Short-Chain Fatty Acids from Periodontal Pathogens Suppress Histone Deacetylases, EZH2, and SUV39H1 To Promote Kaposi's Sarcoma-Associated Herpesvirus Replication

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ABSTRACT

Periodontal pathogens such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* produce five different short-chain fatty acids (SCFAs) as metabolic by-products. We detect significantly higher levels of SCFAs in the saliva of patients with severe periodontal disease. The different SCFAs stimulate lytic gene expression of Kaposi's sarcoma-associated herpesvirus (KSHV) dose dependently and synergistically. SCFAs inhibit class-1/2 histone deacetylases (HDACs) and downregulate expression of silent information regulator-1 (SIRT1). SCFAs also downregulate expression of enhancer of zeste homolog2 (EZH2) and suppressor of variegation 3-9 homolog1 (SUV39H1), which are two histone N-lysine methyltransferases (HMTs). By suppressing the different components of host epigenetic regulatory machinery, SCFAs increase histone acetylation and decrease repressive histone trimethylations to transactivate the viral chromatin. These new findings provide mechanistic support that SCFAs from periodontal pathogens stimulate KSHV replication and infection in the oral cavity and are potential risk factors for development of oral Kaposi's sarcoma (KS).

IMPORTANCE

About 20% of KS patients develop KS lesions first in the oral cavity, while other patients never develop oral KS. It is not known if the oral microenvironment plays a role in oral KS tumor development. In this work, we demonstrate that a group of metabolic by-products, namely, short-chain fatty acids, from bacteria that cause periodontal disease promote lytic replication of KSHV, the etiological agent associated with KS. These new findings provide mechanistic support that periodontal pathogens create a unique microenvironment in the oral cavity that contributes to KSHV replication and development of oral KS.

Kaposi's sarcoma (KS), the most common malignancy in patients infected with human immunodeficiency virus (HIV), is etiologically associated with infection by Kaposi's sarcoma-associated herpesvirus (KSHV) (1). About 20% of KS patients first develop KS lesions in the oral cavity, and up to 70% of KS patients eventually develop concurrent oral and cutaneous KS (2). Through saliva transmission, the oral cavity is a major target of acute KSHV infection and a potential reservoir of latent KSHV as well (3). However, it remains unclear why oral KS occurs only in a subset of patients. Clinically, it has been noticed that HIV patients display a higher prevalence of periodontal disease with more severe symptoms (4–6). Accumulating evidence supports the notion that periodontal disease creates an oral microenvironment that stimulates KSHV replication and infection, contributing to oral KS development. Indeed, a previous study demonstrated that butyric acid produced by anaerobic Gram-negative bacteria such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* induces KSHV lytic gene expression and replication by inhibiting class-1/2 histone deacetylases (HDACs) (7), supporting a link between periodontal disease and KSHV replication in the oral cavity. However, these oral bacteria produce multiple metabolic by-products, such as lipopolysaccharide (LPS), fimbriae, proteinases, and at least five different short-chain fatty acids (SCFAs), including butyric acid, isobutyric acid, isovaleric acid, propionic acid, and acetic acid. In order to further investigate the link between periodontal disease and development of oral KS, it is necessary and

important to analyze the effects of different bacterial metabolites on KSHV replication and decipher the mechanisms involved in the process.

Like other herpesviruses, KSHV enters a latent replication mode following primary infection. Reactivation of the latent virus for lytic replication is necessary and essential for causing *de novo* infection and development of KS tumors (8). During latency, the majority of the KSHV genome is silenced through various epigenetic modifications, including histone deacetylation, repressive histone methylation, and DNA methylation (9, 10). Upon stimulation of KSHV latently infected cells, the viral chromatin undergoes rapid changes, including removal of the silencing/repressive modifications and addition of activating histone marks, leading to transactivation of the viral chromatin and expression of all genes necessary for the viral lytic replication cycle. Very little is known about how different components of the host epigenetic regulatory

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machinery coordinate and execute in concert to achieve the various chromatin modifications to reactivate the viral genome upon stimulation. While the previous study demonstrated that butyric acid induces KSHV reactivation through inhibition of class-1/2 HDACs to result in histone hyperacetylation (7), the roles of other SCFAs and their possible impacts on different epigenetic regulatory components have not been examined.

In the present study, we investigated whether all five SCFAs from periodontal pathogens stimulate KSHV lytic replication and examined how these bacterial metabolic by-products impact different components of the host epigenetic regulatory machinery to transactivate the viral chromatin. We demonstrated that the different SCFAs are prevalent in the saliva of patients with severe periodontal disease and induce KSHV lytic replication in dose-dependent and synergistic manners. These molecules inhibit the activity of class-1/2 HDACs and downregulate expression of sir-tuin-1 (SIRT1), which is a class-3 HDAC (11). SCFAs also down-regulate expression of enhancer of zeste homolog2 (EZH2) (12) and suppressor of variegation 3-9 homolog1 (SUV39H1) (13), which are two histone N-lysine methyltransferases (HMTs) responsible for the repressive histone trimethylation marks H3K27Me3 and H3K9Me3, respectively. As a consequence, SCFA stimulation increases histone acetylation and decreases repressive histone trimethylation at the promoter region of the KSHV immediate-early gene (ORF50), which encodes the viral replication and transcription activator (RTA) protein (14). Our data suggest that SCFAs simultaneously impact multiple components of the host epigenetic regulatory machinery to transactivate the viral chromatin. These new findings provide novel mechanistic support that SCFAs from periodontal pathogens enhance KSHV lytic replication and infection in the oral cavity to promote development of oral KS.

MATERIALS AND METHODS

Reagents. Molecular-grade SCFAs butyric acid, isobutyric acid, isovaleric acid, valeric acid, propionic acid, and acetic acid; SIRT1 inhibitor Sirtinol; class-1/2 HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA); and SUV39H1 inhibitor chaetocin were purchased from Sigma-Aldrich. EZH2 inhibitor UNC was a gift from Jian Jin from the Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, NC, USA. Antibodies against EZH2, SIRT1, RNA polymerase II, acetylated histones, including H4K12-Ac and H2B-Ac, and repressive histones trimethylation marks H3K27Me3 and H3K9Me3 were purchased from Millipore.

Patient recruitment, periodontal disease assessment, and saliva collection. A total of 21 participants were recruited for the study, which included a group of 11 patients with generalized severe chronic periodontitis (7 males and 4 females from 40 to 72 years of age, with a mean age of 50 years) and a group of 10 periodontally healthy controls (6 males and 4 females from 24 to 30 years of age, with a mean age of 25.7 years). We used a protocol that is very similar to what has been reported previously (15) to define and assess the severity of periodontal disease of all recruited participants, which includes (i) probing depth and clinical attachment level (CAL) at six sites per tooth, (ii) gingival recession, and (iii) percentage of sites with bleeding on probing (BOP). Participants having CAL of ≥ 6 mm with at least 5-mm probing depth in 30% of the sites were considered patients with severe periodontal disease. Participants having CAL of ≤ 2 mm with probing depth of ≤ 3 mm on all teeth were selected as healthy controls. All healthy control participants had good general health, practiced good oral hygiene, had lost no teeth, and had no bleeding on probing. The study was performed by fully following an institutional review board (IRB) protocol approved by the Ethics

Committee of Case Western Reserve University and with the written consent of all participants. About 5 ml of saliva was collected from each participant. After centrifugation to remove cells and debris, the supernatant of the collected saliva was sterilized by passing through a 0.22- μ m filter, which either was used immediately for experiment or stored at -80°C . For extracting total DNA from the saliva, 1 ml of the collected saliva without centrifugation was used.

