Discovery of Kaposi's sarcoma herpesvirus-encoded circular RNAs and a human antiviral circular RNA

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Noncoding RNAs have substantial effects in host-virus interactions. Circular RNAs (circRNAs) are novel single-stranded noncoding RNAs which can decoy other RNAs or RNA-binding proteins to inhibit their functions. The role of circRNAs is largely unknown in the context of Kaposi's sarcoma herpesvirus (KSHV). We hypothesized that circRNAs influence viral infection by inhibiting host and/or viral factors. Transcriptome analysis of KSHV-infected primary endothelial cells and a B cell line identified human circRNAs that are differentially regulated upon infection. We confirmed the expression changes with divergent PCR primers and RNase R treatment of specific circRNAs. Ectopic expression of hsa_circ_0001400, a circRNA induced by infection, suppressed expression of key viral latent gene *LANA* and lytic gene *RTA* in KSHV de novo infections. Since human herpesviruses express noncoding RNAs like microRNAs, we searched for viral circRNAs encoded in the KSHV genome. We performed circRNA-Seq analysis with RNase R-treated, circRNA-enriched RNA from KSHV-infected cells. We identified multiple circRNAs encoded by the KSHV genome that are expressed in KSHV-infected endothelial cells and primary effusion lymphoma (PEL) cells. The KSHV circRNAs are located within ORFs of viral lytic genes, are up-regulated upon the induction of the lytic cycle, and alter cell growth. Viral circRNAs were also detected in lymph nodes from patients of KSHV-driven diseases such as PEL, Kaposi's sarcoma, and multicentric Castleman's disease. We revealed new host-virus interactions of circRNAs: human antiviral circRNAs are activated in response to KSHV infection, and viral circRNA expression is induced in the lytic phase of infection.

circular RNA | KSHV | noncoding RNA | circRNA-Seq | herpesvirus

Recent reports have described thousands of naturally occurring circular RNAs (circRNAs) from mammalian genomes (1–3). These circular RNAs usually contain exonic sequences, but have a unique exon order due to a back-splicing event. This back-spliced junction is unique to the specific circular RNA and is not found in the related linear RNA transcripts. Some of these circular RNAs are very abundant, with 10-fold higher levels than their related linear RNAs (3). A recent report has indicated that circular RNAs can be transported by extracellular vesicles (4). These circular RNAs are protected from exonucleases, and some contain multiple predicted miRNA target sites, which are conserved. Furthermore, some circular RNAs can act as sponges or decoys to prevent microRNAs (miRNAs) or RNA-binding proteins (RBPs) from regulating specific mRNA targets (1, 2, 5).

Human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma herpesvirus (KSHV), can cause multiple diseases, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and a plasmablastic form of multicentric Castleman's disease (MCD). Even in the era of effective antiretroviral therapy, KSHV-associated diseases can develop in patients with undetectable HIV viral loads and near normal CD4+ T cell counts (6). KS is the second most common cancer in individuals with AIDS in the United States (7). In addition, 0.5–5% of organ transplant recipients develop KS (8). Furthermore, KSHV-associated diseases are

widespread in regions of sub-Saharan Africa, and in some African countries KS is the most common cancer in men (9, 10). KSHV is also one of a small number of known human cancer viruses and a member of a small group of human viruses that express multiple viral miRNAs. Some of these viral miRNAs are more abundant than human miRNAs in KSHV-infected patient-derived cell lines.

