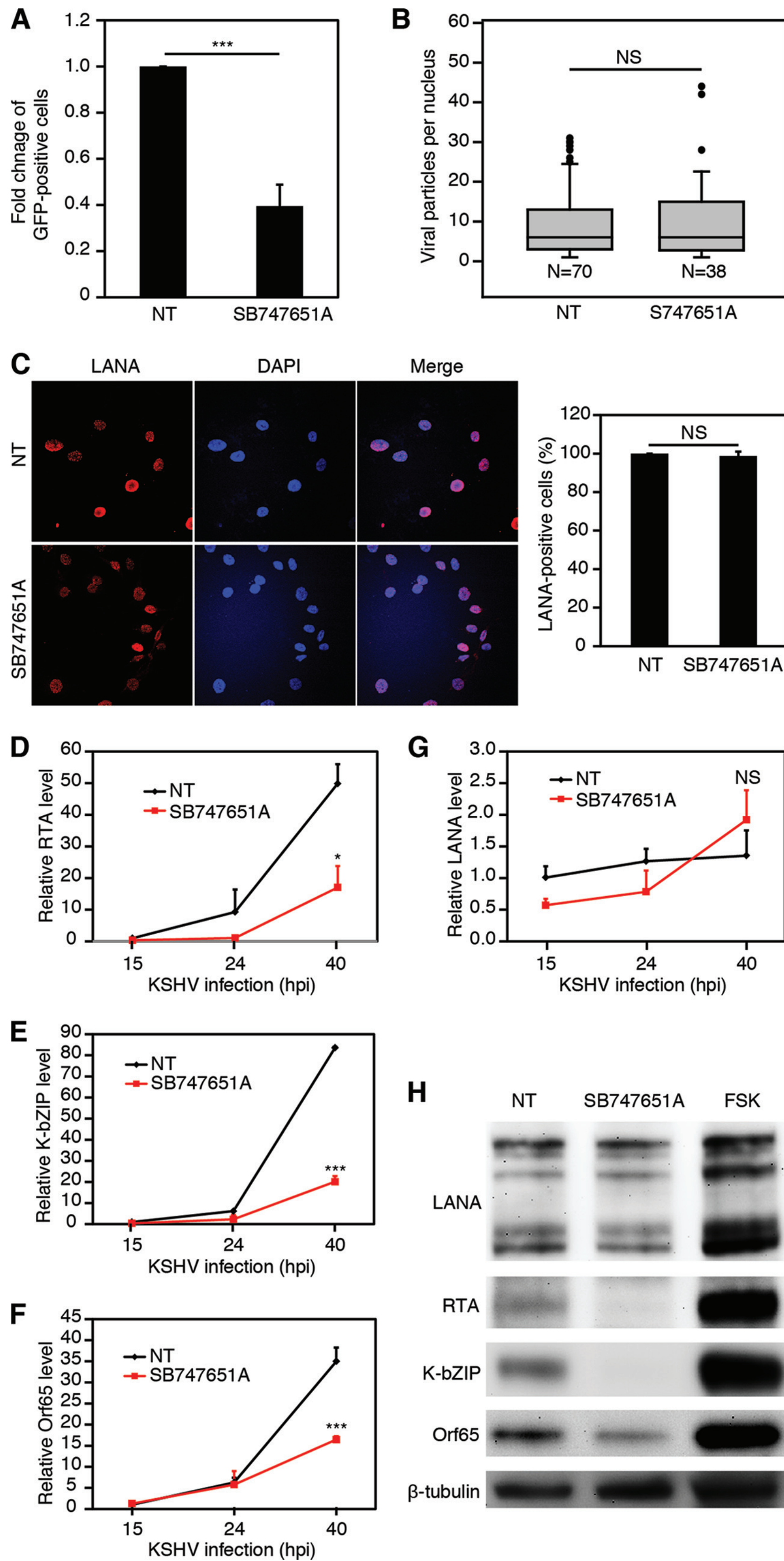

FIG 8 Chemical inhibition of the MSK pathway blocks the KSHV lytic replication program but has no effect on virus entry, trafficking, and infectivity during primary infection. (A) Inhibition of the MSK pathway blocks the production of infectious virions. HUVEC were pretreated with the MSK inhibitor SB747651A for 1 h and then infected with KSHV in the presence of the inhibitor. At 4 h postinfection, cells were extensively washed and replaced with new medium containing

kines, such as IL-6, are known to activate JAK1 and JAK2 (58). However, IL-6 primarily activates the STAT3 pathway (59). We did not observe any significant change in STAT3 phosphorylation during primary KSHV infection, although STAT3 is implicated in KSHV latent infection (60, 61) and can be activated by KSHV vIL6, RTA, vGPCR (ORF74), and kaposin B, as well as through complement activation (61–65). In contrast to that of JAK1/2, the phosphorylation level of STAT5A was significantly reduced. STAT5A is primarily activated by growth factors, such as prolactin (66). Further studies are required to understand how these pathways are regulated during primary KSHV infection.

Several kinases, such as ERK, AKT, and FAK, were reported to be activated during primary KSHV infection but were not identified as hits in the screen. This could be attributed to the limitations of the antibody array approach, which can generate both false-positive and false-negative candidates. Possible limitations of the approach include the following. (i) Antibodies to some targets/phosphorylation sites are absent on the array. (ii) Some antibodies are of poor quality or are not suitable for the antibody array. (iii) Some antibodies have limited sensitivities. (iv) Some antibodies have high variations between adjacent duplicate spots, which could lead to the discard of some hits. For example, the p38 phosphorylation sites T180 and Y182 had the highest signal, with a Z ratio of 6.08, but were discarded due to the high (79.16%) error range (data not shown). (v) Kinase activation is a fast event with rapid kinetics. The collected samples reflect only snapshots of the cells and might not catch all events. Because of the reasons stated above, it is imperative to validate any selected hits following antibody array screening. Despite these limitations, the antibody array assay remains an easy and convenient approach that can generate some potential hits. Combining this method with IPA or similar bioinformatic analyses and other approaches, such as gene expression profiling, should facilitate the mapping of the overall networks and pathways that are activated in the cells.

