PNAS
www.pnas.org

Supplementary Information for

Bile acids and ceramide overcome the entry restriction for GII.3 human norovirus replication in human intestinal enteroids

Kosuke Murakami, Victoria R. Tenge, Umesh C. Karandikar, Shih-Ching Lin, Sasirekha Ramani, Khalil Ettayebi, Sue E. Crawford, Xi-Lei Zeng, Frederick H. Neill, B. Vijayalakshmi Ayyar, Kazuhiko Katayama, David Y. Graham, Erhard Bieberich, Robert L. Atmar and Mary K. Estes.

Mary K. Estes, Ph.D.
E-mail: mestes@bcm.edu

This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1910138117

Supplemental Methods.

Bile. Waste human bile, obtained from adults undergoing hepatobiliary surgery, was collected through the Texas Medical Center Digestive Diseases Center Study Design and Clinical Research Core. The study protocol was approved by the Baylor College of Medicine Institutional Review Board. Bile was incubated with an FDA approved bile acid-sequestrant, cholestyramine (Sigma Aldrich) for 12 h at 4ºC. Concentration of total BA was quantified by a commercial kit (Cell Biolabs) following the manufacturer's instructions.

Western blot. J2 HIE monolayers were differentiated for 5 days and membrane proteins were extracted with the Mem-PER Plus Kit (ThermoFisher) following the manufacturer's protocol. MA104 cells were grown to confluency and monolayers were lysed in Mammalian Cell Lysis Buffer (Abcam) following the manufacturer's protocol. Each cell lysate was clarified by centrifugation and cell lysate was subjected to SDS-PAGE and Western blotting. Proteins were separated on 4-15% SDS-PAGE and then transferred to nitrocellulose membranes. S1PR2 (EDG-5) protein was detected with a mouse primary monoclonal antibody EDG-5 E12 (Santa Cruz). Primary antibody was detected with goat anti-mouse polyvalent immunoglobulin (G, A, M)-peroxidase (Sigma Aldrich) and blots were developed using Amersham ECL Prime Western Blotting Detection reagent (GE Healthcare) according to the manufacturer's instructions then exposing to film.

SphingoStrips. Commercial SphingoStrips (ThermoFisher) were treated as recommended by the manufacturer by blocking with Tris buffered saline with Tween-20 (TBST) containing 3% (wt/vol) BSA. After blocking, the strips were incubated with 1 μg/mL GII.3 TCH04-577 VLPs or CTxB (Sigma Aldrich, C9903) in TBST 3% BSA for 4 h at room temperature (RT). Strips were washed 3 times in TBST 3% BSA and then bound VLP or cholera toxin B subunit (CTxB) was detected with specific primary antibodies: mouse monoclonal NS14 [GII.3; (1)] and goat polyclonal anti-CTxB (Calbiochem). LI-COR anti-mouse or -goat secondary antibodies conjugated to IRDye 800CW were used to detect the primary antibody and stained SphingoStrips were scanned on a LI-COR Odyssey CLx imager.

RNAseq. To assess the transcriptional profile of human intestinal enteroids, monolayers were prepared on transwells and differentiated as previously described (2). J2 monolayers were treated for 3 h with either medium alone or 500 μM GCDCA. Total RNA was isolated using RNeasy mini

kit (Qiagen) followed by rRNA depletion. Quality control and paired-end sequencing (100 bp) using the IlluminaHiSeq 2500 machine was performed by the Genomic and RNA Profiling Core at Baylor College of Medicine. Raw sequence reads were mapped to the human genome. For gene expression analysis, read summarization was performed and genes with less than one read per 10 million sequenced reads in two or more samples were excluded. Gene-expression estimates were normalized for gene length and sequencing depth to generate FPKM values. FPKM value in medium only or GCDCA treated HIEs are indicated in Table S1.

Cell cytotoxicity. HIE monolayers were incubated in differentiation medium containing 500 µM GCDCA and/or 40 µM JTE-013 for 24 h. Cells and medium were used with a commercial lactate dehydrogenase (LDH) assay kit-WST (Dojindo, CK12-01) to assess cell viability following the manufacturer's instructions.

Immunofluorescence and quantitation. FM1-43FX and ceramide staining are described in the main methods section. HBGA expression was evaluated in 5 day differentiated monolayers treated with medium alone or media containing 500 µM GCDCA for 10 mins. GCDCA treated and untreated monolayers were fixed in 4% PFA and stained for 3 h at RT with *Ulex europaeus* agglutinin I (UEA-1) lectin conjugated to rhodamine (Vector Labs Lectin Kit, RLK2200), washed, counterstained with DAPI and imaged with confocal microscopy. The imaging and quantitation was conducted as described in the main methods section.

pHrodo Dextran Uptake. HIE monolayers were incubated with 1 µg/mL of pHrodo labeled dextran (ThermoFisher, P1036) for 30 min at 37ºC. The monolayers were washed twice with differentiation medium, harvested using 0.5 mg/mL trypsin and suspended in cold Fluorbrite medium. A BD Canto flow cytometer equipped with a yellow green laser was used to acquire 10,000 events per sample. The pHrodo positive cells were counted and the number of pHrodo positive cells in the presence of GCDCA, GII.3 stool or both were expressed as a percentage of pHrodo positive cells in medium alone. The data presented are from two independent experiments.

