PNAS

Supporting Information for

A virus-induced circular RNA maintains latent infection of Kaposi's sarcoma herpesvirus.

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Supporting Information Text

Materials and Methods

KSHV lytic Reactivation. Subconfluent monolayers of iSLK-BAC16 (WT) were induced with 1 ug/mL Doxycycline, 1 mM Sodium Butyrate in DMEM media supplemented with 2% Tet-approved FBS. 0 hour time point was when induction media was added and cells were first placed at 37°C to incubate.

KSHV virion production assay. 2×10^5 LECs were depleted for circ_001400 and infected with KSHV as described for three days in 2ml EGM media. Conditioned media was collected and filtered with Minisart 1.2 µm filter (Satorius). 1×10^5 HEK-293 cells are seeded, and media was replaced with 1ml diluted conditioned media (1:1 with DMEM) and incubated for three days. KSHV-infected GFP-positive HEK-293 cells were measured by CytoFlex S (Beckman Coulter) flow cytometer.

Cell growth (cell cycle, apoptosis, and cell death). 2×10^5 HUVECs were depleted for circ_001400 and infected with KSHV as described for three days in 2ml EGM2 media. Cell cycle was measured with Click-iT EdU Pacific Blue Flow Cytometry Assay kit (Thermo Fisher Scientific) and FxCycle PI/RNase staining solution (Thermo Fisher Scientific) following the manufacturer's instructions. Cells were incubated with EdU for 4 hours before fixation and staining. Fluorescence was measure by CytoFlex S (Beckman Coulter) flow cytometer. For cell counts and apoptosis assays, 1×10^4 HUVECs were cultured in EGM2 or dilution with EBM2 (Lonza). Total cells, apoptotic cellsn, and dead cells were stained with Hoechst (Nexcelom), ViaStain Live Caspase 3/7 kit (Nexcelom), and propidium iodide (Nexcelom), respectively. Images were taken and analyzed with Celigo Image Cytometer (Nexcelom). Apoptotic and dead cells numbers were divided by Hoechst-positive cell numbers to calculate percentages.

Immunostaining and flow cytometry. 2×10^5 HUVECs were depleted for circ_001400 and infected with KSHV as described for three days in 2ml EGM media. Cells were washed with PBS, detached with Accutase (BioLegend), and mixed with antibodies in staining buffer (PBS, 0.5% FBS, 2mM EDTA) for 20 minutes at room temperature. Fluorescence was measure by CytoFlex S (Beckman Coulter) flow cytometer. Median fluorescence intensities (MFIs) were quantitated with FlowJo X (BD Biosciences) and MFIs for each antibody is scaled to corresponding isotype controls. Following antibodies were used: PE/Cyanine7 anti-human CD40 antibody (5C3, BioLegend); PE/Cyanine7 Mouse IgG1, κ Isotype ctrl antibody (MOPC-21, BioLegend); Alexa Fluor 647 anti-human CD54 antibody (HCD54, BioLegend); Alexa Fluor 647 Mouse IgG1, κ Isotype ctrl antibody (MOPC-21, BioLegend); PE anti-human CD275 (B7-H2, B7-RP1, ICOSL) antibody (9F.8A4, BioLegend); PE Mouse IgG1, κ Isotype ctrl antibody (MOPC-21, BioLegend).

circRNA/mRNA manipulation. For depletion of circ_0001400, previously reported siRNA, as described as siCirc1400-2, was used (1). This siRNAs target back-splice junctions of circ_0001400 and not affect the linear counterpart RNA, *RELL1*. 2×10^5 of HUVECs or LECs were transfected with 20 nM ON-TARGETplus siRNA (Horizon), 3 ul Dharmafect I (Horizon), and 0.4 ml Opti-MemI (Gibco) in 2ml EGM2 according to manufacturer's guidance, followed by KSHV infection in next day. For 293T cells, 2×10^6 cells were transfected with 20 nM siRNA with 18 µl of RNAiMax (Thermo Fisher Scientific) and 0.6ml Opti-MemI in 3ml DMEM for 48 hours. For a negative control and target mRNAs, ON-TARGETplus Non-targeting Control Pool, and Smart Pool against human *TTI* and human *PNISR* were purchased from Horizon.
Ectopic expression of circ_0001400 in 293T cells was done with a plasmid vector as previously reported (1). 2 µg pcDNA3.1- hsa_circ_0001400 of plasmids was transfected to 2×10^6 cells with 12 µl Transporter 5 (Polysciences) lipofection reagent and 1 ml Opti-MemI in 10 ml DMEM. For primary endothelial cells, lentiviral vectors were used. pcDNA3.1- has_circ_0001400 and pcDNA3.1(+) ZKSCAN1 MCS-WT Split GFP + Sense IRES (Addgene plasmid # 69909) were digested with BamHI and XhoI (NEB) and cloned into pLV-mCherry:T2A:Bsd-CMV plasmid (VectorBuilder). VSV-G pseudotyping and packaging was performed by VectorBuilder. Titration was performed as for KSHV BAC16, but with mCherry reporter. For infection, KSHV and

lentivirus are added to 1×10^6 HUVECs or LECs simultaneously with 8 μg/ml polybrene. Media was replaced after overnight incubation and cells.

RNA extraction and RT-qPCR. Total RNA was extracted with Direct-zol RNA miniprep kit with on-column DNAse I digestion (Zymo Research). 0.5 to 1 μg of total RNA was used for reverse-transcription with ReverTra Ace qPCR RT Master Mix (Toyobo) and qPCR was performed with Thunderbird Next SYBR qPCR Mix (Toyobo) and StepOnePlus Real-Time PCR System (ThermoFisher) following manufacturer's instructions. Primers are sourced from IDT and listed in Table S1.

Microarray. Akata cells were maintained less than 1×10^6 cells/ml. Total RNAs were extracted at log phase of cell growth at three different days. RNAs were extracted with Direct-zol RNA miniprep kit (Zymo Research) with an on-column DNase I digestion following manufacturer's instruction. Total RNAs were subjected to the Human Circular RNA Array (ArrayStar). Total RNA from each sample was treated with RNase R to enrich circular RNA. The enriched circular RNA was reverse transcribed with random primers containing a T7 promoter. The cRNA was linearly amplified and Cy3 labeled by in vitro T7 polymerase transcription using Arraystar Super RNA Labeling protocol (Arraystar). The labeled cRNAs were hybridized onto the Arraystar Human circRNA Arrays V2 (8x15K, Arraystar), and incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The microarray slides were scanned with an Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the R software limma package. Quantitative results are available in Dataset S1. Raw data for Akata cells is available at GSE206824 (Gene Expression Omnibus, GEO). For HUVECs, previously performed microarray (GSE120045, GEO) was used.

