

Supporting Information for Rotavirus-mediated DGAT1 Degradation: a Pathophysiological Mechanism of Viral-induced Malabsorptive Diarrhea

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Materials and Methods

Cell lines and viruses

African green monkey kidney cells (MA104) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Immortalized DGAT1^{-/-} and DGAT1^{+/+} mouse embryonic fibroblasts [(MEFs) kindly provided by Robert V. Farese Jr. (Harvard University, Boston, MA)] (1) were cultivated in DMEM containing 10% FBS, and 1x essential and nonessential amino acids. Human intestinal enteroids (HIEs) were cultured and infected as previously described (2, 3). Simian RV strain SA11 4F (G3P6[1]) and human RV strain Ito (G3[P8]) were propagated in MA104 cells as previously described (4). For all infections, RV was activated for 30 min in 10 µg/mL trypsin (Worthington) and cells were inoculated for 1 h at 37°C and 5% CO₂. The inoculum was removed, the cells washed three times, and media without FBS replaced. Virus titers were determined by focus fluorescent assay (5) and expressed as focus-forming units (FFU)/mL normalized by cell counts prior to infection for MA104 and MEF cells and protein levels assessed by the Red 660 Protein Assay (G Biosciences) for HIEs.

Antibodies and reagents

Antibodies (Abs) to DGAT1, PLIN1, and Ezrin were purchased from Abcam (Cambridge, MA); MGAT3, claudin 4; and Occludin and E-cadherin monoclonal antibodies from Thermo Fisher Scientific; JAM-1 from R&D Systems (Minneapolis, MN). Abs to α/β Tubulin, HA-tag, and GAPDH monoclonal antibody were purchased from Proteintech (Rosemont, IL). Ab to K48 ubiquitin was obtained from Millipore (Burlington, MA). Monoclonal Abs to sucrase-isomaltase (A-17) and villin (B12) were purchased from Santa Cruz Biotechnology (Woburn, MA). Antibodies to IP3R3 from BD Biosciences (Franklin Lakes, NJ) or mCherry (rabbit, E5D8F) used as an irrelevant antibody in immunoprecipitation assays and to P-eIF2α (Ser 51) were from Cell Signaling Technology

(Danvers, MA). Alexa 488-, 549- and 649 conjugated secondary Abs were purchased from Rockland Immunochemicals (Philadelphia, PA). NHE3 Ab was from Novus Biologicals (Littleton, Colorado). IRDye® 800CW and IRDye® 680RD Infrared Dyes conjugated donkey anti-rabbit, anti-mouse and anti-guinea pig Abs were purchased from Li-Cor Biosciences (Lincoln, NE). All commercial antibodies were tested for the presence of RV antibodies by western blot. Only antibodies found to be negative for RV antibodies were used in these studies. Guinea pig polyclonal Abs to purified RV particles (anti-RV), to RV nonstructural protein NSP2 (anti-NSP2) and mouse monoclonal antibodies to the NSP2 isoforms (anti-dNSP2 and anti-vNSP2) were produced in our laboratory (6). MG132 was purchased from BioVision Inc. (Milpitas, CA). Cells were transfected with plasmid DNA, 12-24 hours, or siRNA, 72 hours prior to infection, using Lipofectamine 2000 or lipofectamine RNAiMAX reagent, respectively, following the manufacturer's instructions (Invitrogen).

Immunoprecipitation and western blots

Protein A/G magnetic beads (Pierce) were used to immunoprecipitate cellular or viral proteins from 0.5 mL of mock- or RV-infected cells lysed in RIPA cell lysis buffer (Invitrogen) per the manufacturer's instructions. Western blots were performed as previously described (6).

Immunofluorescence

Mock- or RV-infected MA104 cells seeded on coverslips in 24-well plates, or HIEs seeded on transwells, were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature, and then permeabilized with 0.5% Triton X-100 for viroplasm labeling or 0.1% saponin for LD staining. The cells were blocked in 5% fatty acid-free bovine serum albumin (BSA) for 30 minutes, then incubated in primary and secondary antibodies diluted with 5% BSA. DAPI (NucBlue™ Fixed Cell Stain ReadyProbes™ reagent) was added prior to the final wash to stain the nuclei. LipidTOX™ (Thermo Fisher Scientific) was used per the manufacturer's instructions. Coverslips were mounted on to glass slides for visualization. Images were captured as previously described (6).

Mock- or RV-infected 3D HIEs were placed into Matrigel and fixed in 10% neutral buffered formalin overnight at 4°C. The HIE-containing Matrigel was then dehydrated and embedded in paraffin. Heat-mediated antigen retrieval was performed on 3 µm HIE paraffin sections in 10 mM citrate buffer (pH 6.0). Sections were blocked in 5% BSA, incubated overnight at 4°C in primary antibodies, and then washed three times in PBS containing 0.05% Tween 20 (PBS-T). Fluorescence was visualized by using Alexa Fluor-conjugated secondary antibodies. Cells were imaged on a Nikon A1R confocal laser scanning microscope (6).

