Supplementary Materials for

Two Pore Channels Control Ebolavirus Host Cell Entry and are Drug Targets for Disease Treatment

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Materials and Methods

Cells

HeLa cells (Life Technologies, Grand Island, NY), HEK293 cells (ATCC, Manassas, VA) and Vero-E6 cells (CDC, Atlanta, GA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. 293FT cells were purchased from Life Technologies and cultured in DMEM with 500 ng/ml of G418. Mouse embryonic fibroblasts (MEFs) from WT, Tpcn1$^{-/-}$ and Tpcn2$^{-/-}$ mice were isolated and established as described previously (*25, 32*).

Reagents and antibodies

All pharmacological inhibitors were purchased from Calbiochem (San Diego, CA), Sigma-Aldrich (St. Louis, MO) or Enzo Life Sciences (Farmingdale, NY). Ned19 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For *in vitro* experiments, the chemical stocks were prepared in either molecular-grade water or dimethyl sulfoxide (DMSO) as suggested by the manufacturer, aliquoted and stored at -80°C until use. For *in vivo* experiments, tetrandrine was dissolved in 0.1N HCl at a concentration of 25 mg/ml, filtered through a 0.4-μm filter and stored at -80°C until use. For immunostaining, Hoechst33342 dye and HCS CellMask Blue Stain were purchased from Life Technologies. Mouse anti-ZEBOV GP was purchased from IBT Bioservices (Gaithersburg, MD). Rabbit anti-ZEBOV VP40 was kindly gifted from Dr. Ricardo Carrion Jr. (Texas Biomedical Research Institute, San Antonio, TX). Mouse anti-LAMP1 antibody and rabbit anti-LAMP1 antibody were from Santa Cruz Biotechnology and Abcam (Cambridge, MA), respectively. Rabbit anti-myc antibody was from Cell Signaling (Danvers, MA). Goat anti-mouse Alexa Fluor 488, anti-mouse Alexa Fluor 546, anti-rabbit Alexa Fluor 488, anti-rabbit Alexa Fluor 546, anti-mouse Alexa Fluor 633 and AlexaFluor555-EGF complex were from Life Technologies.

Unless otherwise indicated Ned19 was used at 100 μM, tetrandrine at 2 μM, verapamil at 100 μM or U18666A at 20 μM.

EBOV-GFP cultivation and infection

A recombinant Ebolavirus encoding a GFP reporter gene (EBOV-GFP) was provided by Dr. Heinz Feldman (Rocky Mountain Laboratories, National Institute of Health, Hamilton, MT). The virus was cultivated on Vero-E6 cells by infection at an MOI of approximately 0.1. Culture supernatants were collected after 5 days, when all cells expressed GFP fluorescence and about 80% of the cells showed cytotoxicity, and clarified by centrifugation at 2000 × g for 15 min. The virus was concentrated by ultracentrifugation at 82,700 × g for 2 h pelleting through a 20% sucrose cushion. Virus titer was determined by serial dilution on Vero-E6 cells. After 24 h incubation, the cells were fixed in 10% formalin for > 24 h, washed with PBS, stained with Hoechst33342 dye and imaged by a Nikon Ti Eclipse inverted microscope with a 10× lens. Counting of the cell nuclei and GFP-positive foci was performed using Cell Profiler image analysis software (Broad Institute, MIT, Boston, MA) and customized analysis pipeline (available upon request). For infection assays, HeLa cells, which were pretreated with drugs in 2-fold serial dilutions for 1-5 hours or transfected with siRNAs, or MEFs from WT, Tpcn1^{-}

$^{-/-}$ or Tpcn2$^{-/-}$ mice were plated in 96 well plates and challenged with EBOV-GFP at an MOI of 0.01 for 24 h. This time corresponded to approximately one round of replication and allowed for accumulation of enough GFP to be detected in infected cells. Human primary macrophages were challenged with EBOV-GFP at an MOI of 0.1 for 48 h. All infected cells were fixed and analyzed as described above. All experiments with replication competent EBOV were performed in a biosafety level 4 (BSL4) laboratory at Texas Biomedical Research Institute.

Generation of HIV-based vectors encoding TPCs

To produce an HIV-based vector encoding TPC1 or TPC2, each wild type (WT) TPC open reading frame (ORF) in pCMV-AC-GFP (Origene, Rockville, MD) was inserted into CSII-CMV-MCS-IRES-hrGFP (kindly gifted from Dr. Masao Matsuoka, Kyoto University, Kyoto, Japan) to create CSII-CMV-TPC1-IRES-hrGFP or CSII-CMV-TPC2-IRES-hrGFP, respectively. A dominant negative form of TPC2 (TPC2^{L265P}) was generated by PCR-based site directed mutagenesis. The mutant gene then replaced the wild type TPC2 ORF in pCMV-AC-GFP using In-Fusion HD (Clontech, Mountain View,CA). The TPC2^{L265P} gene was then inserted into CSII-CMV-MCS-IRES-hrGFP to create CSII-CMV-TPC2^{L265P}-IRES-hrGFP. 293FT cells were co-transfected with 5 μg of the pCMV-Δ8/9 packaging vector (kindly gifted from Dr. Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan) and 1 μg of pVSV-G (BD Biosciences, San Jose, CA) as well as 5 μg of CSII-CMV-TPC1-IRES-hrGFP, CSII-CMV-TPC2-IRES-hrGFP, CSII-CMV-TPC2^{L265P}-IRES-hrGFP or CSII-CMV-MCS-IRES-hrGFP using the calcium phosphate method in 10 cm dishes. The supernatant was harvested after 48 h and treated as for pseudotyped VSV. The titer was determined by challenging HeLa cells for 48 h and identifying GFP expression as above.

WT EBOV infection assays

HeLa cells were transduced with the HIV-based vector encoding TPC2^{L265P} with a GFP reporter or just GFP at an MOI of 1.0 in the presence of 8 μg/ml of polybrene (Sigma-Aldrich). After 48 h, cells were reseeded into 96 well plates and then challenged with WT Zaire EBOV (Mayinga strain). After 24 h, cells were fixed in 10% formalin for > 24 h, washed with PBS and blocked with 10% goat serum (Life Technologies). Infected cells were stained with anti-Zaire EBOV GP (IBT Bioservices, Gaithersburg, MD) followed by an anti-mouse Alexa Fluor 546 antibody. Infected and total cells were then counted as above.

For complementation assays, MEFs lacking TPC1 or TPC2 expression were transduced with the HIV-based vector encoding human WT TPC1 or TPC2, respectively, with a GFP reporter. After 48 h, cells were reseeded into 96 well plates and then challenged with WT Zaire EBOV for 24 h. Infected cells were processed and analyzed as above.

siRNA transfection

siRNAs specific for TPC1 or TPC2 were purchased from Qiagen (Valencia, CA) and Life Technologies. As non-targeting controls, AllStars Negative Control siRNA and Negative Control siRNA were used (both from Qiagen). HeLa cells were transfected twice with siRNA at 5 nM (at 24 h and 48 h after seeding) using RNAiMAX (Life

Technologies) following the manufacturer's protocol in 12 well plates. For infection with EBOV-GFP or VSV pseudotyped with GP, the transfected cells were trypsinized and reseeded in 96 well plates 24 h after the second transfection. The cells were also lysed for mRNA isolation and subsequent RT-PCR or immnoblotting 24 h or 48 h post-transfection, respectively.

qRT-PCR

To determine the fold change in the TPC1 and TPC2 mRNA expression in HeLa cells, total RNA was extracted and purified using an Aurum Total RNA kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol. qRT-PCR was performed using an iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) with TPC1 or TPC2 specific primers. GAPDH-specific primer sets (Qiagen) served as internal controls and to calculate $\Delta\Delta CT$ using CFX software (Bio-Rad).

Cytotoxicity measurement

Drug cytotoxicity was measured using CytoTox-Fluor Cytotoxicity Assay (Promega) following the manufacturer's protocol. After HeLa cells were incubated with inhibitors for 24 h, the bis-AAF-R110 fluorogenic substrate was added to the culture media and incubated for additional 2 h. Fluorescence was measured using a GENios microplate reader and Megellan data analysis software (both from TECAN, Männedorf, Switzerland).

