Supplementary Materials for

Drebrin restricts rotavirus entry by inhibiting dynamin-mediated endocytosis

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Materials and Methods

Plasmids. Human drebrin 1 (DBN1) transcript variant 1 (DBN-E, #RC200372) and transcript variant 2 (DBN-A, #RC222368), TMOD3 human cDNA ORF Clone (NM_014547, #RC204417) were purchased from Origene. The GFP-tagged DBN1 mutants were kindly provided by Dr.Tomoaki Shirao (Gunma University) and Dr. Stefan Linder (University of Hamburg).

Plasmids and siRNA Transfection. Plasmids transfection on WT and *DBN1*$^{-/-}$ HEK293 cells was performed using Lipofectamine 3000 reagent (Thermo). siRNA transfection was performed using RNAiMAX (Thermo) via reverse transfection according to the manufacturer's instructions. Briefly, 1.2 µl of 5 µM siRNA was mixed with 1 µl RNAiMAX in 100 µl OptiMEM and incubated in 24-well plate at RT for 15 min. 1×10^5 cells in 500 µl Ab-free DMEM were then added to each well. Viral infection was performed at least 48 hr post transfection. All siRNAs used in this study were SMARTpool ON-TARGET siRNA purchased from Dharmacon and their knockdown efficiency were validated by RT-qPCR analysis of respective genes showing a reduction of more than 80% on mRNA levels.

SDS-PAGE and Western Blot. Protein lysates were harvested in RIPA buffer, mixed with 2×Laemmli Buffer, boiled at 95 °C for 5 min, and separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane. The membrane was blocked with TBST with 5% BSA, and incubated with primary antibodies against DBN1 (ABN207, EMD Millipore); TMOD3 (HPA001849, Sigma); ADD1 (45891S, CST); Flag (clone M2, Sigma); GAPDH (clone poly6314, Biolegend); GFP (#2555, CST); CTTN (#3502S, CST); Secondary incubation was performed with anti-rabbit (#7074), or anti-mouse (#7076) IgG HRP-linked antibodies. Proteins were visualized using Clarity ECL substrate (#170-5061, Biorad), Amersham Hyperfilm (GE Healthcare) and STRUCTURIX X-ray film processor (GE Healthcare). The intensity of bands in WB was quantified by densitometry using ImageJ.

Immunofluorescence. RRV-infected cells were washed with PBS and fixated in 4% PFA at RT for 10 min. Cells were then washed with PBS and incubated with mouse monoclonal antibodies (MAb) against the RV structural proteins VP4 (2G4) and VP7 (159), MAb against dsRNA (J2 clone, Kerafest), MAb against ZO-1 (33-9100, Thermo) or rabbit polyclonal antibody against

DBN1 (ABN207, EMD Millipore) in immunofluorescence assay (IFA) buffer (0.1% saponin, 2% BSA in 1×PBS) at RT for 1 hr. After washing with IFA buffer three times, secondary incubation was performed with chicken anti-mouse Alexa-488 (A21200, Thermo) chicken anti-rabbit Alexa-594 (A21442, Thermo) at RT for 30 min protected from light. Cells were again washed with IFA buffer three times and PBS three times before staining with phalloidin Alexa-555 (#8953, CST) or phallodin Alexa-647 (A22287, Thermo) at RT for 20 min. Stained cells were washed with PBS, mounted with Antifade Mountant with DAPI (P36962, Thermo), and imaged with Zeiss LSM 710 Confocal Microscope. Each experiment was performed at least 5 times, and an average of 5 fields was acquired for each experiment under each condition.

Proximity Ligation Assay (PLA)

PLA was performed according to the manufacturer's instruction (Sigma-Aldrich). In brief, HEK293 cells were infected with RRV at MOI=50 for 30min or MOI=1 for 24 hr. Cells were then fixed with 3% PFA and stained with mouse IgG1 control antibody, mouse anti-VP4 (HS2), mouse anti-VP6 (1E11), mouse anti-VP7 (60) and rabbit anti-DBN1 (ABN207, EMD Millipore) antibodies and incubated with anti-mouse minus and anti-rabbit plus PLA reagents followed by ligation and red amplification reagents. Images were taken with Zeiss LSM 710 Confocal Microscope and analyzed by Duolink ImageTool (Sigma) based on at least 20 micrographs (at least 100 individual cells).

Transferrin uptake. WT and *DBN1*$^{-/-}$ HEK293 cells were incubated on ice for 10 min, washed with cold Live Cell Imaging Solution (LCIS, Thermo), incubated with Alexa-488 conjugated transferrin (#T13342, Thermo) at 20 µg/mL in LCIS containing 20 mM Glucose and 1% BSA at 37°C for 3-30 minutes. Stained cells were washed with LCIS, mounted with Antifade Mountant with DAPI (P36962, Thermo), and imaged with Zeiss LSM 710 Confocal Microscope. At least 20 micrographs were used for quantification with Image J. Fluorescence was measured with BD LSR II flow cytometer and data was analyzed with FlowJo Software v8.8.7 (TreeStar).

Cholera toxin uptake. WT and *DBN1*$^{-/-}$ HEK293 cells were washed with PBS and incubated with 10 µg/ml Alexa-488 conjugated CTxB (#C22841, Thermo) at 37°C for 3-60 minutes. Stained cells were washed with LCIS and analyzed using same methods as described above for transferrin.

Dextran uptake. WT and *DBN1*$^{-/-}$ HEK293 cells were washed with LCIS and incubated with 20 μg/ml pHrodo® Green Dextran, 10,000 MW (#P35368, Thermo) at 37°C for 5-45 minutes. Stained cells were washed with LCIS and analyzed using same methods as described above for transferrin.

***In Vitro* Transcription and Translation.** Full-length VP4, N-terminal VP8* and C-terminal VP5* were PCR-amplified and cloned into the pCMV6-XL6 vector (Promega). 1 μg of plasmid was used in a 50 μl reaction at 30 °C for 90 min using TnT® SP6 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. 5 μl of each reaction was used in the IP experiment.

Immunoprecipitation. Cells were lysed in 1×lysis buffer (#9803, CST) supplemented with 1 mM PMSF. Lysates were first incubated with Pierce Protein A/G Magnetic beads (#88802, Thermo) at 4 °C for 1 hr. Pre-cleared lysates were collected for IP input control and the rest were then incubated with antibody against GFP (ab290, Abcam) or antibody against GST (#2625, CST) at 4 °C overnight. Antibody-lysate complexes were further incubated with magnetic beads at RT for 30 min. The complex was washed with 1×lysis buffer for five times before added to elution buffer, which was prepared using 3×Blue Loading Buffer mixed with 30×DTT at 10:1 ratio (#7722, CST). Samples were boiled at 95 °C for 5 min and supernatants were collected after centrifugation at 14,000 rpm at 4 °C for 1 min. For western blot, anti-rabbit Conformation Specific antibody (clone L27A9, CST) was used instead of traditional secondary antibodies.

Inhibitor treatment. Cells were pretreated with each of the following drugs at the indicated concentrations at 37°C for 30 min prior to RV or VSV infection: 5 μM BTP2 (sc-221441, Santa Cruz), a small-molecule inhibitor of drebrin; 100 μM dynasore (D7693, Sigma), inhibitor of dynamin; 5 μM Dyngo-4a (S7163, Selleck Chemicals), inhibitor of dynamin-2. 1 μM Latrunculin A (ab144290, Abcam), 0.5 μM cytochalasin D (C2618, Sigma) and 1 μM BTP-2 was used to treat HEK293 cells for 24 hr prior to RV infection. Actinomycin D (A1410, Sigma) was used as a positive control for cytotoxicity assay at the concentration of 10 μg/ml for 16 hr.