We have identified MSK1/2-CREB1 as a new essential pathway for KSHV lytic replication during primary infection. Chemical inhibition of MSK1/2 or shRNA knockdown of MSK1/2 or CREB1 substantially impaired the expression of viral lytic genes and the production of infectious virions but had no effect on virus entry, trafficking, latent gene expression, and infectivity. One possible mechanism of CREB1 promotion of KSHV lytic replication is through its binding to the promoters of viral lytic genes to induce their expression. In fact, a CRE site is present from bp −265 to −269 upstream of the transcriptional start site in the RTA gene promoter. As RTA is the major driver of the viral lytic cascade, induction of RTA is sufficient to enhance KSHV lytic replication. The promoters of several other KSHV lytic genes, including the Orf59, K-bZIP, and Orf65 genes, also contain putative CRE sites, which may mediate CREB1's enhancement of KSHV lytic replication. On the other hand, we did not find any potential CRE site in the LANA promoters, which might explain the differential responses between LANA and viral lytic genes. It is worth mentioning that CREB1 regulation of gene expression might be gene or promoter specific and could be influenced by other transcriptional factors. For example, it has been shown that CREB1 activation is enhanced by RTA for promoters containing an RTA-binding site(s); however, it is inhibited by RTA for promoters without an RTA-binding site, in part through competition or sequestration of other transcription coactivators, such as CBP (67). Thus, CREB1 and RTA might synergize to drive the expression of KSHV lytic genes while antagonizing each other to regulate the expression of latent genes during primary infection of HUVEC. However, it has been reported that RTA can activate the LANA promoter through an RBP-Jκ binding site (68). Thus, the lack of responsiveness of LANA expression to CREB1 activation is unlikely to be mediated by RTA.

Another possible mechanism of CREB1 regulation of viral lytic replication is through the induction of proinflammatory cytokines, several of which promote KSHV lytic replication (38). However, it has been reported that CREB1 induces miR-132 to downregulate p300, the proinflammatory cytokines IL-6, IL-1β, and beta interferon, and ISG15, relieving their repression of the expression of KSHV genes and the viral genome copy number in LEC (37). It is interesting that two of the CREB1-downregulated proinflammatory cytokines, IL-6 and IL-1β, can activate the MAPK pathways and promote KSHV lytic replication (38). Another study has also shown that KSHV infection of DMVEC activates CREB1 to induce the proinflammatory factor COX2, which is required for viral latent gene expression (35). Interestingly, during primary KSHV infection of DMVEC, CREB1 activation occurs through the cAMP/PKA pathway but not the MSK pathway (35). It is also worth mentioning that primary KSHV infections in LEC and DMVEC are nonproductive despite the activation of CREB1 (19, 36).

Regardless of the mechanism of CREB1 promotion of KSHV lytic replication, the outcome of CREB1 activation in regard to KSHV lytic replication during primary infection differs depending on the cell type, which might be due to usage of different cellular receptors and entry pathways resulting in the differential activation of signaling pathways. In particular, KSHV induces a high level of activation of the NF-κB pathway in DMVEC (15), which is known to promote KSHV latency by repressing viral lytic replication (22). In contrast, KSHV induces hyperactivation of the ERK, JNK, and p38 MAPK pathways in HUVEC, promoting viral

the inhibitor. At 48 h postinfection, cells were extensively washed and replaced with new medium without the inhibitor. The supernatants were collected at day 4 postinfection and used to titrate infectious virions by infecting fresh HUVEC as described in the legend to Fig. 3B and C. (B) Inhibition of the MSK pathway has no effect on virus entry and trafficking. HUVEC were pretreated with SB747651A for 1 h and then infected with KSHV in the presence of the inhibitor for 6 h. The number of KSHV particles successfully docked at the nucleus was analyzed as described in the legend to Fig. 3D and E. (C) Inhibition of the MSK pathway has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC pretreated with SB747651A for 1 h were infected with KSHV in the presence of the inhibitor and then analyzed for the expression level and pattern of LANA and the number of LANA-positive cells at 48 h postinfection. (D to G) Inhibition of the MSK pathway significantly blocks the mRNA expression of the KSHV RTA (D), K-bZIP (E), and Orf65 (F) lytic genes but has a minimal effect on the latent gene LANA (G). HUVEC pretreated with SB747651A for 1 h were infected with KSHV in the presence of the inhibitor and analyzed for the mRNA expression of viral genes by RT-qPCR at 15, 24, and 40 h postinfection. (H) Inhibition of the MSK pathway blocks the expression of the KSHV RTA, K-bZIP, and Orf65 lytic proteins but has no effect on the LANA protein. HUVEC pretreated with SB747651A for 1 h were infected with KSHV in the presence of the inhibitor and then analyzed for the expression of viral proteins by Western blotting at 48 h postinfection. *, **, and ***, *P* values of <0.05, <0.01, and <0.001, respectively; NS, not significant.

