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Supplemental information

The sphingosine kinase 1 activator, K6PC-5, attenuates Ebola virus infection

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SUPPLEMENTAL FIGURES

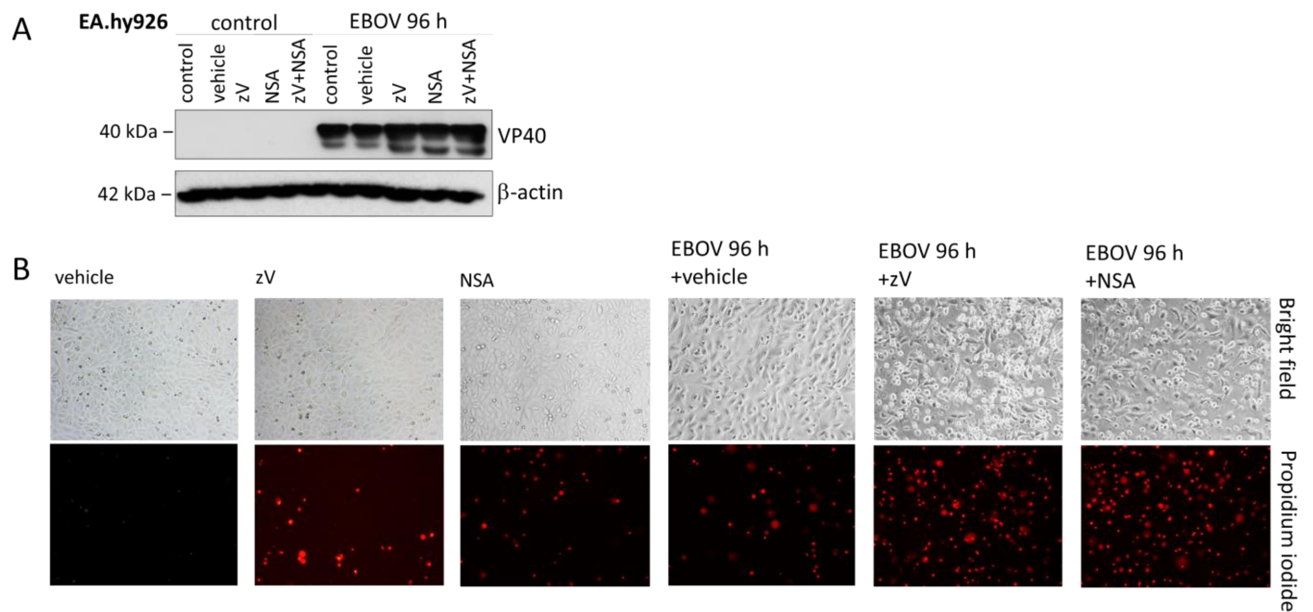

Figure S1: Treatment with zV or NSA has no influence on EBOV infection and infection induced cell death. Related to Figure 1.

(A) Immunoblot of VP40 viral matrix protein 96 h after EBOV infection in EA.hy926 cells. The cells were treated with vehicle (DMSO), 10 µM z-VAD-FMK (zV), 2 µM NSA (necrosulfonamide) or with zV in combination with NSA.

(B) Fluorescence microscopy of propidium iodide-stained EA.hy926 cells 96 h after EBOV infection (MOI=1). The cells were treated with vehicle (DMSO), 10 µM z-VAD-FMK (zV), 2 µM NSA (necrosulfonamide) or with zV in combination with NSA at 0 h. Scale bar: 50µm.