Generation of recombinant VSV pseudotyped with virus glycoproteins

Recombinant vesicular stomatitis virus (rVSV) pseudotyped with Ebolaviurs, Marburgvirus, Lassa virus, Venezuelan equine encephalitis virus or Rabies virus GP (rVSV-EBOV-GP, rVSV-MARV-GP, rVSV-LASV-GP, rVSV-VEEV-GP or rVSV-RABV-GP, respectively) were generated using a recombinant VSV with VSV-G gene replaced by a luciferase reporter gene as described below. For production of rVSV-MARV-GP, rVSV-LASV-GP, rVSV-VEEV-GP or rVSV-RABV-GP, 293FT cells were transfected with 5 μg of pMARV Musoke GP, pLASV GP, pVEEV GP or pRABV GP and 10 μg of pβ-gal plasmid using the calcium phosphate method in 10 cm dishes. Twenty four hours after transfection, the cells were infected with rVSV-VSV-G overnight and washed by PBS twice. The supernatant was harvested 48 h after infection, passed through a 0.4-μm filter and stored at -80°C. For production of rVSV-EBOV-GP, 10 μg of pEBOV Zaire GP and 5 μg of pβ-gal plasmid were cotransfected to HEK293 cells. Twenty four hours after transfection, the cells were challenged with rVSV-MARV-GP overnight and washed twice with PBS. The supernatant was harvested 48 h after infection, passed through a 0.4-μm filter and subjected to ultracentrifugation at 82,700 × g for 2 h pelleting through a 20% sucrose cushion. As a control for contamination of the inoculating virus, the cells were transfected with pβ-gal plasmid alone challenged with each seed virus. Then the culture supernatant was collected and tested for the activity. Virus titers were determined by serial dilution on HeLa cells with luciferase activity measured 8 h post-infection.

In vitro protease cleavage of rVSV-EBOV-GP

Concentrated VSV pseudotyped with EBOV GP were incubated at 37°C for 1 h with 0.5 mg/ml thermolysin (Promega, Madison, WI) in HEPES-MES buffer (40 mM HEPES, 40 mM morpholinepropanesulfonic [MES] acid, 50 mM NaCl, pH 7.5). The reaction was stopped by the addition of 0.5 mM EDTA and protease inhibitors. Mock treatments were conducted in the same buffers without the enzyme. The cleavage efficiencies were confirmed by immunoblotting using anti-Ebolavirus GP antibody as a primary antibody.

Infection assay with pseudotyped VSV

For experiments with drugs, HeLa cells were seeded in white walled 96 well plates (Corning, Lowell, MA) and incubated with each drug in 2-fold serial dilutions. After 1 h, rVSV pseudotyped with each virus glycoproteins was added. The cells were incubated with the drug and virus for 8 h, after which the medium was replaced with luciferase assay buffer (20 mM Tricine-HCl, pH 7.5, 8 mM $MgSO_4$, 0.13 mM EDTA, 0.53 mM ATP, 33 mM DTT 0.47 mM luciferin) containing 0.2% of Triton X-100 detergent. After the cells were incubated with the buffer for 10 min at RT, the luciferase activity was measured using a 96-well plate luminometer (Promega).

Generation of Ebolavirus-like particles (Ebola VLPs)

To visualize EBOV entry into cells, virus-like particles (VLPs) were made with WT VP40, VP40 tagged with GFP or VP40 tagged with the red-fluorescent protein, mKate2. 293FT cells were transfected with 5.0 ug of pVP40 or 4.5 μg of pVP40-GFP or pVP40-mKate2 as well as 0.5 μg of pVP40, together with 5.0 μg of pCAGGS-NP, 1.0 μg of pEBOV GP and 4.0 μg of pβ-gal using the calcium phosphate method with 10 cm dishes. For virus contents release assays, the plasmid encoding VP40 tagged with the fluorescence protein was replaced with one encoding VP40 tagged with β-lactamase; pBLA-EBOV-VP40 (given by Dr. Christopher Basler, Mount Sinai School of Medicine, New York, NY). Culture supernatants were harvested 48 h after transfection, VLPs were pelleted as for virus. Pellets were resuspended in PBS, dissolved overnight at 4°C and stored at -80°C.

VLP infection and antibody staining

For analyzing the effects of drugs on Ebola VLP colocalization with LAMP1, HeLa cells were seeded on 8 well chamber slides (ibidi, Verona, WI). After preincubation with each drug for 1 h, the cells were incubated with VLPs tagged with mKate2 in the presence of the drug for 4 h, washed with PBS, fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton-X 100 in PBS. Goat serum was used to block cells and LAMP1 was stained with a mouse antibody followed by anti-mouse Alexa Fluor 488 antibody. For colocalization analyses in MEFs, the cells were incubated with VLPs tagged with GFP for 4 h. LAMP1 was stained with a rabbit antibody followed by anti-rabbit Alexa Fluor 546 antibody. Cells were stained with HCS CellMask Blue Stain (Life technologies). Using a microscope with a 100× oil objective lens, z-stacks of cell images were captured (0.3 μm slices) and deconvolution was performed using AutoQuant X3 image processing software (MediaCybernetics, Rockville, MD). The deconvolved images were processed by Imaris 3D image analysis software (Bitplane, South Windsor, CT) to reconstruct 3D images.

For determining colocalization of VLPs with TPC1 or TPC2, HeLa cells were transfected with pCMV-AC-GFP encoding TPC1 or TPC2 fused with GFP (OriGene, Rockville, MD) using TransIT LT1 (Mirus Bio LLC, Madison, WI). Forty eight hours after transfection, cells were incubated with WT VLPs for 2 h. VLPs were visualized by staining with an anti-Ebolavirus GP followed by anti-mouse Alexa Fluor 546 antibody. Cells were stained with HCS CellMask Blue Stain, and imaged by a Nikon C2+ confocal microscope with a 100× oil objective lens.

For colocalization of VLPs, TPC2 and NPC1, HeLa cells were transfected with a TPC2-GFP expression plasmid and a plasmid encoding myc-tagged NPC1 (from Dr. Kartic Chandran, Albert Einstein College of Medicine, Bronx, NY). Forty eight hours after transfection, cells were pretreated with drugs and incubated with WT Ebola VLPs in the presence of drugs. VLPs and NPC1 were visualized by staining with an anti-Ebolavirus GP and an anti-myc antibody, respectively, followed by anti-mouse Alexa Fluor 633 and anti-rabbit Alexa Fluor 546 antibody, respectively. Cells were stained with HCS CellMask Blue Stain and imaged by a confocal microscope with a 100× oil objective lens. The ratio of VLPs in TPC2- and/or NPC1-positive compartments in cells expressing both TPC2 and NPC1 was calculated using Cell Profiler image analysis software. All customized Cell Profiler analysis pipelines used in this work are available upon request.

EGF trafficking assays

For detecting accumulation of epidermal growth factor (EGF) in MEFs, cells from WT, Tpcn1-/- or Tpcn2-/- mice were incubated with 1.0 μg/ml of AlexaFluor546-EGF (Life Technologies) in DMEM containing no FBS for 4 h. For analyzing inhibitor effects on EGF trafficking, HeLa cells were pretreated with inhibitors and incubated with 0.3 μg/ml of AlexaFluor546-EGF in the presence of inhibitors. After 30 min, cells were washed with DMEM and incubated with inhibitors for additional 3.5 h. Cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. After visualizing cells by staining with HCS CellMask Blue Stain, cells were imaged by a confocal microscope with a 100× oil objective lens.

For determining colocalization of EGF and Ebola VLPs, HeLa cells were incubated with 1.0 μg/ml of AlexaFluor546-EGF for 30 min. After washing with DMEM, cells were incubated with VLPs tagged with GFP for additional 3.5 h and processed as above.

Virus contents release assay

For detection of virus capsid release into the cell cytoplasm, which requires fusion between the virus and cell membranes, virus contents release assays were performed using LiveBLAzer FRET-B/G Loading Kit with CCF2-AM (Invitrogen) as described previously (24). Briefly, HeLa cells were seeded on 96-well plates 1 day before the assay. After preincubation with each drug for 1 h, cells were inoculated with Ebola VLPs by spinoculation at 2000 × g for 1 h at 4°C and incubated at 37°C for an additional 4 h in the presence of the drug. After loading CCF2-AM substrate, cells were fixed in 10% formalin and imaged with a Nikon Ti Eclipse inverted microscope with a 20× lens. Mean fluorescence intensity of each cell was measured using Cell Profiler image analysis software. The blue and green mean fluorescence intensities were plotted using FCS

Express 4 Image Cytometry software (De Novo Software, Los Angeles, CA) and signals were gated based on uninfected cells.