Viruses have been reported to interact with various noncoding RNAs (ncRNAs). Host miRNAs bind to viral factors; for example, the hepatitis C virus (HCV) RNA genome depends on binding to human miR-122 (11). Many viruses, including KSHV, encode miRNAs and target human or viral transcripts (12). Recently, miR-122 targeting artificial circRNAs was designed and proven to have antiviral capacity (13). These developments raise the possibility that natural human circular RNAs can potentially act as antiviral molecules if they contain binding sites for

Significance

Human herpesviruses are known to interact with non-protein encoding RNAs like microRNAs and long noncoding RNAs. Circular RNAs (circRNAs) are recently discovered noncoding RNAs that are long-lived and resistant to exonucleases, and that bind to other RNAs or RNA-binding proteins. This research aimed to investigate interaction between circRNAs and Kaposi's sarcoma herpesvirus (KSHV). We identified a certain human circRNA that can work as an antiviral molecule by suppressing crucial viral genes. Further, multiple circRNAs were found to be encoded in the KSHV genome and expressed in KSHV-infected cells as well as KSHV-positive patients. We discovered a new layer of host-virus interactions with circRNAs which are potentially applicable to other viruses, and these antiviral circRNAs or viral circRNAs may represent novel therapeutic targets.

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Conflict of interest statement: T.S.U. is a coinventor on a patent application related to the treatment of KSHV-associated diseases with pomalidomide. This invention was made as part of his duties as an employee of the US government, and the patents are or will be assigned to the US Department of Health and Human Services. The government may convey a portion of the royalties it receives from licensure of its patents to its employee inventors. T.S.U. has recently conducted clinical research using drugs supplied to the National Cancer Institute through cooperative research and development agreements with Celgene Corp., Merck and Co., and Hoffman LaRoche.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession nos. GSE119608, GSE120045, and GSE121756).

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proviral miRNAs or RBPs to inhibit functions of viral RNAs or viral proteins. In addition to viral miRNAs, KSHV encodes *PAN*, a long noncoding RNA (lncRNA), which is highly expressed during the lytic phase. KSHV has a relatively large 138-kb genome, and its capacity to encode ncRNAs (analogous to human ncRNAs) prompted us to seek evidence of viral circular RNAs that are similar to cellular RNAs.

We performed de novo KSHV infections and screened for human circRNAs up-regulated in response to viral infection with microarray and circRNA sequencing. We found a human circRNA that has antiviral activity and is induced upon infection. We utilized the same circRNA-Seq technique and found that KSHV expresses multiple circRNAs. The viral circRNAs are encoded in open reading frames (ORFs) of viral lytic genes and are expressed upon induction of the lytic phase in KSHV-infected cells. Some viral circRNAs were detected in clinical samples from KSHV-positive patients, and the expression correlated with viral lytic gene expression. In this study, we revealed a novel antiviral system with human circRNAs, and we reported our discovery of viral circRNAs as a new class of viral ncRNAs.

Results

KSHV Infection Induces Certain Human circRNAs That Harbor KSHV miRNA Binding Sites. We hypothesized that specific human circular RNAs may function as inhibitors of infection. These potentially antiviral circular RNAs may be induced upon infection with KSHV. We searched for human circRNAs that are abundant, contain predicted KSHV miRNA binding sites, and are induced upon KSHV infection. To this end, we used mock- or KSHV-infected primary human umbilical vein endothelial cells (HUVECs) or an infected B cell line, MC116 (14). First, we used new microarrays that measured expression of 5,396 human circular RNAs and found 1,939 circRNAs that were above the detection limit. Of these 1,939 circRNAs, the majority (1,028) were detected in both HUVECs and MC116 cells. We found that 98 and 166 human circRNAs are significantly up-regulated or down-regulated upon infection in HUVECs, respectively, whereas 245 and 40 circRNAs were up-regulated and down-regulated in infected MC116 cells compared with control cells (Fig. 1*A* and [Dataset S1](#)). Only 2% of the up-regulated circRNAs in either HUVECs or MC116 cells (7 out of 336; [Dataset S1](#)) were up-regulated in both cell types after infection. These results suggested that circRNA expression changes due to KSHV infection differ across different cell types.

One possible function of some circular RNAs could be to sponge viral miRNAs and prevent miRNA functions. We tested for enrichment of KSHV miRNA binding sites in human circular RNAs expressed in HUVECs or MC116 cells and found multiple examples of statistically significant enrichment of KSHV miRNA target sites in these circular RNAs ([Dataset S2](#)). These findings suggest that the density of these predicted KSHV miRNA targets is very unlikely to be a result of random sequences in certain circular RNAs.

