Supplemental Information

Porcine Enteric Alphacoronavirus entry through multiple pathways (caveolae, clathrin, and macropinocytosis) and requires Rab GTPases for endosomal transport

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Supplemental Information

Supplemental Experimental Procedures

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, NY, USA), supplemented with 10% fetal bovine serum (GIBCO, Invitrogen). Primary porcine enterocytes were extracted and isolated from the jejunum tissue of 0-day-old piglets. All cells were kept at 37°C in a humidified atmosphere of 5% CO_2. PEAV strain GDS04, a gift from Professor Cao Yongchang of Sun Yat-sen University, was propagated in media containing 0.3% tryptophan phosphate (TPB) and 10 μg/ml trypsin (GBICO) in Vero cells, and P16 was used in the study. The prepared virus supernatant was centrifuged at 10,000 g for 15 min, and the supernatant was centrifuged at 100,000 g at 4°C for 1.5 h with Beckmann SW32Ti rotor, and the virus was suspended in HNE buffer solution (5 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]). The virus suspension was added to a sucrose gradient (10% to 60%) and centrifuged in the same rotor at 120,000 g for 2 h at 4°C. The virus bands were collected from the 40-60% sucrose layer and centrifuged at 40,000 g for 1.5 h to remove sucrose.

Finally, the purified virus was resuspended in HNE buffer and stored at -80°C for future use.

Viral infection. Vero cells were grown to 100% confluence, washed with phosphate-buffered saline (PBS) to remove the serum, and inoculated with different MOI (multiplicity of infection) viruses containing 0.3% TPB (Tryptone Phosphate Broth) and 10 μg/ml trypsin (virus maintenance medium). The virus supernatant (with cell fragments removed) was used in entry experiments in the study.

Antibodies, inhibitors, and reagents. Monoclonal antibody (1:500) was used for the indirect fluorescent antibody (IFA) test, and polyclonal antibody (1:1000) was used for Western blotting against PEAV N protein. Anti-Cytokeratin 18 (A19778) was purchased from Abclonal (1:200). Secondary antibodies (1:300) conjugated to Alexa Fluor 594 and 647 were purchased from Proteintech. Methyl-β-cyclodextrin (MβCD, C4555), chlorpromazine hydrochloride (CPZ, C0982), 5-(N-ethyl-N-isopropyl)amiloride (EIPA, A3085), and proteinase K were purchased from Sigma-Aldrich. Alexa Fluor 488-labeled dextran (dextran, D22910) was purchased from Thermo Fisher Scientific.

Quantitative real-time PCR (qRT-PCR). Viral RNA was extracted using the Fastagen kit according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop 2000 spectrophotometer. According to the manufacturer's instructions, cDNA was synthesized using 500 or 1,000 ng total RNA using iScript reverse transcriptase (Thermo Fisher Scientific). A 1:5 dilution of cDNA was used to perform quantitative real-time PCR (qRT-PCR) using a Light Cycler 480 real-time PCR system (Roche Diagnostics, Indianapolis, IN, USA). Data are presented as the $2^{-\Delta\Delta CT}$ value from quadruplicate samples, and GAPDH was used as a reference gene.

Immunofluorescence assay. Vero cells or Primary porcine enterocytes were first grown on a 14 mm glass bottom cell culture dish (Cellvis) until 90% confluence, then inoculated with PEAV. The cells were fixed with 4% paraformaldehyde (PFA) at different times. The

samples were incubated at 20 ° C for 15 min, washed with PBS three times, and permeabilized with 0.3% Triton X-100 for 5 min. The cells were stained with specific antibodies (2 h, 4°C) overnight, or 37°C. All antibodies were diluted in 2% (w/v) BSA in PBS. Cells were washed thrice with PBS and incubated with the corresponding secondary antibody (1 h, 37 ° C). All secondary antibodies were diluted in PBS. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1 μg/ml). Fluorescent images were acquired using the light-scanning module of a Leica TCS SP8 STED 3× confocal microscope.