HBGA ELISA. Ninety-six well microtiter plates were coated with pig gastric mucin (PGM) (10 µg/ml, 100 µl per well) and incubated overnight at 4ºC. The plates were decanted and then blocked with phosphate-buffered saline (PBS) containing 1% (wt/vol) nonfat dried milk (nfdm) for 2 h at RT. Meanwhile, GII.3 TCH04-577 VLPs were diluted from 100 µg/ml to 1.563 µg/ml into PBS either in the presence or absence of 500 µM GCDCA and incubated for 2 h at RT in the dark. The

microtiter plates were washed with PBS containing 0.05% Tween 20. Each dilution of VLPs with or without GCDCA was transferred to the 96-well microtiter plate previously coated with PGM or PBS only (blank). The plate was incubated at RT for 2 h in the dark. After washes, bound VLPs were detected with rabbit anti-VLP antibodies followed by goat anti-rabbit IgG-peroxidase, both in PBS/1% nfdm. After a final wash, color development was obtained with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate and stopped with phosphoric acid. The PBS (blank) absorbance at 450 nm wavelength at each VLP concentration was subtracted from the corresponding test absorbance. The absorbance of a blank well with no VLP and no PGM was subtracted from all values and these values were graphed.

Supplemental Figures.

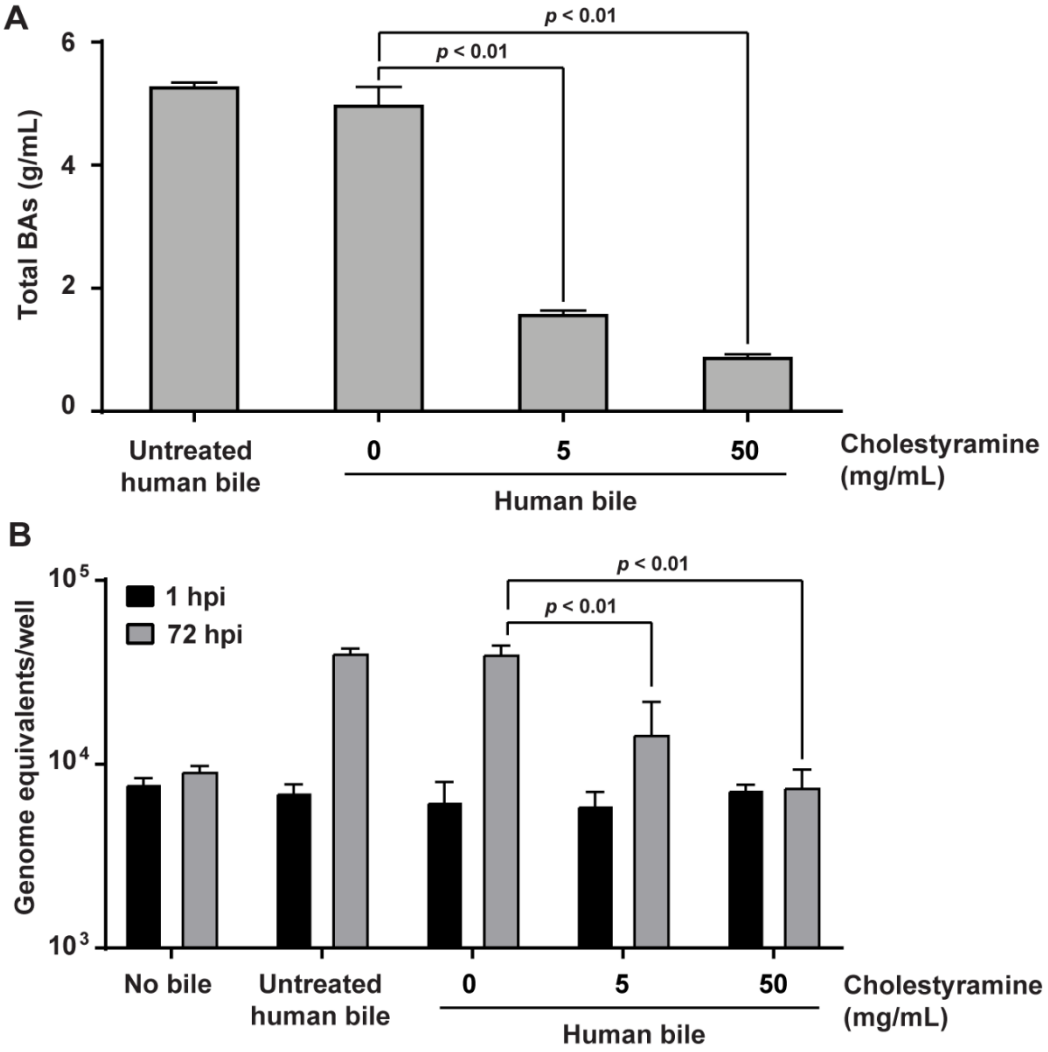

Fig. S1. GII.3 replication is reduced in the presence of BA-depleted human bile. (*A*) Human bile was incubated with 0, 5 and 50 mg/mL of cholestyramine for 12 h at 4°C, and total BAs were quantified by a commercial kit as described in Materials and Methods. Data represent the mean of three wells for each treatment. Error bars denote standard deviation (SD). (*B*) HIE monolayers were infected as in Fig. 1. Cholestyramine treated or untreated bile was added to the medium at the concentration of 5% (vol/vol) during and postinoculation. Viral genome equivalents (GEs) at 1 and 72 hpi were quantified by RT-qPCR. Data represent the mean of three wells for each condition. Error bars denote SD. *P* values between conditions are indicated.