HDAC activity assay. Measurement of the relative activity of class-1/2 HDACs was conducted by using a fluorometric histone deacetylase assay kit from Sigma-Aldrich by following the manufacturer's instructions. Equal numbers (2.5×10^5) of BCBL1 cells were treated with fresh medium (control), supernatant of non-oral *Escherichia coli*, and supernatants of *P. gingivalis* and *F. nucleatum* at a 1:25 dilution (bacterial supernatant volume to total cell culture volume) for 6 h. The cells were harvested and resuspended in the assay buffer and then loaded onto a 96-well plate. Upon incubation with the substrate, the plate was read under a fluorimeter at an excitation wavelength of 380 nm and emission wavelength of 480 nm. The relative HDAC activity of the sample is represented as the mean value of fluorescence intensity from three repeats.

Isolation of total RNAs and quantification of mRNA by qRT-PCR. Total RNAs were isolated using an RNA purification kit from Qiagen, which includes a step to remove residual genomic DNA prior to RNA purification. Reverse transcription (RT) of total RNA was performed by using Superscript Transcriptase II (Invitrogen, Carlsbad, CA). Quantitative RT-PCR (qRT-PCR) was performed to quantify different viral transcripts using primers described previously. The mRNA level of the housekeeping gene β -actin was used as a reference for normalization and determined by using the primers 5'-ATTGCCGACAGGATGCAGA3' (forward) and 5'-GAGTACTTGCCTCAGGAGGA3' (reverse). The mRNA levels of SIRT1, EZH2, and SUV39H1 were determined using the following primers: 5'-AGAGCCTCACATGCAAGCTCTAG3' (SIRT1 forward), 5'-GCCAATCATAAGATGTTGCTGAAC3' (SIRT1 reverse), 5'-GTGGAGAGATTATTTCTCAAGATG3' (EZH2 forward), 5'-CCGACATAC TTCAGGGCATCAGCC3' (EZH2 reverse), 5'-GAGGATACGCACACAC TTGAGATT3' (SUV39H1 forward), and 5'-ATCCGCGAACAGGAATAT TACC3' (SUV39H1 reverse). All qRT-PCRs consisted of three repeats.

Knockdown of SIRT1 and SUV39H1 with specific shRNA. The pLKO.1 lentivirus vector harboring SIRT1-specific short hairpin RNA (shRNA) sequences (Mission shRNA constructs from Sigma-Aldrich) was used to produce a mixture of lentiviral particles for SIRT1 knockdown. Briefly, G-pseudotyped vesicular stomatitis viruses were produced with shRNA sequences that specifically target SIRT1 mRNA in the HEK 293T cell line using Lipofectamine as described previously (16). The sequences of three SIRT1-specific shRNAs were the following: 5'-GTACCG GCATGAAGTGCTCAGATATTACTCGAGTAATATCTGAGGCACT TCATGTTTTTTG3' (shRNA34), 5'-CCGGCCTCGAACAATTCTTAAA GATCTCGAGATCTTTAAGAAATTGTTTCGAGGTTTTT3' (shRNA80), and 5'-CCGGGCGGGAATCCAAAGGATAATTCTCGAGAATTATCCT TTGGATCCCGCTTTTTT3' (shRNA81). A scrambled (control) shRNA lentivirus was made with the following sequence: 5'-ACCGGCGCGAT AGCGCTAATAATTTCTCGAGAAATATTAGCGCTATCGCGCTTT TT3'. Virus titers were determined by infecting 1×10^6 Jurkat T cells with a serial dilution of concentrated and purified virus stocks from harvested culture supernatant by ultracentrifugation. The viral multiplicity of infection (MOI) was determined using a lentivirus qPCR titer kit (Applied Biological Materials Inc.) by following the manufacturer's instructions. Lentiviral particles (Mission shRNA TR0000275322 and TR0000275372) expressing SUV39H1-specific shRNA were purchased from Sigma-Aldrich. To knock down expression of SIRT1 and SUV39H1, BCBL1-BAC36 cells latently infected with KSHV were infected at MOIs of 1.8×10^6 and 3.6×10^6 IU of SIRT1- and SUV39H1-specific or scrambled control shRNA-containing viruses, respectively, followed by culturing the cells in RPMI 1640 plus 10% fetal bovine serum (FBS) for 72 h. Expression of SIRT1 and SUV39H1 after shRNA knockdown was verified by Western blotting with specific antibodies.

Culture of *P. gingivalis* and *F. nucleatum* and preparation of culture supernatants. Periodontopathogens *P. gingivalis* (ATCC 33277) and *F. nucleatum* (ATCC 25586) were maintained in blood agar plates (Fisher) and grown in enriched trypticase soy broth, as described previously (17), in an anaerobic system (5% CO₂, 10% H₂, and 85% N₂ at 37°C) to late log phase. The supernatants were collected by centrifugation at 10,000 × g for 10 min at 4°C to remove bacteria, followed by sterilization through a 0.22-μm-pore-size membrane filter. To prepare culture supernatants with high-molecular-weight bacterial by-products removed, the supernatants were loaded into the column of a YM3 filter (Millipore), followed by high-speed centrifugation (6,000 × g for 20 min) (18). To remove the volatile SCFAs, the filtered supernatants were loaded in conical tubes and heated at 96°C for 4 h, followed by sterilization through 0.22-μm filters. Non-oral *E. coli* bacteria were grown in LB broth at 37°C under aerobic conditions (with shaking), and *E. coli* supernatant was prepared from saturated culture and sterilized by filtration through 0.22-μm filters.

Measurement of SCFAs. Gas-phase chromatography in conjunction with mass spectrometry was used to analyze and determine the types and concentrations of SCFAs in saliva and bacterial culture supernatants, as described previously (19, 20). A series of dilutions for each pure SCFA, including butyric acid, isobutyric acid, propionic acid, isovaleric acid, valeric acid, and acetic acid, were run in parallel to establish a standard curve reflecting the correlation between signal peak area and the concentration of the analyte, and the concentration of the analyte in the sample is determined by comparing its peak area to the standard curve. The concentration of the SCFA in question for a given sample was calculated as the average value from triplicate measurements.

Quantification of periodontal bacteria in saliva by real-time PCR. Total DNA from 1 ml of saliva of each patient was extracted and purified using a genomic DNA isolation kit from Qiagen. Real-time PCR was conducted to determine the relative levels of *P. gingivalis* and *F. nucleatum* in the saliva by using the following specific primers: 5'-CGCAGAAGGTGA AAGTCTGTAT3' (*Fn*-forward), 5'-TGGTCTCACTGATTACACAGA3' (*Fn*-reverse), 5'-TGGTTTCATGCAGCTTCTTT3' (*Pg*-forward), and 5'-TCGGCACCTTCGTAATTCTT3' (*Pg*-reverse). The level of total bacteria in the saliva was determined by using the universal primers for bacterial 16S rRNA: 5'-TCCTACGGGAGGCAGCAGT3' (forward) and 5'-GGACTACCAGGTATCTAATCTGT3' (reverse). All PCRs consisted of triplicate repeats. The relative levels of *P. gingivalis* or *F. nucleatum* in the saliva in question were calculated as the ratios between the levels of the periodontal pathogens and the level of total bacteria.