Cell viability assay. Vero or IPI-2I cells were seeded into 96 well plates and grown to 100% confluence. Different concentrations of drug inhibitors were added, and the cells were incubated at 37 ° C for 165 min. The medium of IPI-2I cells was changed to drug-free media and incubated for a further 6 h. Cell viability was determined by the CCK-8 kit according to the manufacturer's instructions. The absorbance values at 450 nm were measured to determine cell viability. An average of 4-8 readings were taken. Cell survival rate (%) = (OD of experimental group/OD of the control group) × 100%. Similarly, cells inoculated on a 96-well plate were transfected with corresponding siRNAs or siCtrl for 48 h, and a CCK-8 kit was used to determine cell viability. The effect of inhibitors or siRNA cell viability is shown in (Fig. S3).

Entry assay. Vero cells were seeded in a 6-well plate and pre-treated with a sub-toxic dose of drug inhibitor at 37°C for 15 min, then PEAV (MOI=0.1) was inoculated in the presence of drugs at 4°C for 1 h, and then PBS was used to wash the cells three times to remove the nonattached virus. Vero cells were cultured in virus maintenance medium containing drugs, 37°C, 1 h to allow virus internalization. Then, wash the cells with GN buffer (pH=3) for 5 min, remove the non-internalized virus particles on the cell surface, and wash the cells with precooled PBS three times. The cells were lysed according to the manufacturer's instructions, and RNA was extracted.

Primary porcine enterocytes isolation. Porcine enterocytes were extracted and isolated from the jejunum tissue of 0-day-old piglets. Briefly, tissue sections were cut into 5 cm pieces, washed with ice-cold PBS, and placed in 50 ml conical tubes containing 1X antibiotic-antimycotic. The intestinal segment was cut lengthwise, followed by vigorous shaking to remove intestinal contents or fully differentiated enterocytes, and washed with ice-cold PBS. The cleaned intestinal segments were placed in dishes with ice-cold PBS, and the intestinal villi were scraped off with a cell scraper and added to a 50 ml centrifuge tube with ice-cold PBS containing 2% fetal bovine serum. The tubes were centrifuged for 3 min at 500 g, washed with ice-cold PBS 5 times, and then cellular precipitates were collected. Freshly isolated jejunum epithelial cells were seeded at 250,000 cells/well and maintained with Ham's F12 nutrient medium containing murine epidermal growth factor (Sigma) to stimulate epithelial cell growth and differentiation. Differentiated cells were grown on collagen hydrogels for virus infection experiments.

Statistical analysis. All data are presented as the mean ± SD. A Student's t-test was used to compare the data from pairs of treated and untreated groups. Statistical significance is indicated by asterisks (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$) in the figures. All statistical analyses and calculations were performed using Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Supplemental Figures