Establishment of the DGAT^{-/-} human intestinal enteroid (HIE) line

A lentiCRISPRv2 vector expressing Cas9 and guide RNA (5'-GCCAGCTATAGGGATCCTTC-3') targeting human DGAT1 was obtained from GenScript USA Inc (Cat# SC 1805). The DGAT^{-/-} HIE was produced following (7). Immunoblot analysis was performed to confirm the lack of DGAT1 protein expression.

Metabolic labeling with BODIPY™ 558/568 C12

MA104 cells were metabolically labeled for 1 hour with BODIPY™ 558/568 C12 either just prior to RV infection or between hours 2 and 3 post RV infection. At 3 hours post infection, the cells were fixed for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline (PBS) and processed for confocal microscopy.

SDS-PAGE and western blot analysis

Mock- and RV-infected cells were harvested at the indicated times post infection and lysed in RIPA cell lysis buffer (Invitrogen) containing protease inhibitor (complete, mini, EDTA-free protease inhibitor cocktail tablets) and 100 mM N-ethylmaleimide (Sigma). The cell lysates were prepared and analyzed under nonreducing and reducing conditions as required for detecting the different forms of NSP2 with monoclonal antibodies (6). Immunoblots were incubated with primary antibody to DGAT1 (1:5000; Abcam), dNSP2 (1:1000) and vNSP2 (1:1000) (6), P-eIF2 α antibody (9721S, Cell Signaling) and GAPDH antibody (10494-1-AP, Protein Tech) and fluorescent secondary antibodies (1:10,000; LiCor) diluted in 1X Casein buffer at RT for 2 hours or at 4°C overnight. Blots were washed three times with TBS containing 0.1% Tween-20 and once with TBS prior to visualization or quantitation using an Odyssey CLx infrared imaging system (Li-Cor).

Human intestinal enteroid RNA-seq

To assess the transcriptional profile of human intestinal enteroids, monolayers were prepared on transwells and differentiated for 5 days as previously described prior to infection (2). Total cellular RNAs were extracted from 5 day differentiated mock-inoculated and Ito-infected transwells from two independent biological replicates per segment using the RNeasy Mini Kit (Qiagen). Cytoplasmic and mitochondrial ribosomal RNA was removed from the total RNA samples using Illumina's Epidemiology-Specific RiboZero rRNA removal protocol. Then cDNA was generated using the fragmented and rRNA-depleted total RNA using random primers. Sequencing of the cDNA libraries was performed by the Genomic and RNA Profiling Core at Baylor College of Medicine on a high output v4 flow cell (Illumina) using a paired-end 100 cycle run on an Illumina HiSeq 2500 Sequencing System for a depth of 30 million paired end reads (a total of 60 million reads) per RNA sample. Raw sequence reads were checked for quality using FASTQC package ver. 0.11.9 and reads were trimmed with TrimGalore ver. 0.6.5 with default settings for adaptive trimming and for base quality filtering. Trimmed reads were aligned to human genome build GRCh38 using HISAT2, and a count matrix was generated from the aligned reads using featureCounts. Fragments per kilobase of exon per million mapped fragments (FPKM) values for each gene (n=2 biological replicates) are indicated in *SI Appendix*, Fig. 4. The RNA-seq data (GSE168005 and GSE183222) were deposited in the Gene Expression omnibus of the National Center for Biotechnology (8).

Statistical analysis

Each experiment was performed two or more times, with at least three technical replicates. Student's two-tailed t-test was performed to determine the significance unless otherwise indicated. $P < 0.05$ was considered statistically significant. Error bars in graphs of infection experiments denote standard deviation.