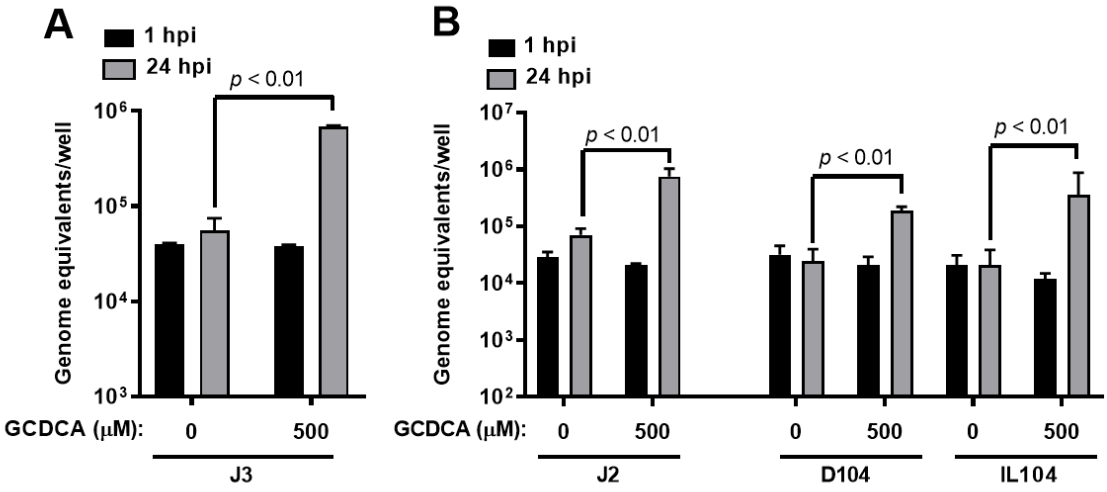

Fig. S2. GII.3 replicates in different secretor positive HIE lines in the presence of GCDCA. *(A)* A secretor positive jejunal culture (J3) was plated as monolayers and infected. Viral genome equivalents (GEs) at 1 and 24 hpi were quantified by RT-qPCR. (*B*) Monolayers of duodenum and ileum HIE cultures derived from a single secretor positive individual were infected for 24 h and subjected to RT-qPCR to assess GII.3 replication. J2 HIEs was infected at the same time as the control. Data represent the mean of three wells for each condition. Error bars denote standard deviation. *P* values between conditions are indicated.

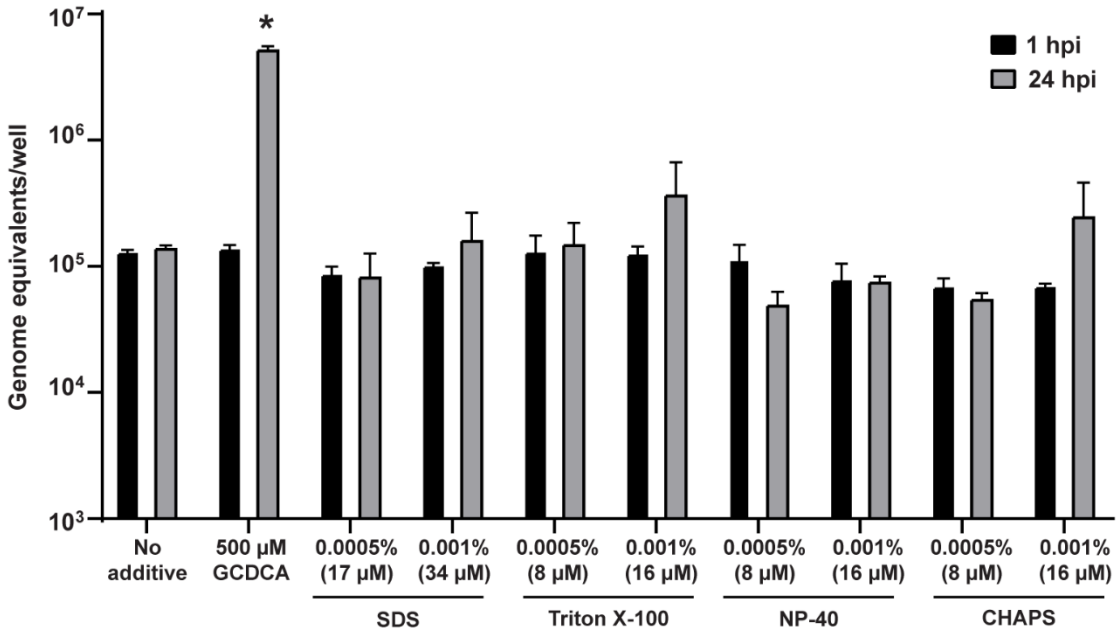

Fig. S3. Detergents have no effect on GII.3 replication in HIEs. HIE monolayers were infected as in Fig. 1 with GII.3 in the presence of detergents (SDS, Triton X-100, NP-40 and CHAPS) and GCDCA at the indicated concentrations for 24 h. *, $P < 0.05$ comparing GEs at 24 hpi to 1 hpi.