Cytotoxicity Measurement

Supernatants from siRNA-transfected HEK293 cells were collected and assayed in triplicate using a Pierce™ lactate dehydrogenase (LDH) cytotoxicity assay kit (Thermo) according to the manufacturer's instruction. The assay was measured by 490nm absorbance with a microplate spectrophotometer (Bio-rad). Percent LDH release was calculated after background subtraction with blank wells.

Fig. S1

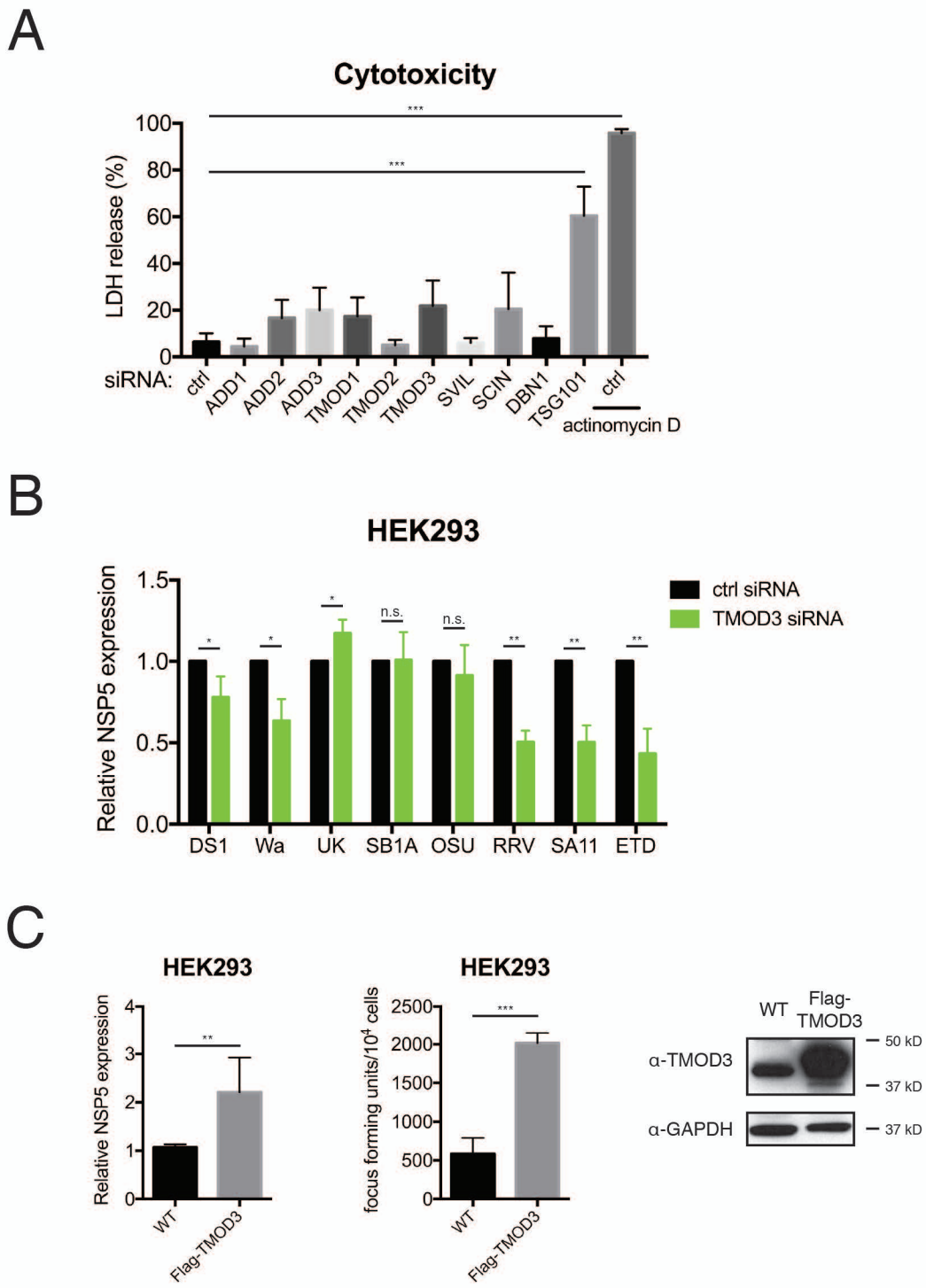


Fig. S1. TMOD3 enhances specific RV infection. (A) HEK293 cells were transfected with indicated siRNA for 96 hr with or without Actinomycin D treatment (10 μg/ml) as a positive control. Cell supernatants were collected and assayed by lactate dehydrogenase (LDH) release as an indicator of cell death. (B) HEK293 cells were transfected with control (ctrl) or TMOD3 siRNA for 48 hr and infected with human and animal RV strains (DS1, Wa: human; UK: bovine; SB1A, OSU: porcine; RRV, SA11: simian; ETD: murine) at MOI=1 for 24 hr. The mRNA level of viral NSP5 was measured by RT-qPCR and normalized to that of GAPDH. (C) WT HEK293 cells and HEK293 cells stably expressing pCMV6-Entry vector encoding Flag-tagged TMOD3 were infected with RRV at MOI=1 for 24 hr. Viral NSP5 level was measured by RT-qPCR and normalized to that of GAPDH to reflect input viral genomes (left panel). Virus titer in the cell supernatants was determined by an FFU assay (middle panel). Cell lysates were harvested for western blot probing for TMOD3 and GAPDH expression (right panel). For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S2