RNA-Seq and data analysis. circ_0001400-manipulated and KSHV (BAC16)-infected HUVECs and LECs, and HSV-1 infected MRC5 cells were harvested for total RNA using the Direct-zol RNA MiniPrep Kit (Zymo), following manufacturer's instructions. For LECs and MRC5 cells, ERCC spike-in controls (ThermoFisher) were added to 500-1000 ng of total RNA. Library preparation and sequencing was performed by Genewiz (circ_0001400 manipulated samples) or NCI CCR-Illumina Sequencing facility (LEC and MRC5). For circ_0001400-depleted HUVECs and ectopically expressed HUVECs, Illumina strand-specific RNA-seq with Poly-A selection and Illumina RNA-seq with rRNA depletion, respectively, were employed. For other samples, RNA was ribominus selected and directional cDNA libraries were generated using either Stranded Total RNA Prep with Ribo-Zero Plus (Illumina # 20040525) or TruSeq Stranded Total RNA Ribo-Zero Gold (Illumina #RS-122-2303). 2-4 biological replicates were sequenced for all samples. Prepared libraries were sequenced with HiSeq 2500 (Illumina), NextSeq 550 (Illumina), or NovaSeq SP (Illumina) in 2×150 bp paired-end conditions. Fastq files are available at GSE206928 (GEO). In addition, previously reported data are used: KSHV-infected LECs (PRJNA851845 (SRR20020770, SRR20020769, SRR20020761, SRR20020757, SRR20020758)), KSHV-infected HUVECs (GSE165328), HCMV-infected MRC5 (GSE155949) (2)).

4SU RNA-Sequencing. iSLK-BAC16 cells were uninduced or induced for lytic cycle as described. At one or three days after induction, 10 mM 4SU (Sigma-Aldrich) was added to cell culture medium. 15 minutes post-4SU addition, cells were collected, and RNAs were extracted. 40-50 ug total RNA was biotinylated in 10 mM Tris pH 7.4, 1 mM EDTA, 0.2 mg/mL EZ-link Biotin-HPDP (Thermo Fisher Scientific). Unbound biotin was removed by performing a chloroform:isoamyl alcohol extraction using MaXtract High Density tubes (Qiagen). RNA was isopropanol precipitated and resuspended in water. Biotinylated RNA was bound 1:1 to Dynabeads My One Streptavidin T1 (Thermo Fisher Scientific) equilibrated in 10 mM Tris pH 7.5, 1 mM EDTA, 2 M NaCl. Bound beads were washed three times with 5 mM Tris pH 7.5, 1 mM EDTA, 1 M NaCl. 4SU-RNA was eluted with 100 mM DTT and isolated using the RNeasy MinElute Cleanup Kit (Qiagen). RNA was sent to the NCI CCR-Illumina Sequencing facility for

library preparation and sequencing. Pulldown RNA was ribominus selected using the NEBNext rRNA Depletion Kit v2 (NEB # E7400L) and RNA-Seq libraries were generated using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB # E7760L). Two biological replicates were sequenced using the Illumina NextSeq 550 platform to generate 150 bp PE reads. Fastq files are available at BioProject #PRJNA851589; SRA accession codes: SRR19792341, SRR19792340, SRR19792338, SRR19792337, SRR19792339, SRR19792336.

Bioinformatics. Sequenced reads were trimmed using cutadapt 1.18 (3) with following parameters: `--pair-filter=any, --nextseq-trim=2, --trim-n, -n 5, -O 5, -q 10,10, -m 35:35, -b, -j 56`, and then mapped to GRCh38.p13 with KSHV genome, NC009333.1, using STAR 2.7.6a (4) with following parmeters: `--outSAMstrandField None, --outFilterMultimapNmax 20, --alignSJoverhangMin 8, --alignSJDBoverhangMin 1, --outFilterMismatchNmax 999, --outFilterMismatchNoverLmax 0.3, --alignIntronMin 20, --alignIntronMax 1000000, --alignMatesGapMax 1000000, --readFilesCommand zcat, --runThreadN 56, --chimSegmentMin 20, --chimMultimapNmax 10, --chimOutType Junctions, --alignTranscriptsPerReadNmax 20000, --outSAMtype None, --alignEndsProtrude 10 ConcordantPair, --outFilterIntronMotifs None`. Reads aligning to human (GENCODE Release 26) and viral (NC009333.1) genes were counted using RSEM (5). On NIDAP (NIH Integrated Data Analysis Platform), after filtering of genes with low sequencing readcounts, upper-quantile normalization was performed followed by DEG analysis using voom (limma package). Ingenuity Pathway Analysis (IPA, Qiagen) was used for downstream pathway enrichment analysis. DEGs from both circ_0001400-depleted HUVECs and ectopically expressed HUVECs were determined by FDR<=0.05. Among them, only genes that were regulated in opposite way by ectopic expression and depletion were further chosen (Dataset S3 and S4) for IPA analysis. To quantify circRNAs and circRNA-to-linear RNA ratios, data from mock and KSHV-infected HUVECs were subjected to CIRCExplorer3 (6). CIRCExplorer3 bases quantitation to intron-spanning reads only, such that reads from forward-splicing (only from linear RNAs) and back-splicing (only from circular RNAs) can be directly compared. 4SU-Seq data was normalized as reads per million total reads per kilobase (RPKM), and Total RNA-Seq data was normalized as reads per million ERCC spike-in reads per kilobase.