Synthesis of NAADP-AM

NAADP was synthesized from NADP (MyBioSource, San Diego, CA) and nicotinic acid (Sigma-Aldrich) by a base-exchange reaction catalyzed by ADP-ribosyl cyclase (Sigma-Aldrich) in the presence of bovine serum albumin. It was purified through an anion-exchange resin (AG MP-1M, Bio-Rad) using a gradient of trifluoroacetic acid (TFA) as described previously (30). NAADP was acetoxymethylated using acetoxymethylene bromide and diisopropylethylamine (DIEA) to make the cell membrane-permeant NAADP-AM, which was then purified by phase seperation as described previously (31). Lyophilized powder was stored at -20°C and dissolved in DMSO immediately before use.

Calcium imaging

For measuring changes in intracellular calcium concentration, HeLa cells were seeded on 8 well chamber slides. After preincubation with each drug for 1 h, the cells were loaded with 4 μM of Fluo-4 AM (Invitrogen) in HBSS buffer at room temperature for 1 h. They were then washed with and incubated in assay buffer (125 mM KCl, 25 mM NaCl, 10 mM HEPES and 0.2 mM MgCl2) at room temperature for at least 10 min. Once mounted on the microscope, the reaction of cells to NAADP-AM (1 μM) or vehicle DMSO was monitored by capturing images every 10 sec.Fluo-4 fluorescence was then measured per cell using Cell Profiler image analysis software.

Patch-clamp electrophysiology

Whole-endolysosome recordings were performed by modified conventional patch-clamp as previously described (*33*-*35*). HEK293T cells stably expressing murine TPC2 or TPC1, both N-terminally fused to GFP were used. HEK293T cells were treated with 1 μM vacuolin-1 overnight. Currents were recorded using an EPC-10 patch-clamp amplifier and PatchMaster acquisition software (HEKA, Lambrecht/Pfalz, Germany). Data were digitized at 40 kHz and filtered at 2.8 kHz. Recording pipettes had a resistance of 10-20 MΩ. Liquid junction potential was corrected. Unless otherwise indicated in the figure legends, the pipette solution (corresponding to luminal endolysosomal solution) contained 140 mM NaMSA, 5 mM KMSA, 2 mM CaMSA, 1 mM CaCl2, 10 mM HEPES and 10 mM MES, pH 4.6. Bath solution (corresponding to cytosolic solution) contained 140 mM KMSA, 5 mM KOH, 4 mM NaCl, 0.39 mM CaCl2, 1 mM EGTA, and 10 mM HEPES, pH 7.2. PI(3,5)P2 was used in a water-soluble diC8 form (from A.G. Scientific). All compounds including PI(3,5)P2 were prepared as high-concentration stock solutions, added to the bath solutions to match the final concentration indicated, and applied via a perfusion pipette. pH of bath solution and pipette solution were adjusted with KOH and MSA, respectively. All recordings were obtained at 21-23℃ and were analyzed using PatchMaster and Origin 6.1 (OriginLab, Northampton, MA) software.

Generation and culture of monocyte-derived macrophages

Human blood from pooled donors was supplied by South Texas Blood Tissue Center (San Antonio, TX). No donor data was obtained. Peripheral blood mononuclear cells

(PBMCs) were first isolated by leucosep tube centrifugation at 1000 × g for 10 min. The PBMC fraction was collected and re-suspended in red blood cell lysis buffer. After 5 min incubation, intact cells were pelleted by centrifugation at 250 × g for 10 min and washed in PBS. The cell pellets were incubated in tissue culture plates and adherent cells were further incubated in IMDM media containing macrophage colony-stimulating factor (750 ng/ml), 2% human serum, antibiotics, non-essential amino acid and 50 μM 2-mercaptoethanol. After 2 days, the media was replaced with fresh media and the cells were incubated for 7 days, at which time cells had differentiated to macrophages.

Infection of mice with mouse-adapted EBOV

EBOV infection of mice was performed in a biosafety level 4 laboratory as described previously (28). All procedures were approved by institutional animal care and use committee. Female Balb/c mice (5-week-old) were obtained from Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. The mice were randomly separated into 2 or 3 groups. All of the mice were injected intraperitoneally with 1000 PFU of mouse-adapted EBOV. Animals were administered intraperioneally with 100 μl of tetrandrine or PBS alone either on the same day or after waiting 1 day and then every 2 days up to 7 days after virus challenge. Mice were observed daily for weight loss and clinical signs. All surviving animals were sacrificed at day 9 or day 10. Blood samples were collected from each animal at day 3 and from surviving animals on day 9, and sera were isolated and stored at -80°C. For quantification of virus loads in the blood, plaque assays were performed. Serum was diluted with PBS in 10-fold serial dilutions and inoculated to Vero-E6 cells seeded in 6 well plates. After 1 h incubation, the supernatant was replaced with DMEM containing methyl cellulose with 2% FBS and incubated for 10 days. Cells were fixed in 10% formalin for > 24 h and stained with crystal violet. Plaques were manually counted.

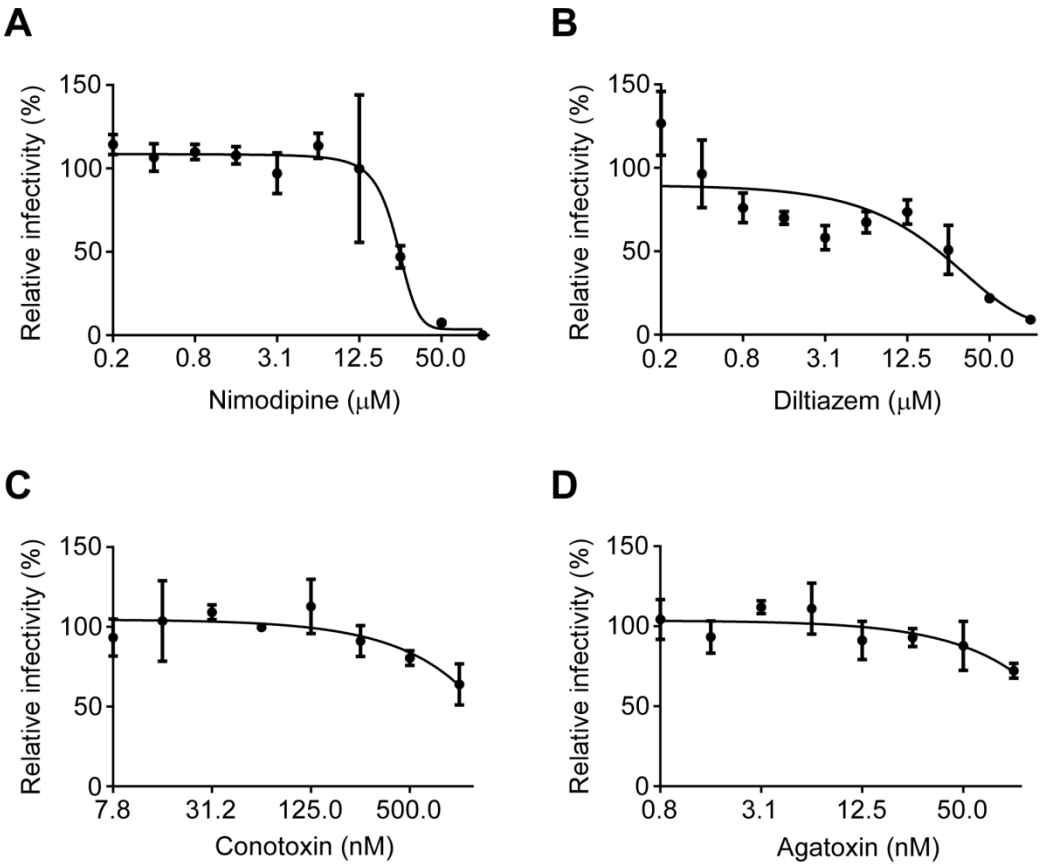


Fig. S1

The effects of calcium channel inhibitors on EBOV-GFP infection. (A to D) Effects of nimodipine (L-type channel inhibitor; A), diltiazem (L-type channel inhibitor; B), ω-conotoxin (N-type channel inhibitor; C) or ω-agatoxin (P/Q-type channel inhibitor; D) on infection efficiency were measured. HeLa cells were pretreated with the compounds and infected with EBOV-GFP for 24 h. The numbers of GFP positive cells were counted and normalized to those of infected samples without drugs. The dose range for each drug was based on reported IC50s for each channel. Each data set is mean ± SD (n = 3) and is representative of at least two independent experiments.