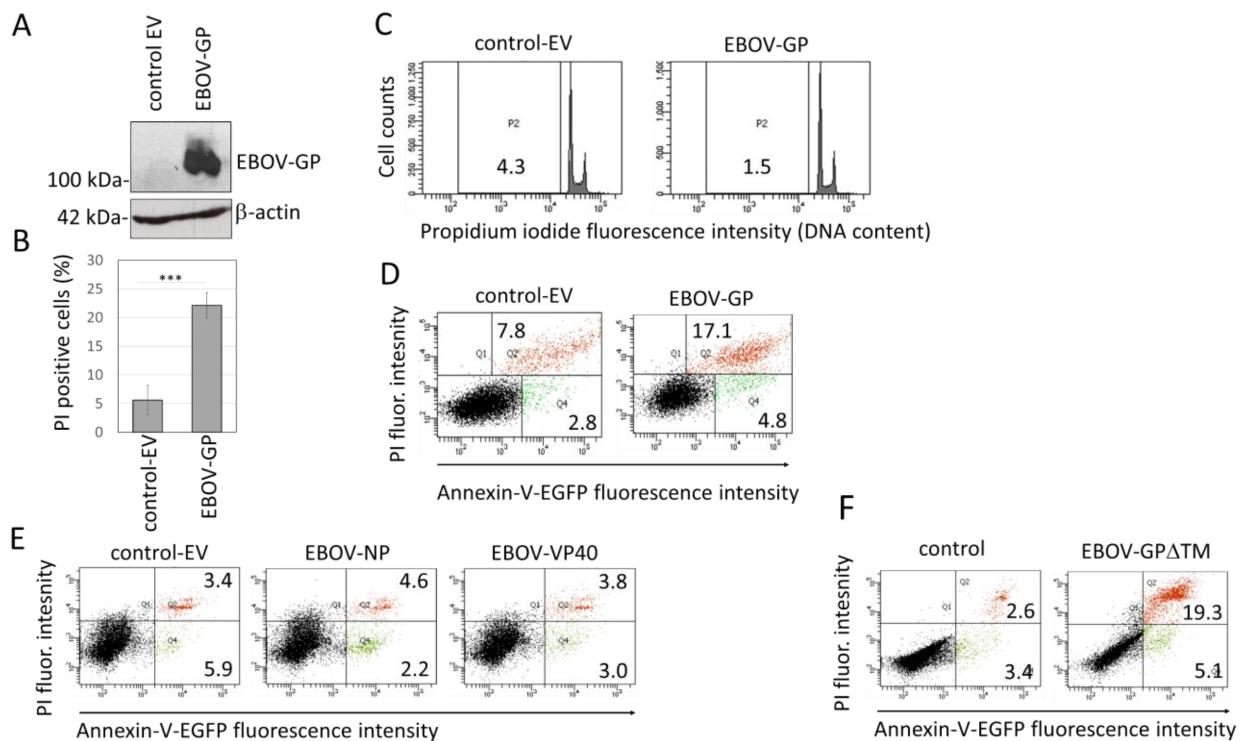

Figure S2. EBOV-GP induces necrotic cell death. Related to Figure 1.

(A) Immunoblot of EBOV-GP expressing cells. EA.hy926 cells were transfected with 1.5 µg/ml pcDNA3.1-empty vector (control EV) or pcDNA3.1-EBOV-GP and harvested for immunoblot 24 h after transfection.

(B) Percentage of propidium iodide positive EA.hy926 cells detected by flow cytometry 24 h after transfection with pcDNA3.1-empty vector (control-EV) or pcDNA3.1-EBOV-GP as in A. Data are presented as mean±SD. n=5; ***p<0.001 in Student´s t-test.

(C) Flow cytometry analysis of pcDNA3.1 empty vector (control-EV) and pcDNA3.1-EBOV-GP (EBOV-GP)-transfected EA.hy926 cells 24 h after transfection. The values indicate the percentage of apoptotic (subG1) population.

(D) Flow cytometry of EA.hy926 cells transfected with 1.5 µg/ml of pcDNA3.1-empty vector (control-EV) or pcDNA3.1-EBOV-GP. Percentage of Annexin-V-positive (green) cells and propidium iodide-Annexin-V double positive (red) cells are indicated.

(E) Flow cytometry of EA.hy926 cells transfected with 1.5 µg/ml of pcDNA3.1-empty vector (control EV), pcDNA3.1-EBOV-NP or -VP40 (24 h after transfection). Percentage of Annexin-V-positive (green) cells and propidium iodide-Annexin-V double positive (red) cells are indicated.

(F) Flow cytometry (as in D) of EA.hy926 cells treated with 40 µg/ml recombinant EBOV-GP without transmembrane domain (EBOV-GPdTM) (24 h).