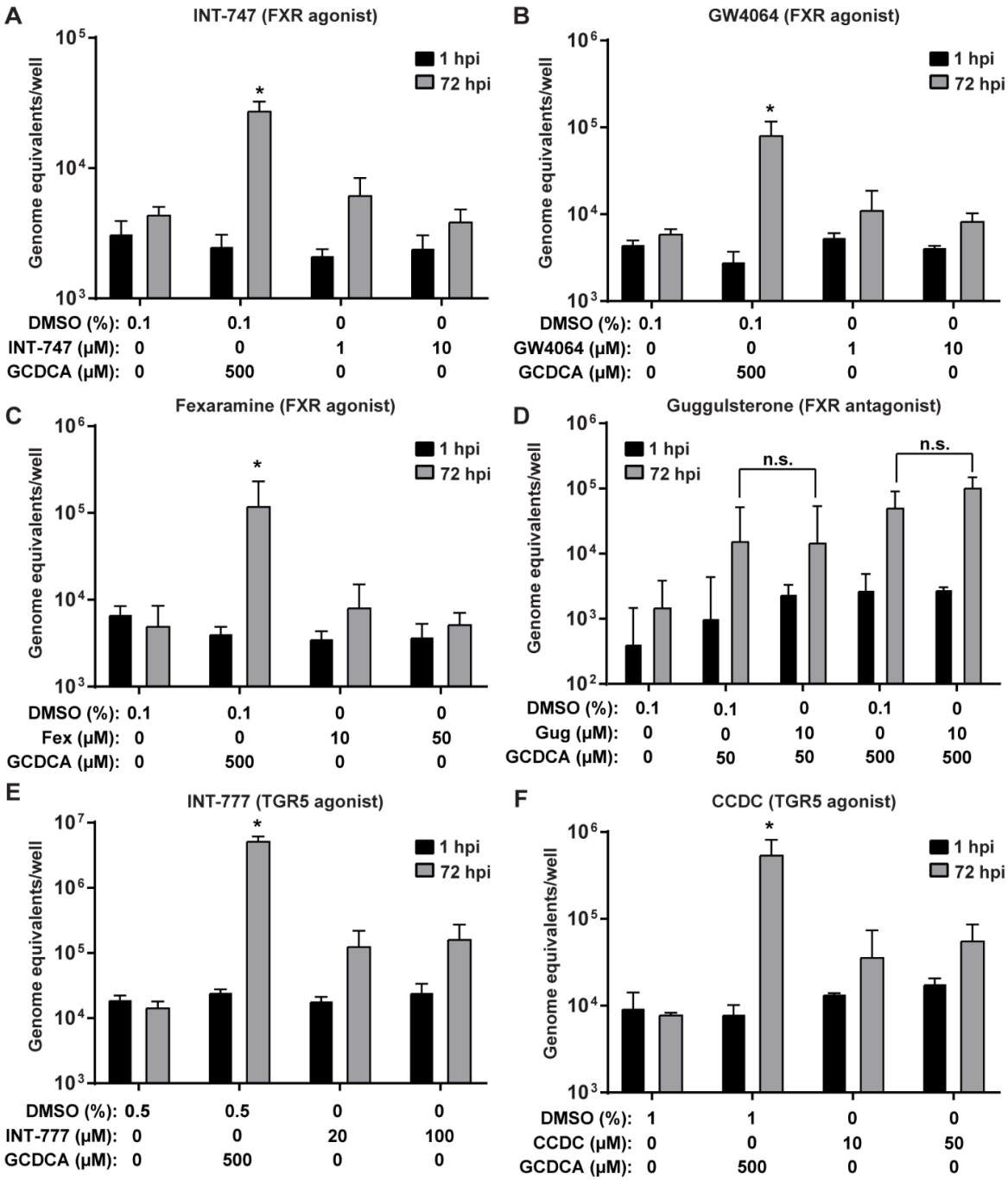

Fig. S4. Classical BA receptors, FXR and TGR5 are not involved in GII.3 replication. (*A-F*) HIE monolayers were infected as in Fig. 1 with GII.3 for 72 h. FXR agonists [INT-747 (A), GW4064 (B) and fexaramine (C)], FXR antagonist [guggulsterone (D)], TGR5 agonists [INT-777 (E) and CCDC (F)] and GCDCA were added to medium during and postinoculation at the indicated concentrations. *, $P < 0.05$ comparing GEs at 72 hpi to 1 hpi. Comparisons of GE at 72 hpi between treatments with and without guggulsterone were not significant (n.s.).