Genome assemblies:

KSHV: NC_009333.1, with the corresponding CDS annotation used for transcript quantification
HSV-1: KT899744.1 modified so only one copy of the joint region was present (Δ1-9603, Δ125,845-126,977, Δ145,361-151,974), with the corresponding CDS annotation used for transcript quantification
Human: hg38, GENCODE Release 36
ERCC: available from ThermoFisher (#4456740)

circRNA-pulldown. The method to pull-down circRNA-RNA complexes was adapted from Ziv et al., 2018 (7). 4×10^6 293T cells were transfected with 2 µg of pcDNA3.1- hsa_circ_0001400 and 12 µl Transporter 5 for 24 hours. Cells were washed and replaced with 5mg of Psoralen (Berry) resolved in 10ml PBS. After 20 minutes incubation, cells were irradiated with UVA for 10 minutes (Stratalinker 1800) to crosslink RNAs. The extracted total RNA was mixed with 100 pmol of biotinylated DNA oligos (IDT) and incubated for 6 hours at 37°C in 1.5 ml hybridization buffer (500mM NaCl, 0.7% SDS, 33 mM Tris-Cl, pH 7, 0.7 mM EDTA, 10% formamide). Add 100 µl of MyOne Streptavidin C1 Dynabeads to RNA (Thermo Fisher Scientific) and incubate for an additional hour. Beads were washed with 2×SSC buffer + 0.5% SDS, and RNA was released by DNase I treatment (Ambion, 0.1 unit/µl) for 30 minutes at 37°C. RNA was purified with RNA Clean&Concentrator-5 kit (Zymo Research) followed by de-crosslinking with UVC exposure (2.5kJ/m^2, Stratalinker 2400). SMART-Seq v4 Ultra Low Input Kit for Sequencing was used for full-length cDNA synthesis and amplification (Clontech), and Illumina Nextera XT library was used for sequencing library preparation. Illumina HiSeq 2500 was used to sequence samples with 2×150 Paired End configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Raw sequence data was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. Fastq files are available at GSE206929 (GEO). Enriched transcripts were identified through DEG analysis on NIDAP platform as described earlier. For these samples, read counts were median scaled ant not normalized before DEG analysis. All

enriched transcripts are available in Dataset S5. Used oligos are as follows: control (sense strand of circ_0001400), 5'- TGGCACAGAGTAGCAGCGAATGCTGATGT -Biotin-TEG-3'; probe (anti sense of circ_0001400), 5'- ACATCAGCATTCGCTGCTACTCTGTGCCA -Biotin-TEG-3'.

CARPID of circ_0001400. CRISPR-assisted RNA–protein interaction detection method (CARPID) was performed as described by Yi et al. (8). 293T cells were chosen for high transfection efficiency with plasmids which will enhance the yield of identified targets by CARPID. 4×10^6 293T cells were transfected with 2 μg of pcDNA3.1- hsa_circ_0001400, 3 μg of CARPID BASU-dCasRx (8), 3 μg of gRNA-expressing plasmids based on pXR004: CasRx pre-gRNA (9), and 48 μl Transporter 5 for 24 hours. CARPID BASU-dCasRx was a gift from Jian Yan & Liang Zhang (Addgene plasmid # 153209 ; http://n2t.net/addgene:153209 ; RRID:Addgene_153209). pXR004: CasRx pre-gRNA cloning backbone was a gift from Patrick Hsu (Addgene plasmid # 109054 ; http://n2t.net/addgene:109054 ; RRID:Addgene_109054). Media was replaced with fresh DMEM and incubate 24 hours more. Media was changed with DMEM + 200 μM biotin (Sigma-Aldrich) and cells were incubated for 15 minutes at 37°C. Cells were washed with 10ml three time and scraped into 1 ml ice-cold PBS. Cells were pelleted and lysed in 1mL of 0.5% NP40 lysis buffer (150 mM NaCl, 50 mM Tris pH ~7, 0.5% NP40, and protease inhibitors) by gently pipetting the solution until fully homogenous. Cell lysates were kept on ice for 30 min followed by pulse sonication to disintegrate the DNA. Lysates were clarified using centrifugation for 10 minutes at 14000 RPM at 4°C. The supernatant was removed and incubated with 30 ul of slurry Pierce Neutravidin Agarose beads (Thermo Fisher Scientific Catalog #29200) overnight at 4°C with rotation. The following day, the beads were washed three times with ice-cold 1x-TBS before resuspending with 25mM ammonium bicarbonate (pH 8.0). The beads were placed on a heat plate for approximately 4 minutes at 95 °C and allowed to cool. Once cooled the beads were digested overnight with 2 μg of Trypsin (Promega Catalog # V5111) at 37 °C and constant rocking.

For clean up and mass spectrometry, the peptides were desalted and eluted using 70% ACN/0.1%TFA, following the procedure for the Pierce C18 spin columns (Thermo Fisher Catalog # 89873) and dried down. The dried eluates were resuspended in 0.1% TFA. Peptides were analyzed on a Q Exactive HF (Thermo Fisher Scientific) mass spectrometer coupled to Easy nLC 1000 system (Thermo Fisher Scientific) fitted with Acclaim PepMap 100 C18 LC column (Thermo Fisher Scientific). The peptides were eluted with a 5% to 36% gradient of Acetonitrile with 0.1% Formic acid over 56 minutes with a flow rate of 300 nl/min. The QE HF was operated with each MS1 scan in the orbitrap at 60,000 resolution with a maximum injection time of 120 ms and an AGC target of 1e6. The MS2 scans had a normalized collision energy of 27 and were run at 15,000 resolution with a maximum injection time of 50 ms and an AGC target of 2e5.
For data analysis, acquired MS/MS spectra were searched against a human uniprot protein database in Proteome Discoverer 3.2 (Thermo Fisher Scientific) using Label free Quantification with Minora feature detection. Significantly enriched proteins were identified with Bioconductor RankProd package (10). Scaled signals for each sample were separated into two groups: enriching gRNAs (gCirc1400_1, gCirc1400_2, gCirc1400_3) and controls (pre-gRNA, negative control gRNA), and rank-product analysis was performed as two-class, unpaired case. Proteins with p-values < 0.05 were classified as enriched. All candidates are listed in Dataset S2. For specific targeting of circ_0001400, we cloned guide RNAs (gRNAs) specific to the circRNAs into CasRx pre-gRNA plasmid. gRNA sequences for circ_0001400 were adapted from Lit et al., 2021 (11). CasRx pre-gRNA was digested with BbsI and oligo DNAs harboring gRNAs and BbsI-compatible overhands were ligated. To test specificities and efficacies, 1×10^5 293T cells were transfecting with 300 ng of cloned gRNA-expression vectors and 300 ng of pXR001- EF1a-CasRx-2A-EGFP for two days. RT-qPCR was performed to confirm the knockdown of circ_0001400 as described. pXR001: EF1a-CasRx-2A-EGFP was a gift from Patrick Hsu (Addgene plasmid # 109049 ; http://n2t.net/addgene:109049 ; RRID:Addgene_109049). Following oligos were used for cloning: negative control, 5'-AAACTCTGTAGTCGTAAGCCTGCTACTCTGTGCC -3', 5'-CTTGGGCACAGAGTAGCAGGCTTACGACTACAGA -3'; gCirc1400_1, 5'-AAACAGACATCAGCATTCGCTGCTACTCTGTGCC -3', 5'-

