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Supplementary Information for

Human cytomegalovirus G-protein coupled receptor US28 promotes latency by attenuating c-fos

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Other supplementary materials for this manuscript include the following:

n/a

www.pnas.org/cgi/doi/10.1073/pnas.1816933116

Supplementary Information Text

SI Materials and Methods

Cells and Viruses

Fibroblasts (primary newborn human fibroblasts, NuFF-1; GlobalStem), retinal pigmented epithelial cells (ARPE19; ATCC), THP-1 (ATCC,), Kasumi-3 (ATCC), 293T (ATCC), S1/S1:MG3 murine stromal cells (described below), and $CD34^+$ hematopoietic progenitor cells (HPCs, described below) were maintained at 37°C, 5% CO_2. NuFF-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM HEPES, and 100 U/ml each of penicillin and streptomycin. ARPE19 cells were maintained in 1:1 DMEM-HAM's medium containing 10% FBS, 2.5 mM L-glutamine, 0.5 mM sodium pyruvate, 15 mM HEPES, 1.2 g/L sodium bicarbonate, and 100 U/ml each of penicillin and streptomycin. Kasumi-3 cells were cultured in RPMI 1640 (ATCC, catalog no. 30-2001) supplemented with 20% FBS, 100 U/ml each of penicillin and streptomycin, and 100 μg/ml gentamicin at a density of $5 \times 10^5 - 1 \times 10^6$ cells/ml. THP-1 cells were maintained at a density between $3 \times 10^5 - 8 \times 10^5$ cells/ml in RPMI 1640 supplemented with 10% FBS and 100 U/ml each of penicillin and streptomycin. 293T cells were maintained in DMEM supplemented with 10% newborn calf serum (NCS), supplemented with 100 U/ml each of penicillin and streptomycin. M2-10B4 (MG3) and S1/S1 murine stromal were a kind gift from Dr. Donna Hogge (Terry Fox Laboratories, BC Cancer Agency, Vancouver, BC, Canada). MG3 were maintained in RPMI 1640, supplemented with 10% FBS and 100 U/ml each of penicillin and streptomycin. S1/S1 cells were maintained in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% FBS, 1 mM sodium pyruvate, and 100 U/ml each of penicillin and streptomycin. MG3 and S1/S1 were plated in a 1:1 ratio (~1.5×10^5 cells of each cell type) onto collagen-coated (1 mg/ml) 6-well plates in human $CD34^+$ long-term culture media (hLTCM), consisting of MyeloCult H5100 (Stem Cell Technologies) supplemented with 1 μM hydrocortisone, and 100 U/ml each of penicillin and streptomycin. The following day, the cells were irradiated using a fixed source ^{137}Cesium, Shepherd Mark I Irradiator at 20 Gy. The cells were then centrifuged and resuspended in fresh hLTCM and returned to culture. Irradiated murine stromal cells were utilized the following day as feeder cells for the primary $CD34^+$ HPCs. Primary $CD34^+$ HPCs were isolated from de-identified cord blood samples (Abraham J. & Phyllis Katz Cord Blood Foundation *dba* Cleveland Cord Blood Center and Volunteer Donating Communities in Cleveland and Atlanta) by magnetic separation, as described elsewhere (1). Isolation and culture methods for the primary $CD34^+$ HPCs are detailed below.

TB40/E*mCherry* (WT), TB40/E*mCherry*-US28-3xF (US28-3xF), and TB40/E*mCherry*-US28Δ (US28Δ) are described elsewhere (2, 3). TB40/E*mCherry*-US28*comp* (US28*comp*) was generated by infecting 2×10^8 NuFF-pBABE-US28-3xF cells (described below) with US28Δ until 100% cytopathic effect (CPE) was observed. To generate stocks of US28*comp*, cell-free virus from the infectious supernatants was then purified by ultracentrifugation through 20% sorbitol cushion after 100% CPE was observed. Virus pellets were resuspended in X-VIVO15 (Lonza) supplemented with 1.5% bovine serum albumin (BSA), and aliquots were flash-frozen in liquid nitrogen prior to long-term storage at -80°C.

TB40/E*mCherry*-US28-R129A (US28-R129A) and TB40/E*mCherry*-US28ΔN (US28ΔN) were each generated by *galK* recombineering, as previously described (4), using TB40/E*mCherry*-US28-3xF as the backbone. The primers used for *galK* insertion, the double

stranded oligonucleotides (ds oligo) used for the reversion, and the primers used to sequence verify the clones are listed in Supplemental Table S1.

Stocks of WT, US28-3xF, US28Δ, US28-R129A, and US28ΔN were generated as described previously [e.g.: ref. (5)]. All viral stocks were titered by 50% tissue culture infectious dose ($TCID_{50}$) assay on NuFF-1 and ARPE19 cells.

Compound treatments

Where indicated, cells were treated with the following compounds. To differentiate THP-1 cells prior to infection, cells were treated with 20 nM 12-*O*-tetradecanoylphorbol-13-acetate TPA for 48 hours (h), and then washed twice in 1X PBS before infection. To inhibit c-fos signaling, cells were treated with T-5224 (BioVision). Cell survival in response to inhibitor treatment was performed to confirm dosage prior to experimentation (Fig S8). To determine a non-toxic concentration of the c-fos inhibitor, T-5224, we treated naïve, uninfected Kasumi-3 cells with T-5224 at varied concentrations (10, 50, and 100 nM) in X-VIVO15. Fresh media containing the inhibitor was exchanged every 2 days (d). Cell viability was assessed 7 d by live-dead discrimination using trypan blue. Cells were counted with a hemocytometer across four quadrants. For all experiments performed, cells were treated with 10 nM T-5224 (BioVision) as indicated in the text, and fresh media supplemented with the inhibitor was added every 48 h for the duration of the experiment.