We focused on de novo infections in the HUVEC cell type to investigate changes in circRNA expression in an early response to KSHV infection in primary cells, as opposed to the MC116 infection, which is a lymphoma cell line with a long-term infection and is maintained by antibiotic selection. In addition to using microarrays, we used circRNA-Seq to measure circular RNA changes in the same samples and to potentially discover new circular RNAs that were not represented on the microarrays. These RNA samples were treated in two ways before RNA sequencing: mock treatment or treatment with RNase R, which cleaves linear mRNAs, but does not cleave circular RNAs, to enrich for circular RNAs (3). The RNase R treatment did enrich human circRNAs that were identified to be highly up-regulated ([SI Appendix, Fig. S1](#)). Many of the circRNAs, such as hsa_circ_0001400 and hsa_circ_0001741, were up-regulated upon infection as found in the microarray expression profiling (Fig. 1*B* and [SI Appendix, Fig. S1](#)). Based on circular RNA abundance, expression changes with infection, and the presence of KSHV

Fig. 1. KSHV induces certain human circular RNAs upon infection. (A) Human circRNA expression levels in KSHV-infected cells were measured by microarrays. Signals for each human circRNA-specific probe from infected cells were normalized with signals from uninfected cells to calculate fold changes. The *P* values were determined by *t* tests. Data are shown as mean values of experiments with three independent experiments. (B) Human circRNA expression levels in RNase R-treated infected HUVECs are shown. circRNAs that were most strongly and consistently up-regulated according to microarray analysis and abundant in circRNA-Seq data were selected. Sequenced reads mapped to back-spliced junctions of known human circRNAs were counted and shown as counts per million reads. Raw values of two independent experiments are shown. (C and D) Expression levels of human circRNAs were assessed with RT-qPCR in KSHV-infected HUVECs (C), 293T cells (D), and MC116 cells (E). Various MOI conditions were used; MOIs were 30 and 100 for HUVECs and 7.5 and 15 for 293T cells. Transcript levels were normalized to *GAPDH* or *ACTB* and uninfected controls. Data are shown as mean values and SD of three independent experiments. **P* < 0.05.

miRNA seed-matching sites, we focused on a subset of human circRNAs (hsa_circ_0001400, hsa_circ_0001741, hsa_circ_0008311, and hsa_circ_0005145). Abundance of a circRNA was the most important criterion for further analysis. One circular RNA of interest, hsa_circ_0001400, is the most abundant circRNA among up-regulated circRNAs and harbors a predicted miR-K12-10b binding site (Fig. 1*B* and [Dataset S2](#)). A linear mRNA transcript that overlaps with hsa_circ_0001400, *RELL1*, was not significantly affected by KSHV infection with fold changes of 0.96 ± 0.09 SD ($n = 4$). Another dataset of circRNA expression (15) reported that hsa_circ_0001400 is the 24th most abundant human circRNA out of 8,143 circRNAs detected in the same primary endothelial cells (HUVECs). Using divergent qPCR primers that cannot amplify linear RNA but can amplify circular transcripts, we found a modest increase in expression levels of these circRNAs with KSHV infection in multiple cell types (Fig. 1*C–E*; HUVEC endothelial, 293T epithelial, and MC116 B).

KSHV Circular RNAs Are Expressed After Lytic Induction. Since human herpesviruses express many types of ncRNA molecules (e.g., miRNAs and lncRNAs) as their human hosts, like miRNAs and lncRNAs, we sought to determine if circRNAs are expressed from the KSHV genome. We utilized the fact that circRNAs