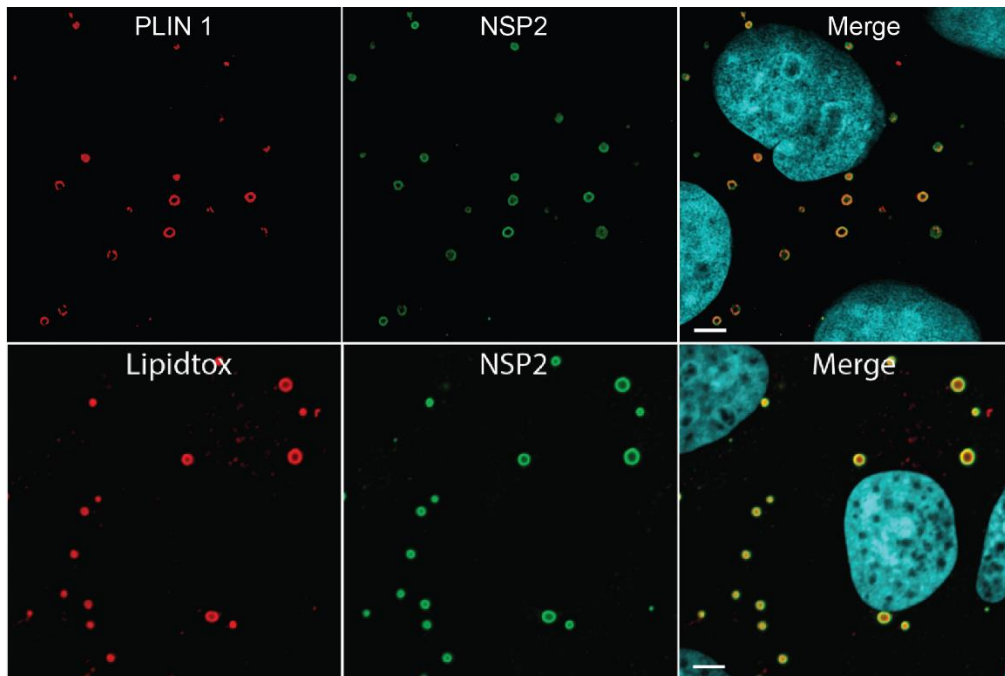
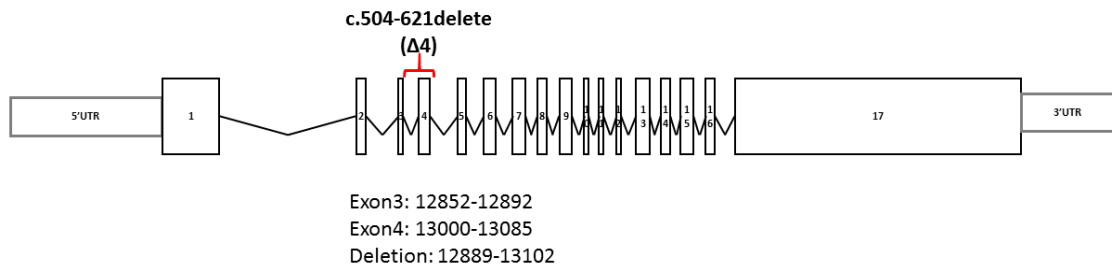


Fig. S1. RV viroplasms colocalize with the LD-specific marker protein PLIN1 and LD neutral lipid. MA104 cells were infected with SA11 4F [multiplicity of infection (MOI) 3] and fixed at 6 hours post infection (hpi). Viroplasms were detected with anti-NSP2 antibody (green), the LD-associated protein PLIN1 was detected with an antibody against PLIN1 (upper panel, red), and the neutral lipid in the LDs was detected with LipidTOX™ (red, lower panel). Scale bar (white) = 5 μ m.

A



B

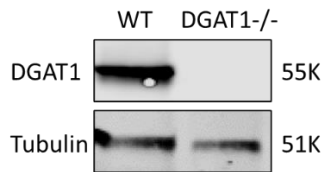


Fig. S2. Schematic of the DGAT1 KO HIE genomic deletion mutation clone. (A) Genomic DNA was extracted from the DGAT1 KO clone for PCR amplification and sequencing. The sequencing result showed a 213 base pair deletion that removed part of exon 3 and the entire exon 4 of DGAT. (B) Western blot showing lack of DGAT1 protein expression in DGAT1^{-/-} compared to wild type HIE lysates.