Fluorescence activated cell sorting (FACS) selection of infected Kasumi-3 cells

Where indicated, Kasumi-3 cells (5 x 10^6 cells/infection) were infected (moi = 1.0) with WT, US28Δ, or US28*comp*. Infected cells (mCherry-positive) were enriched by FACS 1 dpi. Cells were washed twice in 1X PBS, returned to culture in X-VIVO15, and harvested on the days indicated in the text for RT-qPCR analyses.

Generation of pBABE-US28-3xF and pSLIK-US28-3xF

To generate pBABE-US28-3xF, the *US28* ORF with its triple FLAG epitope tag at the C-terminus was amplified from TB40/E-*mCherry*-US28-3xF using the primers listed in Supplemental Table S1. The amplified insert and the pBABE-puro vector [a gift from Hartmut Land, Jay Morgenstern, and Bob Weinberg; Addgene, plasmid #1764; ref. (6)] were each digested with EcoRI and BamHI high fidelity enzymes in cut smart buffer (NEB), purified by ethanol precipitation, and then ligated with T4 ligase (NEB). STABL3 bacterial cells were transformed with the plasmid DNA and resulting transformants were sequenced for genomic integrity.

To generate pSLIK-US28-3xF, the US28 ORF with its triple FLAG epitope at the C-terminus was amplified from pcDNA3-US28 containing the US28 gene from VHL/E (7) and inserted into the SpeI and XbaI sites of pEN_TmiRc3 creating pEN-US28-3xF. The tet-promoter/US28 cassette from pEN-US28-3xF was then recombined into pSLIK-Hygro using LR recombinase according to the manufacturer's recommendations. pSLIK-Hygro [Addgene plasmid # 25737; ref. (8)] and pEN_TmiRc3 [Addgene plasmid #25748; ref. (8)] were gifts from Iain Fraser.

Generation of US28-3xF expressing NuFF-1

To generate the stable NuFF-pBABE-puro and NuFF pBABE-US28-3xF lines, the vectors were transfected into Phoenix (amphotropic) cells using Fugene 6 Transfection Reagent (Promega) in serum-free DMEM according to the manufacturer's protocol and cultured overnight. The media was changed on the cultures, which were then returned to culture an additional 24 h at 32°C/5%

CO_2. Media was then collected from the cultures over the next 2 d, clarified through an 0.45 μM filter, and stored at 4°C until the final day of collection, at which point the media was added to NuFF-1 cells with polybrene (4 μg/ml). After 24 h, the media was removed and the NuFF-1 cells were washed with 1X PBS and cultured in complete media, as described above. Retrovirally transduced cells were selected with puromycin (0.55 μg/ml).

RNA and Protein Analyses

Total RNA was isolated from cells using the High Pure RNA Isolation Kit (Roche), in accordance with the manufacturer's instructions. Next, cDNA (between 250 ng and 1 μg per sample) was reverse transcribed with the TaqMan RT reagent kit (Applied Biosystems), following the manufacturer's protocol. Transcripts were quantified using equal volumes of cDNA template for RTqPCR using SYBR green PCR mix (Applied Biosystems). All samples were analyzed in triplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). To determine the ratio of *UL138*/*UL123, UL123* was first normalized to *GAPDH*, after which *UL138* was then normalized to *UL123*/*GAPDH* and presented as *UL138*/*UL123*. All primers are shown in Table S1.

The PDGF Signaling via STATs and NFkB PrimePCR Panel (BioRad) in 96-well format for the CFX Connect Real Time instrument (BioRad) was used to assess cellular gene expression regulated by pUS28 expression. To this end, THP-1-pSLIK-hygro or THP-1-pSLIK-US28-3xF cells were treated with DOX (1.0 μg/ml) for 24 h. Cells were harvested and total RNA was extracted using the Aurum total RNA mini kit (BioRad), according to the manufacturer's instructions. Equal concentrations (1.0 μg) were used in the cDNA reaction, in which RNA was reversed transcribed using the iScript cDNA synthesis kit (BioRad), according to the manufacturer's protocol. Equal volumes of the cDNA reaction were then loaded into the PrimePCR plate, in which each well contained lyophilized primers (20X) for the cellular genes included in the pathway, in addition to negative, positive, and quality controls. SsoAdvanced universal SYBR Green (BioRad) was used in the reaction, and gene expression was determined using the CFX Connect Real Time machine (BioRad) using the cycling parameters recommended for PrimePCR assay plates. The data was analyzed in CFX Manager.