KSHV production and titration. The culture supernatants of BCBL1-BAC36 cells were collected 5 days after stimulation, followed by low-speed centrifugation (4,000 × g for 15 min) to remove cellular debris. To determine the relative titers of KSHV in the supernatants, 1 ml of the supernatant was used to infect telomerase-immortalized human microvascular endothelial cells (TIME) that were placed in 6-well plates. At 72 h postinfection, cells were harvested and counted with a hemocytometer under a fluorescence microscope. The numbers of KSHV-infected green fluorescent protein (GFP)-positive cells and the numbers of total cells from 8 independent readings were used to calculate the average percentage of GFP-positive cells, which was used as the relative viral titer of the supernatant in question.

ChIP assay. Equal numbers of BCBL1-BAC36 cells (8 × 10⁶ cells) were treated with culture supernatant of *P. gingivalis* or fresh medium (control) at a 1:25 (supernatant volume to total cell culture medium volume) dilution for 6 h or 18 h, respectively, followed by fixation with 0.5% formaldehyde for 15 min. Chromatin suspensions were prepared and chromatin immunoprecipitation (ChIP) assays were performed by following the instructions of the ChIP assay kit from Invitrogen. Antibodies against EZH2, SIRT1, RNA polymerase II (RN Pol II), acetylated histone-4 (H4K12-Ac), the repressive histone trimethylation marks H3K27Me3 and H3K9Me3, activating histone trimethylation mark H3K4Me3, and control IgG from rabbit, all from Millipore, were used for the procedure. DNA from the input and the end ChIP products was iso-

lated by using the PCR product purification kit from Qiagen. The purified DNA was resuspended in 200 μl sterile water and used for real-time PCR quantification of specific viral chromatin by using the following primers: 5'-CTCATCGTCGGAGCTGTCACACG3' (RTA promoter-forward), 5'-TCTCCCGATGGCGACGTGCACTAC3' (RTA promoter-reverse), 5'-CATTGCCCGCCTCTATTATCA3' (vFLIP-forward), and 5'-ATGACGTTG GCAGGAACCA3' (vFLIP-reverse).

RESULTS

Culture supernatants of *P. gingivalis* and *F. nucleatum* stimulate KSHV lytic replication. The culture supernatants of anaerobic Gram-negative periodontal pathogens *P. gingivalis* and *F. nucleatum* have been shown previously to stimulate KSHV lytic replication in cultured primary effusion lymphoma (PEL) cell lines (7). Here, we used a recently developed quantitative system (21) to study the effects of bacterial metabolic by-products on KSHV lytic replication. As shown in Fig. 1A, the open reading frame 65 (ORF65), encoding a KSHV small capsid protein in the recombinant KSHV, BAC36 (22), was deleted and replaced with the firefly luciferase gene. With expression of the luciferase gene driven by the ORF65 promoter, this recombinant virus was reconstituted and stably maintained in BCBL1 cells to generate the BCBL1-BAC36-ORF65Δ/lucif cell line for quantitative measurement of KSHV lytic replication. Under normal culture conditions, over 98% of the cells have latent KSHV infection. We treated equal numbers of these cells with fresh medium for culturing *P. gingivalis* and *F. nucleatum* (control), culture supernatant of the non-oral pathogen *E. coli*, culture supernatants of *P. gingivalis* and *F. nucleatum* at 1/500, 1/100, 1/50, and 1/25 dilutions (volume of bacterial supernatant to total cell culture volume), and a combination of 12-O-tetradecanoyl-phorbol-13-acetate (TPA; 20 ng/ml) plus sodium butyrate (NaB; 1 mM) as a positive control for 72 h, followed by measuring luciferase activities of the differently treated cells. As shown in Fig. 1B, treatment with TPA plus NaB gave rise to the strongest luciferase activity. Compared to fresh medium (control), treatment with *E. coli* supernatant had little effect on luciferase activity. In contrast, treatment with the supernatants of *P. gingivalis* and *F. nucleatum* increased the luciferase activity in a dose-dependent manner. By conducting a trypan blue staining of cells at 24 h posttreatment, we observed dose-dependent cell death rates by the supernatants of *P. gingivalis* and *F. nucleatum*, as well as the combination of TPA plus NaB (Fig. 1C), suggesting significant toxicity of these different treatments. Accordingly, the luciferase activities shown in Fig. 1B were the values after normalization to the percentages of viable cells from each treatment. Consistent with the luciferase reporter results, the mRNA levels of KSHV lytic genes ORF50, ORF65, and ORF59 increased in response to stimulation with the supernatants of *P. gingivalis* and *F. nucleatum* in a dose-dependent manner (Fig. 1D). Treatment with the supernatants of *P. gingivalis* and *F. nucleatum* strongly induced expression of KSHV small capsid protein (ORF65) in BCBL1 cells (Fig. 1E) and increased viral production from BCBL1-BAC36 cells (Fig. 1F). These results are consistent with the previous report that culture supernatants of *P. gingivalis* and *F. nucleatum*, but not that of the non-oral pathogen *E. coli*, strongly stimulate KSHV lytic replication.

Oral transmission is a major route of KSHV infection, and the oral mucosa is the primary target for acute KSHV infection. Previous studies suggest that KSHV undergoes productive lytic replication in human oral epithelial cells (HOECs) following acute

FIG 1 Supernatants of *P. gingivalis* and *F. nucleatum* induce KSHV lytic gene expression and replication. (A) Schematic illustration of the mutant KSHV BAC36-ORF65 Δ /lucif in which the open reading frame (ORF) of the viral small capsid protein (ORF65) was replaced with the firefly luciferase gene. (B) Firefly luciferase activities from identical numbers of BCBL1-BAC36-ORF65 Δ /lucif that were treated with fresh medium (control) or supernatants of *E. coli* (Ec), *P. gingivalis* (Pg), or *F. nucleatum* (Fn) at different dilutions or a combination of TPA (20 ng/ml) plus NaB (1 mM) for 72 h. (C) Percentages of dead cells from equal numbers of BCBL1-BAC36-ORF65 Δ /lucif cells that were treated with fresh medium (control), supernatant of *E. coli* (Ec), *P. gingivalis* (Pg), or *F. nucleatum* (Fn) at different dilutions, or a combination of TPA (20 ng/ml) plus NaB (1 mM) for 24 h. Dead cells were revealed by 0.4% trypan blue staining and counted under a microscope. (D) Relative mRNA levels (fold) of KSHV lytic genes ORF50, ORF59, and ORF65 from BCBL1-BAC36 cells that were treated with fresh medium (control) or supernatant of *E. coli* (Ec), *P. gingivalis* (Pg), or *F. nucleatum* (Fn) at different dilutions for 24 h. (E) Western blot detection of KSHV small capsid protein (ORF65) from BCBL1-BAC36 cells that were treated with fresh medium (control), supernatant of *P. gingivalis* (Pg) or *F. nucleatum* (Fn) at a 1:25 dilution, or a combination of TPA (20 ng/ml) plus NaB (1 mM) for 72 h. β -Tubulin was used as a reference to calibrate loading. (F) Relative viral titers in the supernatants of equal numbers of BCBL1-BAC36 cells that were stimulated with fresh medium (Med) and supernatants of *P. gingivalis* (Pg) and *F. nucleatum* (Fn) at a 1:25 dilution for 5 days. Viral titers were determined by counting percentages of GFP-positive cells upon infecting telomerase-immortalized human microvascular endothelial (TIME) cells with supernatants of BCBL1-BAC36 cells for 48 h.

infection (23). To examine how periodontal pathogens influence KSHV replication in HOECs, we infected HOECs with a high titer of the recombinant KSHV BAC16 (24), which resulted in infection of virtually all cells. Three days after infection, we treated the cells with fresh medium (control) and *F. nucleatum* supernatant. As shown in Fig. 2A, the transcript levels of KSHV lytic genes ORF50 and ORF57 were substantially higher in cells treated with *F. nucleatum* supernatant than in cells treated with fresh medium (control) at 24 h posttreatment. Treatment with *F. nucleatum* supernatant also resulted in higher expression of viral small capsid protein (ORF65) (Fig. 2C). Similarly, stimulation with *P. gingivalis* supernatant substantially increased the mRNA levels of KSHV lytic genes ORF50 and ORF57 (Fig. 2B) and the protein level of ORF65 (Fig. 2C) in telomerase-immortalized human umbilical

vein endothelial cells latently infected with KSHV (TIVE-KSHV) (25). Together, these results suggest that the supernatants of *P. gingivalis* and *F. nucleatum* stimulate KSHV lytic replication in different types of cells in the oral cavity, which may include B cells, vascular endothelial cells, and oral epithelial cells.