form back-spliced junctions, which are distinct from the linear genomic order of sequences, and detected such chimerically mapped reads in RNA-Seq data from RNase R-treated and RNase R-untreated KSHV-infected cells (Fig. 2*A* and *B*). We used divergent primers and RNase R resistance assays to confirm these back-spliced junctions in viral circular RNAs in these regions (Fig. 2*B–E* and *SI Appendix*, Fig. S24). We discovered multiple back-spliced junctions on coding sequences of viral lytic genes (Fig. 2*B*, Table 1, and *Dataset S3*). The cloning of these amplicons revealed that back-spliced junctions have variability (Table 1, *SI Appendix*, Fig. S2, *Dataset S4*), suggesting unconventional splicing events or modifications upon ligation. Normally, the majority of cells after infection become latently infected by KSHV (16). We used a cell line that contains an inducible form of *RTA*, a KSHV gene that can induce the lytic cycle. We measured these KSHV circular RNAs in latent or lytic infection cycles and found that these viral circRNAs are more abundant in lytic infection compared to latent infection cycles (Fig. 2*C*). However, human *hsa_circ_0001400* expression did not change between latent and lytic infection cycles. In separate assays, we found that these viral circRNAs are similarly resistant to

RNase R as the human circRNA (*hsa_circ_0001400*). The RNase R treatment of extracted total RNA did not affect human and viral circRNAs' transcript levels, whereas the transcript level of a negative control linear mRNA, *GAPDH*, was reduced to less than 1% by the RNase R treatment (Fig. 2*D*).

In addition to patient-derived cell lines and de novo infections, we also detected KSHV circRNAs in fresh lymph node biopsies from KSHV-infected individuals enrolled in separate clinical trials. We measured expression of two KSHV protein-encoding genes, *LANA* (marker for latent infection) and *RTA* (critical regulator of the lytic phase), in addition to KSHV circRNAs. One negative control sample was from a patient's lymph node that was negative for *LANA* expression by immunohistochemistry. As expected, no KSHV genes were detected in this sample by qPCR. In contrast, multiple KSHV circRNAs were detected in the other lymph node samples that were positive for KSHV protein-encoding genes. In addition, increased expression of *RTA* correlated best with detection of various KSHV circRNAs (Fig. 2*E*). This finding is consistent with increased expression of these viral circRNAs in cells when the lytic cycle is induced (Fig. 2*C*). Taken together, the results from RNA sequencing from

Fig. 2. KSHV lytic genes encode circular RNAs. (A) Schematic overview of the circRNA discovery analysis. Numbers of back-spliced junctions of viral origins are shown. (B) KSHV circRNAs identified by circRNA-Seq are shown. All back-spliced junctions detected in KSHV-infected HUVECs (with RNase R, n = 2; without RNase R, n = 4) were accumulated in 200 nucleotide bins and plotted. Viral coding sequences and *PAN* RNA are shown to describe genomic loci. Amplicons of divergent primers are shown. (Right inset) A partial genomic map of identified KSHV circRNAs. Positions of coding sequences (CDSs) of viral genes, ncRNAs, and divergent primers are shown. The genomic positions are according to the KSHV reference genome, NC_009333. (C) Viral circRNA levels were assessed with RT-qPCR using divergent primers in doxycycline-treated TReX-BCBL1 and TReX-BCBL1-RTA cells. Transcript levels were normalized to *GAPDH* and TReX-BCBL1 controls. Samples with a Ct value of more than 35 were determined to be under the detection limit and designated as nondetected (n.d.). (D) Viral circRNAs were tested for RNase R resistance. Total RNA from doxycycline-treated TReX-BCBL1-RTA cells was treated with RNase R and was subjected to RT-qPCR. Transcript levels of RNase R-treated samples were normalized to mock-treated controls and are shown as percentages. Data are shown as raw and mean values and SD of three independent experiments. (E) Viral transcripts and circRNAs were assessed from lymph node samples from five KSHV-positive donors (Patients 1 to 5). Diagnosis (lymph nodes and patients) and *LANA* detection (lymph nodes) results are shown below each donor. Transcript levels were normalized to *ACTB* and donor 5. Samples with a Ct value of more than 35 were determined to be under the detection limit and designated as not detected (n.d.). FH, follicular hyperplasia; KS, Kaposi's sarcoma; MCD, multicentric Castleman's disease; PEL, primary effusion lymphoma.