pUS28 expression was detected using the FLAG epitope during infection, in THP-1-pSLIK-US28-3xF cells, or in NuFF-pBABE-US28-3xF cells. In all cases, total cell lysates were collected, lysed in RIPA buffer, and equal protein amounts, as indicated in the text, were analyzed by western blot. The detection of pUS28 incorporated into the mature viral particle in US28*comp* is described in detail elsewhere (9). In brief, infectious media was collected and pre-cleared of cellular debris twice by centrifugation (3000 x *g*, 4 min, room temperature), and the virus-containing media was purified by ultracentrifugation through a 20% sorbitol cushion. Viral particles were concentrated 280X in 10 mM Tris-Cl, pH 7.5, 400 mM NaCl, and 10 mM EDTA, and $1/20^{th}$ of the concentrated particles were then analyzed by western blot. In all cases, proteins were denatured for 10 min at 42°C prior to SDS-PAGE separation. Antibodies used herein include: anti-IE1 [clone 1B12; ref. (10)] and anti-pp71 [clone 2H10-9; ref. (11)] both diluted at 1:100, anti-phospho-c-Fos (Ser32, Cell Signaling, 5348T), anti-c-Fos (Cell Signaling, 4384T) both diluted 1:1000, anti-tubulin (Sigma) diluted at 1:5000, anti-FLAG M2 (Sigma) diluted 1:7500, and horseradish peroxidase (HRP)-conjugated goat-anti-mouse and goat-anti-rabbit secondary antibodies (Jackson Immuno Research Labs) diluted 1:10000. Where indicated, protein expression was quantified by densitometry using Image J software (NIH).

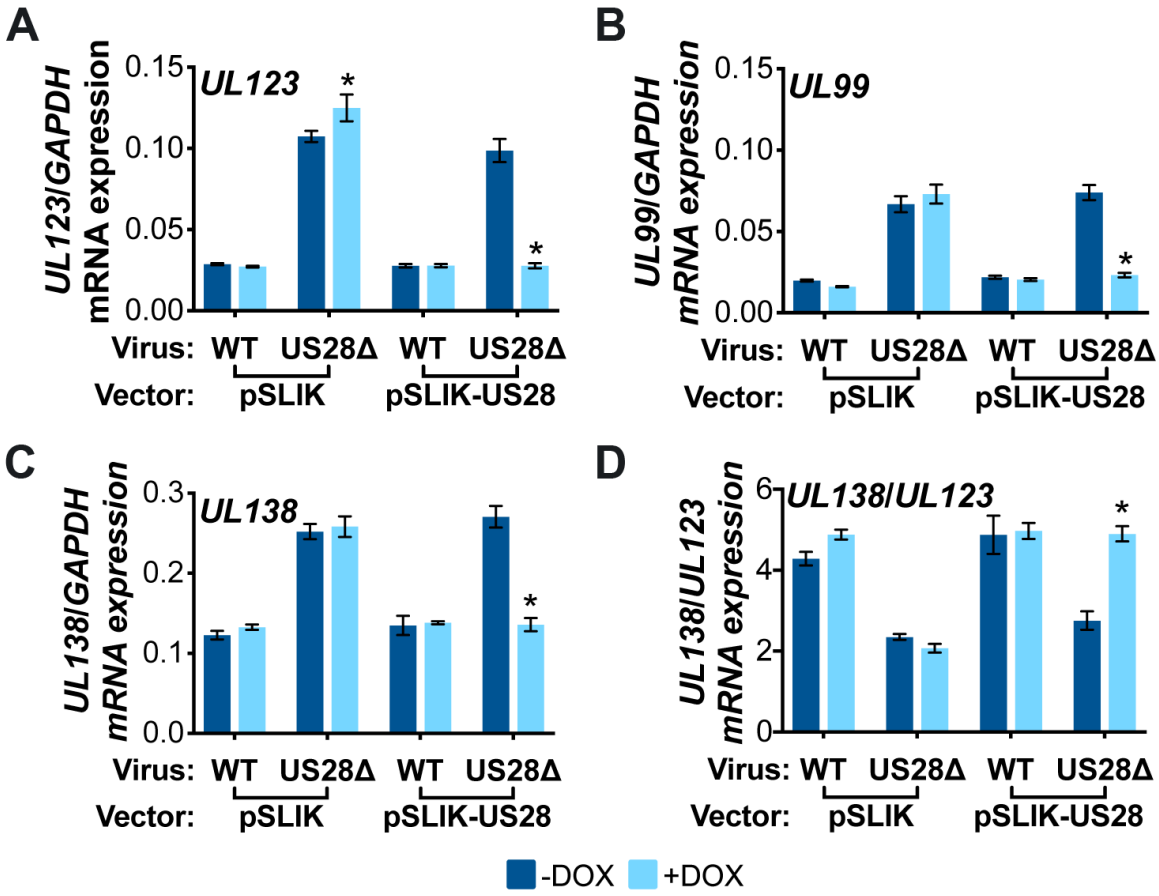

Fig. S1. DOX treatment does not alter viral gene expression. THP-1-pSLIK or THP-1-pSLIK-US28 were treated +/- DOX (24h) and then infected with either WT or US28Δ (moi = 1.0). Total RNA was harvested 7 dpi, and the following transcripts were assessed by RTqPCR: (**A**) *UL123*, (**B**) *UL99*, (**C**) *UL138*, and (**D**) the ratio of *UL138*/*UL123*. Samples were normalized to *GAPDH*, and each sample was analyzed in triplicate. Errors bars indicate standard deviation. Statistical significance was calculated using two-way ANOVA and Sidak post-hoc analysis relative to -DOX at each time point; *$p < 0.05$