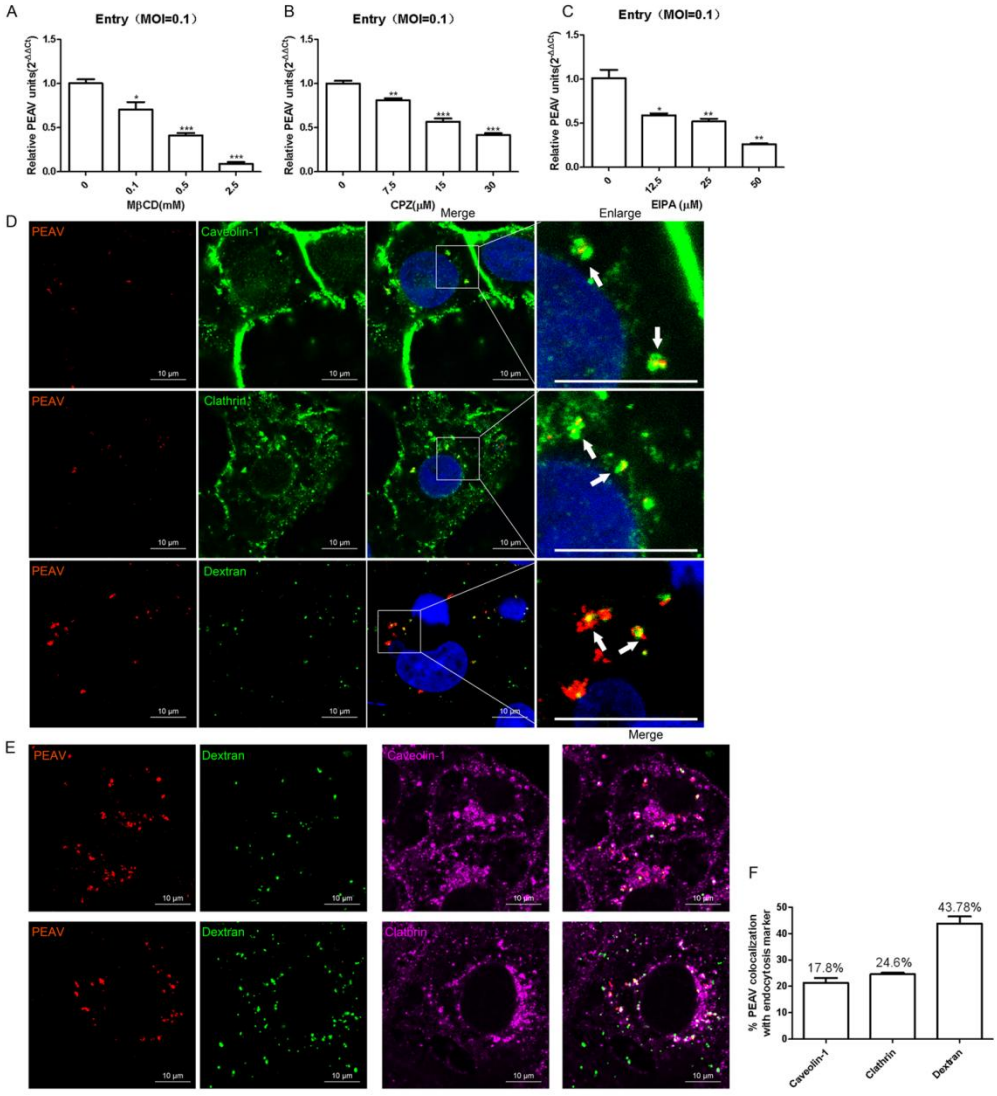


FIG S1. PEAV enters into Vero cells via multiple pathways (caveolae-mediated endocytosis, clathrin-mediated endocytosis, and macropinocytosis) at different MOI. (A to C) In order to measure the effect of low infection dose on virus entry. Vero cells were pretreated with MβCD, CPZ, or EIPA (15 min, 37°C), followed by PEAV (MOI=0.1) inoculation to allow entry (1 h, 37 °C) in the presence of drugs. (D) Vero cells were inoculated with PEAV (MOI=5) (1 h, 4°C), washed, incubated (15 min, 37°C), and fixed (4% PFA, 15 min, RT). Vero cells were stained with anti-PEAV N, anti-clathrin, and anti-caveolin-1 monoclonal antibodies, respectively, and their respective localizations were observed with a confocal microscope. The white arrow shows that PEAV

co-localizes with endocytosis vesicles; scale bar=10 μm. (E) Vero cells were inoculated with PEAV (MOI=10) (1 h, 4 ° C), washed, incubated in a maintenance medium containing 0.5 mg/ml Alexa Fluor 488-labeled dextran (15 min, 37 °C), and fixed (4% PFA, 15 min, RT). Vero cells were stained with anti-PEAV N, anti-clathrin, and anti-caveolin-1 monoclonal antibodies, respectively, and their respective localizations were observed with a confocal microscope. The co-location ratio of dextran and PEAV was used as a reference to determine the main infection route. (F) As described above, co-localization proportions were determined using ImageJ software with Mander's co-localization coefficient. Over 250 co-localizing virus particles were included in the quantification. The mean value ± SEM is representative of three individual enlarged pictures.