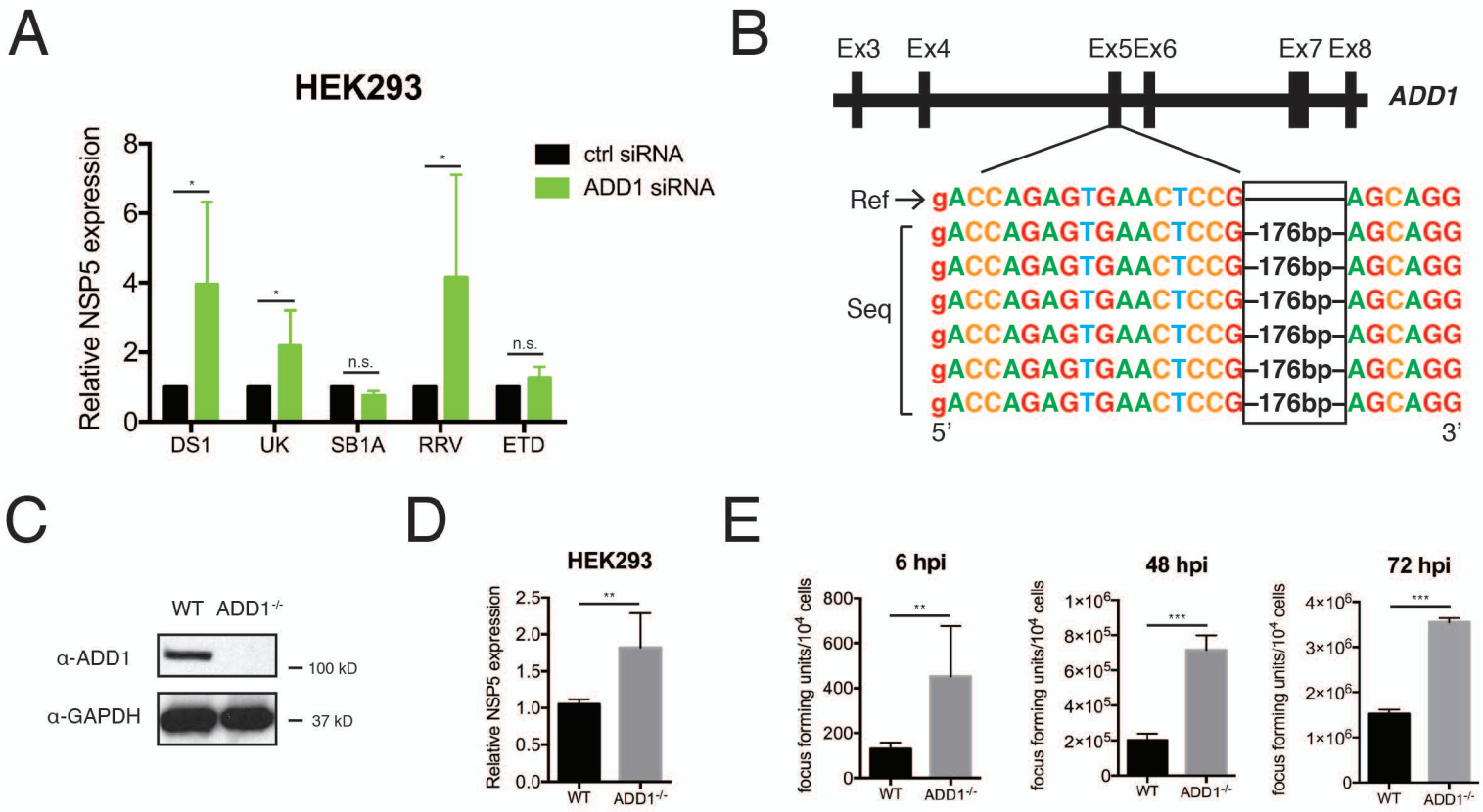


Fig. S2. ADD1 inhibits specific RV infection. (A) HEK293 cells were transfected with control (ctrl) or ADD1 siRNA for 48 hr and infected with human and animal RV strains (DS1: human; UK: bovine; SB1A: porcine; RRV: simian; ETD: murine) at MOI=1 for 24 hr. Viral NSP5 level was measured by RT-qPCR and normalized to that of GAPDH. (B) Genotyping of CRISPR-induced ADD1 knockout (KO) HEK293 cells by Sanger sequencing showing the mutated locus (insertion of a long stretch of sequences) in multiple alleles and the wild-type reference. (C) WT and ADD1 KO HEK293 cell lysates were harvested for western blot probing for ADD1 and GAPDH expression. (D and E) WT and ADD1 KO HEK293 cells were infected with RRV at MOI=1. Viral NSP5 expression at 24 hpi was measured by RT-qPCR (D) and virus titer in the cell supernatants at indicated time points was determined by an FFU assay (E). For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S3

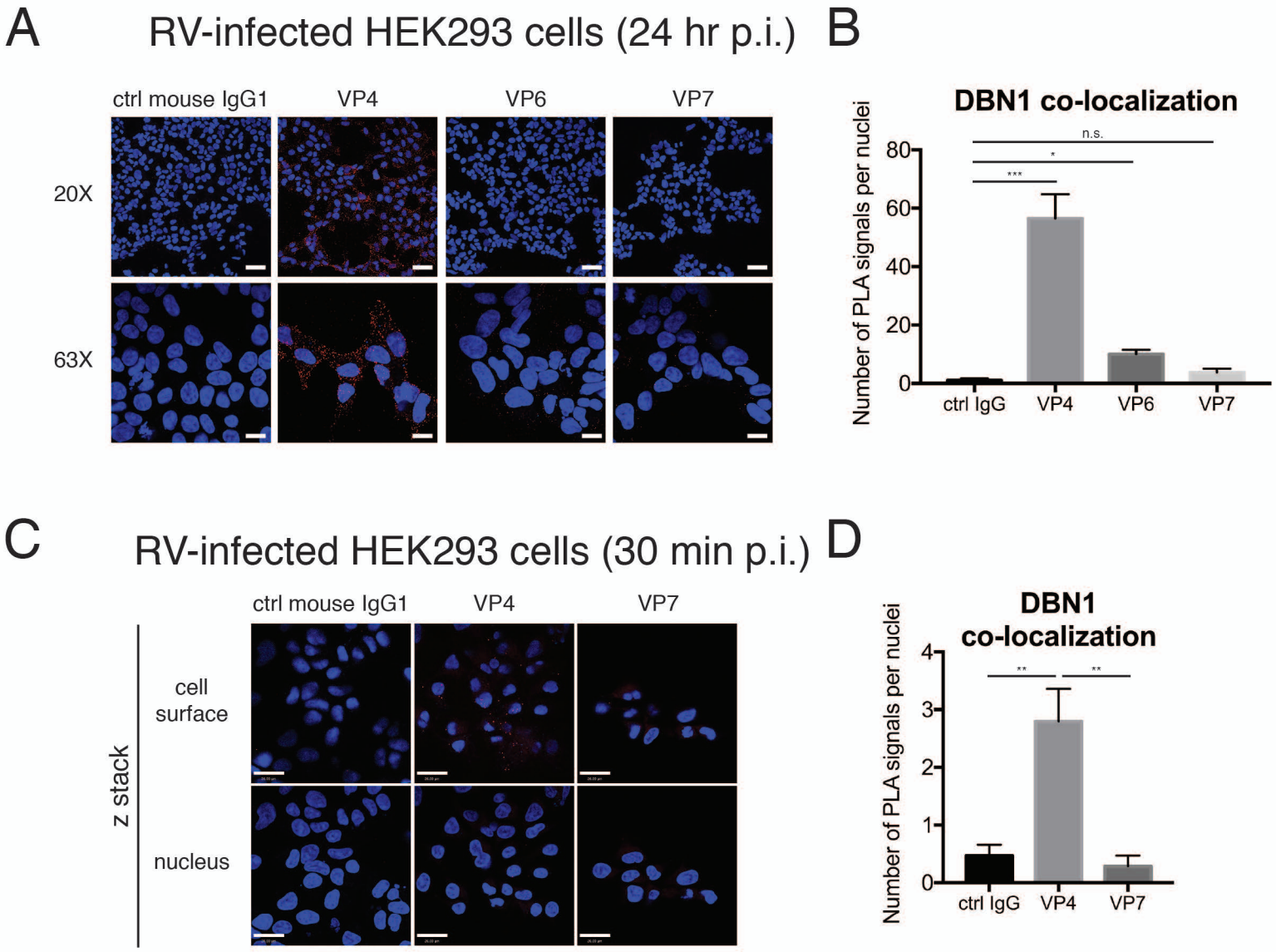


Fig. S3. DBN1 co-localizes with VP4 during RV infection. (A) WT HEK293 cells infected with simian RV RRV strain at MOI=1 for 24 hr were examined in a Duolink proximity ligation assay (PLA). Infected cells were examined at low magnification (20X, top panel) and high magnification (63X, bottom panel) for co-localization of indicated viral antigens and DBN1. Each red spot represents for a single point of interaction and nucleus was stained with DAPI. Panels are single z slices with a scale bar of 20 µm. Experiments were repeated twice. (B) Quantification of (A) was performed using the Duolink ImageTool software. The analysis was based on at least 20 micrographs (at least 100 cells). (C) Same experiment as (A) except that RRV infection was performed at MOI=50 and PLA assay was conducted at 30 min post infection. (D) Quantification of (C) was performed using the Duolink ImageTool software. The analysis was based on at least 15 micrographs (at least 80 cells). Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S4