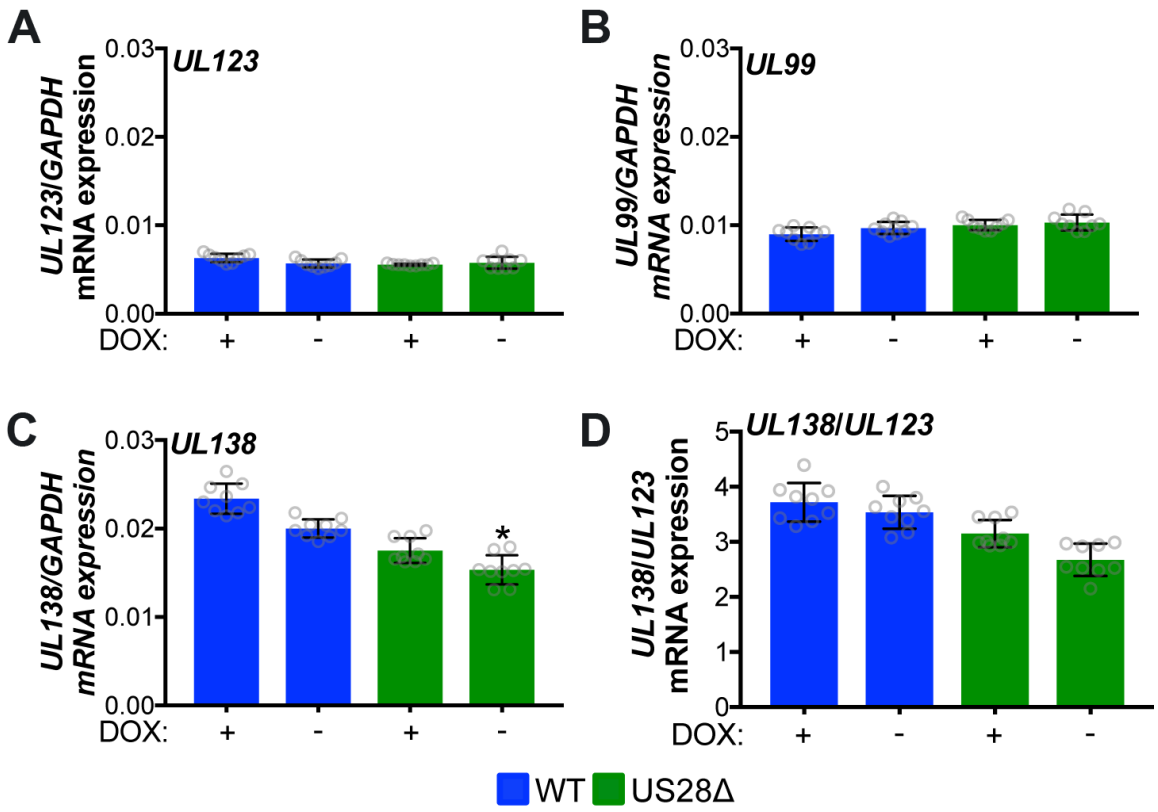

Fig. S2. pUS28 does not suppress lytic expression in differentiated THP-1 cells. THP-1-pSLIK-US28-3xF cells were treated with TPA (20 nM) for 48 h to promote differentiation. Cells were then treated with (+) or without (-) DOX (1 μg/ml) for 48 h to induce pUS28 expression. Next, the cells were infected with WT (blue) or US28Δ (green) and cultured for 7 d, during which the indicated DOX treatment was maintained. Total RNA from each culture was harvested and (**A**) *UL123*, (**B**) *UL99*, (**C**) *UL138*, and (**D**) the ratio of *UL138*/*UL123* gene expression were measured by RT-qPCR. Each sample was normalized to *GAPDH* and analyzed in triplicate. Errors bars indicate standard deviation; *$p < 0.05$, calculated using one-way ANOVA and Dunnett's post-hoc analysis.