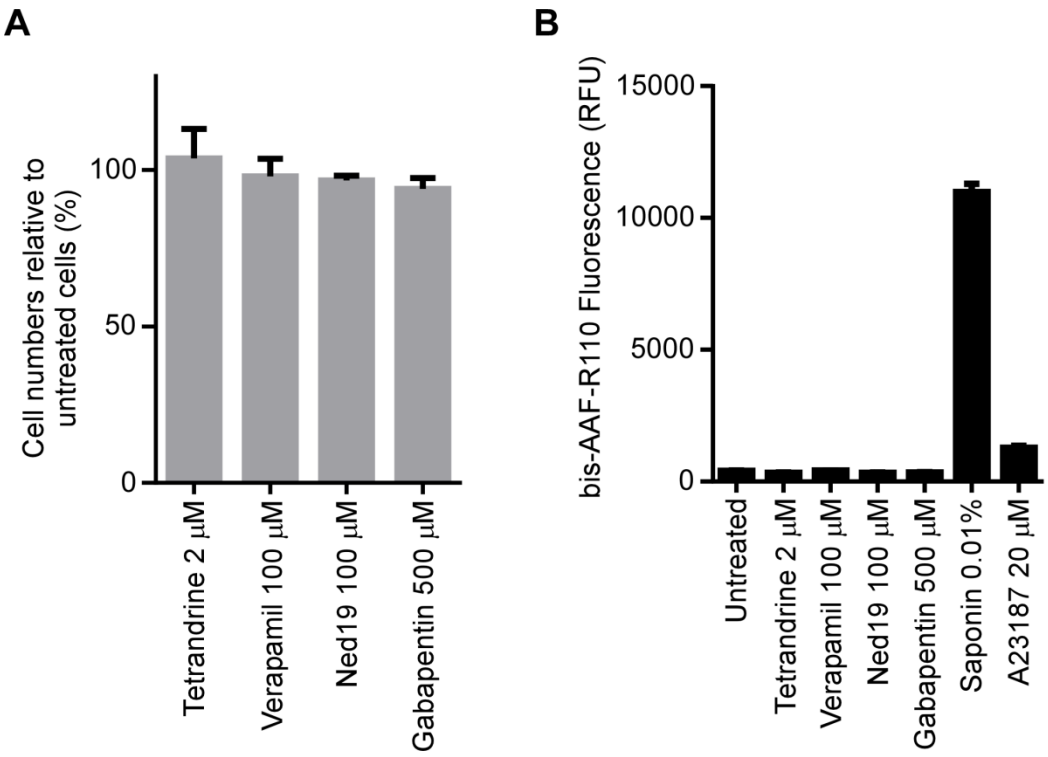


Fig. S2

Measurement of inhibitor cytotoxicity. Cytotoxicity of inhibitors was assessed by counting cell numbers or measuring dead-cell protease activity released from cells that have lost membrane integrity. Data were shown with highest concentrations of inhibitors used in Fig. 1. (A) HeLa cells were treated with indicated inhibitors and incubated for 24 h. The cells were fixed, stained by Hoechst33342 dye to visualize the nuclei and analyzed by microscope. The numbers of cells was normalized to that of untreated cells. (B) HeLa cells were treated with indicated inhibitors and a calcium ionophore A23187 as a positive control, and incubated for 24 h. After cells were mixed with a fluorogenic substrate and incubated for additional 2 h, the fluorescence was measured by a plate reader. As another positive control, cells were mixed with saponin before being mixed with the substrate. Each data set is mean ± SD (n = 3) and is representative of three independent experiments.

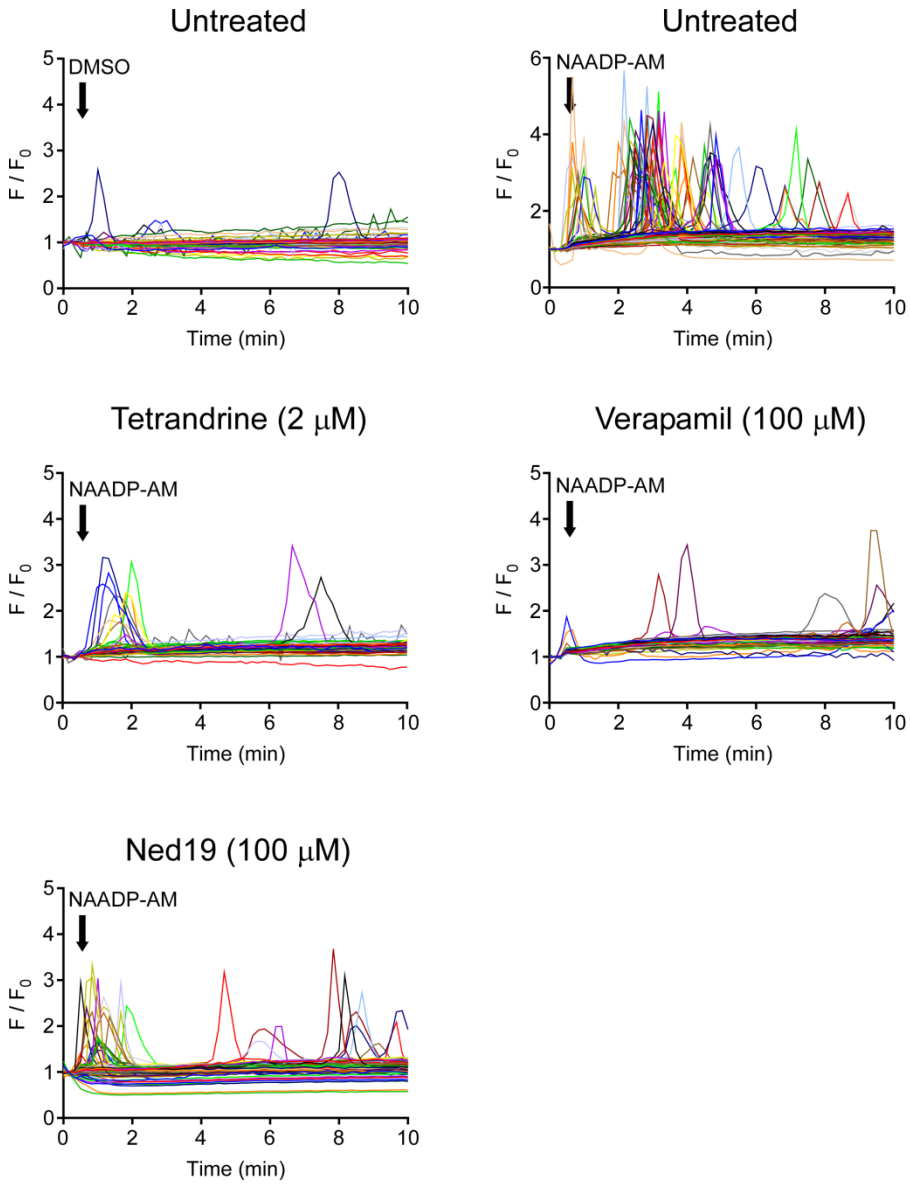


Fig. S3

Time-lapse analyses of NAADP-stimulated calcium influx. After pretreatment with indicated compounds, HeLa cells were loaded with Fluo-4 AM dye to visualize cytoplasmic calcium flux. The images of the cells were captured by microscope every 10 sec for 10 min. Thirty sec after starting the imaging, NAADP-AM (1 μM) or vehicle DMSO was added to the cells. For each cell, the mean fluorescence intensity at each time point (F) was measured and normalized to the average of the mean fluorescence intensities before stimulation (F_0) to give F/F_0.