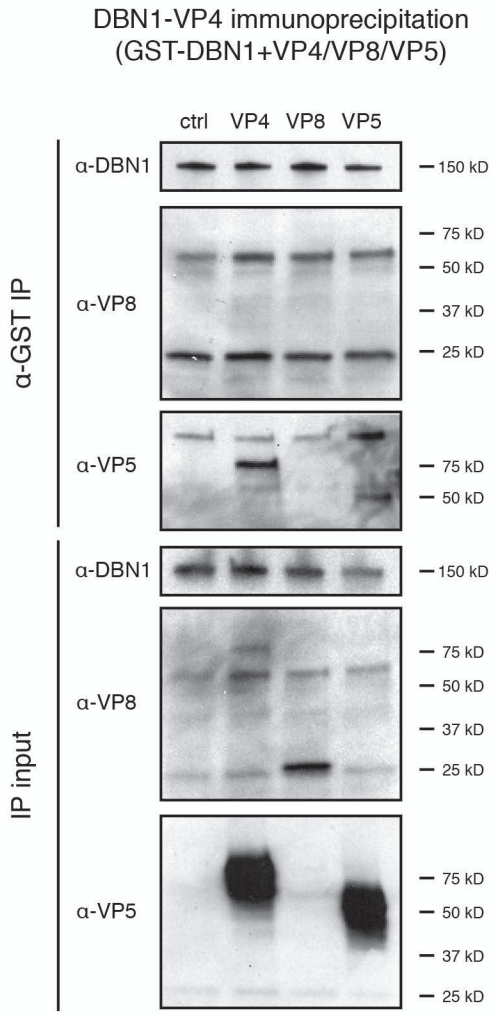


Fig. S4. DBN1 co-precipitates with the C-terminal VP5* region of VP4. Full-length VP4, N-terminal VP8*, and C-terminal VP5* proteins were generated using rabbit-reticulocyte lysate based in vitro transcription and translation reaction. The lysates were incubated with GST-tagged human DBN1 recombinant protein (5 μg/lane) and subjected to IP using α-GST antibody. Lysates input and IP products were analyzed by Western blot using indicated antibodies.

Fig. S5

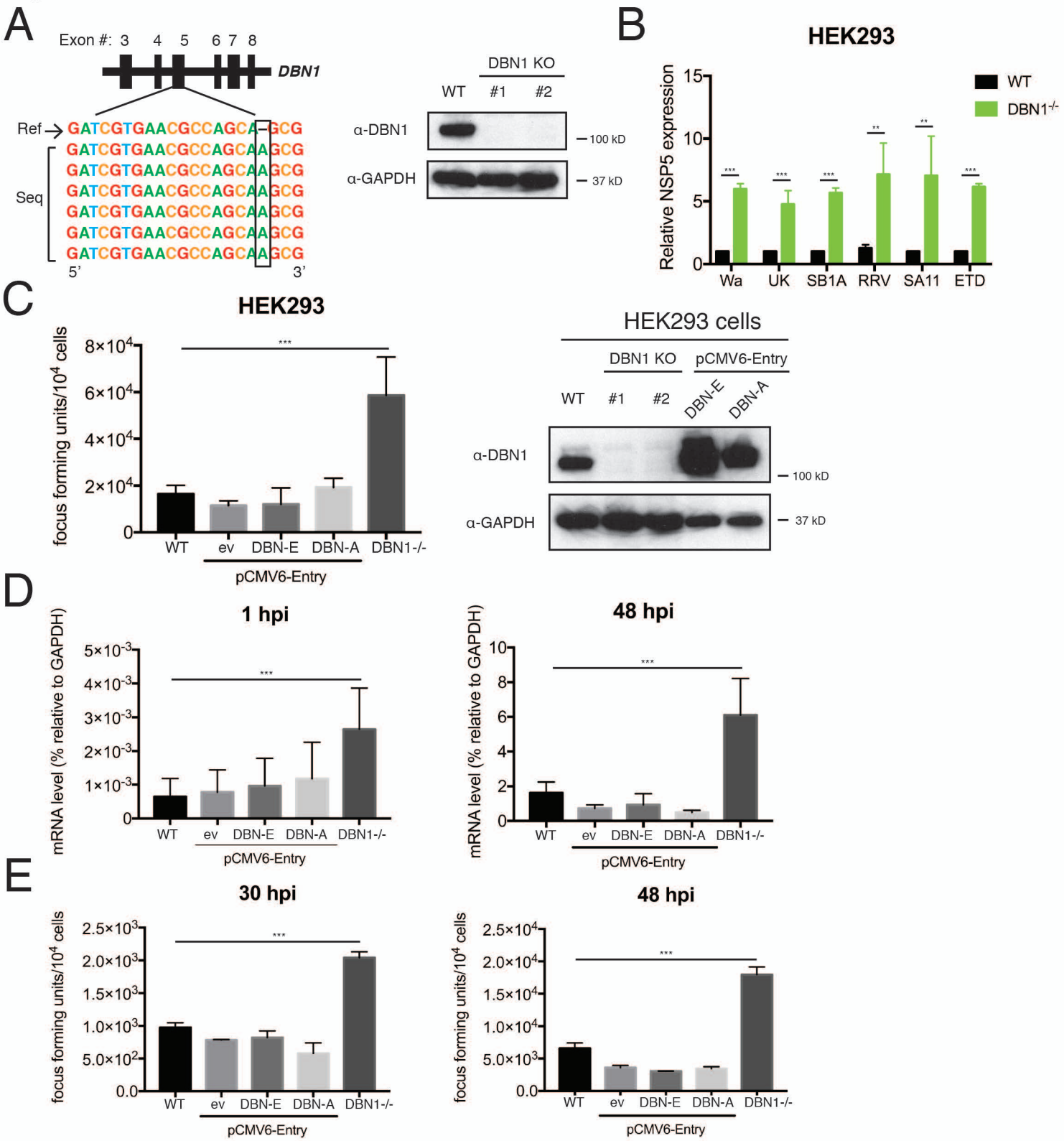
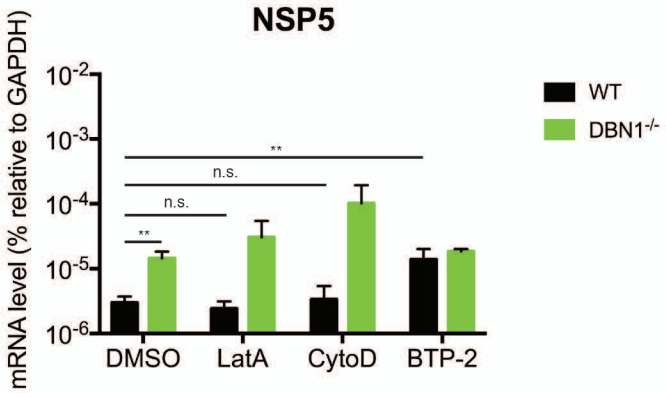


Fig. S5. DBN1 broadly restricts RV infection. (A) Genotyping of CRISPR-induced DBN1 knockout (KO) HEK293 cells by Sanger sequencing showing the mutated locus (insertion mutation) in multiple alleles and the wild-type reference (left panel). DBN1 depletion was further confirmed in two individual KO clones by western blot (right panel). (B) WT and DBN1 KO HEK293 cells were infected with human and animal RV strains (Wa: human; UK: bovine; SB1A: porcine; RRV, SA11: simian; ETD: murine) at MOI=1 for 24 hr. Viral NSP5 level was measured by RT-qPCR and normalized to that of GAPDH to reflect input viral genomes. (C) WT, DBN1 KO HEK293 cells or HEK293 cells overexpressing Flag-tagged full-length DBN1 (DBN-A and DBN-E) were infected with RRV (MOI=1) for 24 hr and supernatants were harvested and examined by an FFU assay (left panel) (ev: empty vector); cells were also harvested for western blot analysis probing for the protein levels of DBN1 and GAPDH (right panel). (D and E) WT, DBN1 KO, DBN1 over-expression HEK293 cells were infected with RRV (MOI=1) for indicated time points and RNA was harvested to measure viral NSP5 level by RT-qPCR (D) or supernatants were collected for virus titration by an FFU assay (E). For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S6