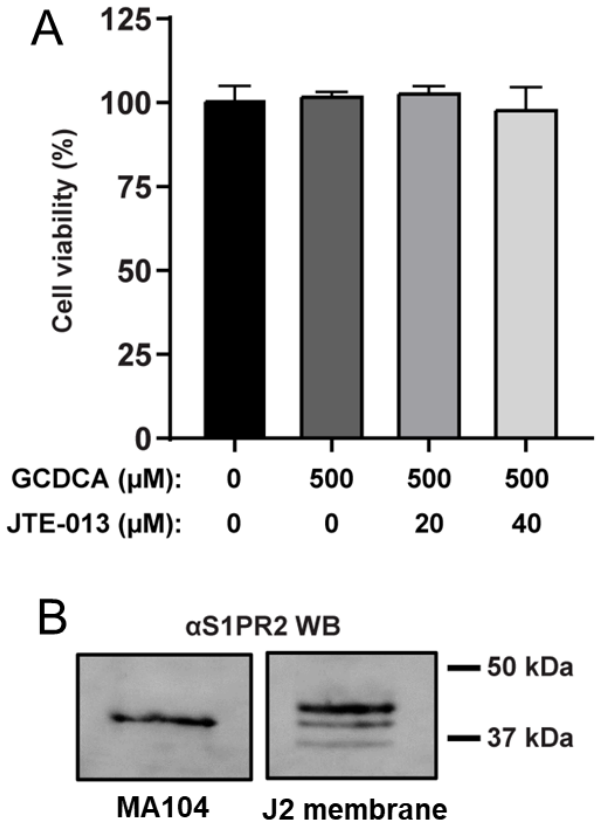

Fig. S5. Cell viability of HIEs treated with the S1PR2 inhibitor JTE-013 and expression of S1PR2 in HIEs. *(A)* J2 HIE monolayers were incubated with differentiation medium containing the indicated additives. Cell viability of the cells at 24 h posttreatment was measured by lactate dehydrogenase assay. Data represent the mean of three wells for each condition. Error bars denote standard deviation. *(B)* Western blot detecting S1PR2 protein. Multiple bands detected in J2 membrane lysate likely indicate post translational modification.

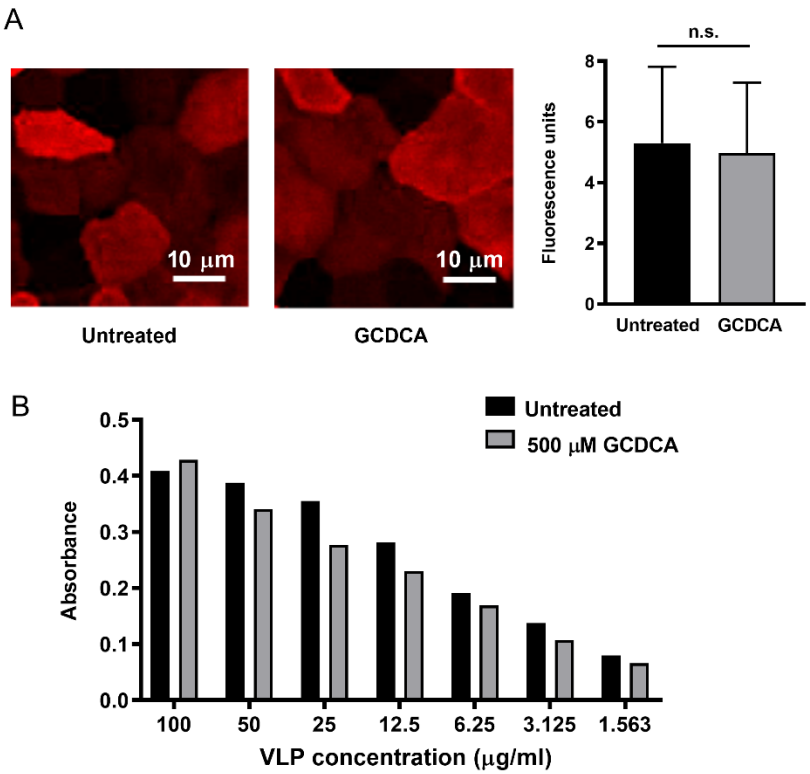

Fig. S6. GCDCA treatment does not alter HBGA levels of HIEs or increase GII.3 VLP binding to porcine gastric mucin. (*A*) The effect of GCDCA on the HBGA expression was analyzed using UEA-1 lectin (red), on HIE monolayers. A representative confocal image shows UEA-1 staining on either untreated or 500 μM GCDCA treated HIE monolayers. The graph shows quantitation of the fluorescence staining. (*B*) ELISA detection of GII.3 VLP binding to porcine gastric mucin in the presence or absence of 500 μM GCDCA. There were no difference in VLP binding to untreated or GCDCA-treated HIEs.