Fig. 3. Circular RNAs regulate viral gene expression and cell growth. (A) Transcript levels of *hsa_circ_0001400* were assessed in SLK cells stably expressing *hsa_circ_0001400* with RT-dPCR (digital PCR). Data were normalized to *ACTB* and are shown as mean values and SD of three independent experiments. (B) Transcript levels of viral genes were assessed in SLK cells stably expressing *hsa_circ_0001400* with RT-qPCR 3 d after KSHV de novo infection. Data were normalized to *ACTB* and shown as mean values and SD of four independent experiments. * $P < 0.05$. (C) Transcript levels of human and viral genes were assessed in BAC16 KSHV-infected SLK (SLK-K) cells transfected with siRNAs for 48 h with RT-qPCR. Transcript levels were normalized to *ACTB* and to siNT (nontargeting negative control) transfected SLK-K cells. Data are shown as mean values and SD of three independent experiments. * $P < 0.05$. siNT, nontargeting siRNA; siCirc1400-1/2, *hsa_circ_0001400* targeting siRNA 1 and 2. (D) Transcript levels of human and viral genes were assessed in RTA-induced BCBL1 cells and in de novo infected SLK cells stably expressing circRNAs with RT-dPCR. Data were normalized to *ACTB* and are shown as mean values and SD of three independent experiments. n.d., not detected. (E) Viability of SLK cells stably expressing circRNAs was assessed after BAC16 KSHV infections by measuring reducing potentials of living cells. Cell viabilities were normalized to circGFP-expressing SLK cells. Data are shown as mean values and SD of three experiments.

What is the benefit for viruses in encoding circRNAs? In addition to the benefit described for human circRNAs, viral circRNAs are likely nonantigenic, as in the case of viral miRNAs, and may provoke less antiviral immunity. Recently, others demonstrated that exogenous circRNAs can activate immune responses through RIG-I because of the lack of RBPs normally bound to endogenous circRNAs (20). However, viral circRNAs made inside the infected cells using the host machinery likely avoid being sensed as foreign circRNAs. Furthermore, as we observed, some viral circRNAs can inhibit viral gene expression and thus may reduce immunogenicity of infected cells and help immune evasion. In this case, a function of viral circRNAs is analogous to that of viral miRNAs, and has been reported to help immune evasion (21, 22). Finally, circRNAs are also cost-efficient, for they arise by back-splicing of already existing transcribed RNAs and do not usurp translation machinery from other viral transcripts.

Decoying specific RNAs to alleviate targeted genes from repression is a known function of human noncoding RNAs (1, 2, 23). To analyze potential purposes of viral circRNAs, miRNA binding sites were predicted, and known target genes of those miRNAs were enriched with overrepresentation analysis and grouped according to functional pathway categories (Dataset S5). Pathways involved in cancer or p53 signaling were enriched, suggesting that viral circRNAs function in apoptosis. Some lncRNAs are known to bind complementary mRNAs and regulate translation of target mRNAs (23). We found that genes whose transcripts have strong complementarity to both *kcirc55* and *kcirc97*, which have progrowth functions, are highly enriched for cell cycle-related genes (Dataset S5). These predictions are consistent with our observations that exogenously expressed viral circRNAs showed progrowth phenotypes (Fig. 3E and SI Appendix, Fig. S3F). Not only human miRNAs but also multiple viral miRNAs were predicted to bind viral circRNAs (SI Appendix, Fig. S5). It is therefore possible that viral circRNAs sequester functions of viral miRNAs that are unnecessary or adversarial upon lytic infection. The KSHV SOX protein shuts down expression of human and viral messages (24), and these viral circRNAs may regulate expression and activity of both human and viral gene products. On the other hand, analysis of

human mRNA that had predicted binding interactions with *hsa_circ_0001400* revealed an enrichment of transcription factors related to chromatin modification (Dataset S5). These findings may suggest that some human circRNAs regulate viral transcription by altering chromatin that leads to how *hsa_circ_0001400* can inhibit expression of both KSHV genes, *RTA* and *LANA*.