A



B

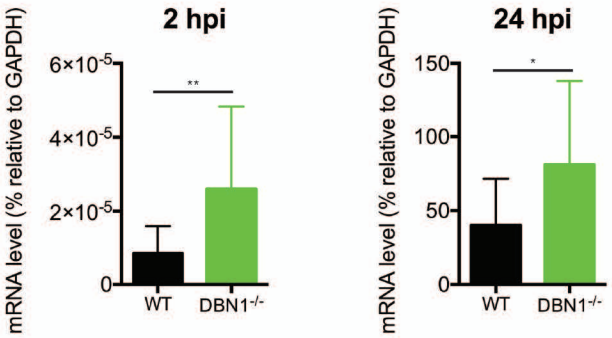


Fig. S6. DBN1 inhibits virus entry independent of actin stabilization. (A) WT and DBN1 KO HEK293 cells were pre-treated with indicated chemical inhibitors (Latrunculin A, LatA, 1 μM; Cytochalasin D, CytoD, 0.5 μM; BTP-2, 1 μM) for 24 hr and infected with RRV for 1 hr. The level of NSP5 was measured by RT-qPCR and normalized to that of GAPDH as an indicator of input viral genomes. (B) WT and DBN1 KO HEK293 cells were infected with VSV at MOI=1 for 2 or 24 hr. The mRNA level of VSV gene N was measured by RT-qPCR and normalized to that of GAPDH. For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

Fig. S7

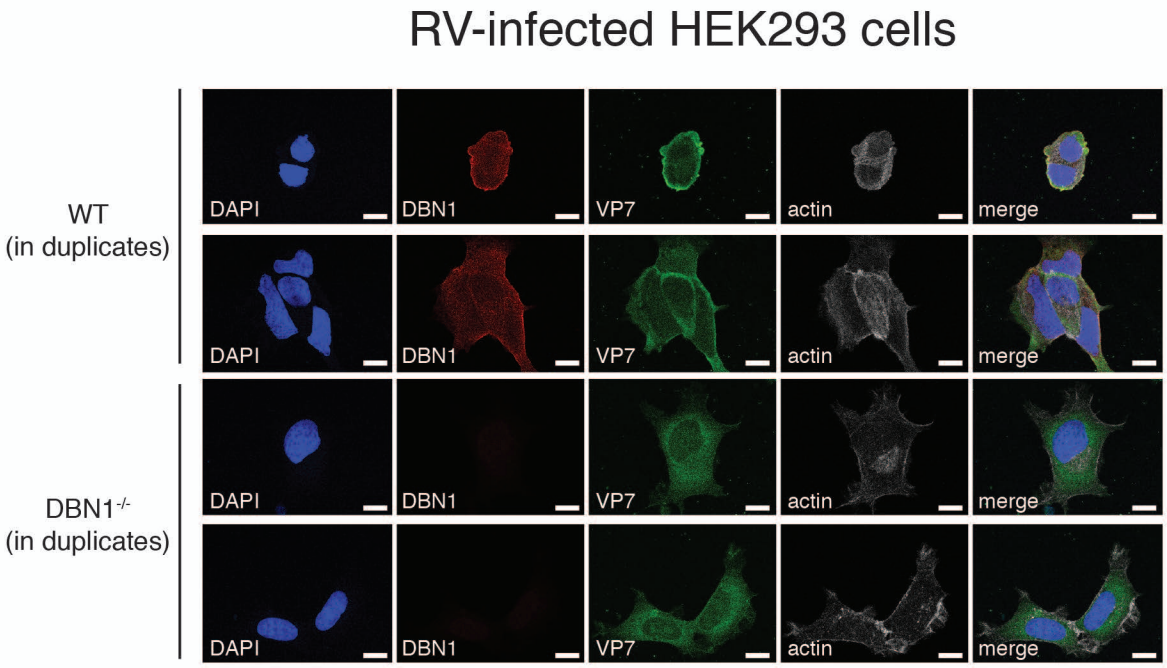


Fig. S7. DBN1 depletion accelerates RV particle trafficking. WT and DBN1 KO HEK293 cells were infected with RRV at MOI=10 for 15 min and analyzed by confocal microscopy for the localization of VP7 trimer (green), DBN1 (red), actin (phalloidin, white), and nucleus (DAPI, blue). Panels are single z slices with a scale bar of 8 μm. Two sets of images were duplicates of the same experiment and experiments were repeated twice.