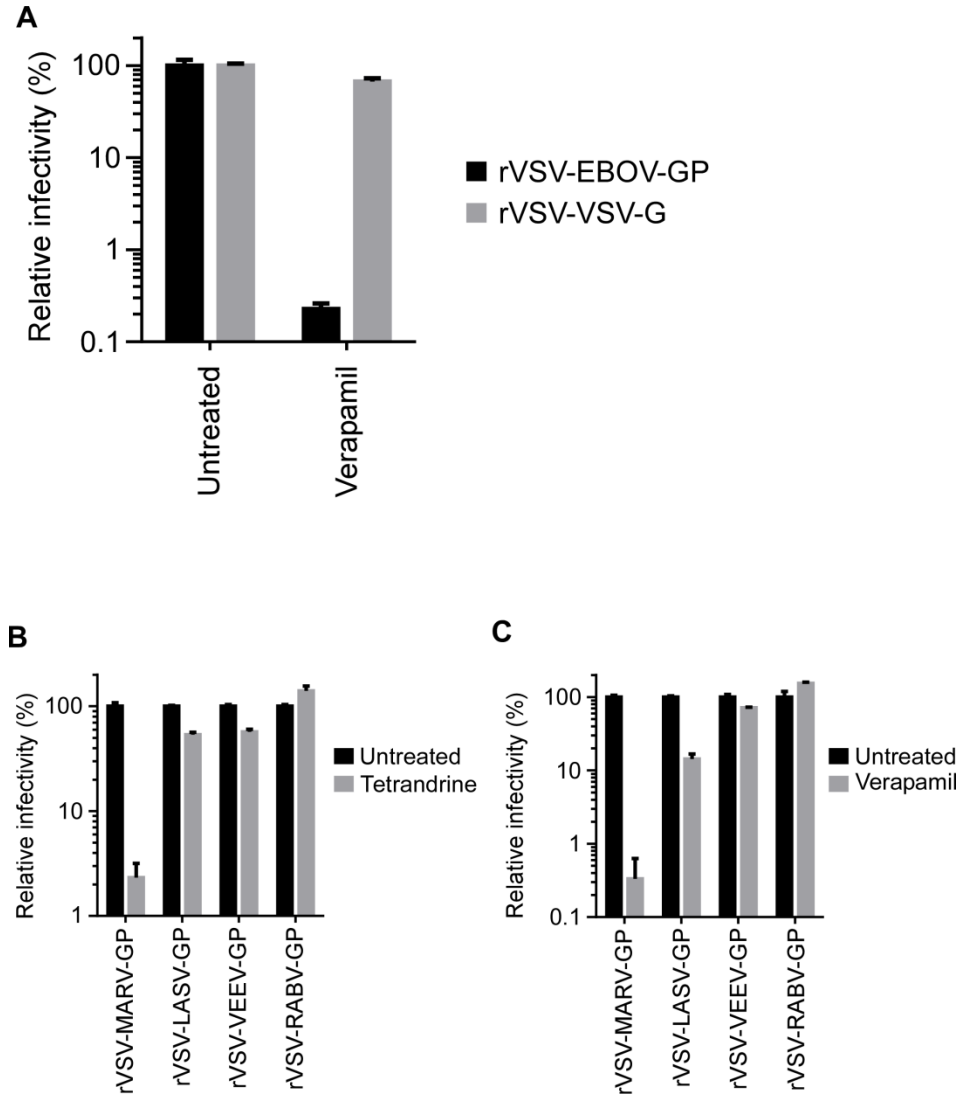


Fig. S4

Impact of verapamil and tetrandrine on infection of VSV-pseudotypes. (A) After pretreatment with verapamil (50 μM), the cells were challenged with rVSV-EBOV-GP or rVSV-VSV-G. (**B** and **C**) After pretreatment with tetrandrine (2 μM, B) or verapamil (50 μM, C), the cells were challenged with recombinant VSV bearing Marburg virus (rVSV-MARV-GP), Lassa virus (rVSV-LASV-GP), Venezuelan equine encephalitis virus (rVSV-VEEV-GP) or Rabies virus (rVSV-RABV-GP) glycoproteins. The luciferase activities normalized to those of untreated samples are shown. Each data set is mean ± SD (n = 3) and is representative of at least two independent experiments.

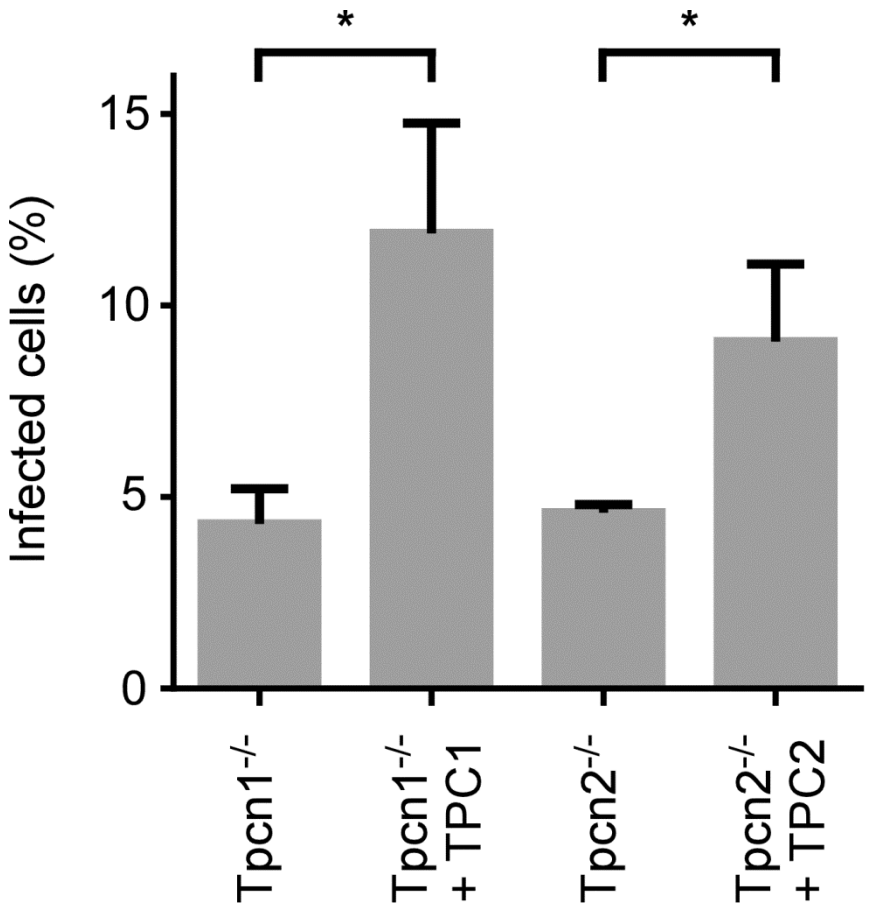


Fig. S5

Complementation of TPC knockout MEFs. Mouse embryonic fibroblasts (MEFs) from $Tpcn1^{-/-}$ or $Tpcn2^{-/-}$mice were transduced with an HIV-based vector encoding WT human TPC1 or TPC2, respectively, and challenged with WT EBOV. Infected cells were detected using anti-EBOV GP antibody. The proportion of cells with or without GFP fluorescence that were infected was calculated. Data are mean ± SD (n = 3) and representative of two independent experiments. * $P < 0.05$ by unpaired Student's t test.