RNA-binding proteins are other candidates to be sequestered by viral circRNAs. Prediction of RBP binding sites on these KSHV circRNAs identified HNRNPA1, a regulator of certain human miRNAs (25), and FUS and QKI, a negative and a positive regulator of the circRNA biogenesis (26, 27) (Dataset S6). Together with direct-binding to miRNAs, it is thus possible that viral circRNAs contribute to the proviral environment controlling the biosynthesis step of particular noncoding RNA such as proinflammatory miRNAs.

Herpesviruses have been utilizing ncRNA functions (miRNA, long noncoding RNA) by either interacting with human factors or encoding viral ncRNA themselves with their relatively large genomes. Indeed, in very recent studies, Epstein-Barr virus and KSHV were reported to encode circRNAs (28, 29). One of the reported KSHV viral circRNAs, *circvIRF4*, was also detected in our circRNA-Seq of infected HUVECs (Dataset S3). It is expected that other viruses that utilize splicing may also express circular RNAs. In this work, we explored circular RNAs in the context of KSHV infection. We identified an antiviral host circRNA induced upon infection and discovered viral circRNAs expressed in the lytic phase of KSHV. This finding of novel layers of host-virus interaction will advance our understanding of this oncovirus and may reveal new potential therapeutic targets.

Methods

Preparation of Infectious KSHV Stocks and de Novo Infection. For BCBL-1 virus stock, BCBL-1 cells were induced for the lytic cycle with valproic acid (300 μ M; Sigma Aldrich) and incubated for 5 d. For BAC16 virus stock, iSLK cells were induced for the lytic cycle with doxycycline (1 μ g/mL; Thermo Fisher Scientific) and sodium butyrate (1 mM; Sigma Aldrich) for 3 d. For infection with HUVECs and 293T cells, collected supernatants were cleared of debris by centrifugation at $500 \times g$ for 5 min, filtered (Rapid-Flow 0.45- μ m filter; Thermo Fisher Scientific), and concentrated with Vivaflow 50 (Sartorius). For infection of SLK cell lines, supernatants of BAC16 were filtered and pelleted with ultracentrifugation (2.5 h at 4 $^{\circ}$ C at 50,000 $\times g$ with SW 32 Ti; Beckman

Coulter) and finally resuspended with Dulbecco's modified Eagle's medium without any supplementation (Thermo Fisher Scientific).

De novo infections in HUVECs and 293T cells were carried out by diluting concentrated supernatant in endothelial cell growth medium-2 (EGM2) at a multiplicity of infection (MOI) of 40, as determined by LANA copy number, unless otherwise mentioned. Polybrene (Millipore) was added at 8 $\mu\text{g}/\text{mL}$. Virus supernatant was washed off after 6 h and replaced with fresh EGM2. HUVECs were refed every 2 d until harvest. For SLK cell de novo infection, BAC16 virus supernatant concentrated with pelleting was used at an MOI of 15 with Polybrene (8 $\mu\text{g}/\text{mL}$) and media were replaced 16 h after infection.

Clinical Samples. Lymph node biopsies were obtained from patients with a confirmed history of HIV and KSHV coinfection. Plasma HIV-1 mRNA was measured by real-time quantitative RNA PCR using Roche Amplicor HIV-1 monitoring kits (Roche Diagnostic Systems). All patients were enrolled in an Institutional Review Board-approved protocol at the National Cancer Institute (NCTNCT00006518). All patients gave written informed consent. KSHV tumor status was confirmed by staining for latency-associated nuclear antigen (LANA) (anti-ORF73 rat mAb; Advanced Biotechnologies). Portions of the same patient specimens were used to purify total RNA (miRNeasy Kit; QIAGEN).