***P. gingivalis* and *F. nucleatum* produce multiple SCFAs to synergistically induce KSHV lytic replication.** As mentioned earlier, *P. gingivalis* and *F. nucleatum* produce multiple metabolic by-products, including LPS, fimbriae, proteinases, and five different SCFAs. Since the supernatant of *E. coli*, which also produces LPS and fimbriae, did not enhance KSHV lytic replication (Fig. 1B and D), we suspected that SCFAs were the major bacterial metabolic by-products responsible for inducing KSHV lytic replication. To test this hypothesis, we passed the supernatants of *P.*

FIG 2 *F. nucleatum* and *P. gingivalis* supernatants induce KSHV lytic gene expression in acutely infected HOECs and latently infected and telomerase-immortalized human umbilical vein endothelial cells (TIVE-KSHV). (A and B) Relative mRNA levels of KSHV lytic genes ORF50 and ORF57 in acutely infected HOECs (A) and TIVE-KSHV cells (B) upon treatment with medium (control) and *F. nucleatum* or *P. gingivalis* supernatant at a 1:25 dilution for 24 h. (C) Immunofluorescence antibody (IFA) staining of KSHV small capsid protein (ORF65, red) in acutely infected HOECs and TIVE-KSHV cells that were treated with medium (control) and *F. nucleatum* or *P. gingivalis* supernatant at a 1:25 dilution for 72 h. All GFP-positive cells were KSHV infected.

gingivalis and *F. nucleatum* through the YM3 filter, which only allows molecules with a molecular mass smaller than 3 kDa through while excluding all other bacterial by-products from the filtered supernatants (18). Since SCFAs are volatile, we heated the filtered supernatants at 100°C for 2 h to remove SCFAs. We analyzed and measured the concentrations of all SCFAs in the supernatants of *P. gingivalis* and *F. nucleatum* before and after heating by using gas-phase chromatography coupled with mass spectrometry. As summarized in Table 1, both *P. gingivalis* and *F. nucleatum* produce millimolar levels of acetic acid, butyric acid, and propionic acid. *P. gingivalis* also produces millimolar levels of isobutyric acid and isovaleric acid. After heating, only residual levels of butyric acid and acetic acid were detected in the supernatants. We

TABLE 1 Concentration of individual SCFAs in the supernatants of *P. gingivalis* and *F. nucleatum* before and after heating at 100°C for 2 h^a

SCFA	Concn (mM) of:			
	<i>P. gingivalis</i>	<i>F. nucleatum</i>	<i>P. gingivalis</i> heated	<i>F. nucleatum</i> heated
Acetic acid	24.5	12.1	2.4	1.3
Butyric acid	7.4	16.5	0.1	0.1
Isobutyric acid	1.4	0	0	0
Isovaleric acid	3.6	0	0	0
Propionic acid	0.2	0.6	0	0

^a Late-log-phase cultures of the bacteria were used for gas-phase chromatography detection and measurement of SCFAs. The concentration of each SCFA was the average value from three readings.

then treated equal numbers of BCBL1-BAC36-ORF65Δ/lucif cells with fresh medium (control), supernatants of *P. gingivalis* and *F. nucleatum*, and filtered supernatant of *P. gingivalis* and *F. nucleatum* with and without heating for 72 h. As shown in Fig. 3A, both the filtered and unfiltered supernatants of *P. gingivalis* and *F. nucleatum* increased the luciferase activity of the treated cells at equal levels of efficiency. However, after heating, the bacterial supernatants lost their abilities to stimulate luciferase expression. These results confirm that SCFAs in the bacterial supernatants are responsible for inducing KSHV replication.

To further examine the effects of SCFAs on KSHV lytic replication, we treated equal numbers of BCBL1-BAC36-ORF65Δ/lucif cells with different doses of pure individual SCFAs that were detected in the supernatants of *P. gingivalis* and *F. nucleatum*. As shown in Fig. 3B, all SCFAs except acetic acid dose dependently increased luciferase activity of the treated cells. Among the five SCFAs, butyric acid was the most potent, while acetic acid had little effect. At low concentrations (0.5 mM), an additive effect was seen when combinations of the different SCFAs were used to treat the cells (Fig. 3C). Data from qRT-PCR and Western blot analysis confirmed that the different SCFAs induced transcription of KSHV lytic gene RTA (ORF50) and expression of the viral small capsid protein (ORF65) dose dependently and collectively (Fig. 3D and E) in BCBL1-BAC36 cells. Consistent with the luciferase activity patterns, butyric acid had the strongest effect while acetic acid had little effect on viral gene expression. The different SCFAs also increased viral production in a pattern similar to that of viral gene expression (Fig. 3F). Altogether, these results suggest that the

FIG 3 SCFAs from periodontal pathogens induce KSHV lytic gene expression dose dependently and additively. (A) Firefly luciferase activities from identical numbers of BCBL1-BAC36-ORF65Δ/lucif cells that were stimulated with fresh medium (control) or with supernatant of *F. nucleatum* (Fn) or *P. gingivalis* (Pg), with and without passing through the YM3 filter to remove other bacterial by-products and with and without heating to remove SCFAs, at a 1:25 dilution for 72 h. (B and C) Firefly luciferase activities from equal numbers of BCBL1-BAC36-ORF65Δ/lucif cells that were treated with different doses of individual SCFAs, alone (B) or in different combinations (C), for 72 h. (D) Relative mRNA levels of RTA (ORF50) in BCBL1-BAC36 cells that were treated with different SCFAs at 0.5 mM, alone or in combination, for 24 h. (E) Western blot detection of KSHV small capsid protein (ORF65) from equal numbers of BCBL1-BAC36 cells that were treated with different SCFAs at 0 to 5 mM for 72 h. (F) Relative titers (percentage of GFP-positive cells upon infection of TIME cells for 48 h) of KSHV produced from equal numbers of BCBL1-BAC36 cells that were stimulated with different SCFAs, alone or in combination, for 5 days. B, butyric acid; IB, isobutyric acid; IV, isovaleric acid; P, propionic acid; A, acetic acid.

different SCFAs from periodontal pathogens stimulate KSHV lytic replication dose dependently and collectively.