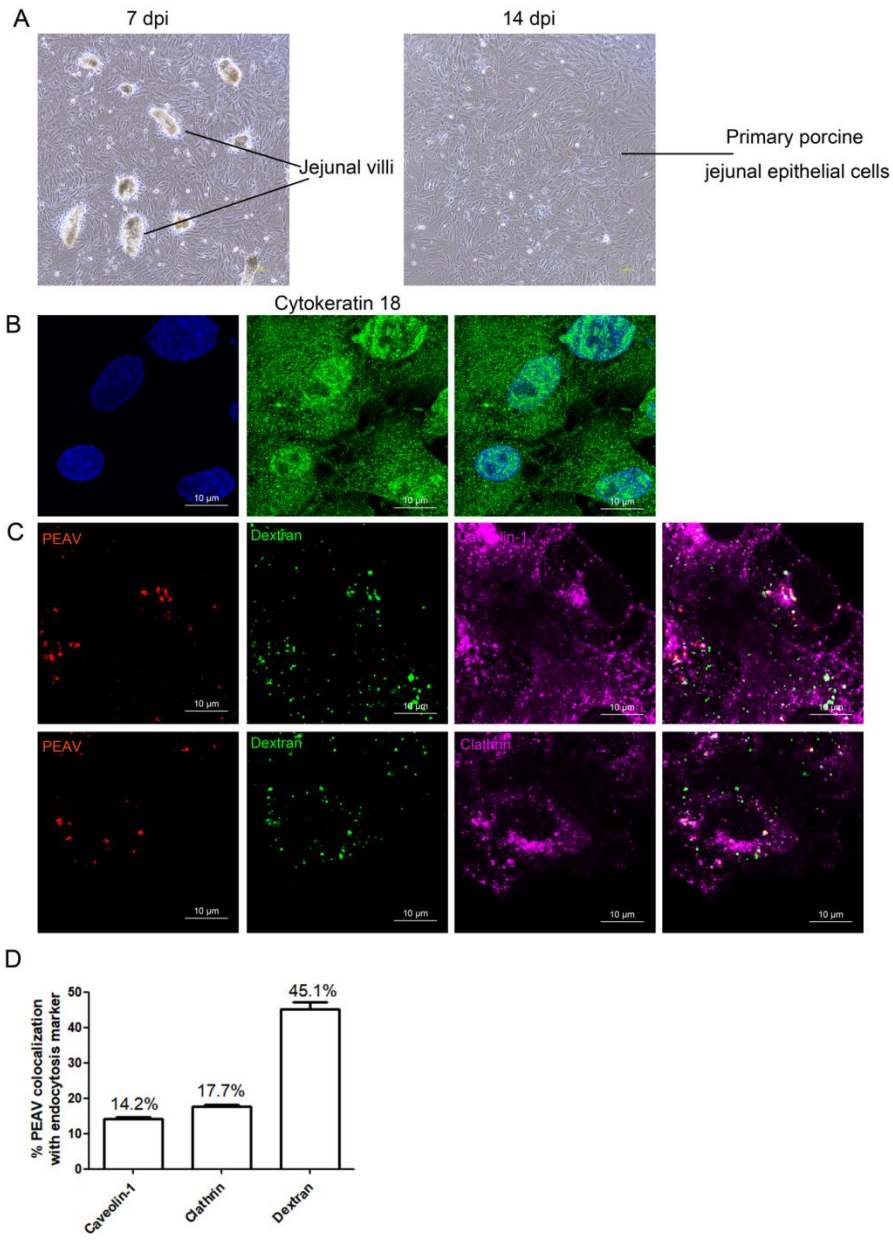
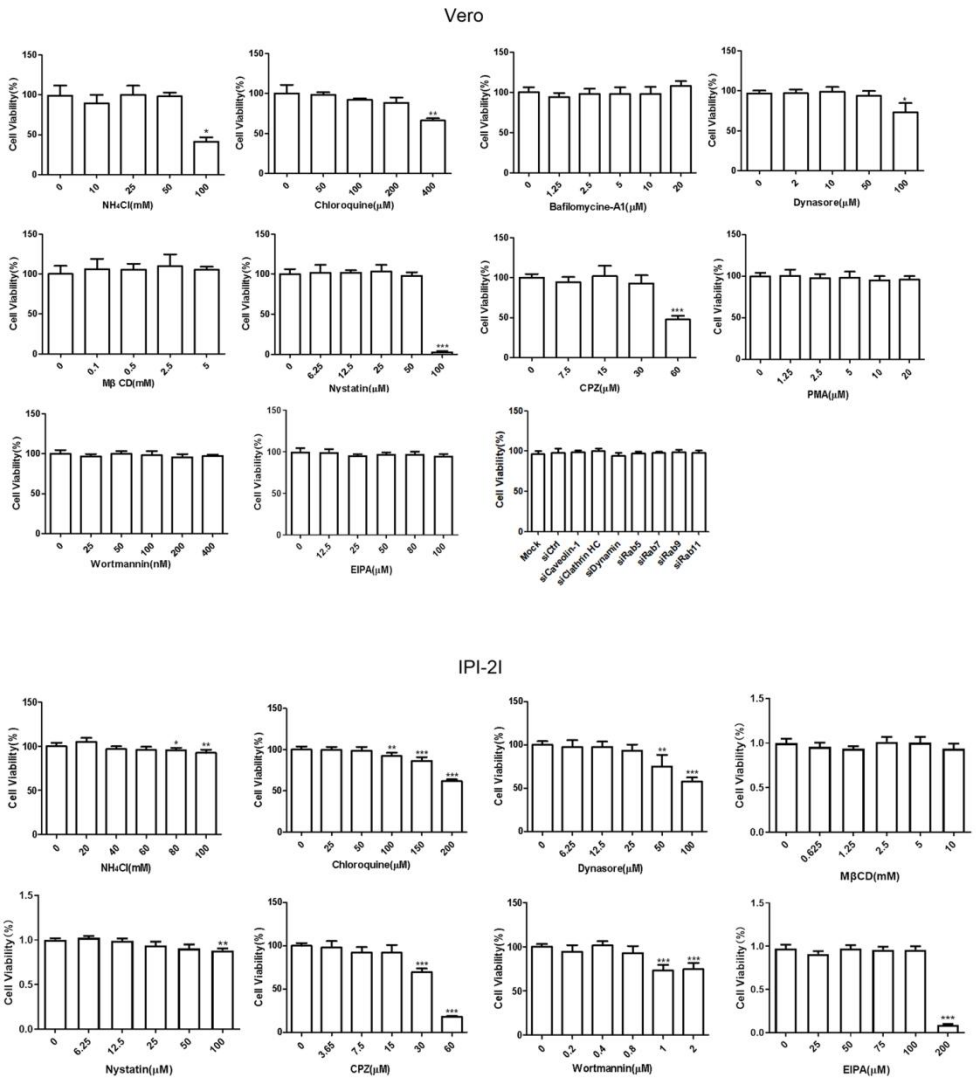