CTTGGGCACAGAGTAGCAGCGAATGCTGATGTCT -3'; gCirc1400_2, 5'-AAACTCAGCATTCGCTGCTACTCTGTGCCACTGC -3', 5'-CTTGGCAGTGGCACAGAGTAGCAGCGAATGCTGA -3'; gCirc1400_3, 5'-AAACCTTTAAGACATCAGCATTCGCTGCTACTCT -3', 5'-CTTGAGAGTAGCAGCGAATGCTGATGTCTTAAAG -3'.

RNA-IP of circ_0001400. 12×10^6 293T cells were transfected with 8 µg of pcDNA3.1-hsa_circ_0001400 and 48 µl Transporter 5 for 24 hours. Cells were detached and 10 million cells was used per antibody. Cells were washed with PBS twice, lysed in 500 µl IP buffer (20 mM Tris-CL, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitor), incubated for 10 minutes at 4°C, and pelleted. Supernatant was collected pre-cleared with 20 µl Protein A/G MagBeads (GenScript) per 1.5 ml lysate for 30 minutes, 4°C. Beads were removed and supernatant was incubated with 4 µg antibody/400 µl lysate overnight at 4°C. 70 µl magbeads were added and beads were incubated for 4 hours at 4°C. Beads were washed three times with 1ml PBS and RNAs were eluted with Trizol and extracted with Direct-zol RNA miniprep kit (Zymo Research) followed by RT-qPCR. Following antibodies were used: α-SFRS18/SRrp130 (A301-609A, Thermo Fisher Scientific); α-eIF3k (PA5-98862); Rabbit IgG Control (GenScript).