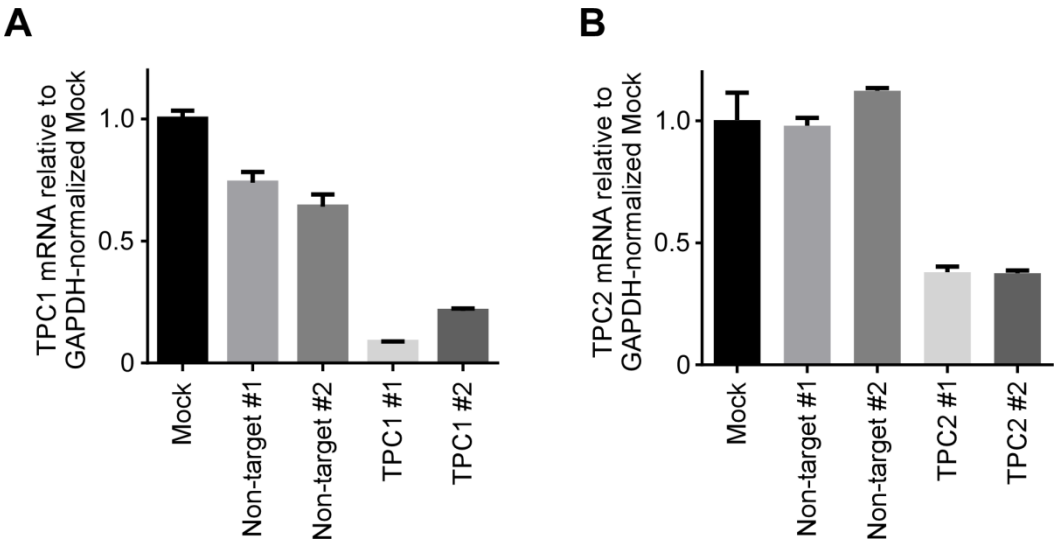
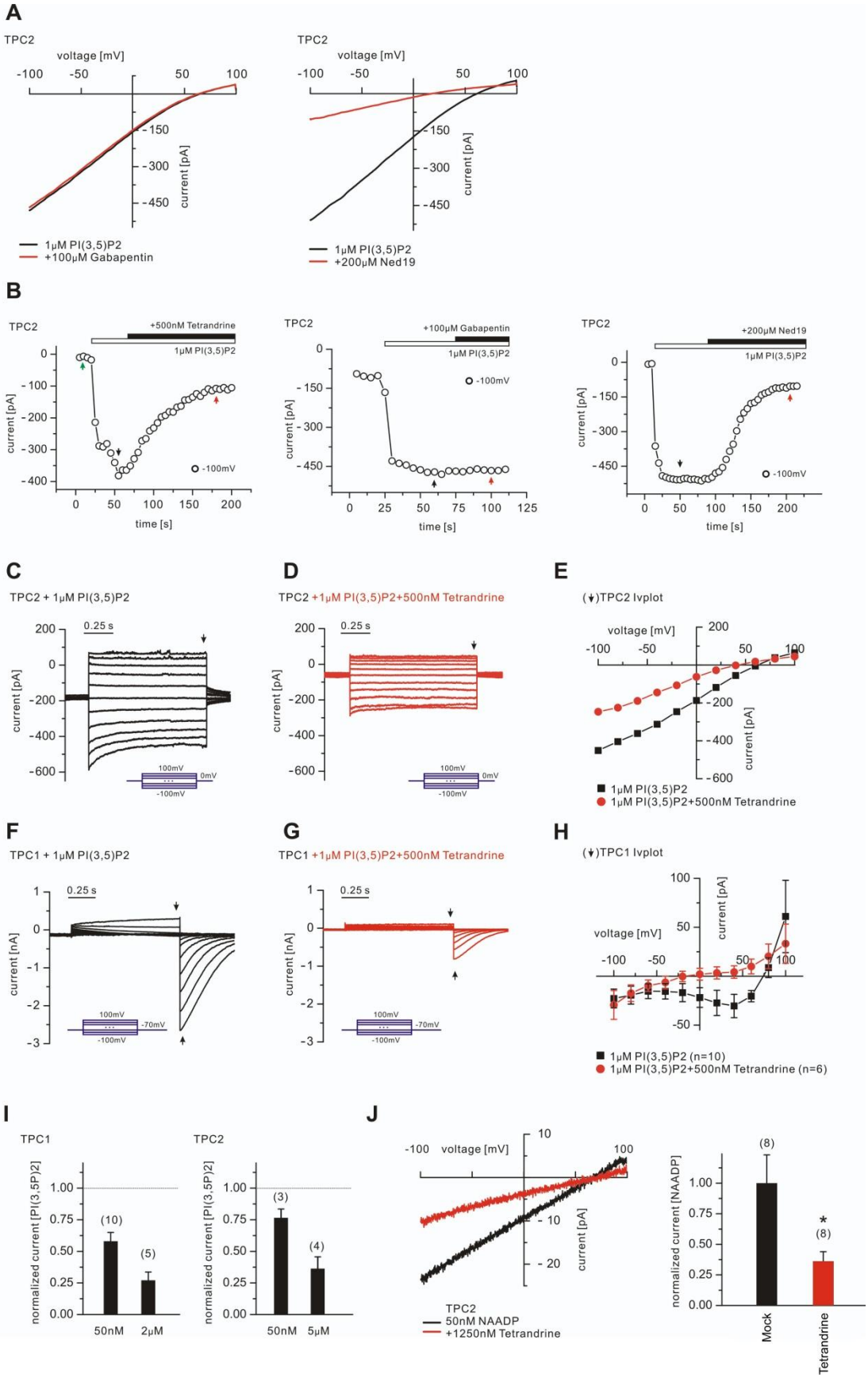


Fig. S6

Expression levels of TPCs in siRNA-transfected cells. The expression levels of TPC1 and TPC2 were analyzed by qRT-PCR using specific primers 24 h after siRNA transfection. GAPDH expression levels were analyzed as an internal control. Data are mean ± SD (n = 3) and are representative of at least two independent experiments.


A
TPC2
voltage [mV]
-100 -50 50 100
-150
-300
-450
current [pA]
1μM PI(3,5)P2
+100μM Gabapentin
TPC2
voltage [mV]
-100 -50 50 100
-150
-300
-450
current [pA]
1μM PI(3,5)P2
+200μM Ned19
B
TPC2
+500nM Tetrandrine
1μM PI(3,5)P2
current [pA]
0 -100 -200 -300 -400
-100mV
0 50 100 150 200
time [s]
TPC2
+100μM Gabapentin
1μM PI(3,5)P2
current [pA]
0 -150 -300 -450
-100mV
0 25 50 75 100 125
time [s]
TPC2
+200μM Ned19
1μM PI(3,5)P2
current [pA]
0 -150 -300 -450
-100mV
0 50 100 150 200
time [s]
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TPC2 + 1μM PI(3,5)P2
0.25 s
current [pA]
200 0 -200 -400 -600
100mV
0mV
-100mV
D
TPC2 +1μM PI(3,5)P2+500nM Tetrandrine
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current [pA]
200 0 -200 -400 -600
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voltage [mV]
-100 -50 50 100
200
-200
-400
-600
current [pA]
1μM PI(3,5)P2
1μM PI(3,5)P2+500nM Tetrandrine
F
TPC1 + 1μM PI(3,5)P2
0.25 s
current [nA]
1 0 -1 -2 -3
100mV
-70mV
-100mV
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TPC1 +1μM PI(3,5)P2+500nM Tetrandrine
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100mV
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100
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-50
current [pA]
voltage [mV]
-100 -50 50 100
1μM PI(3,5)P2 (n=10)
1μM PI(3,5)P2+500nM Tetrandrine (n=6)
I
TPC1
normalized current [PI(3,5P)2]
1.00 0.75 0.50 0.25 0.00
(10)
(5)
50nM 2μM
TPC2
normalized current [PI(3,5P)2]
1.00 0.75 0.50 0.25 0.00
(3)
(4)
50nM 5μM
J
-100 voltage [mV] 100
10
-10
-20
current [pA]
TPC2
50nM NAADP
+1250nM Tetrandrine
normalized current [NAADP]
1.00 0.75 0.50 0.25 0.00
(8)
*
(8)
Mock
Tetrandrine

Fig. S7

Whole endolysosomal currents from TPC-expressing HEK293T cells. (A) Currents were activated by voltage ramps from +100 mV to -100 mV applied from a holding potential of 0 mV (ramp duration: 500 ms, inter ramp duration: 5 s). From these recordings current-voltage relations were determined. Experiments in HEK293 cells expressing TPC2 demonstrate that TPC2 is inhibited by Ned-19 (200 μM), but not by gabapentin (100 μM). (B) Current amplitudes measured at -100 mV were used to plot the time course of activation and inhibition. Application of PI(3,5)P2 and inhibitors is indicated by open and closed bars, respectively. Representative I-V curves (Fig. 2E and fig. S7A) at different time points, as indicated by arrows. (C-H) Family of current traces activated in lysosomes expressing TPC2 (C-E) and TPC1 (F-H) by voltage steps ranging from +100 mV to -100 mV as illustrated. These experiments demonstrate that TPC2 and TPC1 were sensitive to low concentrations of tetrandrine. The amplitudes of the step currents (indicated by ↓ in C, D, F and G) were used to reconstruct the current-voltage relations (E and H). (I) Left: Bar diagram summarizing data of TPC1 amplitudes from inward tail currents (indicated by ↑ in F and G and normalized to those before different concentration of tetrandrine application). Right: Bar diagram summarizing data of TPC2 current amplitudes determined at -100 mV in the presence of channel blockers applied from the cytoplasmic side normalized to those before different concentration of tetrandrine application. (J) (Left) Whole endolysosomal currents were recorded from TPC2-expressing HEK293T cells using the planar patch-clamp technique with NAADP. Solutions and voltage-ramp protocols are as previously described (*25*). (Right) Bar diagram summarizing data of NAADP-evoked TPC2 current amplitudes determined at -100 mV. Data of bar diagram are given as mean ± SEM. $P<0.05$ by Student's t test.