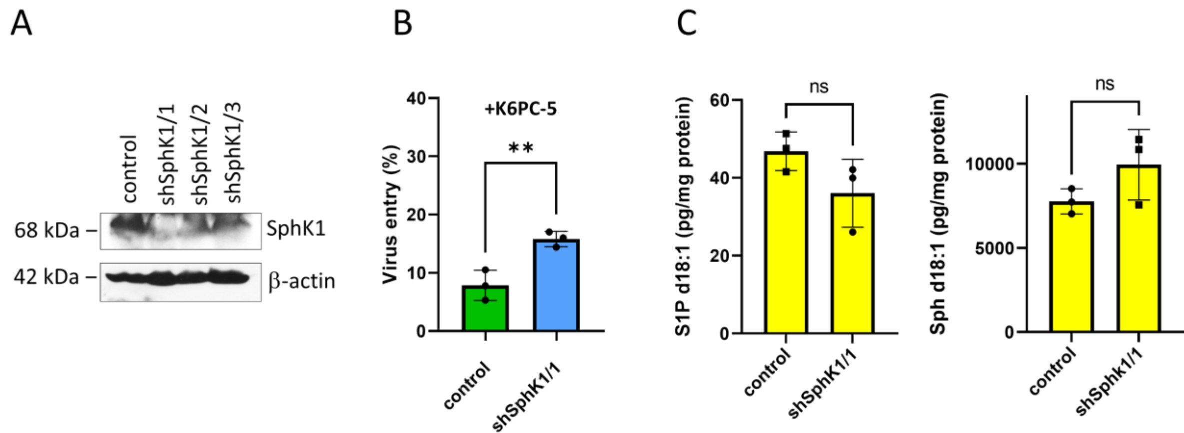

Figure S3. The effect of stable genetic depletion of SphK1 in MLV-EBOV-GP transduced cells. Related to Figure 2.

(A) Western blot analysis of EA.hy926 cells (control) and stable Sphk1 knock down EA.hy926 cells (shSphK1/1; -2; -3).

(B) Luciferase activity of K6PC-5 (25 µM) treated and MLV-EBOV-GP transduced EA.hy926 cells (control) and Sphk1 knock down EA.hy926 cells (shSphK1/1) 24 h after transduction. The values indicate the percentage of viral entry, measured as luciferase activity, relative to the vehicle (DMSO) treated samples. Data are presented as means±SD, n=3; **p<0.01 in one-sample t-test.

(C) Mass spectrometry-based determination (lower panel) of the concentration of S1P and sphingosine (Sph) d18:1 in EA.hy926 (control) and Sphk1 knock down EA.hy926 cells (shSphK1/1). The samples were normalized to total protein concentration. The values represent the amount of S1P (pg) in the relation of 1 mg total protein of the corresponding lysate. Data are shown as means±SD, n=3, ns, not significant in Student´s t-test.

TRANSPARENT METHODS

Cell lines and reagents

HeLa (American Type Culture Collection (ATCC; CCL-2)) and EA.hy926 (Edgell et al., 1983) cell lines were cultured in RPMI-1640 medium (Gibco), and HEK293T (ATCC CRL-11268) cells were cultured in DMEM (Gibco), both containing 10 % fetal bovine serum (FBS; Sigma-Aldrich) and 1 % penicillin-streptomycin (Sigma-Aldrich) and incubated at 37 °C and in 5 % CO_2. Human dermal microvascular endothelial cells (HMEC-1 cells) were from ATCC (CRL-3243) and cultured in MCDB-131 medium (Gibco) supplemented with glutamine (10 mM), epidermal growth factor (10 ng/ml), hydrocortisone (1 µg/ml), and 10 % FBS. For inhibitor experiments, cells were cultured in 6wel or 12 well plates. K6PC-5 (Sigma-Aldrich) (dissolved in dimethyl sulfoxide (DMSO)) was added to the cells in concentrations indicated in the figure legends. Sphingosine-1-Phosphate (d18:1) (S1P) (Avanti Polar Lipids) was dissolved in methanol and was added in combination with 100 ng/ml fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich) to the cells at 1 μM final concentration. The solvent was used as control. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-fmk) (Biozol) and necrosulfonamide (Biotechne) were dissolved in DMSO and were used in concentrations indicated in the figures. $S1P_1$ agonist SEW2871 (ENZO), $S1P_2$ agonist CYM5520, and FTY720-phosphate (FTYp) (both from Cayman) were dissolved in DMSO. $S1P_{1/3}$ antagonist VPC23019 (Tocris) was dissolved in DMSO. Pertussis toxin (PTX) (Calbiochem) was dissolved in sterile ddH2O.