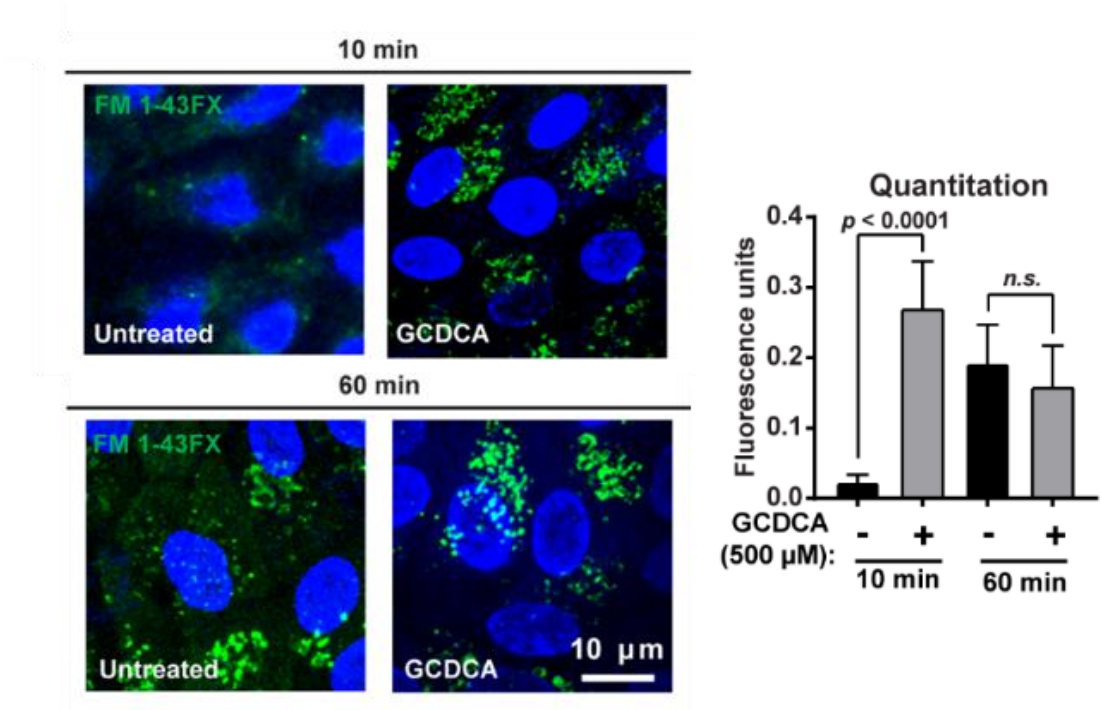

Fig. S7. Elevated endocytosis due to GCDCA treatment was transient. Endocytosis in HIE monolayers untreated or treated with 500 µM GCDCA was analyzed by confocal laser-scanning microscopy (left panels). The endocytic vesicles (green) and nuclei (blue) were visualized by FM1-43FX and DAPI, respectively. Scale bar 10 µm. Right panel shows quantitation of the FM1-43FX fluorescence.

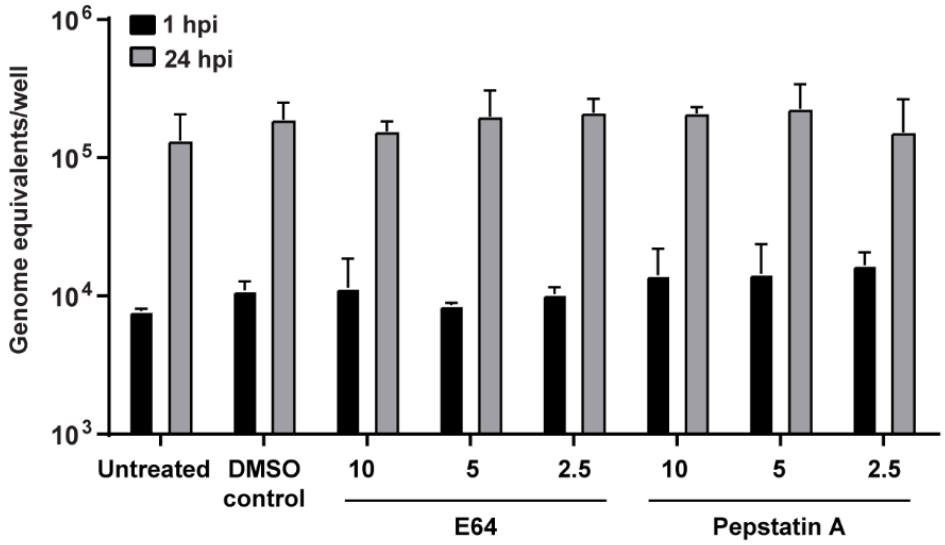

Fig S8. Inhibitors of cathepsins do not alter GII.3 replication. HIE monolayers were pretreated for 1 h with inhibitors for cathepsins B and L (pepstatin A) or cathepsins D and E (E64) and inhibitors were present during the 1 h inoculation with GII.3. After washing infection was continued without inhibitors as in Fig. 1 for 24 hrs. GCDCA was present throughout inoculation and infection. All comparisons between different additive conditions at 24 h were not significant.