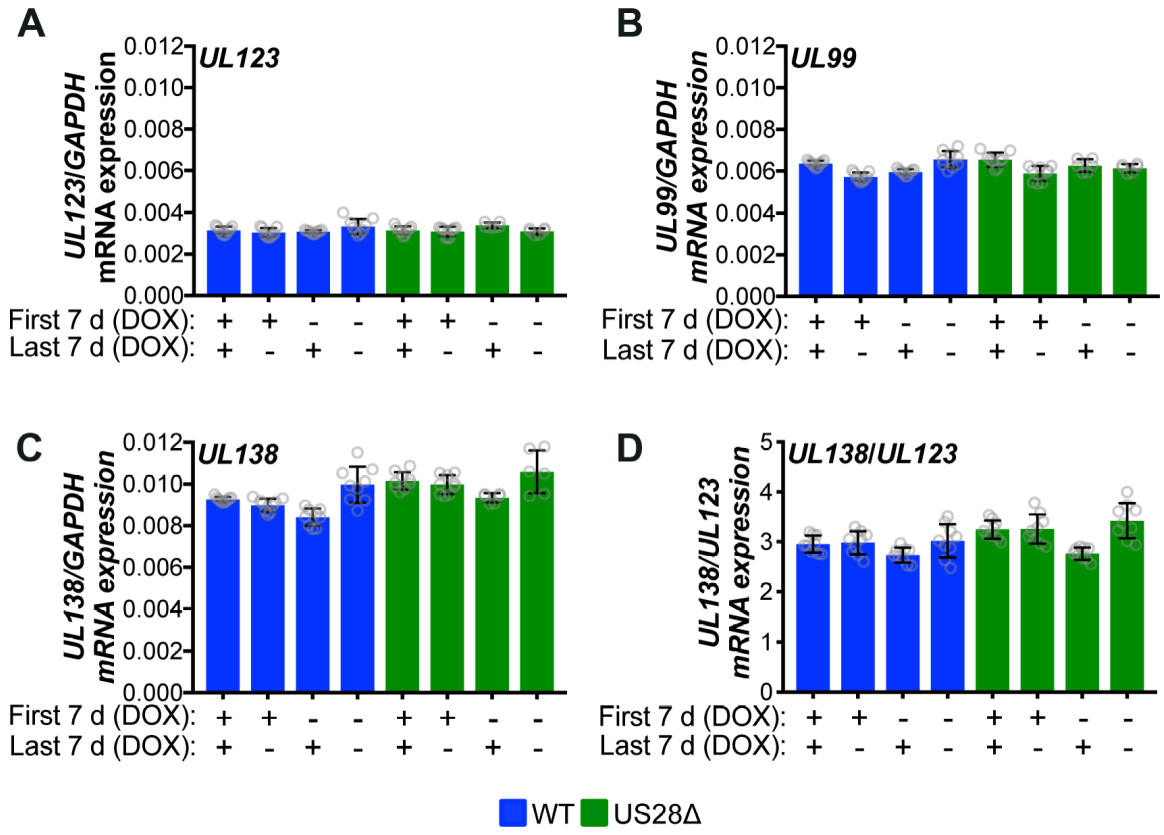

Fig. S3. pUS28 protein does not suppress lytic gene expression at later time points post-infection in differentiated THP-1 cells. THP-1-pSLIK-US28-3xF cells were differentiated with TPA (20 nM) for 48 h. Cells were then treated with (+) or without (-) DOX (1 µg/ml) for an additional 48 h to induce pUS28 expression and next infected with WT (blue) or US28Δ (green) (moi = 1.0). Cells were cultured for 7 d, during which cells were maintained under their original treatment conditions (First 7 d). At 7 dpi, cells were washed in PBS and treated with (+) or without (-) DOX (Last 7 d). Total RNA was harvested and (**A**) *UL123*, (**B**) *UL99*, (**C**) *UL138*, and (**D**) the ratio of *UL138*/*UL123* expression were measured by RT-qPCR. Each sample was normalized to *GAPDH* and analyzed in triplicate. Errors bars indicate standard deviation. No results were significant, as tested using one-way ANOVA and Dunnett's post-hoc analysis.