Saliva from patients with severe periodontal disease contains significantly higher levels of SCFAs. Previously, it has been reported that millimolar levels of SCFAs are present in the gingival crevicular fluid of patients with severe periodontal disease but are undetectable in the gingival crevicular fluid of healthy people (20). We collected saliva and measured the levels of SCFAs from 11

patients with severe periodontal disease and 10 healthy controls. Despite tremendous variations among individuals, the average concentrations of all five SCFAs were significantly higher in the saliva of patients with periodontal disease (Table 2). On average, the saliva of patients with periodontal disease contained about 5.4, 7.8, 2.3, and 1.6 times higher levels of butyric acid, isobutyric acid, isovaleric acid, and propionic acid than the healthy controls. In contrast to the previous report, lower levels of SCFAs were also

TABLE 2 Concentration of individual SCFAs in the saliva of healthy participants (control) and patients with severe periodontal disease

SCFA	Concn. (mM) (no. of patients) by patient group		Difference	P value ^a
	Control	PD		
Acetic acid	6.305 ± 1.744 (10)	7.406 ± 1.542 (11)	1.101 ± 2.339	0.643 (NS)
Butyric acid	0.058 ± 0.015 (10)	0.305 ± 0.083 (11)	0.247 ± 0.089	0.012 (S)
Isobutyric acid	0.029 ± 0.010 (10)	0.228 ± 0.107 (11)	0.198 ± 0.113	0.047 (S)
Isovaleric acid	0.064 ± 0.008 (10)	0.150 ± 0.056 (11)	0.086 ± 0.059	0.163 (NS)
Propionic acid	0.715 ± 0.108 (10)	1.353 ± 0.322 (11)	0.637 ± 0.354	0.043 (S)

^a NS, not statistically significant; S, statistically significant. P values were determined with the unpaired t test. P < 0.05 is significant.

detected in the saliva of healthy controls, and millimolar levels of acetic acid were found in the saliva of both groups of participants. By using specific primers for different periodontal pathogens and universal 16S rRNA primers for all bacteria, we conducted real-time PCR to determine the relative levels of *P. gingivalis* and *F. nucleatum* in the saliva of the two different groups of participants. In full agreement with the SCFA results, we detected *P. gingivalis* and *F. nucleatum* in both groups of participants. However, the average levels of *P. gingivalis* and *F. nucleatum* in the saliva of patients with periodontal disease were remarkably higher than those in the saliva of healthy controls (Fig. 4A).

To examine how saliva from the two groups of participants influences viral replication, we treated equal numbers of BCBL1-BAC36 cells with the saliva from 11 patients with periodontal disease and 10 healthy controls at a 1:2 dilution (saliva volume

versus total culture medium volume) for 24 h. Data from qRT-PCR analysis showed that the mRNA level of KSHV lytic gene RTA (ORF50) was significantly higher in cells treated with saliva from the patients with periodontal disease than in cells treated with saliva from the healthy controls (Fig. 4B). We also detected higher expression of RTA protein from cells that were treated with saliva from patients with periodontal disease (Fig. 4C). Notably, there were tremendous variations among the different oral samples in their abilities to induce viral gene expression. In general, strong correlations between the levels of *P. gingivalis* and *F. nucleatum*, the levels of SCFAs, and induction of KSHV gene expression were observed. These results consistently demonstrate that the different SCFAs from periodontal pathogens dose dependently and collectively promote KSHV lytic replication.

SCFAs simultaneously suppress class-1/2 HDACs, SIRT1, EZH2, and SUV39H1. The majority of the KSHV genome is silenced during latency through multiple epigenetic modifications, such as histone deacetylation and repressive histone methylations. During reactivation, the levels of histone acetylation increase, while repressive histone methylation marks are removed from the viral genome and replaced with activating histone methylation marks (9, 10, 26, 27). In a pilot study, we observed that SCFA stimulation of BCBL1 cells not only increased the overall levels of acetylated histone marks, such as H4K12-Ac and H2B-Ac, but also decreased the levels of the constitutive repressive histone trimethylation mark H3K9Me3 and the facultative repressive histone trimethylation mark H3K27Me3 (data not shown). Therefore, we decided to examine how SCFA treatment impacts the enzymes that are responsible for the different histone modifications. Previously, it was reported that SCFAs inhibit the activity of class-1/2 HDACs (28). We found that the relative activities of class-1/2 HDACs were indeed significantly reduced in BCBL1 cells that were treated with supernatants of *P. gingivalis* and *F. nucleatum* at 1:50 dilutions for 6 h compared to cells treated with fresh medium and *E. coli* supernatant (Fig. 5A). By Western blotting, no significant change in the level of HDAC1 protein was seen in the differently treated cells (Fig. 5B), suggesting that SCFAs do not affect HDAC1 expression despite inhibiting its activity. In contrast, the protein levels of the class 3 HDAC SIRT1 and the two HLMTs, EZH2 and SUV39H1, were significantly reduced in cells treated with supernatants of *P. gingivalis* and *F. nucleatum* (Fig. 5B). As shown in Fig. 5C, in cells treated with *P. gingivalis* supernatant, downregulation of SIRT1, EZH2, and SUV39H1 started as early as 3 h posttreatment. The level of the activating histone trimethylation mark H3K4Me3 was increased at 6 h posttreatment with *P. gingivalis* supernatant. However, the level of SETD1A, the enzyme responsible for H3K4Me3 (29), was reduced by treatment with *P. gingivalis* supernatant, suggesting that SCFAs engage other mech-

FIG 4 Saliva from patients with severe periodontal disease contains higher levels of SCFAs. (A) Relative levels of *F. nucleatum* (Fn) and *P. gingivalis* (Pg) in the saliva of patients with severe periodontal disease (PD) and healthy controls (Ctl). (B) Relative mRNA levels of KSHV lytic gene RTA (ORF50) in BCBL1-BAC36 cells that were stimulated with saliva from 11 patients with severe periodontal disease (PD) and 10 healthy controls (Ctl) for 24 h. (C) Western blot detection of KSHV RTA protein from equal numbers of BCBL1-BAC36 cells that were stimulated with saliva from 8 patients with severe periodontal disease (PD) and 8 healthy controls (Ctl) for 24 h. β -Tubulin was used as a reference to calibrate loading. Concentrations (mM) of butyric acid (B), isobutyric acid (IB), propionic acid (P), and isovaleric acid (IV) in each of the saliva samples used for treating BCBL1-BAC36 cells are shown below the corresponding protein bands.

FIG 5 SCFAs inhibit activity of class-1/2 HDACs, suppress transcription of EZH2 and SUV39H1, and cause degradation of SIRT1 and EZH2. (A) Average fluorescence intensity from three replicates of HDAC activity assays, representing the relative activity of class-1/2 HDACs from equal numbers of BCBL1 cells that were treated with control medium (Ctl) or supernatant of *E. coli* (Ec), *P. gingivalis* (Pg), or *F. nucleatum* (Fn) at a 1:50 dilution for 24 h. (B) Western blot detection of SIRT1, EZH2, SUV39H1, HDAC1, and β -tubulin in BCBL1-BAC36 cells that were treated with control medium (Ctl) or supernatants of *E. coli* (Ec), *P. gingivalis* (Pg), or *F. nucleatum* (Fn) for 24 h. (C) Western blot detection of SIRT1, EZH2, SUV39H1, SETD1A, H3K4Me3, and β -tubulin in BCBL1-BAC36 cells that were treated with control medium (Ctl) or supernatant of *P. gingivalis* (Pg) for 3 h or 6 h. (D) Relative mRNA levels of SIRT1, EZH2, and SUV39H1 in BCBL1 cells that were treated with control medium (Ctl) or *P. gingivalis* (Pg) supernatant at a 1:50 dilution for 24 h. (E) Western blot detection of SIRT1 and EZH2 in BCBL1-BAC36 cells and KSHV acutely infected HOECs (HOECs-BAC16) that were treated with control medium (Ctl) and *P. gingivalis* (Pg) supernatant at a 1:50 dilution for 24 h. Small fragments resulting from protein degradation are indicated with an arrow.

anisms to increase the level of H3K4Me3. Similar downregulation of SIRT1, EZH2, and SUV39H1 was also seen in KSHV-infected HOECs and TIVE-KSHV upon treatment with *P. gingivalis* supernatant (data not shown), suggesting that the bacterial by-products have similar epigenetic effects on different types of cells.