Fig. S8

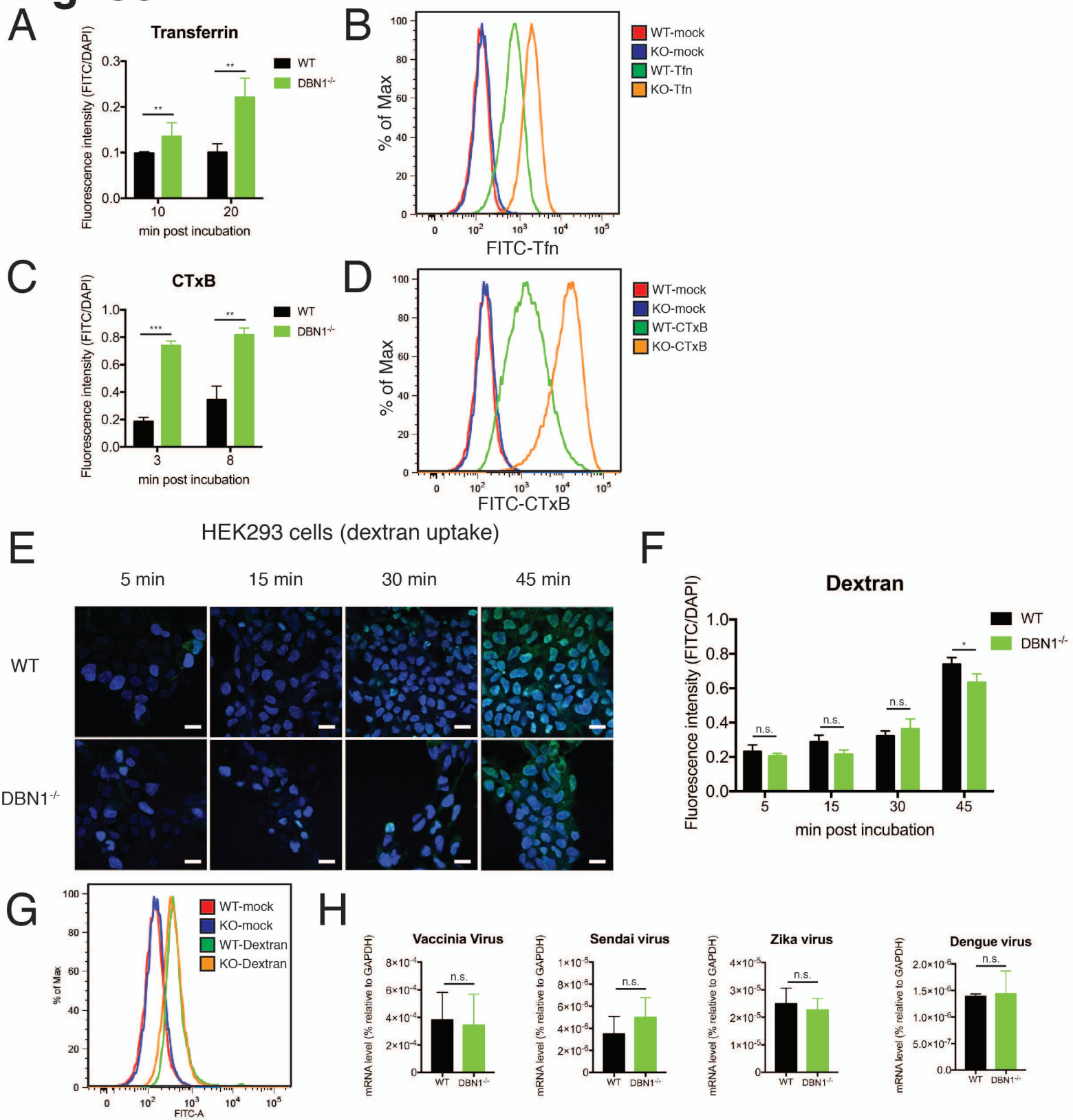


Fig. S8. Loss of DBN1 results in increased uptake of dynamin-dependent substrates. (A) Quantification of the transferrin micrographs in Fig. 3A. (B) WT and DBN1 KO HEK293 cells were incubated with FITC-conjugated transferrin (Tfn, 20 µg/ml), fixed at 8 min post incubation and analyzed by flow cytometry. (C) Quantification of the cholera toxin subunit B (CTxB) micrographs in Fig. 3C. (D) WT and DBN1 KO HEK293 cells were incubated with FITC-conjugated CTxB (10 µg/ml), fixed at 5 min post incubation and analyzed by flow cytometry. (E) WT and DBN1 KO HEK293 cells were incubated with FITC-conjugated high-molecular-weight dextran and fixed at indicated time points for confocal microscopy examining intracellular dextran (green), and nucleus (DAPI, blue). Panels and single z slices with a scale bar of 20 µm. (F) Quantification of the dextran micrographs in (C). (G) WT and DBN1 KO HEK293 cells were incubated with FITC-conjugated high-molecular-weight dextran (20 µg/ml), fixed at 8 min post incubation and analyzed by flow cytometry. (H) WT and DBN1 KO HEK293 cells were infected with Vaccinia or Sendai virus at MOI=0.1 for 1 hr, or Zika or Dengue virus at MOI=0.01 for 3 hr. Expression of respective viral genes was measured by RT-qPCR and normalized to that of GAPDH. For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S9

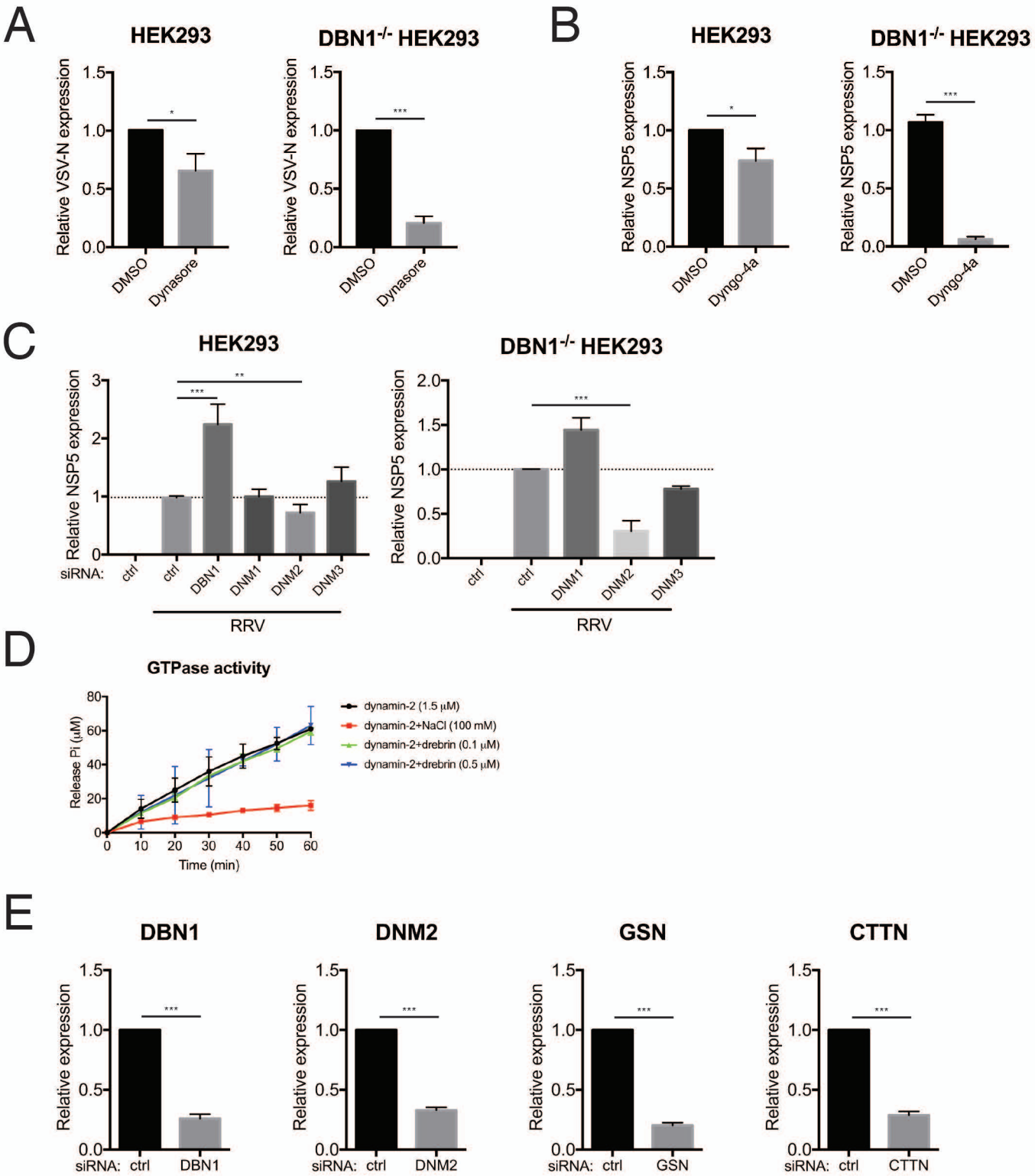


Fig. S9. Chemical inhibition of dynamin significantly reduces virus infection in DBN1 KO cells. (A) WT and DBN1 KO HEK293 cells were treated with either vehicle control (DMSO) or dynasore (100 μM) for 30 min prior to VSV infection (MOI=1). Total RNA was harvested at 1 hpi and viral gene N expression measured by RT-qPCR and normalized to that of GAPDH. (B) WT and DBN1 KO HEK293 cells were treated with either vehicle control (DMSO) or Dyngo-4a (5 μM) for 30 min prior to RRV infection (MOI=1). Total RNA was harvested at 1 hpi and NSP5 level measured by RT-qPCR and normalized to that of GAPDH. (C) WT and DBN1 KO HEK293 cells were transfected with scrambled siRNA (ctrl) or siRNA against dynamin (DNM)-1, 2, 3 for 48 hr prior to infection with RRV at MOI=1 for 1 hr. Viral NSP5 level was measured by RT-qPCR and normalized to that of GAPDH. (D) Non-radioactive GTPase assay for dynamin-2 with or without drebrin. The addition of NaCl served as a positive control. (E) WT HEK293 cells were transfected with scrambled siRNA (ctrl) or specific siRNA against DBN1, DNM2, GSN (gelsolin) and CTTN (cortactin) for 48 hr. Total RNA was harvested and indicated gene expression was measured by RT-qPCR and normalized to that of GAPDH. For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S10