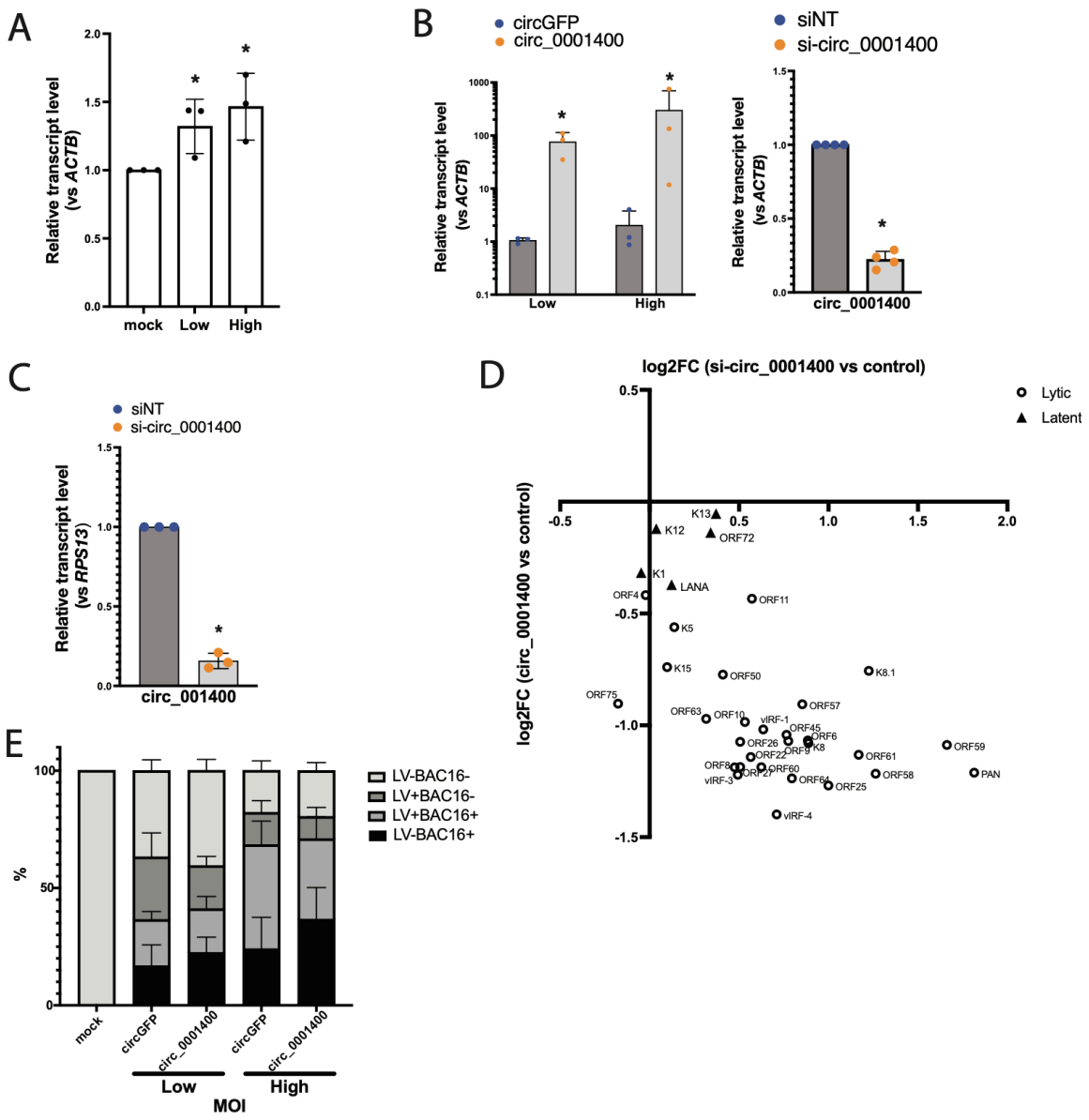

Fig. S1. circ_0001400 affects mainly viral lytic gene expression.
(A) circ_0001400 induction with KSHV infection in LECs. LECs were infected with KSHV at low (0.5 i.u.) or high (1.0 i.u.) MOI for 3 days followed by RT-qPCR. n=3. (B) circ_0001400 transcript levels after manipulation of circ_0001400. circ_0001400 was ectopically expressed with lentivirus (left) or treated with siRNA (right) in KSHV-infected HUVECs. MOIs were 0.5 (Low) or 1.0 (High) i.u. for KSHV and 1.0 for lentivirus. Total RNAs were extracted followed by RT-qPCR at 3 days post infection (n=3). (C) circ_0001400 transcript levels after depletion of circ_0001400 by siRNAs in KSHV-infected LECs. Total RNAs were extracted followed by RT-qPCR at 3 days post infection (n=3). (D) Log_2 fold changes of KSHV transcript levels after manipulation of circ_0001400 in HUVECs. circ_0001400 was ectopically expressed with lentivirus or depleted by siRNAs in KSHV-infected HUVECs. At 3 days post infection, total RNAs were extracted for RNA-Seq. x-axis shows the effect of circ_0001400 depletion while y-axis describes the result of circ_0001400 ectopic expression. The list of fold changes is available in Dataset S3. (E) Flow cytometry analysis of HUVECs dually infected with KSHV and lentiviruses. MOIs were 0.5 (Low) or 1.0 (High) i.u. for KSHV (n=3). Infectivity of HUVECs was measured using GFP (KSHV) and mCherry (lentivirus) at 3 days post infection with KSHV BAC16. For A-C, significances were calculated by T-test. *: $p<0.05$.

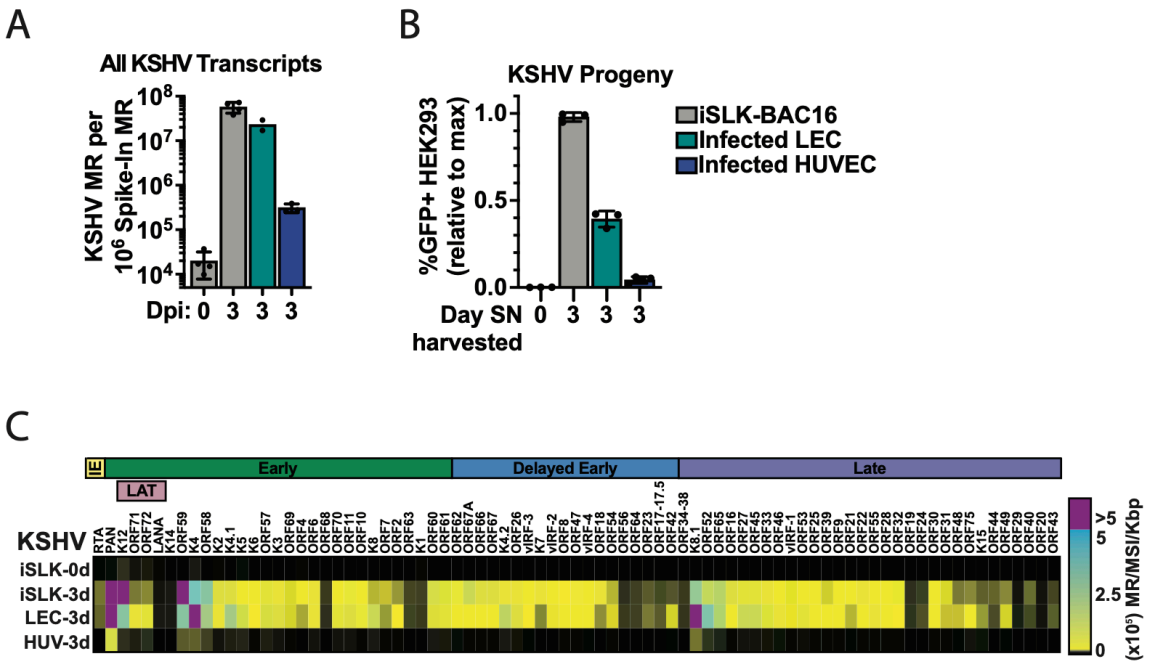

Fig. S2. Comparison of KSHV de novo infection and reactivation models.
(A) Accumulated viral transcripts three days after de novo infection (HUVEC and LEC) or reactivation (iSLK-BAC16). Data is normalized as reads mapped to the viral genome per million ERCC spike-in reads. Each dot is a biological replicate, columns are the average, and error bars are standard deviation (n=2-4). (B) Infectious viral progeny produced from KSHV-infected cells. Supernatant was collected from latent (0 d) and reactivated (3 d) iSLK-BAC16, or infected endothelial cells (3 d). HEK293 were infected with supernatant and after 3 days the percentage GFP positive cells was measure by flow cytometry. HEK293 infection was performed at the same time for all supernatant collected and data is plotted as the relative maximum among all samples (n=3). (C) A heatmap of viral gene expression, the average from biological replicates is plotted. Data is normalized as mapped reads per million spike-in reads per kilobase pair (MR/MSI/Kbp). Transcripts are color coded by transcript class with IE: Immediate early, E: Early, DE: Delayed early, L: Late, LAT: Latent transcripts.