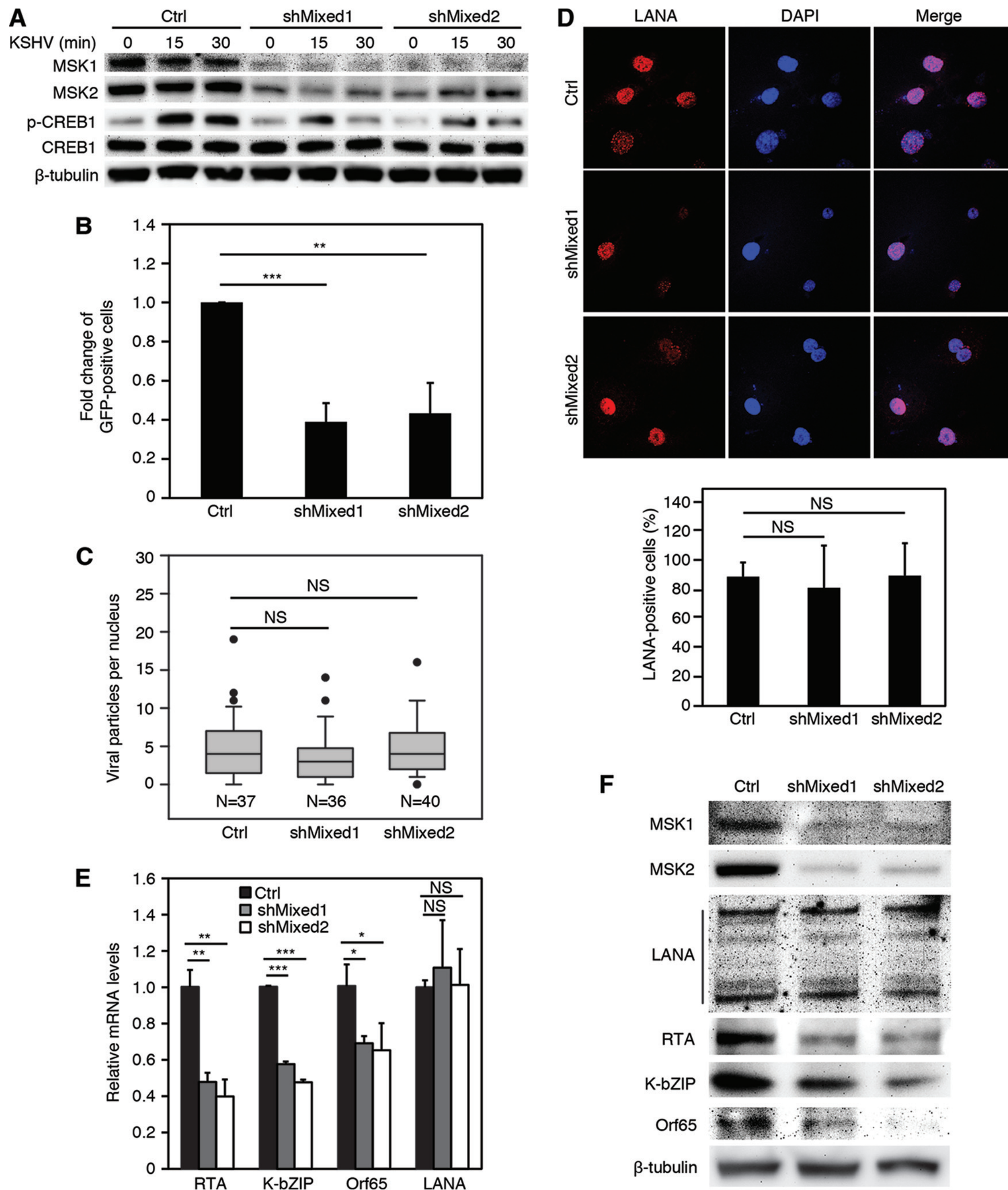

FIG 9 MSK1 and -2 are required for CREB1 activation and KSHV lytic replication but not for virus entry, trafficking, and infectivity during primary infection. (A) shRNA knockdown of MSK1/2 prevents CREB1 activation. HUVEC were infected with lentiviruses expressing 2 sets of combined shRNAs targeting both MSK1 and MSK2 (shMixed1 and shMixed1) or the control shRNA (Ctrl). Stable cell populations selected with puromycin (1 μg/ml) were infected with KSHV and analyzed by Western blotting for total MSK1, total MSK2, phospho-CREB1 (S133), and total CREB1 at the indicated times. (B) shRNA knockdown of MSK1/2 inhibits the production of infectious virions. HUVEC with knockdown of MSK1/2 were infected with KSHV. At 4 h postinfection, cells were extensively washed and replaced with new medium. At day 4 postinfection, the supernatants were collected and used to titrate infectious virions by infecting fresh HUVEC as described in the legend to Fig. 3B and C. (C) shRNA knockdown of MSK1/2 has no effect on virus entry and trafficking. HUVEC with knockdown of MSK1/2 were infected with KSHV for 6 h. The number of KSHV particles successfully docked at the nucleus was analyzed as described in the legend to Fig. 3D and E. (D) shRNA knockdown of MSK1/2 has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC with knockdown of MSK1/2 were infected with KSHV and analyzed for the expression level and pattern of LANA and the number of LANA-positive cells at 48 h postinfection. (E) shRNA knockdown of MSK1/2 significantly blocks the mRNA expression of the KSHV RTA, K-bZIP, and Orf65 lytic genes but has a minimal effect on the latent gene LANA. HUVEC with knockdown of MSK1/2 were infected with KSHV and analyzed for the mRNA expression of viral genes by RT-qPCR at 40 h postinfection. (F) shRNA knockdown of MSK1/2 blocks the expression of the KSHV RTA, K-bZIP, and Orf65 lytic proteins but has no effect on the latent protein LANA. HUVEC with knockdown of MSK1/2 were infected with KSHV and analyzed for the expression of viral proteins by Western blotting at 48 h postinfection. *, **, and ***, P values of <0.05, <0.01, and <0.001, respectively; NS, not significant.

lytic replication (9, 10). The interaction of the MAPK and NF-κB pathways might be one of the factors that determines the impact of MSK1/2-CREB1 activation on the KSHV replication program. In this case, the level of MSK1/2-CREB1 activation might have only a minimal impact on the KSHV replication program. Alternatively, the KSHV replication program could be determined entirely by the extent of MSK1/2-CREB1 activation. The MSK1/2-CREB1 proteins could be the critical mediators of the hyperactivated MAPK pathways, in addition to the AP-1 complexes, which control KSHV lytic replication in HUVEC. In this scenario, MSK1/2-CREB1 could also be activated by other pathways/signals. Indeed, besides MSK1/2, CREB1 can also be activated directly by cAMP/PKA, PI3K/AKT, MK2, and RSK pathways. As a result, intracellular and extracellular signals that can activate CREB1 are likely to promote KSHV lytic replication. As predicted, we have shown that chemical activation of CREB1 with FSK, an artificial activator of the cAMP/PKA pathway, can robustly enhance viral lytic replication (Fig. 5). Our works have clearly identified CREB1 as an essential factor for KSHV lytic replication during primary infection of HUVEC. Because phosphorylation is required for CREB1 activation, further demonstration of the role of CREB1 phosphorylation in KSHV lytic replication would require mutation of the specific CREB1 residue that mediates its phosphorylation.