FIG S2. PEAV enters primary porcine jejunal epithelial cells via multiple pathways (caveolae-mediated endocytosis, clathrin-mediated endocytosis, and macropinocytosis). (A)The morphology of primary porcine jejunum villi cultured after 7 dpi and 14 dpi (10×magnification); scale bar=200 μm. (B) Epithelial cell surface marker Cytokeratin 18 was used to determine cell purity. (C) Jejunal epithelial cells were inoculated with PEAV (MOI=10) (1 h, 4°C), washed, incubated in a maintenance medium containing 0.5 mg/ml Alexa Fluor 488-labeled dextran (15 min, 37°C), and fixed (4%

PFA, 15 min, RT). Vero cells were stained with anti-PEAV N, anti-clathrin, and anti-caveolin-1 monoclonal antibodies, respectively, and their respective localizations were observed with a confocal microscope. The co-location ratio of dextran and PEAV was used as a reference to determine the main infection route. (D) As described above, co-localization proportions were determined using ImageJ software with Mander's co-localization coefficient. Over 250 co-localizing virus particles were included in the quantification. The mean value ± SEM is representative of three individual enlarged pictures.



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153 **FIG S3.** Vero or IPI-2I cell viabilities following incubation with each drug and siRNA
154 duplex tested in this work, as assessed using the CCK8 cell viability detection kit. All
155 results are presented as the mean ± SD from three independent experiments (*, $P<0.05$;
156 **, $P<0.01$; ***, $P<0.001$).