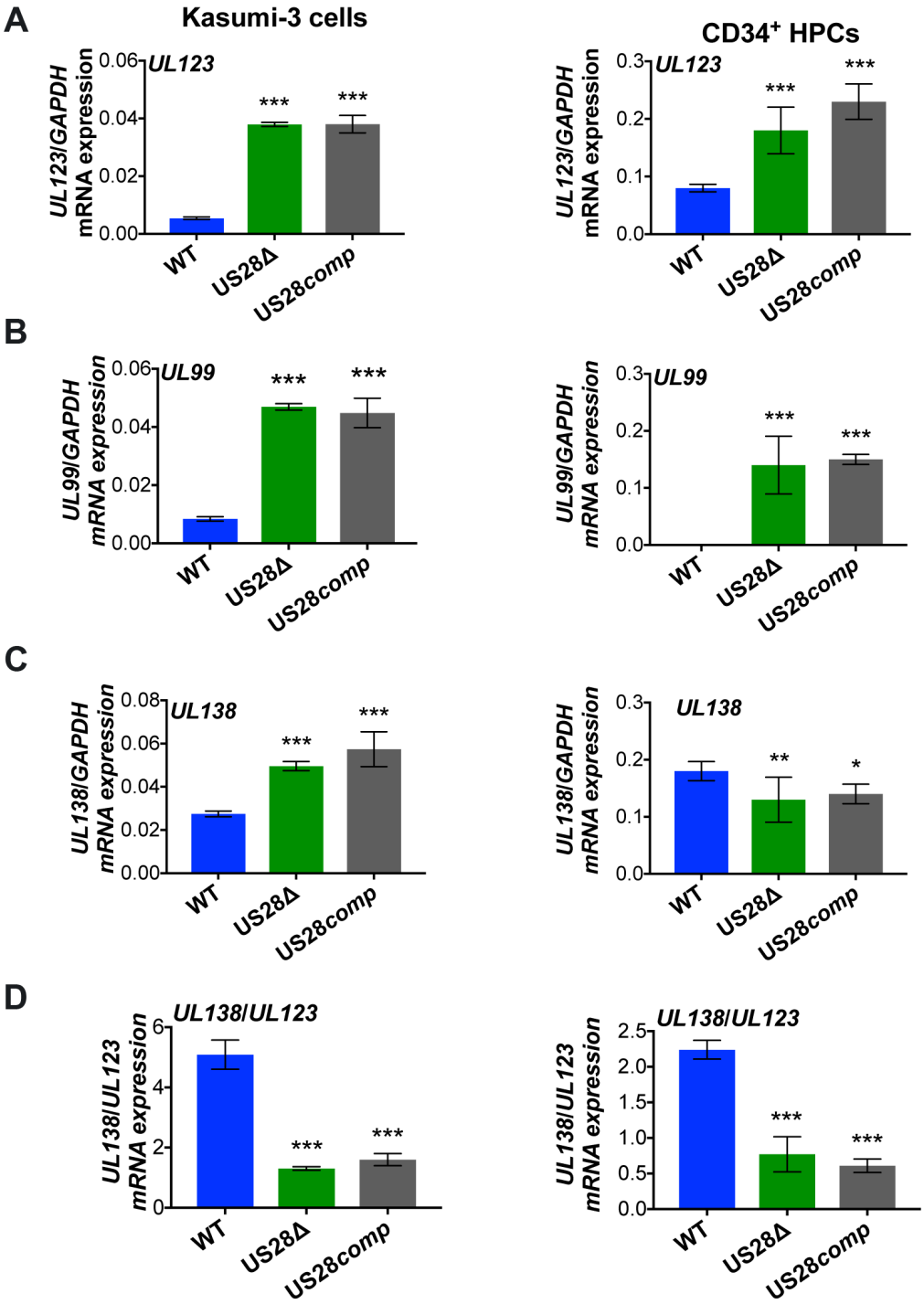

Fig. S4. Sustained pUS28 expression is required to maintain viral latency in Kasumi-3 cells CD34+ HPCs. Kasumi-3 cells (left; moi = 1.0) or CD34$^+$ HPCs (right; moi = 2.0) were infected with WT, US28Δ, or US28*comp*. Cells were harvested 12 dpi and (**A**) *UL123*, (**B**) *UL99*, (**C**) *UL138*, and (**D**) the ratio of *UL138*/*UL123* gene expression were measured by RT-qPCR. Each sample was normalized to *GAPDH* and analyzed in triplicate. Errors bars indicate standard deviation, and statistical significance was calculated using one-way ANOVA and Dunnett's post-hoc analysis; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