While our results have demonstrated only that the MSK1/2-CREB1 pathway promotes KSHV lytic replication during primary infection, it is likely that it also enhances KSHV reactivation from latency. In fact, CREB1 has been reported to mediate reactivation of several other herpesviruses from latency (44, 69, 70). In LPS-induced HCMV reactivation, activated CREB1 induces the expression of the viral immediate early gene by binding directly to the promoter (44). MSKs can also phosphorylate histone H3 at serine, which might contribute to the expression of the viral immediate early gene. In HSV-1-induced Epstein-Barr virus (EBV) reactivation, the PKA/CREB1 pathway mediates the activation of Zta, a major transcriptional transactivator of EBV lytic replication (69). A previous study of KSHV demonstrated that activation of the cAMP/PKA pathway alone is sufficient to reactivate KSHV from latency (34). Because CREB1 is one of the primary transcription factors that mediate PKA signaling (39), deletion of CRE from the RTA promoter reduced PKA-induced RTA expression by 50% (34). Thus, the cAMP/PKA/CREB1 pathway is essential for KSHV reactivation. It is interesting that the cAMP/PKA pathway is not activated during primary KSHV infection of HUVEC. Instead, the MSK1/2-CREB pathway is activated and contributes to the viral lytic replication program. Because MSKs can activate multiple transcription factors, including CREB and AP1, it would be worthwhile to study the role of the MSK pathway in KSHV reactivation.

In KS lesions, most of the spindle-shaped tumor cells are latently infected by KSHV, indicating an essential role of viral latent infection in KSHV-induced malignant cell proliferation (71–73). However, a small subset of KS tumor cells also undergo spontaneous viral lytic replication, which might contribute to KS development (51). In patients, KSHV lytic activity and the viral load are strongly correlated with the progression of KS tumors (74–77). Our results suggest that the MSK1/2-CREB1 pathway might be a potential target for inhibiting KSHV lytic replication. Since CREB1 signaling can also induce proinflammatory cytokines, including IL-6 and tumor necrosis factor alpha (TNF-α) (25), targeting the MSK1/2-CREB1 pathway could have dual inhibitory effects on KSHV lytic replication and inflammation by preventing the establishment of a vicious amplification cycle, particularly during the early stage of tumor development.

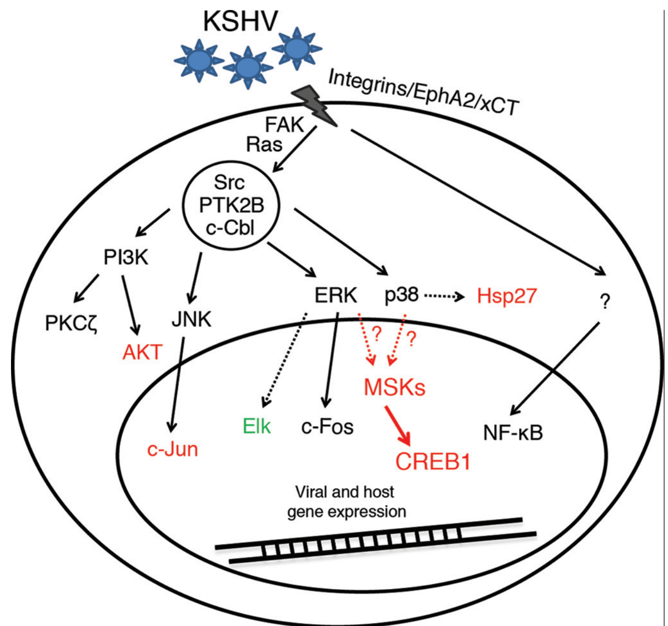

FIG 10 Schematic illustration of activated signaling pathways that regulate KSHV lytic replication during primary infection. The binding of KSHV virions to cellular receptors activates multiple signaling pathways, including FAK, PI3K/AKT, ERK, JNK, p38, and NF-κB pathways. MAPKs activate AP-1 factors, including c-Jun and c-Fos, as well as MSK1/2 and the downstream target CREB1. The interactions of the activated signaling pathways and their downstream transcriptional factors determine KSHV gene expression and lytic replication. The MAPK–AP-1 and MAPK-MSK-CREB1 pathways promote KSHV lytic replication, while the NF-κB pathway inhibits KSHV lytic replication. The phosphorylation statuses of the kinases or molecules are shown in green if not yet confirmed, in black if confirmed in previous studies, and in red if confirmed in the present study.

Figure 10 illustrates the confirmed cellular signaling pathways that are hijacked by KSHV for the optimal expression of viral genes and productive viral lytic replication during primary KSHV infection.

ACKNOWLEDGMENTS

We thank members of S.-J. Gao's laboratory for technical assistance and helpful discussions.

This work was supported by grants from the NIH (CA096512, CA124332, CA132637, CA177377, DE025465, and CA197153) to S.-J.G. and from the NIH (CA180779, CA082057, CA31363, CA115284, CA180779, AI105809, and AI073099), the Hastings Foundation, and the Fletcher Jones Foundation to J.U.J.

REFERENCES

1. **Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869. http://dx.doi.org/10.1126/science.7997879.
2. **Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med **332:**1186–1191. http://dx.doi.org/10.1056/NEJM199505043321802.
3. **Soulier J, Grollet L, Oksenhendler E, Cacoub P, Cazals-Hatem D, Babinet P, d'Agay MF, Clauvel JP, Raphael M, Degos L, Sigaux F.** 1995.

Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood **86:**1276–1280.

4. **Zhang W, Gao SJ.** 2012. Exploitation of cellular cytoskeletons and signaling pathways for cell entry by Kaposi's sarcoma-associated herpesvirus and the closely related rhesus rhadinovirus. Pathogens **1:**102–127. http://dx.doi.org/10.3390/pathogens1020102.
5. **Akula SM, Naranatt PP, Walia NS, Wang FZ, Fegley B, Chandran B.** 2003. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) infection of human fibroblast cells occurs through endocytosis. J Virol **77:**7978–7990. http://dx.doi.org/10.1128/JVI.77.14.7978-7990.2003.
6. **Chakraborty S, Veettil MV, Chandran B.** 2012. Kaposi's sarcoma associated herpesvirus entry into target cells. Front Microbiol **3:**6. http://dx.doi.org/10.3389/fmicb.2012.00006.
7. **Dutta D, Chakraborty S, Bandyopadhyay C, Valiya Veettil M, Ansari MA, Singh VV, Chandran B.** 2013. EphrinA2 regulates clathrin mediated KSHV endocytosis in fibroblast cells by coordinating integrin-associated signaling and c-Cbl directed polyubiquitination. PLoS Pathog **9:**e1003510. http://dx.doi.org/10.1371/journal.ppat.1003510.
8. **Greene W, Gao SJ.** 2009. Actin dynamics regulate multiple endosomal steps during Kaposi's sarcoma-associated herpesvirus entry and trafficking in endothelial cells. PLoS Pathog **5:**e1000512. http://dx.doi.org/10.1371/journal.ppat.1000512.
9. **Pan H, Xie J, Ye F, Gao SJ.** 2006. Modulation of Kaposi's sarcoma-associated herpesvirus infection and replication by MEK/ERK, JNK, and p38 multiple mitogen-activated protein kinase pathways during primary infection. J Virol **80:**5371–5382. http://dx.doi.org/10.1128/JVI.02299-05.
10. **Xie J, Pan H, Yoo S, Gao SJ.** 2005. Kaposi's sarcoma-associated herpesvirus induction of AP-1 and interleukin 6 during primary infection mediated by multiple mitogen-activated protein kinase pathways. J Virol **79:**15027–15037. http://dx.doi.org/10.1128/JVI.79.24.15027-15037.2005.
11. **Naranatt PP, Krishnan HH, Smith MS, Chandran B.** 2005. Kaposi's sarcoma-associated herpesvirus modulates microtubule dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. J Virol **79:**1191–1206. http://dx.doi.org/10.1128/JVI.79.2.1191-1206.2005.
12. **Greene W, Zhang W, He M, Witt C, Ye F, Gao SJ.** 2012. The ubiquitin/proteasome system mediates entry and endosomal trafficking of Kaposi's sarcoma-associated herpesvirus in endothelial cells. PLoS Pathog **8:**e1002703. http://dx.doi.org/10.1371/journal.ppat.1002703.
13. **Valiya Veettil M, Sadagopan S, Kerur N, Chakraborty S, Chandran B.** 2010. Interaction of c-Cbl with myosin IIA regulates Bleb associated macropinocytosis of Kaposi's sarcoma-associated herpesvirus. PLoS Pathog **6:**e1001238. http://dx.doi.org/10.1371/journal.ppat.1001238.
14. **Naranatt PP, Akula SM, Zien CA, Krishnan HH, Chandran B.** 2003. Kaposi's sarcoma-associated herpesvirus induces the phosphatidylinositol 3-kinase-PKC-zeta-MEK-ERK signaling pathway in target cells early during infection: implications for infectivity. J Virol **77:**1524–1539. http://dx.doi.org/10.1128/JVI.77.2.1524-1539.2003.
15. **Sadagopan S, Sharma-Walia N, Veettil MV, Raghu H, Sivakumar R, Bottero V, Chandran B.** 2007. Kaposi's sarcoma-associated herpesvirus induces sustained NF-kappaB activation during de novo infection of primary human dermal microvascular endothelial cells that is essential for viral gene expression. J Virol **81:**3949–3968. http://dx.doi.org/10.1128/JVI.02333-06.
16. **Gao SJ, Deng JH, Zhou FC.** 2003. Productive lytic replication of a recombinant Kaposi's sarcoma-associated herpesvirus in efficient primary infection of primary human endothelial cells. J Virol **77:**9738–9749. http://dx.doi.org/10.1128/JVI.77.18.9738-9749.2003.
17. **Yoo SM, Zhou FC, Ye FC, Pan HY, Gao SJ.** 2005. Early and sustained expression of latent and host modulating genes in coordinated transcriptional program of KSHV productive primary infection of human primary endothelial cells. Virology **343:**47–64. http://dx.doi.org/10.1016/j.virol.2005.08.018.
18. **Grossmann C, Ganem D.** 2008. Effects of NFkappaB activation on KSHV latency and lytic reactivation are complex and context-dependent. Virology **375:**94–102. http://dx.doi.org/10.1016/j.virol.2007.12.044.
19. **Krishnan HH, Naranatt PP, Smith MS, Zeng L, Bloomer C, Chandran B.** 2004. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. J Virol **78:**3601–3620. http://dx.doi.org/10.1128/JVI.78.7.3601-3620.2004.