Virus infection and titration

The Zaire Ebola virus (EBOV) (GenBank accession number: NC_002549) was propagated in Vero C1008 cells (ATCC CRL-1586). EA.hy926 cells were pre-treated with inhibitors (specified in the figures) or with the vehicle (DMSO) one h before infection. The cells were infected with EBOV at a multiplicity of infection (MOI) of 1. After virus adsorption at 37 °C for 1 h, the inoculum was removed, cells were washed with PBS and incubated in DMEM with 2 % fetal calf serum. The inhibitors were added again in the same concentration as before the infection. Samples from the supernatant were taken at the indicated time points post infection. The samples were clarified by centrifugation for 5 minutes at 8,000 rpm and titrated onto Vero C1008 cells.

Virus titer was determined by $TCID_{50}$/ml analysis. Briefly, Vero cells were cultured in 96 well plates to 50 % confluence and infected with 10-fold serial dilutions of supernatants from infected cells. At 10 to 14 days post infection, when the cytopathic effect had stabilized, cells were analysed by light microscopy. The $TCID_{50}$/ml was calculated using the Spearman-Kärber method (Hierholzer and Killington, 1996). All work with Ebola virus was performed in the biosafety level-4 (BSL4) laboratory of the Institute of Virology, Philipps-University Marburg, Germany.

VP40 /DAPI staining immunofluorescence

Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for at least 30 min. The fixative was removed, and free aldehydes were quenched with 100 mM glycine in PBS. Afterwards, the samples were washed with PBS, the cells were

permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated in blocking solution (2% bovine serum albumin, 0.2% Tween 20, 5% glycerol, and 0.05% sodium azide in PBS). and stained with a monoclonal anti-VP40 antibody (1:50). After washing steps, the cells were incubated with an anti-mouse Alexa-594 secondary antibody and co-stained with DAPI. The samples were visualized by fluorescence microscopy (Zeiss Axiomat).

Cell transfection with plasmids

Cells were cultured in 6 or 12 well plates until 70 % confluence. 100 µl/ml OptiMEM (Gibco) was mixed with GeneJuice (Merck-Millipore) (DNA : GeneJuice ratio = 1µg : 3µl) and incubated at room temperature for 5 minutes. Then, the plasmid (pcDNA3.1-empty-vector, -zEBOV-GP, -zEBOV-VP40, -zEBOV-NP, pmCherry-C1, pmCherry-C1-SphK1, pEYFP-N1 and pAcGFP-C2-hSphK2) was added to the mix, incubated for further 15 minutes and added to the cells. The cells were incubated in 5 % CO_2 at 37 °C as long as indicated in the figure legends. The cells transfected with empty vector or pmCherry-C1-SphK1 were treated with 1 mg/ml G418 (Sigma-Aldrich) 6 h after transfection for positive selection, and kept in culture for additional 6 days with complete medium change (with 1 mg/ml G418) on every second day. Six days after treatment, cells that had survived were plated on 12 well plates. On the next day, the cells were transduced with murine leukemia virus (MLV) virions as described in the section “MLV pseudotyped viral particles”. Luciferase activity detection/harvesting for immunoblot analysis was performed 24 h post infection.

Generation of stable knock down cells using lentiviral shRNA particles.