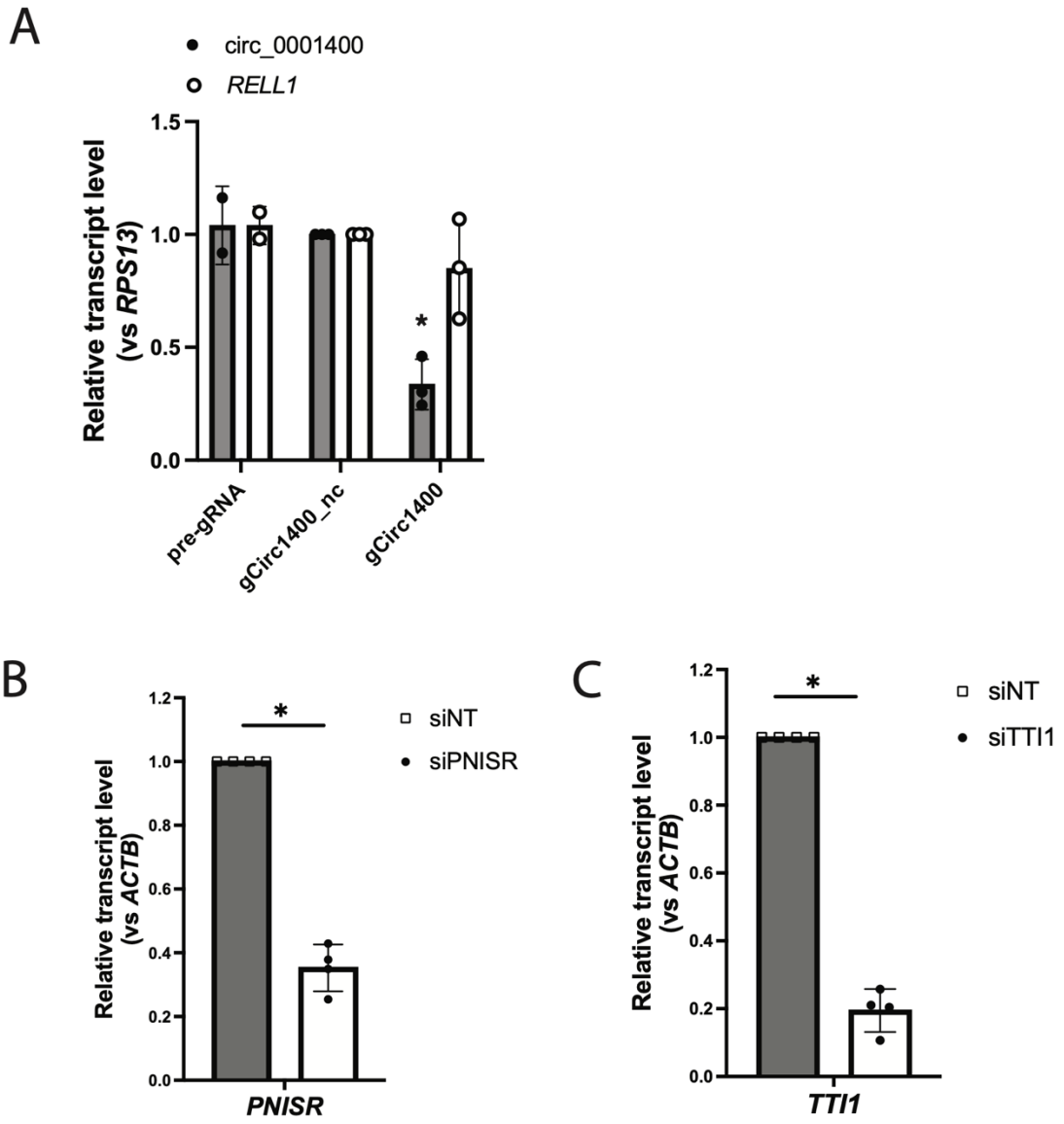

Fig. S3. Knockdown of *circ_0001400*, *PNISR*, and *TTI1*.
(A) Confirmation of specificity and efficacy of guide RNAs. 293T cells were transfected with expression plasmids harboring a pre-gRNA, a negative control gRNA, or a circ_0001400-specific gRNA and CasRx-expression plasmid for 48 hours. RT-qPCR was performed to measure transcript levels and normalized to the negative control gRNA result (n=2-3). Significances were calculated with paired-t tests to the control when n=3. *:p-value < 0.05. (B) RNAi-mediated knockdown of *TTI1*. LECs were transfected with siRNAs targeting *TTI1* followed by KSHV infection at MOI of 1.0 i.u. RNA was extracted 3 days post infection and transcript levels of *TTI1*. N=4. (C) RNAi-mediated knockdown of *PNISR*. LECs were transfected with siRNAs targeting *PNISR* followed by KSHV infection at MOI of 1.0 i.u. RNA was extracted 3 days post infection and transcript levels of *PNISR*. N=4.
Significances were calculated by paired t-tests. *: p<0.05.