To investigate how SCFAs downregulate SIRT1, EZH2, and SUV39H1, we first conducted qRT-PCR to measure the mRNA levels of these genes in BCBL1 cells that were treated with *P. gingivalis* supernatant or fresh medium for 12 h. As shown in Fig. 5D, the levels of SUV39H1 and EZH2 mRNAs in cells treated with *P. gingivalis* supernatant were reduced to 50.6% and 67.3%, respec-

tively, of that in cells treated with fresh medium, suggesting that SCFAs suppress transcription of these two HLMTs. In BCBL1 cells treated with 1 mM butyric acid, isovaleric acid, propionic acid, isobutyric acid, and acetic acid, the levels of SUV39H1 are 59.2%, 68.7%, 72.1%, 89.4%, and 96.8% of that in untreated cells, respectively, further confirming that SCFAs inhibit transcription of SUV39H1. In contrast, the mRNA of SIRT1 is 1.72 times higher in cells treated with *P. gingivalis* supernatant than in cells treated with fresh medium, suggesting that SCFA downregulation of SIRT1 is not at the transcriptional level. Since downregulation of SIRT1 occurred as early as 3 h posttreatment, we suspected that SCFAs treatment causes degradation of this protein. By using a different anti-SIRT1 antibody, we found that treatment with *P. gingivalis* supernatant substantially reduced the level of the SIRT1 protein band while generating two smaller fragments (Fig. 5E), indicating that SCFAs indeed cause degradation of SIRT1. The previously used monoclonal anti-SIRT1 antibody did not detect these two fragments, probably because the specific epitope was located outside these fragments. Similarly, a small fragment was also detected from cells treated with *P. gingivalis* supernatant with the anti-EZH2 antibody, suggesting that SCFAs not only suppress EZH2 transcription but also cause its protein degradation. We also detected similar degradation of SIRT1 and EZH1 in KSHV-infected HOECs (Fig. 5E) that were treated with *P. gingivalis* supernatant, suggesting that SCFAs have similar effects on these proteins in different types of cells.

We next examined how individual SCFAs impact the different epigenetic regulators. As shown in Fig. 6A, butyric acid had the strongest dose-dependent effects on downregulation of SIRT1, EZH2, and SUV39H1. As a consequence, butyric acid substantially increased the levels of histone acetylation represented by H4-K12-Ac but decreased the levels of repressive histone methylation marks H3K27Me3 and H3K9Me3. Propionic acid and isovaleric acid also strongly reduced the levels of SIRT1, EZH2, and SUV39H1 in a dose-dependent manner. Isobutyric acid had weaker effects on these proteins. In contrast, acetic acid had little effect on the levels of SIRT1 and EZH2. Butyric acid, isovaleric acid, and propionic acids also increased the level of H3K4Me3 dose dependently (Fig. 6B). At low concentrations (0.5 mM), additive effects on downregulation of SIRT1, EZH2, and SUV39H1 were seen when combinations of SCFAs were applied to BCBL1-BAC36 cells (Fig. 6C), which is consistent with their additive effect on induction of viral gene expression shown previously.

SCFAs simultaneously increase histone acetylation and decrease repressive histone methylation to transactivate viral chromatin. To examine if SCFAs increase histone acetylation and decrease repressive histone methylation on KSHV chromatin to induce viral gene expression, we performed chromatin immunoprecipitation (ChIP) assays by using specific antibodies to SIRT1, EZH2, acetylated histone mark H4K12-Ac, repressive histone trimethylation marks H3K27Me3 and H3K9Me3, activating histone trimethylation mark H3K4Me3, RNA polymerase II (RN Pol II), and control IgG to pull down specific chromatin from BCBL1-BAC36 cells that were treated with fresh medium (control) and *P. gingivalis* supernatant for 6 h and 18 h, respectively. We focused on the promoter of the KSHV immediate-early gene RTA (ORF50), which is not expressed during latency due to promoter silencing. RTA expression is essential for transcriptional activation of downstream genes to complete the viral lytic replication cycle (14). As indicated in Fig. 7A, a pair of primers specific for the

FIG 6 SCFAs downregulate SIRT1, EZH2, and SUV39H1 to increase histone acetylation and decrease repressive histone trimethylation. Western blot analysis was performed to detect protein levels of SIRT1, EZH2, SUV39H1, acetylated histone-4 (H4K12-Ac), repressive histone trimethylation marks H3K27Me3 and H3K9Me3 (A), and activating histone trimethylation mark H3K4Me3 (B) in BCBL1-BAC36 cells that were treated with different doses of butyric acid (B), propionic acid (P), isobutyric acid (IB), isovaleric acid (IV), and acetic acid (A), individually (A and B) or in different combinations at 0.5 mM each (C) for 24 h.

RTA promoter was designed to quantify DNA from the resulting ChIP products. A pair of primers from the coding region of the latent gene vFLIP (ORF71) was used to study the effects of SCFAs on KSHV chromatin at the latency locus. The relative amounts of viral chromatin at the two different regions resulting from the ChIP assay with a given antibody were determined by real-time PCR with the specific primers and read as ratios (percentages) to the corresponding amounts of input DNA that were initially used for each ChIP assay.

As shown in Fig. 7B, both SIRT1 and EZH2 were present in the RTA promoter and vFLIP coding region during latency, but their presence was rapidly reduced from the viral chromatin upon treatment with *P. gingivalis* supernatant. The relative level of H4K12-Ac in the RTA promoter region was 1.83 times higher in cells that were treated with *P. gingivalis* supernatant for 18 h than in cells that were treated with medium. In the vFLIP coding region, the level of H4K12-Ac increased 1.19 times only. In contrast, the levels of the repressive histone marks H3K27Me3 and H3K9Me3 in both RTA promoter and the vFLIP coding region rapidly decreased upon treatment with *P. gingivalis* supernatant. The level of H3K4Me3 in the RTA promoter increased 1.33 and 4.36 times at 6 h and 18 h posttreatment with *P. gingivalis* supernatant, respectively. The level of H3K4Me3 in the vFLIP coding

region slightly increased upon treatment with *P. gingivalis* supernatant but was substantially higher than that in the RTA promoter with and without treatment with *P. gingivalis* supernatant. Consistent with the increased levels of H4K12-Ac and H3K4Me3 but decreased levels of H3K27Me3 and H3K9Me3, the level of RNA polymerase II in the RTA promoter increased by 6.02 times. The level of RNA polymerase in the vFLIP coding region slightly decreased upon treatment with *P. gingivalis* supernatant. Notably, the relative level of RNA polymerase II in the vFLIP coding region was significantly higher than that in the RTA promoter in cells that were treated with fresh medium (control), suggesting that the RTA promoter is indeed silenced while the latent locus is active during latency. Much lower levels of DNA were pulled down with the control IgG, possibly due to nonspecific antibody binding to chromatin, and no significant differences were seen between cells with the different treatments, suggesting that all ChIP assays were antibody specific. Collectively, these results demonstrate that SCFAs simultaneously increase histone acetylation and decrease repressive histone trimethylation on KSHV chromatin to induce viral gene expression.