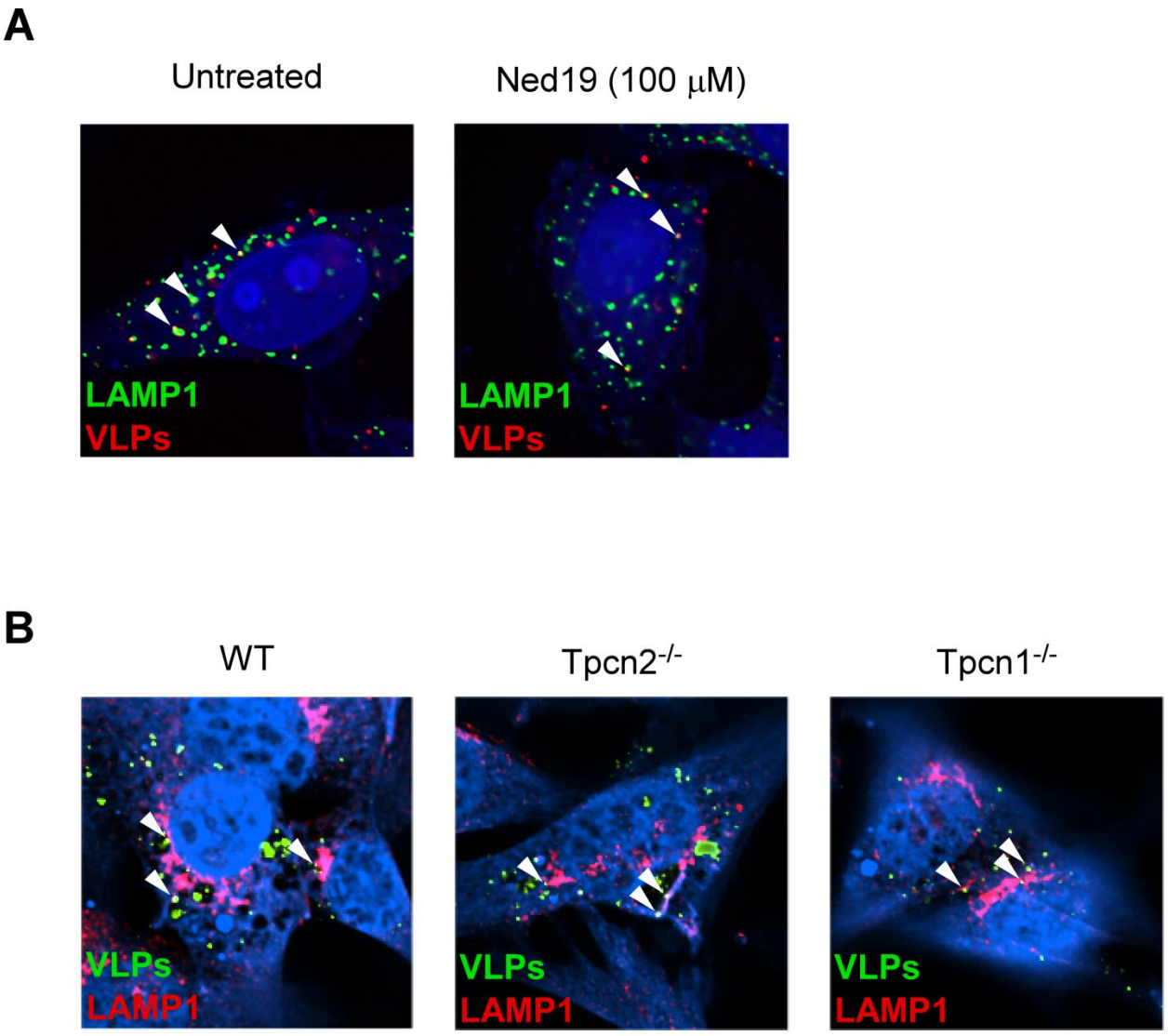

Fig. S8

Colocalization of VLPs with LAMP1. (A) Untreated or Ned19-treated cells were incubated with Ebola VLPs tagged with mKate2 (red) for 4 h. LAMP1 (green) was stained with a specific antibody and images captured. (B) WT, Tpcn1$^{-/-}$ or Tpcn2$^{-/-}$ MEFs were incubated with Ebola VLPs tagged with GFP (green) for 4 h. LAMP1 (red) was stained with a specific antibody and images captured. Examples of colocalized particles are indicated by arrowheads.

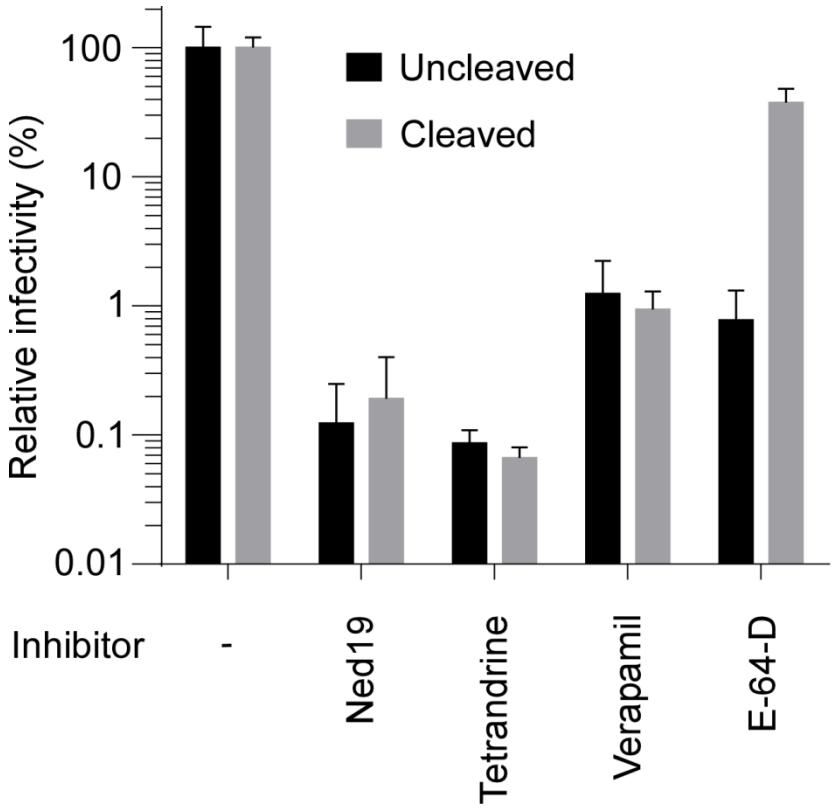

Fig. S9

Impact of inhibitors on infection of *in vitro*-cleaved rVSV-EBOV-GP. To determine if calcium channel inhibitors prevent cleavage of GP by endosomal proteases, HeLa cells were pretreated with the indicated compounds (100 μM of Ned19, 2 μM of tetrandrine, 100 μM of verapamil or 50 nM of E-64-D) and incubated with pre-cleaved or uncleaved rVSV-EBOV-GP. Luciferase activities were normalized to those of untreated controls. Data are mean ± SD (n = 3) and are representative of three independent experiments.