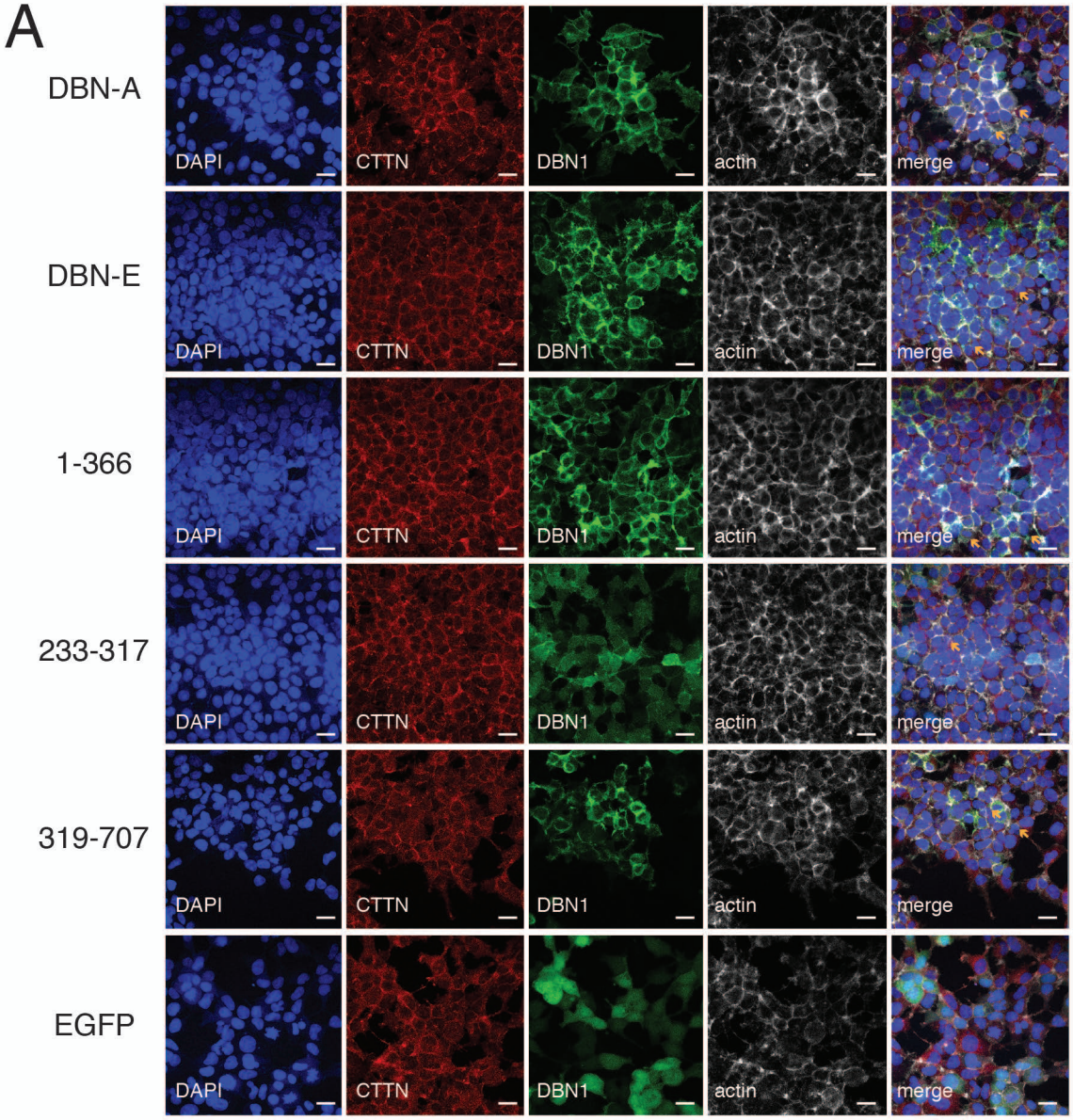


Fig. S10. DBN1 co-localizes with CTTN at the cytoskeleton. (A) DBN1 KO HEK293 cells stably expressing indicated GFP-tagged DBN1 mutants or control EGFP were analyzed by confocal microscopy for the localization of DBN1 (green), CTTN (red), actin (phalloidin, white) and nucleus (DAPI, blue). Co-localization (yellow) is highlighted by white arrowheads. Panels and single z slices with a scale bar of 40 μm. (B) Reconstituted DBN1 KO HEK293 cells were examined by western blot for protein levels of indicated DBN1 mutants. (C) Reconstituted DBN1 KO HEK293 cells were infected with RRV at MOI=1 for 24 hr and NSP5 levels were examined by QPCR (left panel) or lysates harvested for western blot (right panel). Experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Fig. S11

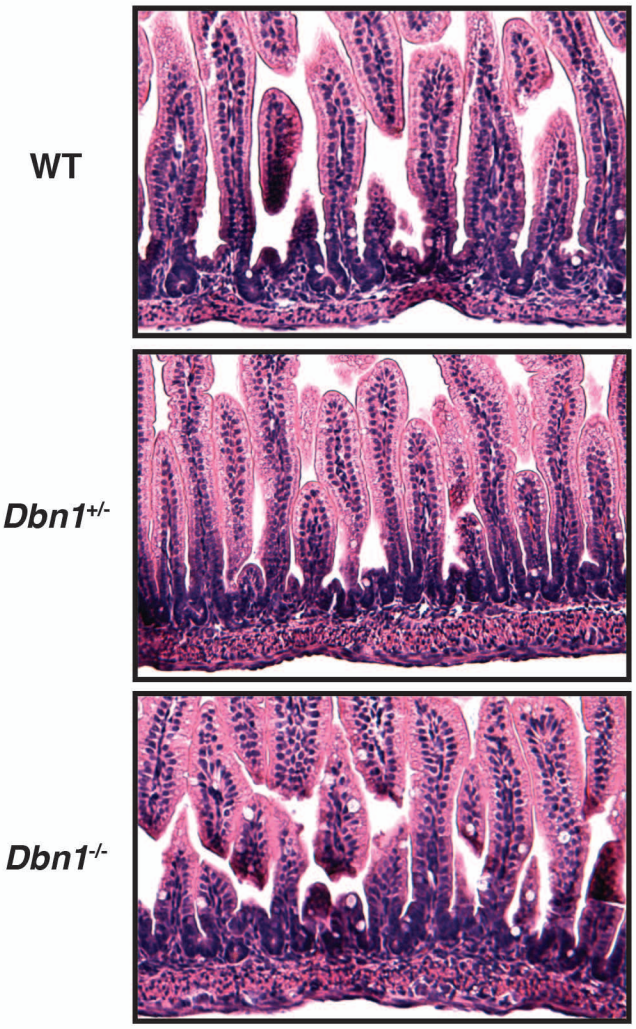


Fig. S11. Dbn1 deficiency does not alter intestinal permeability. Hematoxylin-and-eosin staining of ileal sections of 5-day old wild-type, Dbn1 heterozygous and Dbn1 knockout suckling mice.