In order to achieve a stable knock down, EA.hy926 cells were seeded in 96 well plate format at 40,000 cells/100 µl medium. On the next day, the medium was changed to polybrene (hexadimethrine bromide, final concentration: 8 µg/ml) containing medium and the cells were infected with shRNA-carrying lentiviral particles (Sigma-Aldrich) at a MOI of 6. The medium was changed 24 h later. At 48 h post-infection, the cells were trypsinized, resuspended in puromycin-containing medium and seeded onto 12 well plates. To avoid clonal-specific effects, a pool of infected cells was used for the subsequent experiments after validating the knock down of the respective genes by immunoblot analysis. The following sequences of *SphK1* were employed for accomplishing knock down:

shSphk1/1 (TRCN0000344943):

CCGGACCTAGAGAGTGAGAAGTATCCTCGAGGATACTTCTCACTCTCTAGGTTTTTG

shSphK1/2 (TRCN0000333028):

CCGGCGCTGTGCCTTAGTGTCTACTCTCGAGAGTAGACACTAAGGCACAGCGTTTTTG

shSphK1/3 (TRCN0000219838):

CCGGAGAAGGTGGAGGCTATGCTTTCTCGAGAAAGCATAGCCTCCACCTTCTTTTTG

and

ShControl (SHC002V): non-target shRNA

Immunoblot assay

Cells seeded on 6 well plates were harvested by scratching, 50 µl 2x Laemmli (Sigma-Aldrich) was added and the samples were incubated at 99 °C for 5 minutes. 20 µl sample/ condition was subjected to SDS/PAGE. Next, the samples were transferred to a nitrocellulose membrane (0.45 µm, Biorad). The membrane was blocked for 1 h with 5 % milk or BSA TBS/0.1 % Tween at room temperature, washed three times for 5 minutes with TBS/0.1 % Tween, and then incubated with the respective primary antibody in 5 % milk TBS/0.1 % Tween overnight at 4 °C. Next, the membrane was washed three times for 5 minutes with TBS/0.1 % Tween, incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and washed again three times for 5 minutes. Then, the membrane was incubated 5 minutes with enhanced chemiluminescence solution (GE Healthcare), and luminescence was detected using a light sensitive X-ray film (Amersham hyperfilm, GE healthcare).

Visualization of necrotic cell death in cell culture by fluorescence microscopy

To detect necrotic cell membrane integrity loss in cell culture, cells grown in 12 well plates were incubated for 15 minutes with 2 µg/ml propidium iodide (Sigma-Aldrich). In figure 1, the propidium iodide fluorescence (excitation: 534 nm; emission: 617 nm) was imaged using a fluorescence cell culture microscope. The propidium iodide positive cells (red fluorescence) and the total number of cells (bright field) were quantified by employing the ImageJ analysis software.

Cell death detection by flow cytometry

For cell death detection, $0.5x10^6$ cells per well were seeded onto 12 well plates. After treatment as indicated in the figure legends, they were washed with 150 µl Dulbecco´s phosphate buffered saline (DPBS; Gibco) and incubated for 5 minutes with 150 µl trypsin/EDTA (Gibco). The collected cells were washed, resuspended in 300 µl resuspension buffer (ENZO) with 3 µl propidium iodide (PI) (ENZO) and 3 µl Annexin-V-EGFP (ENZO). Cells were analysed on FACSCanto-II flow cytometer (BD Bioscience) with FACSDiva, by employing the Annexin-V-EGFP/FL1 (488-nm blue laser/530-nm band-pass filter) and PI/FL2 channel (488-nm blue laser/585-nm band-pass filter). The debris (population exhibiting low FSC/FL2 intensity) was excluded from the analysis by employing the FSC/FL2 dot-plot.

Detection of the sub-G1 population by flow cytometry

The appearance of sub-G1 (under G1 phase) population of cells was monitored to measure apoptosis. Samples were prepared by employing a modified protocol of Gong et al (Gong J. et al., 1994). The treated cells were centrifuged and the pellets were resuspended in 1 ml of 70 % ethanol (−20 °C). The cells were fixed at room temperature for 30 minutes and stored at −20 °C overnight. Oligonucleosomal DNA fragments were extracted from ethanol-fixed cells in 1 ml of extraction buffer containing 200 mM Na_2HPO_4/citric acid (pH 7.8) and 10 µg/ml RNAse A (Sigma-Aldrich) for 15 min, then stained with propidium iodide (5 µg/ml final concentration) for 15 minutes before measurement. Cells were analysed on FACSCanto-II flow cytometer (BD Bioscience) with FACSDiva. Cells were gated to exclude the debris by using the FSC/FL2 dot-plot. The sub-G1 population was analysed by employing the FL2 histogram.