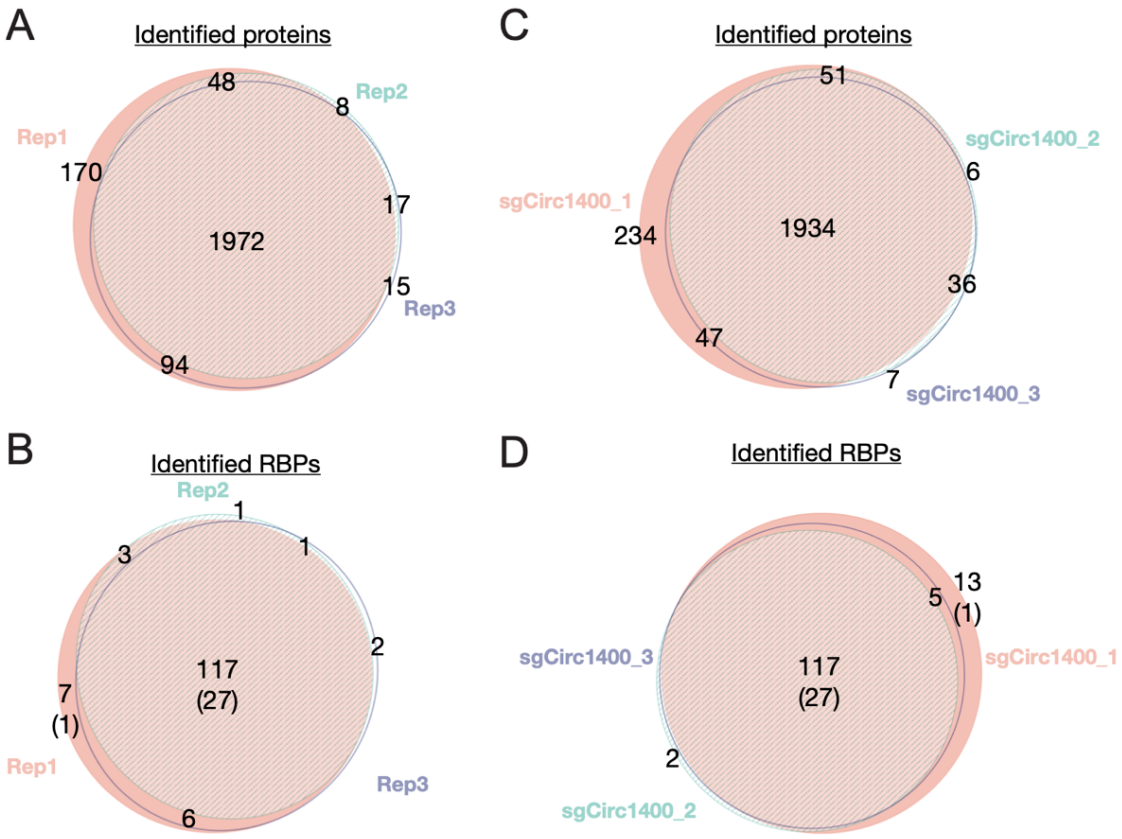

Fig. S4. Reproducibility and RBP enrichments of CARPID.
(A) Identified proteins by CARPID were compared between biological replicates. (B) Among proteins shown in (A), RNA-binding proteins based on RBPDB (12) are shown. Among RBPs, splicing factors based on SpliceAid-F (13) are shown in parenthesis. (C) Identified proteins by CARPID were compared between guide RNAs specific to circ_0001400. (D) Among proteins shown in (B), RNA-binding proteins based on RBPDB are shown. Among RBPs, splicing factors based on SpliceAid-F are shown in parenthesis.

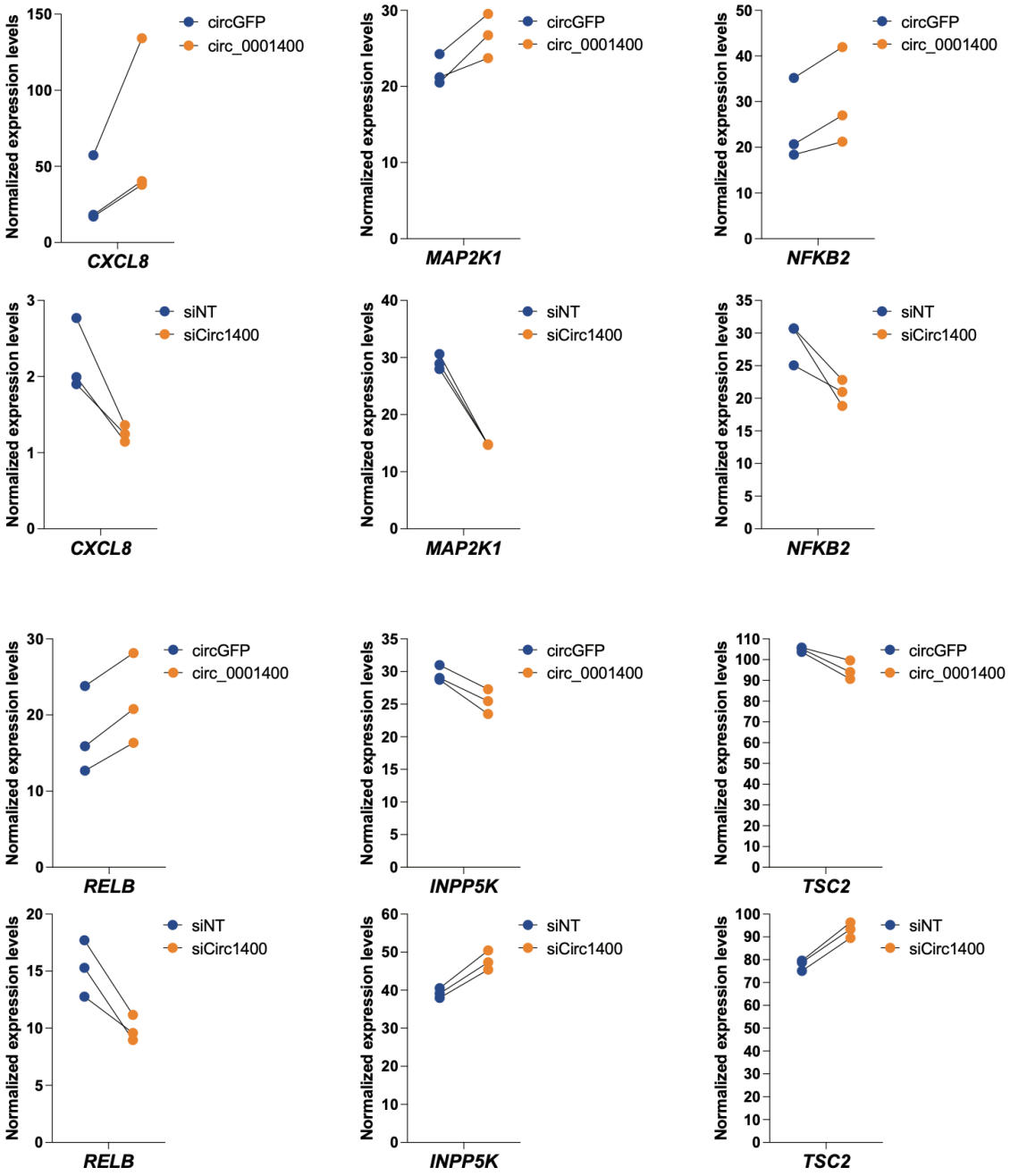

Fig. S5. Regulations of genes involved in PI3K/AKT pathways by has_circ_0001400. Expression levels of select genes from KSHV-infected HUVECs transcriptomes are shown. Transcript levels of genes that are mediating PI3K/AKT signaling (*MAP2K1*, *NFKB2*, *RELB*), downstream (*CXCL8*), and inhibitors (*INPP5K* and *TSC2*) after gain-of-function (circGFP or circ_0001400) and loss-of-function (siNT or siCirc1400) are shown. Sequenced reads were scaled, and quantile normalized. All genes above are differentially regulated ($p<0.05$) as determined by voom in limma package.