20. **Li X, Feng J, Sun R.** 2011. Oxidative stress induces reactivation of Kaposi's sarcoma-associated herpesvirus and death of primary effusion lymphoma cells. J Virol **85:**715–724. http://dx.doi.org/10.1128/JVI.01742-10.
21. **Xie J, Ajibade AO, Ye F, Kuhne K, Gao SJ.** 2008. Reactivation of Kaposi's sarcoma-associated herpesvirus from latency requires MEK/ERK, JNK and p38 multiple mitogen-activated protein kinase pathways. Virology **371:**139–154. http://dx.doi.org/10.1016/j.virol.2007.09.040.
22. **Ye FC, Zhou FC, Xie JP, Kang T, Greene W, Kuhne K, Lei XF, Li QH, Gao SJ.** 2008. Kaposi's sarcoma-associated herpesvirus latent gene vFLIP inhibits viral lytic replication through NF-kappaB-mediated suppression of the AP-1 pathway: a novel mechanism of virus control of latency. J Virol **82:**4235–4249. http://dx.doi.org/10.1128/JVI.02370-07.
23. **Deutsch E, Cohen A, Kazimirsky G, Dovrat S, Rubinfeld H, Brodie C, Sarid R.** 2004. Role of protein kinase C delta in reactivation of Kaposi's sarcoma-associated herpesvirus. J Virol **78:**10187–10192. http://dx.doi.org/10.1128/JVI.78.18.10187-10192.2004.
24. **Johannessen M, Delghandi MP, Moens U.** 2004. What turns CREB on? Cell Signal **16:**1211–1227. http://dx.doi.org/10.1016/j.cellsig.2004.05.001.
25. **Wen AY, Sakamoto KM, Miller LS.** 2010. The role of the transcription factor CREB in immune function. J Immunol **185:**6413–6419. http://dx.doi.org/10.4049/jimmunol.1001829.
26. **Altarejos JY, Montminy M.** 2011. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol **12:**141–151. http://dx.doi.org/10.1038/nrm3072.
27. **Kandel ER.** 2012. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. Mol Brain **5:**14. http://dx.doi.org/10.1186/1756-6606-5-14.
28. **Zirpel L, Janowiak MA, Veltri CA, Parks TN.** 2000. AMPA receptor-mediated, calcium-dependent CREB phosphorylation in a subpopulation of auditory neurons surviving activity deprivation. J Neurosci **20:**6267–6275.
29. **Quasdorff M, Protzer U.** 2010. Control of hepatitis B virus at the level of transcription. J Viral Hepat **17:**527–536. http://dx.doi.org/10.1111/j.1365-2893.2010.01315.x.
30. **Millhouse S, Kenny JJ, Quinn PG, Lee V, Wigdahl B.** 1998. ATF/CREB elements in the herpes simplex virus type 1 latency-associated transcript promoter interact with members of the ATF/CREB and AP-1 transcription factor families. J Biomed Sci **5:**451–464. http://dx.doi.org/10.1007/BF02255935.
31. **Schwartz R, Helmich B, Spector DH.** 1996. CREB and CREB-binding proteins play an important role in the IE2 86-kilodalton protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter. J Virol **70:**6955–6966.
32. **Vendel AC, McBryant SJ, Lumb KJ.** 2003. KIX-mediated assembly of the CBP-CREB-HTLV-1 Tax coactivator-activator complex. Biochemistry **42:**12481–12487. http://dx.doi.org/10.1021/bi0353023.
33. **Langlois M, Audet B, Legault E, Pare ME, Ouellet M, Roy J, Dumais N, Mesnard JM, Rothstein DM, Marriott SJ, Tremblay MJ, Barbeau B.** 2004. Activation of HTLV-I gene transcription by protein tyrosine phosphatase inhibitors. Virology **329:**395–411. http://dx.doi.org/10.1016/j.virol.2004.09.003.
34. **Chang M, Brown HJ, Collado-Hidalgo A, Arevalo JM, Galic Z, Symensma TL, Tanaka L, Deng H, Zack JA, Sun R, Cole SW.** 2005. Beta-adrenoreceptors reactivate Kaposi's sarcoma-associated herpesvirus lytic replication via PKA-dependent control of viral RTA. J Virol **79:**13538–13547. http://dx.doi.org/10.1128/JVI.79.21.13538-13547.2005.
35. **Sharma-Walia N, George Paul A, Patel K, Chandran K, Ahmad W, Chandran B.** 2010. NFAT and CREB regulate Kaposi's sarcoma-associated herpesvirus-induced cyclooxygenase 2 (COX-2). J Virol **84:**12733–12753. http://dx.doi.org/10.1128/JVI.01065-10.
36. **Sivakumar R, Sharma-Walia N, Raghu H, Veettil MV, Sadagopan S, Bottero V, Varga L, Levine R, Chandran B.** 2008. Kaposi's sarcoma-associated herpesvirus induces sustained levels of vascular endothelial growth factors A and C early during in vitro infection of human microvascular dermal endothelial cells: biological implications. J Virol **82:**1759–1776. http://dx.doi.org/10.1128/JVI.00873-07.
37. **Lagos D, Pollara G, Henderson S, Gratrix F, Fabani M, Milne RS, Gotch F, Boshoff C.** 2010. miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nat Cell Biol **12:**513–519. http://dx.doi.org/10.1038/ncb2054.
38. **Ye F, Zhou F, Bedolla RG, Jones T, Lei X, Kang T, Guadalupe M, Gao SJ.** 2011. Reactive oxygen species hydrogen peroxide mediates Kaposi's