Production of murine leukemia virus (MLV) pseudotyped viral particles

MLV-pseudotyped viral particles were produced in HEK-293T cells. The cells were seeded onto 6 well plates with 1 ml medium per well at 70% confluence. The transfection reagent contained 100 µl/ml OptiMEM (Gibco) and 7.2 µl/ml GeneJuice (Merck-Millipore), mixed and incubated at room temperature for 5 minutes. Then, 1.2 µg/ml pTG-Luc, 0.6 µg/ml pCMV-MLV-gag-pol and 0.6 µg/ml pcDNA3.1-empty-vector (EV) or -zEBOV-GP was added, incubated for further 15 minutes and finally given to the cells. The medium was replaced 6 h post-transfection. Supernatant was harvested after 24 h, the cells were again filled up with 1 ml complete DMEM medium and harvested a second time 24 h later. The collected viral supernatant was filtered using a 0.45 µM filtertube (Merck-Millipore) and kept at -80 °C.

MLV luciferase assay

The cells were cultured in 12 well plates to 80 % confluence. After removing the supernatant, 1000 µl MLV viral supernatant was added and incubated at 37 °C and in 5 % CO_2 for 24 or 48 h. Next, the supernatant was collected, the plate was washed with 500 µl DPBS (Gibco), incubated for 5 minutes with 300 µl Trypsin-EDTA (Gibco) and the detached cells were pooled together with the collected supernatant and DPBS of the corresponding samples. The cell suspension was centrifuged at 2000 rpm for 4 minutes, the pellet was resuspended in 50 µl cell lysis buffer (Promega) and incubated for 15 minutes. After transferring the lysate into a 96 well white microplate (Corning-Costar), the luminescence intensity was detected (integration time 500 ms) before and after adding 90 µl Luciferase-reagent (Promega) by employing a SpectraMax luminometer (Molecular Devices) with SoftMax Pro software.

Sphingolipid analysis

$8x10^6$ cells were washed 2x in PBS and were harvested by scraping and mixing with 500 µl methanol (-20 °C). The protein measurement was performed parallel on a portion ($2,67x10^6$ cells) of the same sample by employing the Bradford method. The samples were collected in 1.5 ml tubes and the methanol was removed by employing speedvac. The sphingolipid analysis was done using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described elsewhere (Talanta, 2020). In brief, cell samples were resuspended in 200 µl extraction buffer (citric acid 30 mM, disodium hydrogen phosphate 40 mM) and mixed with 20 µl internal standard solution.

Then, the samples were extracted once with 600 µl methanol:chloroform:HCl (15:83:2, v/v/v). The collected lower organic phases were evaporated at 45 °C under a gentle stream of nitrogen and reconstituted in 50 µl methanol containing 5 % formic acid. For calibration standards and quality control samples preparation, 20 µl of the working solution were processed as stated instead of sample.

The quantification of all analytes was performed using a hybrid triple quadrupole-ion trap mass spectrometer QTRAP 5500 (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in positive ESI mode. Sphingolipids were separated using an Agilent 1290 HPLC system equipped with a Zorbax C8 Eclipse Plus UHPLC column (2.1 * 30 mm, 1.8 µm, Agilent technologies, Waldbronn, Germany). Quality control samples of three different concentration levels (low, middle, high) were run at the beginning and end of each run. Samples were processed using Analyst software 1.6 and the obtained concentrations were evaluated using MultiQuant 3.0 (both Sciex, Toronto, Canada) using the internal standard method (isotope-

dilution mass spectrometry). Calibration curves were calculated by linear or quadratic regression with 1/x weighting or 1/x2 weighting, respectively. Variations in accuracy of the calibration standards were lower than 15 % over the range of calibration, except for the lower limit of quantification (LLOQ), where a limit of 20 % was accepted.