Inhibition or knockdown of SIRT1, EZH2, and SUV39H1 suffices to induce KSHV lytic replication. The role of EZH2 in KSHV chromatin silencing has been previously suggested (10),

FIG 7 SCFAs increase histone acetylation and decrease repressive histone trimethylation to transactivate KSHV chromatin. (A) Locations of primers used for real-time PCR quantification of DNA in the promoter of KSHV lytic gene RTA (ORF50) and coding region of latent gene vFLIP (ORF71) following ChIP assays. (B) Relative amounts of DNA resulting from ChIP assays with specific antibodies to SIRT1, EZH2, H4K12-Ac, H3K27Me3, H3K9Me3, RNA polymerase II (RN Pol II), H3K4Me3, and control rabbit IgG. Chromatin suspensions were isolated from equal numbers of BCBL1-BAC36 cells that were stimulated with fresh medium (control) or *P. gingivalis* supernatant for 6 h or 18 h. The relative amount of DNA in the RTA promoter (RTA) or vFLIP coding region (VF) from each ChIP reaction was determined by real-time PCR and calculated as the average ratio between the level of ChIP product and the level of input DNA from three repeats.

while the ways SIRT1 and SUV39H1 contribute to viral genome silencing have not been reported. To demonstrate that suppression of SIRT1 and SUV39H1 induces KSHV lytic gene expression and replication, we either knocked down expression or inhibited their activities with specific inhibitors in BCBL1-BAC36 cells. As shown in Fig. 8A, both knockdown of SIRT1 expression with specific shRNAs and treatment with the SIRT1-specific inhibitor Sirtinol dose dependently induced expression of KSHV small capsid protein (ORF65) in BCBL1-BAC36 cells. Similarly, knockdown of SUV39H1 with specific shRNA also induced expression of ORF65 (Fig. 8B). In agreement with the previous report of EZH2 involvement in KSHV genome silencing, treatment of BCBL1-BAC36 cells with the EZH2 inhibitor UNC clearly induced ORF65 expression (Fig. 8C). A slight increase in ORF65 expression was also seen when a combination of Sirtinol and UNC was used to treat the cells.

The results shown above demonstrate that suppressing either one of these three proteins is sufficient to induce KSHV lytic gene expression, which is very intriguing, because each of these proteins is responsible for a different type of histone modification, and the latent KSHV genome is silenced through all types of histone modifications by each of these proteins. Because we observed that the class-1/2 HDAC inhibitor SAHA also downregulates expression of SIRT1 and EZH2 (data not shown), we suspected that class-1/2 HDACs, SIRT1, EZH2, and SUV39H1 mutually regulate each other. To test this hypothesis, we treated BCBL1 cells with another class-1/2 HDAC inhibitor, trichostatin A (TSA), the

SIRT1 inhibitor Sirtinol, the EZH2 inhibitor UNC, and the SUV39H1 inhibitor chaetocin for 24 h. As shown in Fig. 8D, similar to SAHA, TSA substantially reduced the level of EZH2 and, to a lesser degree, lowered the levels of SIRT1 and SUV39H1. Similarly, the SIRT1 inhibitor Sirtinol substantially reduced the levels of EZH2 and SIRT1 and weakly lowered the level of SUV39H1. EZH2 inhibitor UNC had no effect on EZH2 but reduced the levels of SIRT1 and SUV39H1. The SUV39H1 inhibitor chaetocin significantly reduced the level of SIRT1. These results indicate that the different epigenetic components/pathways of the host epigenetic regulatory machinery mutually regulate each other, and that inhibition of either one also suppresses the others. Notably, all inhibitors induced expression of KSHV lytic protein RTA, further supporting the notion that suppression of either class-1/2 HDACs, SIRT1, EZH2, or SUV39H1 is sufficient to induce KSHV lytic gene expression. Collectively, these results strongly suggest that the different epigenetic regulators cross-talk by regulating expression of each other. Through such coordinated cross-talk, upon stimulation, cells simultaneously achieve different histone modifications on the viral chromatin to induce gene expression.

DISCUSSION

KS is the most common malignancy in HIV patients. The oral cavity is the first site of KS lesions in 20% of KS patients, and up to 70% of KS patients eventually develop oral KS concurrent with skin and visceral KS (2). Development of oral KS suggests active KSHV replication and infection in the oral cavity of the patients.

FIG 8 Functional inhibition or expressional knockdown of SIRT1, EZH2, and SUV39H1 suffices to induce KSHV lytic gene expression. (A and B) Western blot detection of SIRT1 (A), SUV39H1 (B), KSHV small capsid protein (ORF65), and β -tubulin in BCBL1-BAC36 cells that were transduced with lentiviruses expressing SIRT1- and SUV39H1-specific shRNAs at an MOI of 1.8×10^6 or 3.6×10^6 IU or treated with SIRT1 inhibitor Sirtinol at 5 nM and 25 nM for 72 h. Cells transduced with lentivirus expressing control shRNA or treated with placebo (dimethylsulfoxide [DMSO]) were used as controls. (C) Western blot detection of ORF65 protein in BCBL1-BAC36 cells that were treated with EZH2 inhibitor UNC and SIRT1 inhibitor Sirtinol at 5 nM each, alone or in combination, for 72 h. (D) Western blot detection of EZH2, SIRT1, SUV39H1, KSHV lytic protein RTA, and β -tubulin in BCBL1-BAC36 cells that were treated with class-1/2 HDAC inhibitor TSA (10 μ M), Sirtinol (25 nM), or UNC (5 nM) or the SUV39H1 inhibitor chaetocin (50 nM) for 24 h.

Like all herpesviruses, KSHV establishes a persistent latent infection following acute primary infection. In HIV patients, the latent virus in the viral reservoir reactivates to undergo lytic replication, producing new virions to cause *de novo* infection of endothelial cells to promote KS tumor growth. The fact that only a subset of HIV patients develops oral KS suggests that the unique microenvironment in the oral cavity of these patients plays an important role in promoting KSHV replication and infection.

Periodontal disease is a common condition that results from infection by multiple Gram-negative anaerobic bacteria and subsequent chronic inflammation. Higher prevalence of periodontal disease has been observed in HIV patients (4, 5). The symptoms of periodontal disease in HIV patients are very distinctive and more severe (6). Accumulating evidence supports the notion that periodontal pathogens and viruses in the oral arena interact with each other to exert synergistic effects on the diseases they cause (30–32). Viral infection may weaken the host antimicrobial ability in favor of infection by periodontal pathogens, while periodontal pathogens may promote viral infection and reactivation for lytic replication. Indeed, a previous study showed that the culture supernatants of periodontal pathogens *P. gingivalis* and *F. nucleatum* promote lytic replication of KSHV (7). Butyric acid, a metabolic by-product of these bacteria, was found to be responsible for pro-

moting viral replication by inhibiting class-1/2 HDACs, leading to histone hyperacetylation and induction of viral gene expression and replication. In the present study, we quantitatively showed that the supernatants of *P. gingivalis* and *F. nucleatum* promote KSHV lytic replication in latently infected BCBL1 cells as well as in acutely infected primary oral epithelial cells. *P. gingivalis* and *F. nucleatum* produce at least five different SCFAs, including butyric acid, isobutyric acid, isovaleric acid, propionic acid, and acetic acid. All of the SCFAs except acetic acid promote KSHV lytic gene expression dose dependently. The different SCFAs also act in concert to elicit an additive and much stronger effect on viral replication. Saliva from patients with severe periodontal disease contains significantly higher levels of these different SCFAs and induces KSHV lytic gene expression much more efficiently. Collectively, these results strongly support the link between chronic periodontitis and increased risks of KSHV replication and infection in the oral cavity for development of oral KS.