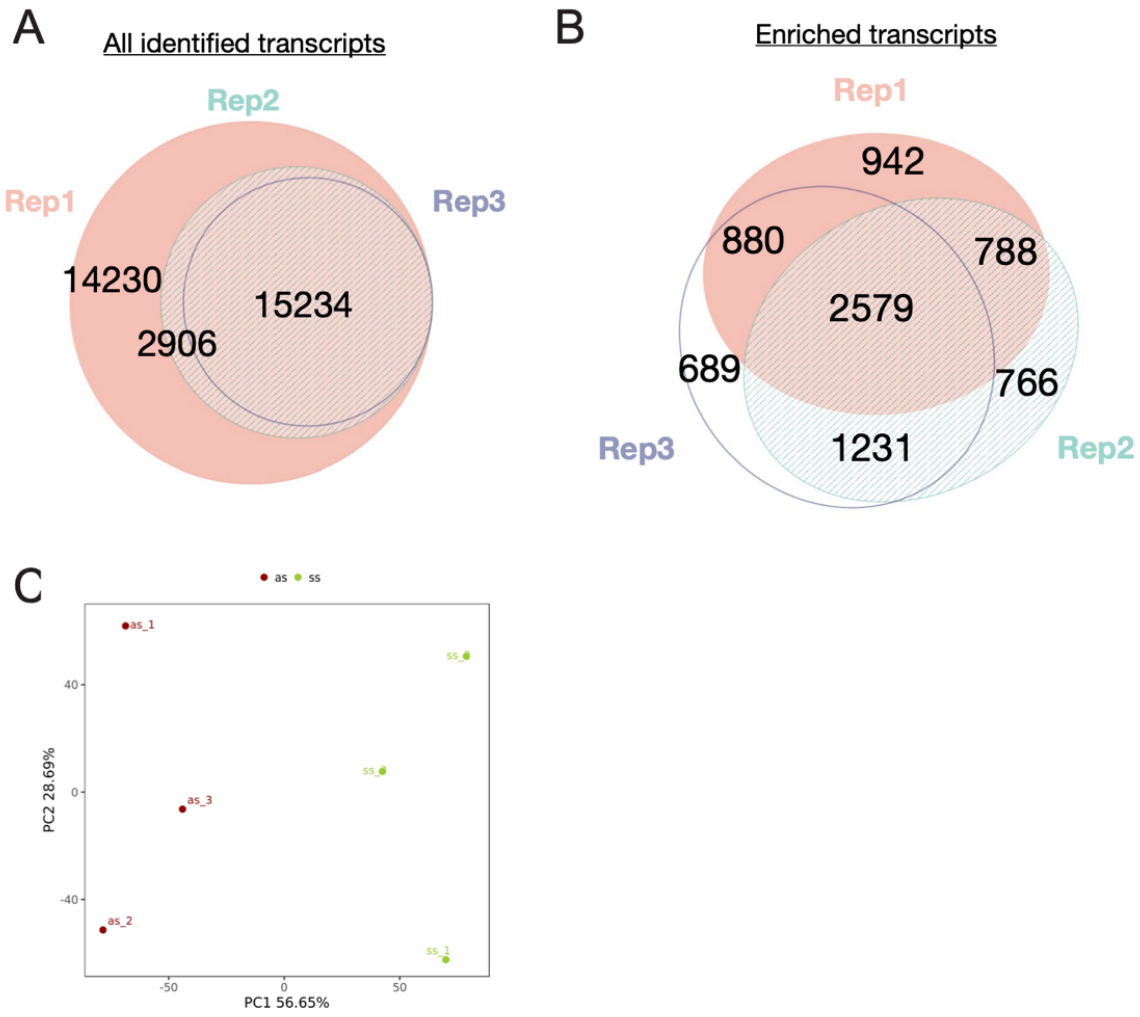

Fig. S6. Reproducibility and specificity of the circRNA-pulldown assay.
(A) Identified RNAs by circ_0001400-pulldown assays were compared between biological replicates. (B) Among RNAs shown in (A), transcripts that showed enrichment (higher amount by a circ_0001400-specific probe compared to a control probe) are shown and compared between biological replicates. (C) Primary component analysis of identified RNA. Percentages of variances for each primary component are shown on axes. as: anti-sense probe (specific to circ_0001400), ss: control probe, PC: primary component.

Table S1. Primers used for RT-qPCR.

Target	Forward	Reverse
hsa_circ_0001400	ATGTCTGTTAGTGGGGCTGA	TATCTGCTACCATCGCCTTT
RELL1	GCAGTGGCACAGAGTAGCAG	CAGTGCAGCCTTACCAGTTG
LANA	GTGACCTTGGCGATGACCTA	CAGGAGATGGAGAATGAGTA
RTA	CTGACGTCATGTCACCCTTG	TCTCTACACGGCACACCTTG
TTI1	CCCTCCATTCTGCCACGTTTA	ACACTGCAAGAGTAGAACCTGTA
CNOT10	CAGTCTTCGGCCATTCCTGT	GCCCCATTTTCCTGCTTTGG
PNISR	AGATGCACACCGCAAAGCAACG	GACTCAGATCCTCTGTCACTCC
ACTB	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG
GAPDH	TTCACACCCATGACGAACAT	TTCACACCCATGACGAACAT
RPS13	TCGGCTTTACCCTATCGACGCAG	ACGTACTTGTGCAACACCATGTGA
ETS1	TCCTGCAGAAAGAGGATGTG	GCTCTGAGAACTCCGATGGT
ICAM1	GGCCTCAGTCAGTGTGA	AACCCCATTCAGCGTCA
VCAM1	CCGGATTGCTGCTCAGATTGGA	AGCGTGGAATTGGTCCCCTCA
SELE	GGCAGTGGACACAGCAAATC	TGGACAGCATCGCATCTCA
CCNA1	GCACACTCAAGTCAGACCTGCA	ATCACATCTGTGCCAAGACTGGA
CD40	CAGCCAGGACAGAAACTGGTGAGT	CTTCTTCACAGGTGCAGATGGTGTC
CD83	GGTGAAGGTGGCTTGCTCCGAAG	GAGCCAGCAGCAGGACAATCTCC

Legends for Tables S1 to S5

Dataset S1. circRNA expression changes upon herpesvirus infections and circRNA-linearRNA ratios in KSHV-infected HUVECs.

Dataset S2. All candidates of circ_0001400-interacting proteins.

Dataset S3. KSHV transcriptome of circ_0001400-manipulated KSHV-infected HUVECs.

Dataset S4. Differentially expressed genes by circ_0001400 in KSHV-infected HUVECs.

Dataset S5. All candidates of circ_0001400-interacting transcripts.

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