RNAse R Treatment and RT-qPCR. Total RNA was extracted using Qiazol (QIAGEN) and Direct-zol (Zymo Research). For select samples, 3 μg of total RNA were treated with 20 U of RNAse R (Lucigen) and 20 U of Ribolock RNAse (Thermo Fisher Scientific) for 30 min at 37 $^{\circ}\text{C}$, followed by purification with a Direct-zol kit. cDNA was prepared using whole RNAse R-treated RNA or 2 μg of total RNA, random primers, and a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Quantitative PCR was performed with FastStart Universal SYBR Green Master Mix (Roche) or a THUNDERBIRD Probe qPCR kit (Toyobo) on an ABI StepOnePlus real-time PCR system (Applied Biosystems). Relative transcript levels were computed using the threshold cycle ($\Delta\Delta\text{Ct}$) method with transcripts of genes coding for β -actin or GAPDH as references. For quantifications of *ACTB*, *IL6*, *IFNB*, *CXCL8*, *TNF*, and *GADD45B*, TaqMan assays (Thermo Fisher Scientific) were used. Divergent primers to quantify circRNAs were designed with Circular RNA Interactome (30). Synthesized DNA oligos including primers are listed in [Dataset S4](#).

De Novo Infection of Stable SLK Cell Lines Expressing Circular RNAs. For plasmids harboring human or viral circRNA (pcDNA3.1-kcirt54, pcDNA3.1-kcirt55, and pcDNA3.1-kcirt97; see [SI Appendix, Methods](#) for details), 0.4 μg of each plasmid were transfected into 1×10^5 SLK cells with Transporter 5 (Poly-science) according to the manufacturer's instructions. SLK cells were selected with G418 (250 $\mu\text{g}/\text{mL}$) for 4 wk. Expression of circRNAs was confirmed with RT-PCR. The resulting cell lines are called SLK-circGFP [with pcDNA3.1(+)

ZKSCAN1 MCS-WT Split GFP + Sense IRES], SLK-circ_0001400 (with pcDNA3.1-hsa_circ_0001400), SLK-kcirt54 (with pcDNA3.1-kcirt54), SLK-kcirt55 (with pcDNA3.1-kcirt55), and SLK-kcirt97 (with pcDNA3.1-kcirt97). Stable SLK cells were infected with BAC16 KSHV at an MOI of 15 (prepared with ultracentrifugation), and total RNA was extracted at 3 d postinfection followed by RT-PCR. Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific), and transcripts were quantified with quantitative PCR or digital PCR.

Establishing KSHV-Infected SLK Cell Lines and Knockdown of Specific circRNAs.

SLK cells were infected with BAC16 KSHV at an MOI of 15 and selected with 0.5 mg/mL Hygromycin (Corning) for 4 wk; the resulting cells are called SLK-K cells. Next, 5×10^4 SLK-K cells were transfected with 20 nM of siRNA (Dharmacon), 4 μL RNAiMax (Thermo Fisher Scientific), and Opti-Mem I (Thermo Fisher Scientific) according to the manufacturers' guidance and incubated for 48 h. ON-TARGETplus nontargeting pool (Dharmacon) was used as a negative control. The following sequence was designed with Circular RNA Interactome (30) and used to synthesize custom ON-TARGETplus siRNAs (Dharmacon) targeting hsa_circ_0001400: siCirc1400_1 sense: AGAGUAGCAGCGAAGCUGAUUUU, antisense: 5'-P-AUCAGCAUUCGUGCUACUCUUU; siCirc1400_2 sense: AGUAGCAGCGAAGCUGAUGUUU, antisense: 5'-P-ACAUCAGCAUUCGUGCUACUUU. P: phosphate.

Cell Growth of SLK Cells. For cell growth, 2×10^4 stable SLK cells expressing human and viral circRNAs were seeded in black-walled, clear-bottom 96-well assay plates (Corning). Cells were infected with BAC16 KSHV at a MOI of 15 (prepared with ultracentrifugation) and incubated overnight. Growth of cells was measured continuously with RealTime-Glo MT Cell Viability Assay (Promega) and Modulus II Microplate Multimode Reader (Turner Biosystems).

Statistics. Most graphs contain plots with each data point represented and also include the mean and SDs. For testing of significance, *t* tests were performed with Prism (GraphPad) and presented with asterisks indicating *P* values.

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