Fig. S12

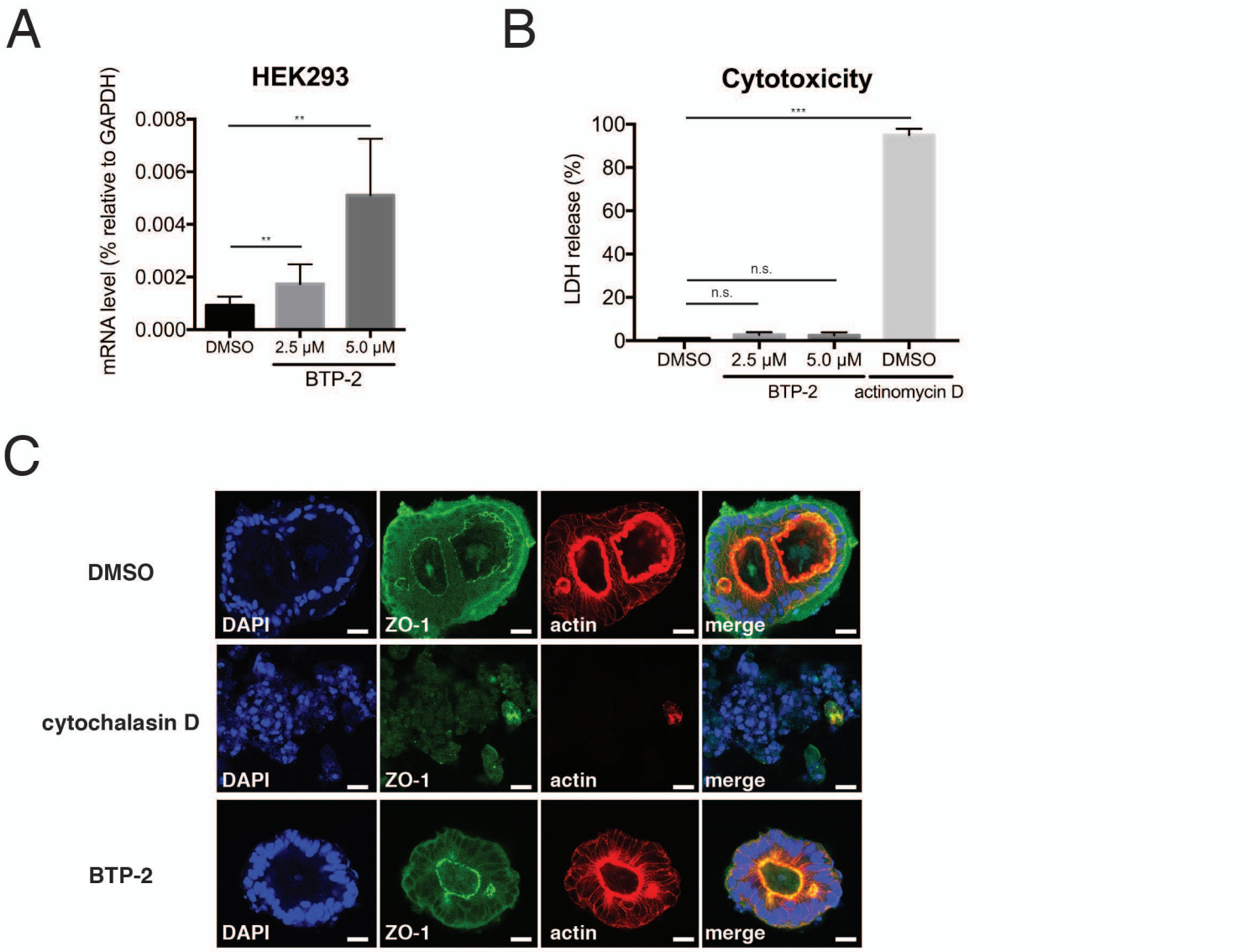


Fig. S12. Chemical inhibition of DBN1 enhances RV infection without disruption of tight junctions. (A) WT HEK293 cells were treated with either vehicle control (DMSO) or DBN1 inhibitor BTP-2 at indicated concentrations for 30 min prior to RRV infection (MOI=1). Total RNA was harvested at 1 hpi and NSP5 level was measured by RT-qPCR and normalized to that of GAPDH. (B) HEK293 cells were treated with DMSO or BTP-2 for 16 hr in the presence or absence of Actinomycin D treatment (10 μg/ml). Cell supernatants were collected and assayed by lactate dehydrogenase (LDH) release as an indicator of cell death. (C) Human ileal enteroids were treated with cytochalasin D (0.5 μM) or BTP-2 (1 μM) for 24 hr, and analyzed by confocal microscopy for the localization of ZO-1 (green), actin (phalloidin, red), and nucleus (DAPI, blue). The apical surface of the epithelium was shown in yellow. Panels and single z slices with a scale bar of 40 μm. For all figures, experiments were repeated at least three times. Data are represented as mean ± SEM. Statistical significance is determined by Student's t test (*p≤0.05; **p≤0.01; ***p≤0.001).

Table S1. Oligonucleotide Sequences

	Primer 5'-3'
RV-NSP5	F: CTGCTTCAAACGATCCACTCAC R: TGAATCCATAGACACGCC Probe: Cy5/TCAAATGCAGTTAAGACAAATGCAGACGCT/IAbRQSp
RV-VP7	F: ACGGCAACATTTGAAGAAGTC R: TGCAAGTAGCAGTTGTAACATC
GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG
ZIKV	F: TTGGTCATGATACTGCTGATTGC R: CCTTCCACAAAGTCCCTATTGC
DENV	F: GCATATTGACGCTGGGARAGAC R: TTCTGTGCCTGGAATGATGCTG
SV40	F: TTAGCAATTCTGAAGGAAAGTCCTTG R: AGCAGTGGTGGAATGCCTTTCATGAGG
VV	F: GGCAATGGATTCAGGGATATAC R: ATTTATGAATAATCCGCCAGTTAC
HAdV5	F: GACATGACTTTCGAGGTCGATCCCATGGA R: CCGGCTGAGAAGGGTGTGCGCAGGTA
SeV	F: CAAAAGTGAGGGCGAAGGAGAA R: CGCCCAGATCCTGAGATACAGA
GSN	F: GGT GTG GCA TCA GGA TTC AAG R: TTT CAT ACC GAT TGC TGT TGG A
DBN1	F: AGG AGC TTT CGG GAC ACT TTG R: GAG CAC GTA TTT TGG CAG AGC
CTTN	F: GTGGTTTTGGCGGCAAGTATG R: CTCTCTGTGACTCGTGCTTCT
DNM2	F: CCGCTGGTCAACAAACTGC R: CGGGTGACGATTCCTGAACC
ADD1 CRISPR KO 20-nt target sequence	GACCAGAGTGAACTCCGAGC
DBN1 CRISPR KO 20-nt target sequence #1	GATCGTGAACGCCAGCAGCG
DBN1 CRISPR KO 20-nt target sequence #2	TACTGAGGTCACCCTGGCAG
ADD1 KO sequencing primer	F: GGTCATTACATCCTCCTGTTG R: GATGTCTGATAACATGAACTGAACTG
DBN1 KO sequencing primer #1	F: TGGGCGAAGATGTGCCTGATG R: CCACAGCTGCATCCGTCTTCTGGTA
DBN1 KO sequencing primer #2	F: TAGACAGTGAGGAGACCAGAGCAGC R: CCTGCTCTTGCTCCAGCTCCTCAA

DBN1 mice genotyping forward primer	GGAGGATTCAGAGGCAAAGA
DBN1 mice genotyping WT reverse primer	CTGGAGAAACCAGGGAGATG
DBN1 mice genotyping KO reverse primer	CCAACCGTACCCACTTCAGA