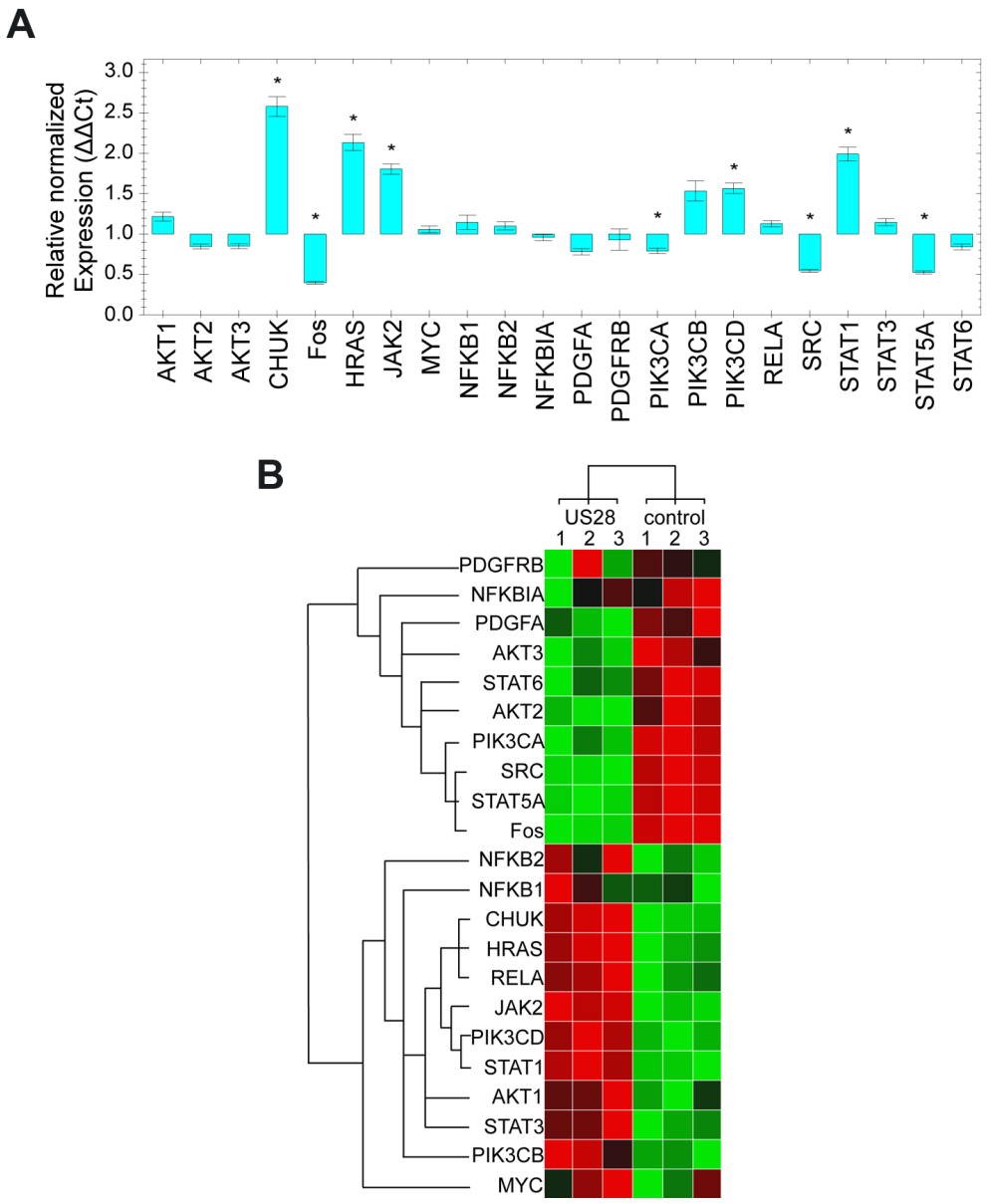

Fig. S5. pUS28 expression reveals dysregulated cellular genes. THP-1-pSLIK-US28-3xF (US28) or THP-1-pSLIK-hygro (control) cells were treated with DOX for 24 h, after which total RNA was harvested from each culture. Equal concentrations of RNA (1.0 µg) were used to generate cDNA, after which an equal volume of cDNA was loaded onto PrimePCR plates. (**A**) Expression of each gene is shown as fold-change in DOX-treated THP-1-pSLIK-US28-3xF cells relative to its expression in the control cells (set to 1.0). Each sample was assessed in triplicate and normalized to *GAPDH*. Error bars represent standard deviation; *$p < 0.001$. (**B**) Clustergram of the replicates for each sample reveal the most significant down-regulated (green) and up-regulated (red) genes in response to pUS28 expression. Two genes (platelet derived growth factor subunit B [*PDGFB*] and platelet derived growth factor receptor alpha [*PDGFRA*]) were excluded from the analyses because their quantitation cycles were within three cycles of the genomic DNA control.

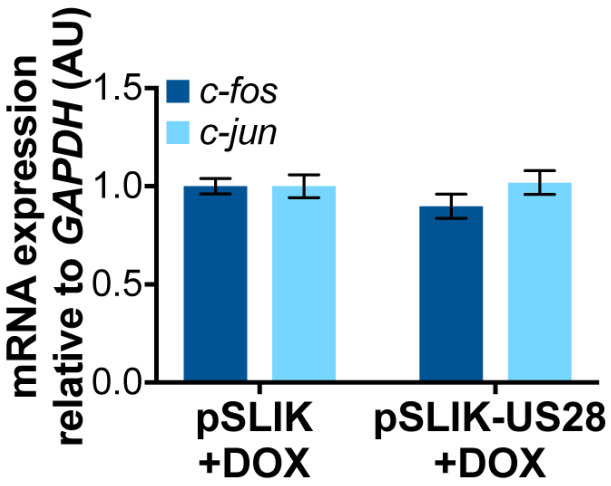

Fig. S6. pUS28 signaling does not impact c-fos expression in differentiated cells that support lytic infection. THP-1-pSLIK-hygro (pSLIK) and THP-1-pSLIK-US28-3xF (pSLIK-US28) were differentiated with TPA (20 nM) for 48 h and then treated with DOX for an additional 24 h. Cells were collected and *c-fos* (dark blue) and *c-jun* (light blue) expression were measured relative to *GAPDH*. Each sample was analyzed in triplicate. Errors bars indicate standard deviation. None of the samples were considered significant by Welch's t-test.

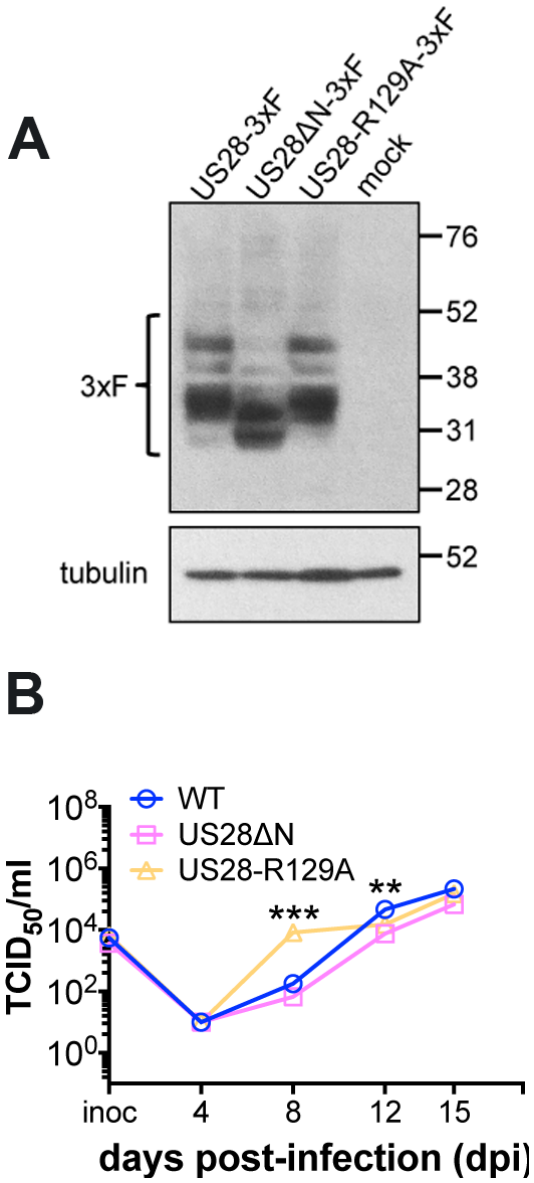

Fig. S7. pUS28 signaling mutants are expressed and display wild type growth kinetics in fibroblasts. (**A**) NuFF-1 cells were infected (moi = 0.5) for 96 h and total cell lysates were harvested for immunoblot analyses. pUS28 was detected using an antibody directed at the FLAG epitope tag. Cellular tubulin is shown as a control. (**B**) NuFF-1 cells were infected (moi = 0.01) with the indicated viruses, and cell-free supernatants were collected over a 15 d time course. All samples were analyzed in triplicate by $TCID_{50}$. Error bars indicate standard deviation. Statistical significance was calculated using the Student's T-test; $^{**}p < 0.01$ (WT vs. US28ΔN; WT vs. US28-R129A), $^{***}p < 0.001$ (WT vs. US28-R129A).