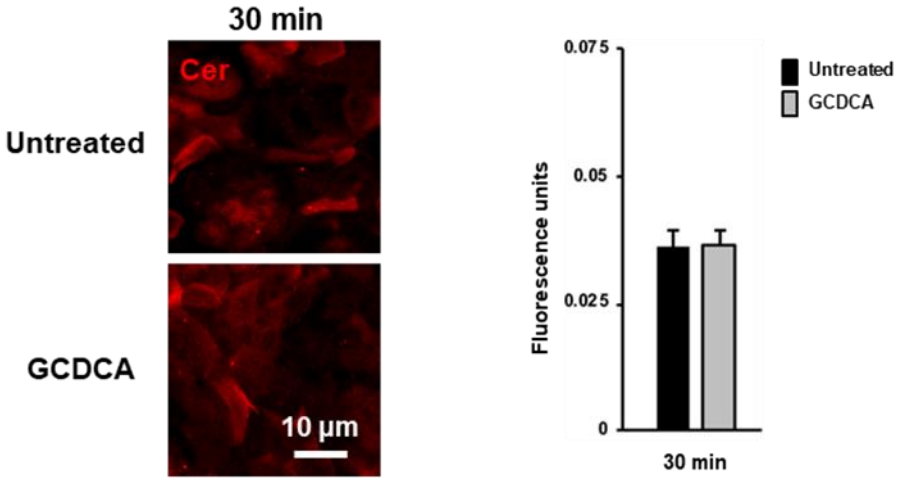

Fig. S9. Elevated ceramide levels in GCDCA-treated HIE monolayers are transient and normalize by 30 min posttreatment. Ceramide levels in HIE monolayers treated with medium alone or GCDCA for 10 min (shown in Fig. 6) or 30 min were analyzed by immunofluorescence microscopy (left panels). Scale bar is 10 µm. Right panel shows quantification of fluorescence intensity as described in the Materials and Methods section. Error bars denote SD.

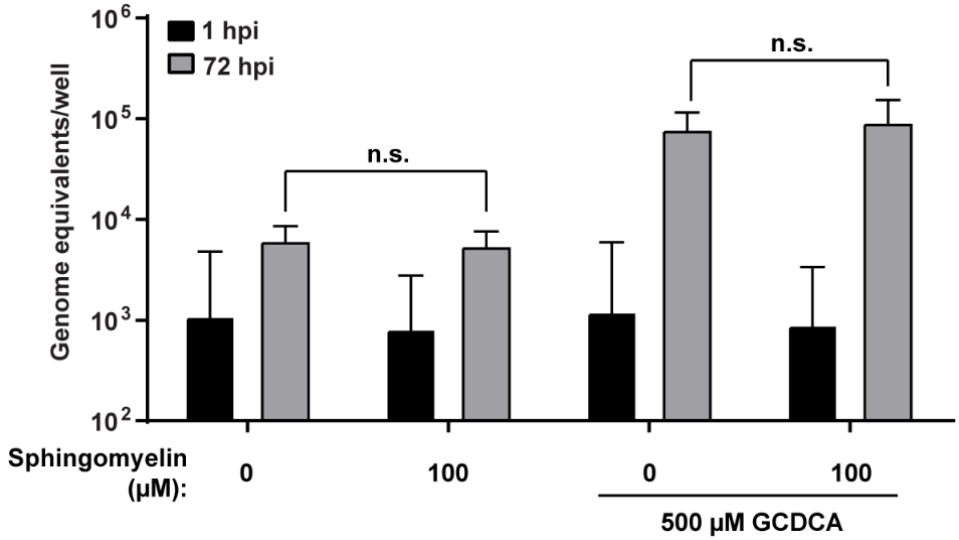

Fig. S10. Exogenous addition of the ceramide precursor-phospholipid, sphingomyelin, does not alter GII.3 replication. HIE monolayers were infected with GII.3 in the presence of sphingomyelin at the indicated concentrations for 72 h. The indicated additives were added to the medium during and postinoculation. Viral GEs at 1 and 72 hpi were quantified by RT-qPCR. n.s., not significant between indicated samples.

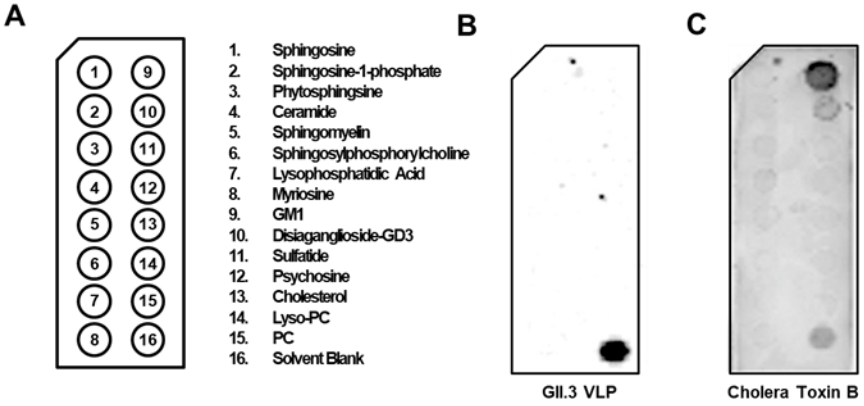

Fig. S11. GII.3 VLPs do not directly bind to phospholipids or sphingolipids, including ceramide. Commercial SphingoStrips were blocked with TBST containing 3% (wt/vol) BSA. After blocking, strips were incubated with 1 μg/mL GII.3 VLP or cholera toxin subunit B (CTxB; positive control) in TBST 3% BSA for 4 h at RT. Strips were washed 3 times and then bound VLP or CTxB was detected with specific primary antibodies and LI-COR secondary antibodies conjugated to IRDye 680RD and scanned on a LI-COR Odyssey CLx imager.