SCFAs are natural inhibitors of class-1/2 HDACs. We unexpectedly found that these molecules also downregulate expression of the class-3 HDAC SIRT1 and the HMTs EZH2 and SUV39H1. As a consequence of simultaneous suppression of both class-1/2 HDACs and SIRT1, SCFA treatment substantially increases histone acetylation. SCFAs also increase the level of the activating

histone trimethylation mark H3K4Me3 while reducing the protein level of SETD1A, which is the enzyme responsible for H3K4Me3. This suggests that SCFAs increase the level of H3K4Me3, possibly by engaging other mechanisms, such as enhancing the activity of SETD1A and/or inhibiting the activity or downregulating the expression of enzymes that demethylate H3K4Me3 (33). Further studies are needed to decipher the mechanisms of increased levels of H3K4Me3 by SCFAs. Meanwhile, SCFAs downregulate EZH2 and SUV39H1 to decrease the levels of the facultative histone trimethylation mark H3K27Me3 and the constitutive histone trimethylation mark H3K9Me3. Data from ChIP assays confirm that SCFA treatment indeed increases the levels of histone acetylation and the activating histone trimethylation mark H3K4Me3 but decreases the levels of the repressive histone methylation marks H3K27Me3 and H3K9Me3 in the promoter region of the KSHV immediate-early gene RTA (ORF50). These new findings suggest that SCFAs do not act simply as class-1/2 HDAC inhibitors as previously reported but impact multiple components of the host epigenetic regulatory machinery to transactivate the KSHV genome and promote viral gene expression and lytic replication.

Different from other HDACs that simply hydrolyze acetyllysine residues, SIRT1 is a member of the class-3 HDACs and the Sirtuin protein family, which couples lysine deacetylation to NAD hydrolysis (34–36). The dependence of SIRT1 on NAD links its enzymatic activity directly to the energy status of the cells via the cellular NAD/NADH ratio, the absolute levels of NAD, NADH, or nicotinamide, or a combination of these variables. SIRT1 is well known for its antiaging, antioxidative stress, and anti-inflammation properties (37–39). However, its role in silencing the KSHV genome has not been well defined. In this study, we found that SIRT1 is present in the RTA promoter during latency but quickly disassociates from the viral chromatin upon SCFA stimulation. Functional inhibition with Sirtinol or expressional knockdown with specific shRNAs of SIRT1 suffices to induce KSHV gene expression. These results suggest that SIRT1 indeed plays an important role in silencing the latent KSHV genome. With regard to EZH2, which is also a subunit of the polycomb repressive complex2 (PRC2) (40), previous studies demonstrate that it is present along the entire genome of KSHV during latency but rapidly disassociates from the RTA promoter upon stimulation for lytic replication (10). Our results demonstrate that EZH2 is indeed present in the RTA promoter during latency and quickly disassociates upon SCFA stimulation. Chemical inhibition of EZH2 leads to enhanced KSHV lytic gene expression, confirming an important role of EZH2 in KSHV genome silencing through the facultative histone methylation mark H3K27Me3. Previous studies reported that the RTA promoter has a bivalent structure with concomitant and extensive presence of the activating histone mark H3K4Me3, as well as the facultative repressive mark H3K29Me3, but a very limited level of the constitutive repression mark H3K9Me3 (9, 10). In contrast to the previous reports, our data suggest that the RTA promoter is also subjected to silencing by the HLMT SUV39H1 with the extensive presence of the constitutive repression mark H3K9Me3 during KSHV latency. Upon SCFA stimulation, the level of H3K9Me3 in the RTA promoter decreases rapidly. Consistent with these results, chemical inhibition or knockdown of SUV39H1 enhances RTA gene expression. Collectively, our data clearly demonstrate that SCFAs simultaneously modulate both histone acetylation and repressive histone methyl-

ation to efficiently induce KSHV gene expression and lytic replication.

It is important to note that butyric acid has also been shown to induce reactivation of other herpesviruses, such as Epstein-Barr virus (EBV) and latent HIV-1, for lytic replication through inhibition of class-1/2 HDACs (41, 42). The new mechanistic findings from the present work also may apply to other herpesviruses and HIV-1 reactivation induced by SCFAs. It is not known if high levels of SCFAs contribute to any human diseases. Recent studies suggest that SCFAs suppress macrophages to inhibit innate immunity against bacteria and promote differentiation of regulatory T cells in mice (43, 44), suggesting that these bacterial by-products exert much broader effects on the host. Since HIV patients display higher prevalence and more severe symptoms of periodontal disease, it would be interesting to investigate whether SCFAs contribute to oral KS through other mechanisms, such as promoting *de novo* KSHV infection and the growth and progression of KS tumors in the oral microenvironment in the future.

During latency, the majority of the KSHV genome is silenced through multiple epigenetic regulatory mechanisms, including histone deacetylation, repressive histone methylation (9, 45, 46), DNA methylation (26, 47), and repression of specific chromatin regions by the insulator CCCTC-binding factor (CTCF) and the cohesin complex, among others (48–50). Reactivation of the latent virus for lytic replication may require removal of all of the silencing factors and addition of chromatin-activating elements to the viral genomes simultaneously. It is our speculation that SCFAs affect many additional epigenetic regulators, such as those responsible for different types of activating histone methylation marks, DNA methylation/demethylation, etc., and insulator CCCTC-binding factor (CTCF) and the cohesin complex that silence the KSHV genome at specific regions. Further investigation of the global effects of these bacterial by-products on host epigenetic regulatory machinery is needed to fully understand how these bacterial by-products stimulate KSHV gene expression and replication.

It is unclear how the different components of the host epigenetic regulatory machinery coordinate with each other to accomplish the different histone modifications to induce viral gene expression. Cross-talk among the different epigenetic regulators might be one mechanism for such coordination. Indeed, SIRT1 has been shown to recruit and interact with EZH2 and SUV39H1, respectively, to result in histone deacetylation and repressive histone trimethylation simultaneously (51, 52). Similar interaction between class-1/2 HDACs and EZH2 has also been observed (53). In the present study, we found that treatment of cells with the class-1/2 HDAC-specific inhibitors SAHA and TSA reduces the protein levels of SIRT1, EZH2, and SUV39H1. Similarly, inhibition of SIRT1 with Sirtinol downregulates EZH2 and SUV39H1, and suppression of SUV39H1 leads to reduced levels of SIRT1 and EZH2 as well. These data strongly suggest that class-1/2 HDACs control expression of SIRT1, EZH2, and SUV39H1, and that SIRT1, EZH2, and SUV39H1 also mutually regulate expression of each other. Suppression of either one of these different epigenetic regulators/pathways affects expression and activities of the other pathways. Through such coordinated cross-talk among the different epigenetic regulators, cells achieve the different histone modifications simultaneously to transactivate the viral chromatin. With such mechanisms, it is not surprising that suppression of either class-1/2 HDACs, SIRT1, EZH2, or SUV39H1 suffices to

induce RTA expression. At this point, the mechanisms of SCFA downregulation of SIRT1, EZH2, and SUV39H1 remain to be further determined. Given that SCFAs are well-known class-1/2 HDAC inhibitors, we speculate that SCFA downregulation of SIRT1, EZH2, and SUV39H1 is mediated by inhibition of class-1/2 HDACs.

In summary, we have demonstrated that periodontal pathogens, such as *P. gingivalis* and *F. nucleatum*, produce multiple SCFAs to synergistically affect different components of the host epigenetic machinery to transactivate the viral chromatin and induce viral gene expression and lytic replication in the oral cavity. These new findings underscore the potential risks of these bacterial metabolic by-products in promoting KSHV replication and infection in the oral cavity for the development of oral KS. Discovery of these new mechanisms of SCFA induction of viral gene expression should also shed light on developing novel strategies to prevent and treat oral KS in HIV patients.

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We declare no conflict of interest.

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