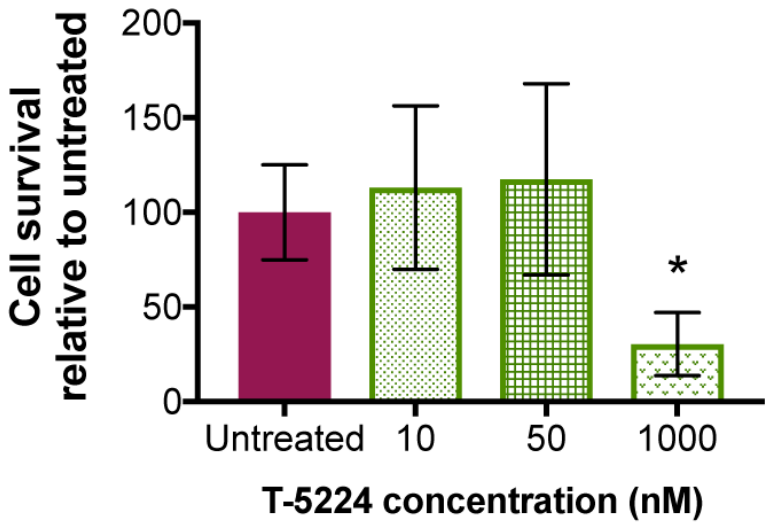

Fig. S8. The c-fos inhibitor, T-5224, is not toxic to Kasumi-3 cells below 50 nM. Kasumi-3 cells were treated with the c-fos inhibitor, T-5224, at the concentrations indicated. Fresh media with continued T-5224 was provided every 2 d. Cell survival was measured on day 7 by counting live cell numbers using trypan blue with a hemocytometer. Data is shown as percentage cell survival relative to untreated control. Error bars represent standard deviation, calculated using one-way ANOVA and Dunnett's post-hoc analysis; $*p < 0.05$.

Table S1. Primers used in this study.

Primer Use	Sequence	Target
galK insertion	TACGTGGCTATGTTTGCCAGTTTGTGTTTTATCACGG AGATTGCACTCGATCCTGTTGACAATTAATCATCGGC A	US28-R129A*galK* FWR
galK insertion	AAGGCAGGCCTGTTTTACAGGCCGATATCTCATGTAA ACAATAGCGTAGTATCAGCACTGTCCTGCTCCTT	US28-R129A*galK* REV
ds oligo	ATGTTTGCCAGTTTGTGTTTTATCACGGAGATTGCAC TCGATGCCTACTACGCTATTGTTTACATGAGATATCG GCCTGTAAAACAG	US28-R129A ds oligo
seq (R129A)	ATGACGCCGACGACGACGACCACGG	US28 5' FWR
seq (R129A)	GCAGTAGCTGATGACACTGAGC	US28 internal-2 REV
galK insertion	GCGTGGACCAGGCGGTGTCCATGCACCGAGGGCAGA ACTGGTGCTACCATGCCTGTTGACAATTAATCATCGG CA	US28ΔN*galK* FWR
galK insertion	CTTTGACTGATTAAGCACGTCGGTGAAGGTACAAGG GGTTGCTCCAAGGTCTCAGCACTGTCCTGCTCCTT	US28ΔN*galK* REV
ds oligo	GCGGTGTCCATGCACCGAGGGCAGAACTGGTGCTAC CATGGACCTTGGAGCAACTCCTTGTACCTTCACCGAC GTGCTTA	US28ΔN ds oligo
seq (US28ΔN)	CCGCACATCTATTTTTGCTAATTGC	US28 upstream FWR
seq (US28ΔN)	CGTACACGGCACGCTGGCTAGGGAG	US28 internal REV
qPCR	GCC TTC CCT AAG ACC ACC AAT	UL123 FWR
qPCR	ATT TTC TGG GCA TAA GCC ATA ATC	UL123 REV
qPCR	GTG TCC CAT TCC CGA CTC G	UL99 FWR
qPCR	TTC ACA ACG TCC ACC CAC C	UL99 REV
qPCR	GGT TCA TCG TCT TCG TCG TC	UL138 FWR
qPCR	CAC GGG TTT CAA CAG ATC G	UL138 REV
qPCR	ACC CAC TCC TCC ACC TTT GAC	GAPDH FWR
qPCR	CTG TTG CTG TAG CCA AAT TCG T	GAPDH REV
qPCR	CAG TCC AAG CGG AGA CAG AC	Fos FWR
qPCR	AGG TCA TCA GGG ATCTTG CAG	Fos REV
qPCR	TCG ACA TGG AGT CCC AGG A	Jun FWR
qPCR	GGC GAT TCT CTC CAG CTT CC	Jun REV
qPCR	AAC AGC GTG GAT GGC GTC TCC	MIEP FWR
qPCR	GGC ACC AAA ATC AAC GGG ACT TT	MIEP REV
qPCR	CTC GTC GTG TGA CAG CAG GAT G	UL69 non-promoter region
qPCR	GAA CTA CAG CAA CTC AGC CGT TTG A	UL69 non-promoter region
pBABE-US28 cloning	CGC GGA TCC GCC ACC ATG ACG CCG ACG ACG ACG A	US28 5' with BamH1
pBABE-US28 cloning	CGC GAA TTC TTA CTT GTC GTC GTC GTC CTT GTA GTC GAA TTC C	US28 3' with EcoR1

FWR, forward primer; REV, reverse primer; seq, sequencing primer; ds oligo, double stranded oligonucleotide. Underlined sequences bind the pGalK plasmid, described elsewhere (4).

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