sarcoma-associated herpesvirus reactivation from latency. PLoS Pathog **7:**e1002054. http://dx.doi.org/10.1371/journal.ppat.1002054.

39. **Mayr B, Montminy M.** 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol **2:**599–609.
40. **Montminy M.** 1997. Transcriptional regulation by cyclic AMP. Annu Rev Biochem **66:**807–822. http://dx.doi.org/10.1146/annurev.biochem.66.1.807.
41. **Arthur JS.** 2008. MSK activation and physiological roles. Front Biosci **13:**5866–5879.
42. **Thomas GM, Huganir RL.** 2004. MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci **5:**173–183. http://dx.doi.org/10.1038/nrn1346.
43. **Lonze BE, Ginty DD.** 2002. Function and regulation of CREB family transcription factors in the nervous system. Neuron **35:**605–623. http://dx.doi.org/10.1016/S0896-6273(02)00828-0.
44. **Kew VG, Yuan J, Meier J, Reeves MB.** 2014. Mitogen and stress activated kinases act co-operatively with CREB during the induction of human cytomegalovirus immediate-early gene expression from latency. PLoS Pathog **10:**e1004195. http://dx.doi.org/10.1371/journal.ppat.1004195.
45. **Zhao Q, Liang D, Sun R, Jia B, Xia T, Xiao H, Lan K.** 2015. Kaposi's sarcoma-associated herpesvirus-encoded replication and transcription activator impairs innate immunity via ubiquitin-mediated degradation of myeloid differentiation factor 88. J Virol **89:**415–427. http://dx.doi.org/10.1128/JVI.02591-14.
46. **Brulois KF, Chang H, Lee AS, Ensser A, Wong LY, Toth Z, Lee SH, Lee HR, Myoung J, Ganem D, Oh TK, Kim JF, Gao SJ, Jung JU.** 2012. Construction and manipulation of a new Kaposi's sarcoma-associated herpesvirus bacterial artificial chromosome clone. J Virol **86:**9708–9720. http://dx.doi.org/10.1128/JVI.01019-12.
47. **Zhou FC, Zhang YJ, Deng JH, Wang XP, Pan HY, Hettler E, Gao SJ.** 2002. Efficient infection by a recombinant Kaposi's sarcoma-associated herpesvirus cloned in a bacterial artificial chromosome: application for genetic analysis. J Virol **76:**6185–6196. http://dx.doi.org/10.1128/JVI.76.12.6185-6196.2002.
48. **Gao SJ, Kingsley L, Li M, Zheng W, Parravicini C, Ziegler J, Newton R, Rinaldo CR, Saah A, Phair J, Detels R, Chang Y, Moore PS.** 1996. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat Med **2:**925–928. http://dx.doi.org/10.1038/nm0896-925.
49. **He M, Zhang W, Bakken T, Schutten M, Toth Z, Jung JU, Gill P, Cannon M, Gao SJ.** 2012. Cancer angiogenesis induced by Kaposi sarcoma-associated herpesvirus is mediated by EZH2. Cancer Res **72:**3582–3592. http://dx.doi.org/10.1158/0008-5472.CAN-11-2876.
50. **Rose PP, Carroll JM, Carroll PA, DeFilippis VR, Lagunoff M, Moses AV, Roberts CT, Jr, Fruh K.** 2007. The insulin receptor is essential for virus-induced tumorigenesis of Kaposi's sarcoma. Oncogene **26:**1995–2005. http://dx.doi.org/10.1038/sj.onc.1210006.
51. **Ye F, Lei X, Gao SJ.** 2011. Mechanisms of Kaposi's sarcoma-associated herpesvirus latency and reactivation. Adv Virol **2011:**193860.
52. **Sharma-Walia N, Krishnan HH, Naranatt PP, Zeng L, Smith MS, Chandran B.** 2005. ERK1/2 and MEK1/2 induced by Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) early during infection of target cells are essential for expression of viral genes and for establishment of infection. J Virol **79:**10308–10329. http://dx.doi.org/10.1128/JVI.79.16.10308-10329.2005.
53. **Ye FC, Zhou FC, Nithianantham S, Chandran B, Yu XL, Weinberg A, Gao SJ.** 2013. Kaposi's sarcoma-associated herpesvirus induces rapid release of angiopoietin-2 from endothelial cells. J Virol **87:**6326–6335. http://dx.doi.org/10.1128/JVI.03303-12.
54. **Qian LW, Xie J, Ye F, Gao SJ.** 2007. Kaposi's sarcoma-associated herpesvirus infection promotes invasion of primary human umbilical vein endothelial cells by inducing matrix metalloproteinases. J Virol **81:**7001–7010. http://dx.doi.org/10.1128/JVI.00016-07.
55. **Ye FC, Blackbourn DJ, Mengel M, Xie JP, Qian LW, Greene W, Yeh IT, Graham D, Gao SJ.** 2007. Kaposi's sarcoma-associated herpesvirus promotes angiogenesis by inducing angiopoietin-2 expression via AP-1 and Ets1. J Virol **81:**3980–3991. http://dx.doi.org/10.1128/JVI.02089-06.
56. **Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoel M, Raffelsberger W, Poch O, McKeating JA, Brino L, Baumert TF.** 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat Med **17:**589–595. http://dx.doi.org/10.1038/nm.2341.
57. **Hahn AS, Kaufmann JK, Wies E, Naschberger E, Panteleev-Ivlev J, Schmidt K, Holzer A, Schmidt M, Chen J, Konig S, Ensser A, Myoung J, Brockmeyer NH, Sturzl M, Fleckenstein B, Neipel F.** 2012. The ephrin receptor tyrosine kinase A2 is a cellular receptor for Kaposi's sarcoma-associated herpesvirus. Nat Med **18:**961–966. http://dx.doi.org/10.1038/nm.2805.
58. **Sansone P, Bromberg J.** 2012. Targeting the interleukin-6/Jak/Stat pathway in human malignancies. J Clin Oncol **30:**1005–1014. http://dx.doi.org/10.1200/JCO.2010.31.8907.
59. **Garbers C, Aparicio-Siegmund S, Rose-John S.** 2015. The IL-6/gp130/STAT3 signaling axis: recent advances towards specific inhibition. Curr Opin Immunol **34C:**75–82. http://dx.doi.org/10.1016/j.coi.2015.02.008.
60. **Punjabi AS, Carroll PA, Chen L, Lagunoff M.** 2007. Persistent activation of STAT3 by latent Kaposi's sarcoma-associated herpesvirus infection of endothelial cells. J Virol **81:**2449–2458. http://dx.doi.org/10.1128/JVI.01769-06.
61. **Lee MS, Jones T, Song DY, Jang JH, Jung JU, Gao SJ.** 2014. Exploitation of the complement system by oncogenic Kaposi's sarcoma-associated herpesvirus for cell survival and persistent infection. PLoS Pathog **10:**e1004412. http://dx.doi.org/10.1371/journal.ppat.1004412.
62. **Molden J, Chang Y, You Y, Moore PS, Goldsmith MA.** 1997. A Kaposi's sarcoma-associated herpesvirus-encoded cytokine homolog (vIL-6) activates signaling through the shared gp130 receptor subunit. J Biol Chem **272:**19625–19631. http://dx.doi.org/10.1074/jbc.272.31.19625.
63. **Gwack Y, Hwang S, Lim C, Won YS, Lee CH, Choe J.** 2002. Kaposi's sarcoma-associated herpesvirus open reading frame 50 stimulates the transcriptional activity of STAT3. J Biol Chem **277:**6438–6442. http://dx.doi.org/10.1074/jbc.M108289200.
64. **Burger M, Hartmann T, Burger JA, Schraufstatter I.** 2005. KSHV-GPCR and CXCR2 transforming capacity and angiogenic responses are mediated through a JAK2-STAT3-dependent pathway. Oncogene **24:**2067–2075. http://dx.doi.org/10.1038/sj.onc.1208442.
65. **King CA.** 2013. Kaposi's sarcoma-associated herpesvirus kaposin B induces unique monophosphorylation of STAT3 at serine 727 and MK2-mediated inactivation of the STAT3 transcriptional repressor TRIM28. J Virol **87:**8779–8791. http://dx.doi.org/10.1128/JVI.02976-12.
66. **Furth PA, Nakles RE, Millman S, Diaz-Cruz ES, Cabrera MC.** 2011. Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer. Breast Cancer Res **13:**220. http://dx.doi.org/10.1186/bcr2921.
67. **Brown HJ, Peng L, Harada JN, Walker JR, Cole S, Lin SF, Zack JA, Chanda SK, Sun R.** 2010. Gene expression and transcription factor profiling reveal inhibition of transcription factor cAMP-response element-binding protein by gamma-herpesvirus replication and transcription activator. J Biol Chem **285:**25139–25153. http://dx.doi.org/10.1074/jbc.M110.137737.
68. **Lan K, Kuppers DA, Verma SC, Sharma N, Murakami M, Robertson ES.** 2005. Induction of Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen by the lytic transactivator RTA: a novel mechanism for establishment of latency. J Virol **79:**7453–7465. http://dx.doi.org/10.1128/JVI.79.12.7453-7465.2005.
69. **Wu H, Li T, Zeng M, Peng T.** 2012. Herpes simplex virus type 1 infection activates the Epstein-Barr virus replicative cycle via a CREB-dependent mechanism. Cell Microbiol **14:**546–559. http://dx.doi.org/10.1111/j.1462-5822.2011.01740.x.
70. **Murata T, Sato Y, Nakayama S, Kudoh A, Iwahori S, Isomura H, Tajima M, Hishiki T, Ohshima T, Hijikata M, Shimotohno K, Tsurumi T.** 2009. TORC2, a coactivator of cAMP-response element-binding protein, promotes Epstein-Barr virus reactivation from latency through interaction with viral BZLF1 protein. J Biol Chem **284:**8033–8041. http://dx.doi.org/10.1074/jbc.M808466200.
71. **Deng JH, Zhang YJ, Wang XP, Gao SJ.** 2004. Lytic replication-defective Kaposi's sarcoma-associated herpesvirus: potential role in infection and malignant transformation. J Virol **78:**11108–11120. http://dx.doi.org/10.1128/JVI.78.20.11108-11120.2004.
72. **Fakhari FD, Jeong JH, Kanan Y, Dittmer DP.** 2006. The latency-associated nuclear antigen of Kaposi sarcoma-associated herpesvirus induces B cell hyperplasia and lymphoma. J Clin Invest **116:**735–742. http://dx.doi.org/10.1172/JCI26190.
73. **Staudt MR, Dittmer DP.** 2003. Viral latent proteins as targets for Kaposi's sarcoma and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)