Statistical analysis

Student`s t test, one way ANOVA or one sample t test was performed to test for statistical significance of the results as indicated in the corresponding figure legends. (*P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.*0001*, ns= not significant). The sample size (number of independent experiments) is indicated in the figure legends as *n=x*.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-tubulin	Sigma-Aldrich	
α-Beta-Actin-Mouse Monoclonal (AC-15)	Sigma-Aldrich	Lot#026M4780V
α-SphK1-Mouse	Abnova	H000056848B01P
α-SphK2-Mouse	Abnova	H00008877-M01
Living colors	Clontech	632377
α-mCherry	Abcam	#43590
α-Mouse-HRP IgG Goat	Sigma-Aldrich	#12349
α-PARP 46D11 Rabbit mAb	Cell Signaling	#9532
α-Rabbit-HRP IgG Goat	Sigma-Aldrich	#A0545
α-zEBOV-GP 4F3 Mouse mAb	IBT Bioservices	Lot#1501002
α-zEBOV-VP40 Rabbit pAb	IBT Bioservices	Lot#1107001S
Plasmids		
pcDNA3.1	Invitrogen	
pmCherry-C1	Clontech/Takara Bio Europe France	
pEYFP-N1	Clontech	
pmCherry-C1_Sphk1	described in Blankenbach et al. 2020	NCBI reference sequence NM_001142601.2
pcDNA3.1_zEBOV-GP	Prof. Dr. Fatah Kashanchi	
pcDNA3.1_zEBOV-NP	Prof. Dr. Fatah Kashanchi	

pcDNA3.1_zEBOV-VP40	Prof. Dr. Fatah Kashanchi	
pCMV-MLV-gag-pol	Prof. Dr. Pöhlmann	Bartosch B. et al., 2003
pTG-luc	Prof. Dr. Pöhlmann	Bartosch B. et al., 2003
pAcGFP-C2_SphK2	A. Don & H. Rosen	Addgene plasmid #84370
Cell lines		
EA.hy926	Prof. Dr. Pfeilschifter	Edgel J. et. al., 1983
HEK-293T	ATCC	CRL-11268
HeLa	ATCC	CCL-2
HMEC-1	ATCC	CRL-3243
Viruses		
Zaire Ebola virus (EBOV)	Prof. Dr. Stephan Becker	GenBank accession number NC_002549
Chemicals, Commercial Assays		
Amersham ECL Western Blotting Detection Reagents	GE Healthcare	Lot#16809358
Annexin V-EGFP Apoptosis Detection Kit	ENZO	Lot#08081911
GeneJuice Transfection Reagent	MERCK-Millipore	Lot#3248274
z-VAD-fmk	Biozol	APE-A1902-1MG
Necrosulfonamide	Biotechne	5025/10
K6PC-5	Sigma-Aldrich	SML1709
CYM5520 (CYM)	Cayman	1449747-00-5
SEW2871 (SEW)	Enzo Life Sciences	BML-LPI06-0050
FTY720-phophate (FTYp)	Cayman	10008639
VPC23019 (VPC)	Tocris	4195
Pertussis toxin (PTX)	Calbiochem	516560
Sphingosine-1-Phosphate (d18:1) (S1P)	Avanti Polar Lipids	860492
Luciferase Assay System	Promega	Lot#000392448
Propidium iodide solution	Sigma-Aldrich	Lot#MKBV9923V
Sample Buffer Laemmli 2x Concentrate	Sigma-Aldrich	Lot#SLBX2653
Software and Algorithms		
FACSDiva	BD Bioscience	

SoftMaxPro	Molecular Devices	
ImageJ	ImageJ	https://imagej.nih.gov/ij/
GraphPad Prism 9.0.0	GraphPad Software	http://graphpad.com

SUPPLEMENTAL REFERENCES

Gong, J., Traganos, F., Darzynkiewicz Z. (1994). A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal Biochem. 218, 314–319.

Hierholzer JC., Killington RA. (1996) Virus isolation and quantitation. In: Kangro BW., Ma HO., editor. Virology methods manual. London, United Kingdom: Academic Press Limited. 36–38.

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