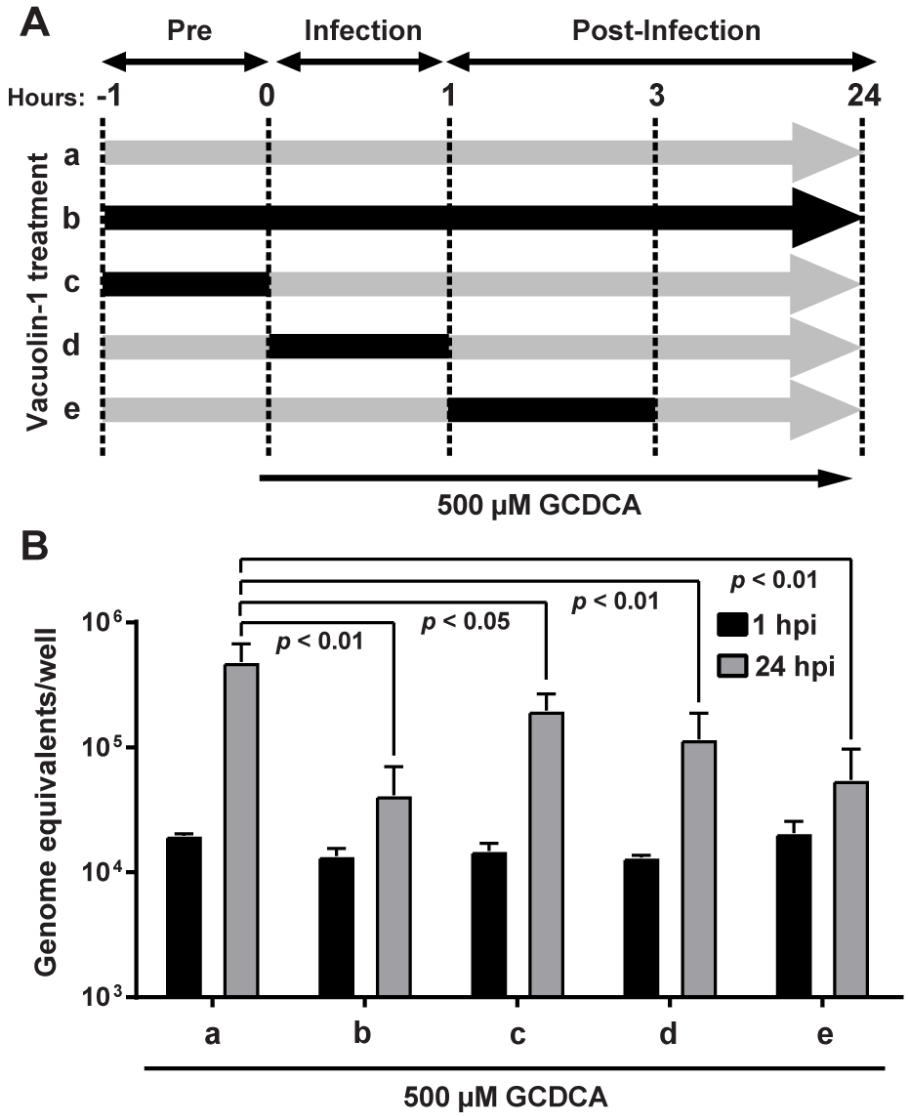

Fig. S12. Vacuolin-1 inhibits GII.3 infection at early time points. *(A)* Schematic showing with black arrows when 10 µM vacuolin-1 was added to the medium during GII.3 infection of HIEs. *(B)* HIE monolayers were infected as in Fig. 1 with GII.3 in the presence of vacuolin-1 during time points indicated in (A). 500 µM GCDCA was maintained in the medium from inoculation and on. *P* values between conditions are indicated.

Supplemental Tables.

Table S1. mRNA expression of BA-related proteins in GCDCA treated and untreated jejunal HIEs.

Protein Name (gene symbol)	FPKM	
	Untreated	GCDCA
FXR (NR1H4)	22.2	22.8
FGF19 (FGF19)	0.3	2.5
FABP6 (FABP6)	0.4	1.9
TGR5 (GPBAR1)	0.05	0.18
S1PR2 (S1PR2)	0.09	0.15

Table. S2. HIE lines used in this study.

Line	Secretor Status	HBGA Type	Segment
J2	Positive	B Lewis b	Jejunum
J3	Positive	O Lewis b	Jejunum
D104	Positive	A Lewis b	Duodenum
IL104	Positive	A Lewis b	Ileum

Supplemental References.

1. Kou B*, et al.* (2015) Characterization of cross-reactive norovirus-specific monoclonal antibodies. *Clin Vaccine Immunol* 22(2):160-167.
2. Zou WY*, et al.* (2017) Human Intestinal Enteroids: New Models to Study Gastrointestinal Virus Infections. *Methods Mol Biol*. DOI: 10.1007/7651_2017_1.