induced lymphoma. Curr Drug Targets Infect Disord **3:**129–135. http://dx.doi.org/10.2174/1568005033481150.
74. **Brown EE, Whitby D, Vitale F, Fei PC, Del Carpio C, Marshall V, Alberg AJ, Serraino D, Messina A, Gafa L, Goedert JJ.** 2005. Correlates of human herpesvirus-8 DNA detection among adults in Italy without Kaposi sarcoma. Int J Epidemiol **34:**1110–1117. http://dx.doi.org/10.1093/ije/dyi131.
75. **Dagna L, Broccolo F, Paties CT, Ferrarini M, Sarmati L, Praderio L, Sabbadini MG, Lusso P, Malnati MS.** 2005. A relapsing inflammatory syndrome and active human herpesvirus 8 infection. N Engl J Med **353:**156–163. http://dx.doi.org/10.1056/NEJMoa042850.
76. **Duprez R, Kassa-Kelembho E, Plancoulaine S, Briere J, Fossi M, Kobangue L, Minsart P, Huerre M, Gessain A.** 2005. Human herpesvirus 8 serological markers and viral load in patients with AIDS-associated Kaposi's sarcoma in Central African Republic. J Clin Microbiol **43:**4840–4843. http://dx.doi.org/10.1128/JCM.43.9.4840-4843.2005.
77. **Guadalupe M, Pollock BH, Westbrook S, Redding S, Bullock D, Anstead G, Agan BK, Marconi VC, Barbieri S, Sankar V, Rebeles J, Flahive Y, Schoolfield J, Wang L, Lei X, Dow D, Yeh CK, Dang H, Infante AJ, Gao SJ.** 2011. Risk factors influencing antibody responses to Kaposi's sarcoma-associated herpesvirus latent and lytic antigens in patients under antiretroviral therapy. J Acquir Immune Defic Syndr **56:**83–90. http://dx.doi.org/10.1097/QAI.0b013e3181fdc928.