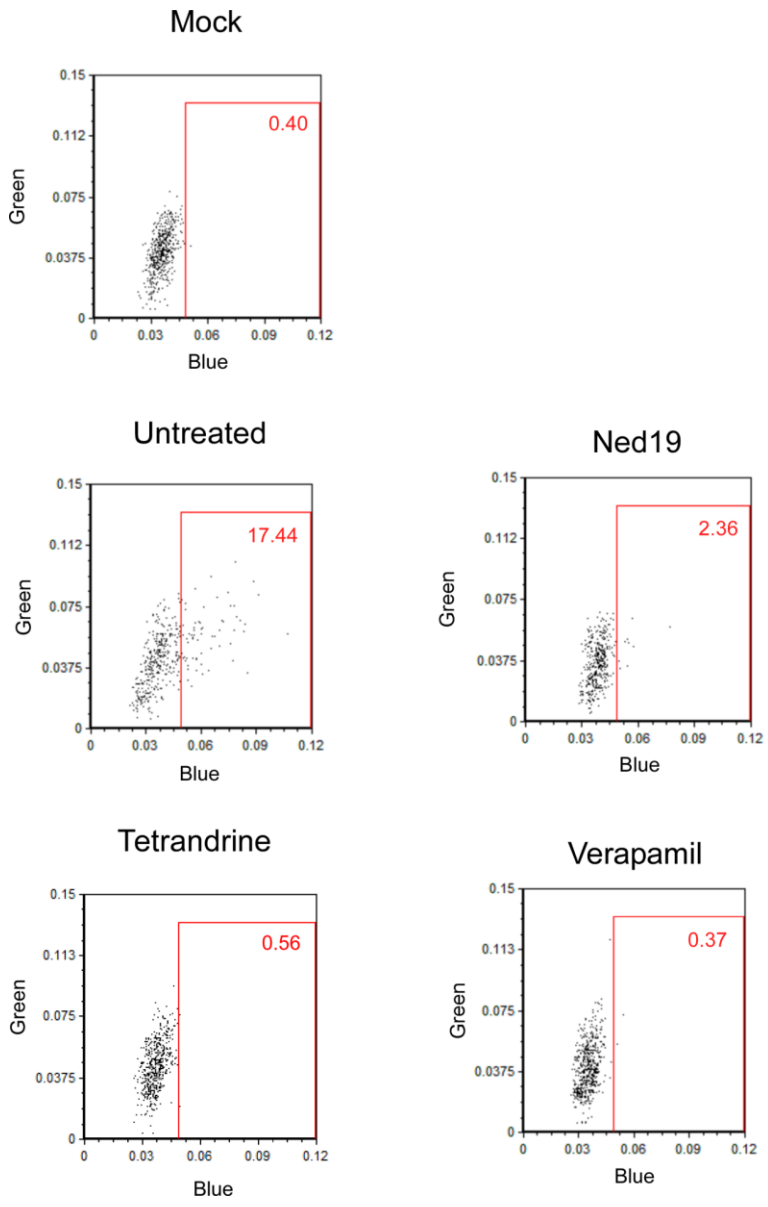

Fig. S10

Representative image cytometry plots from virus contents release assays. Virus capsid release into the cell cytoplasm was measured in the presence of each indicated compound (100 μM of Ned19, 2 μM of tetrandrine or 100 μM of verapamil). VLPs were generated using VP40 fused to β-lactamase. The β-lactamase enzyme cleaves the green fluorescent substrate, CCF2, which was loaded into the cell cytoplasm, to give a blue fluorescent product. After imaging of infected cells in both blue (excitation 400 nm) and green (excitation 488 nm) fluorescence channels, mean fluorescence intensity for each cell was measured and plotted. Samples not incubated with VLPs (mock) were used to gate background fluorescence signals and to set thresholds for positive signals. The numbers shown represent the percentages of each population that had signal above the background threshold. The plots were generated using FCS Express 4 Image Cytometry software.

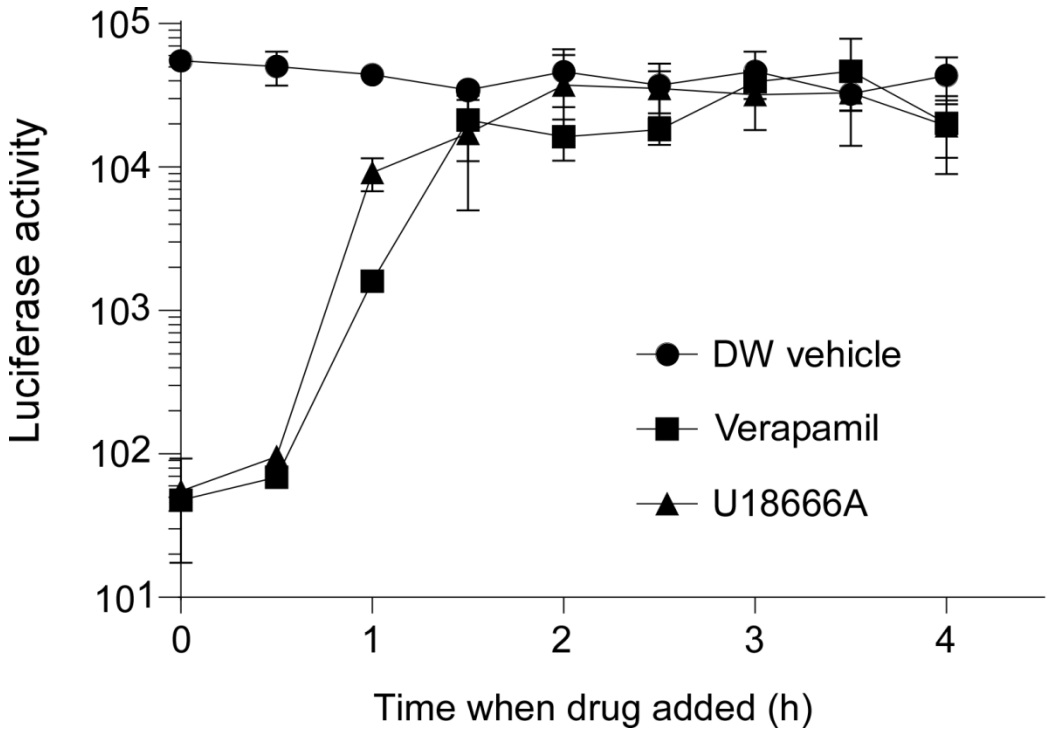

Fig. S11

Time of drug addition analyses. The time at which drug treatment became ineffective to block infection was determined. After rVSV-EBOV-GP infection for 30 min, verapamil (100 μM), U18666A (20 μM) or medium alone was added at indicated time points. Eight hours after infection, luciferase activity was measured. Data are mean ± SD (n = 3) and are representative of three independent experiments.

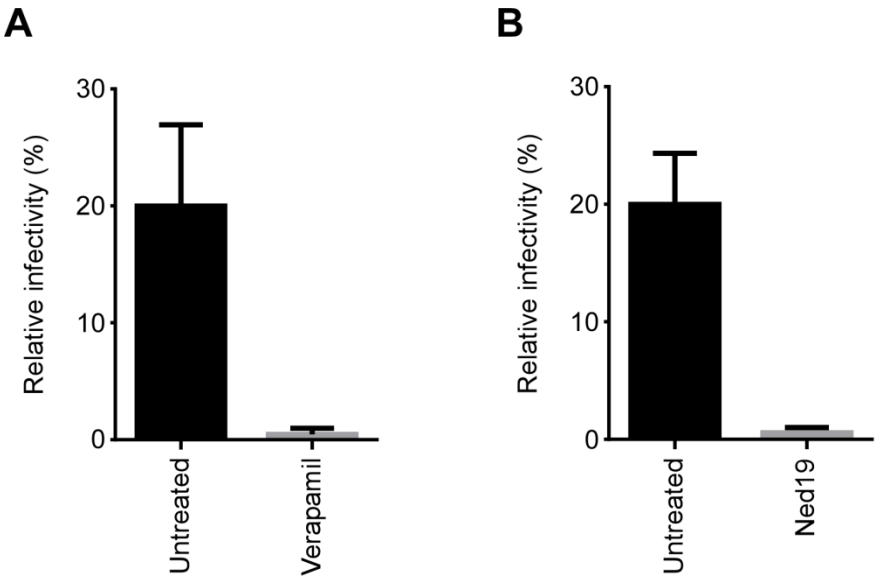

Fig. S12

The effects of inhibitors on EBOV infection into primary human macrophages. Primary human macrophages were pretreated with verapamil (200 μM) or Ned19 (200 μM), infected with EBOV-GFP for 48 h and imaged. The numbers of GFP positive cells normalized to those of untreated samples are shown. Each data set is mean ± SD (n = 3) and is representative of two independent experiments.

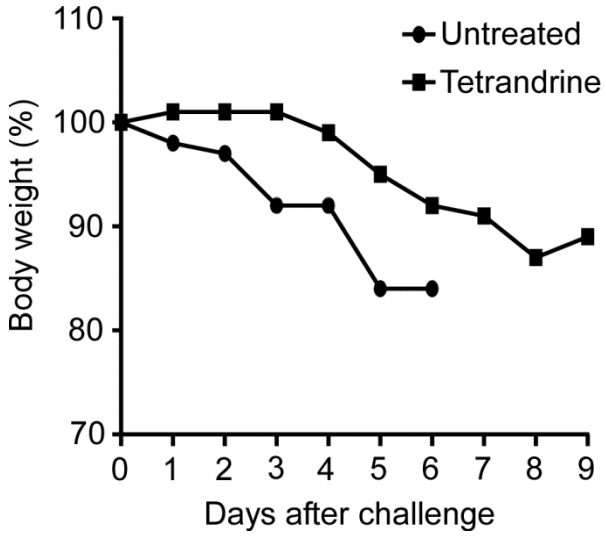

Fig. S13

Body weight of EBOV-infected mice. Balb/c mice challenged with mouse-adapted EBOV were monitored for 9 days. The average body weight of each group was measured and normalized to that of day 0.