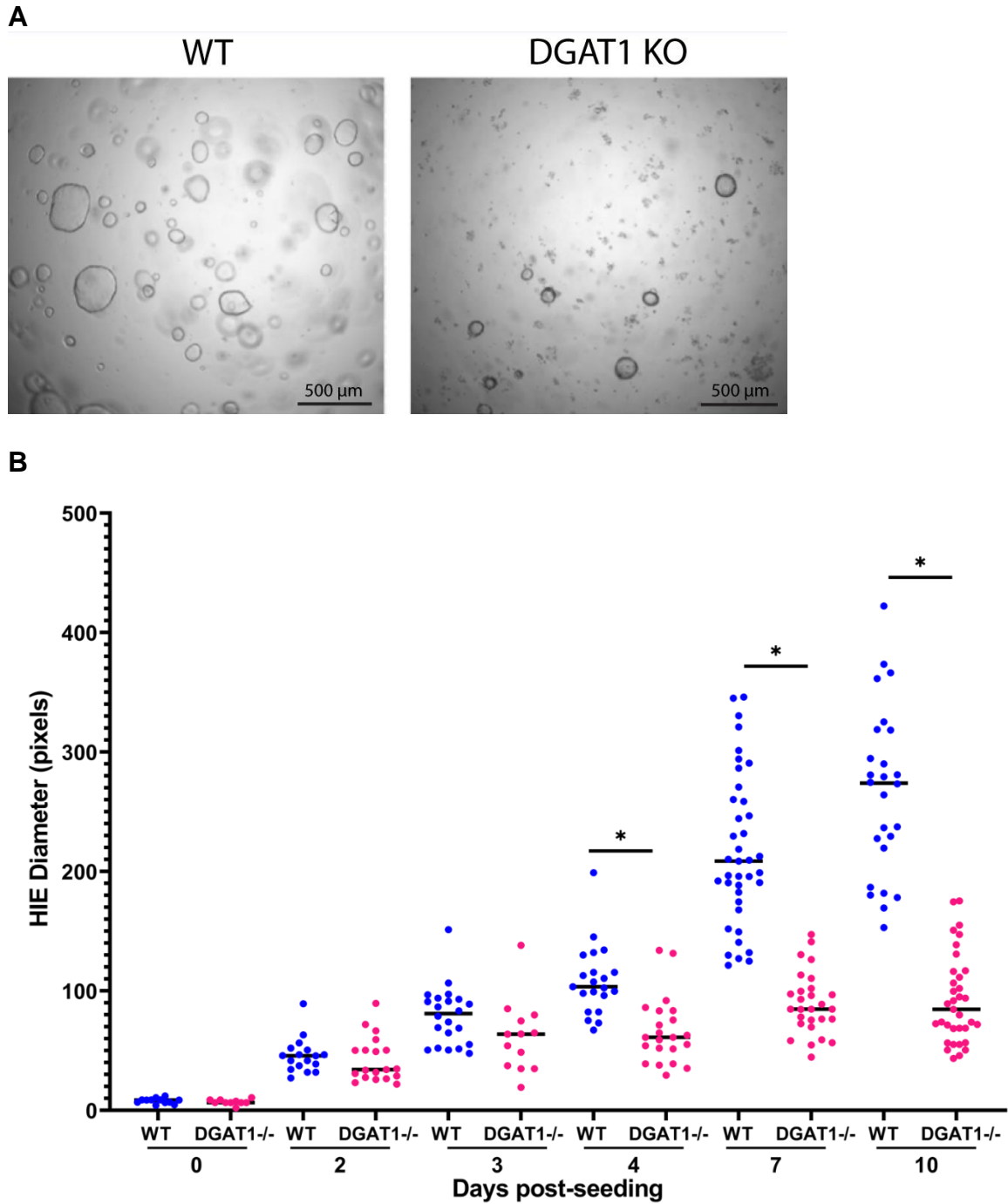
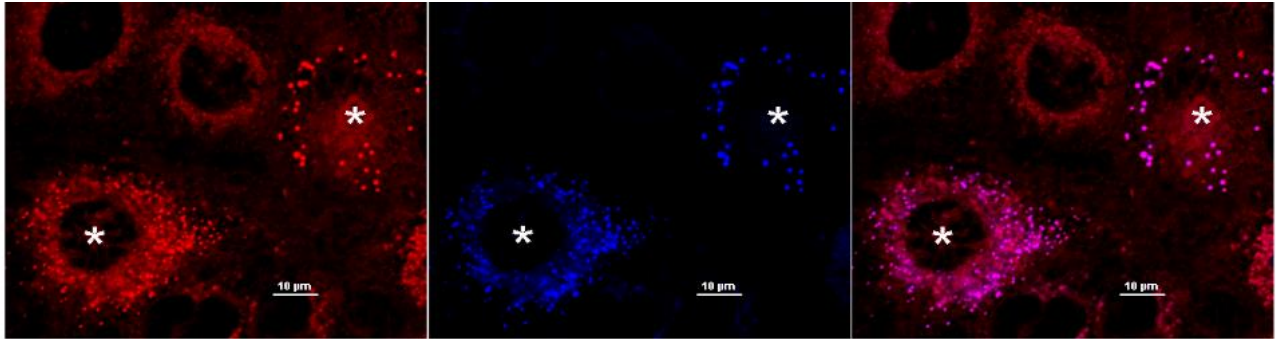


Fig. S3. Growth of DGAT1 KO HIEs is slower than parental WT HIEs. (A) Representative bright field microscopy images on day 7 post-seeding. Bar, 500 μ m. (B) Comparison of the diameter of 3D WT and DGAT1 KO HIEs following seeding. Each symbol represents a single HIE. *, $P < 0.05$, Student t test.

A



B

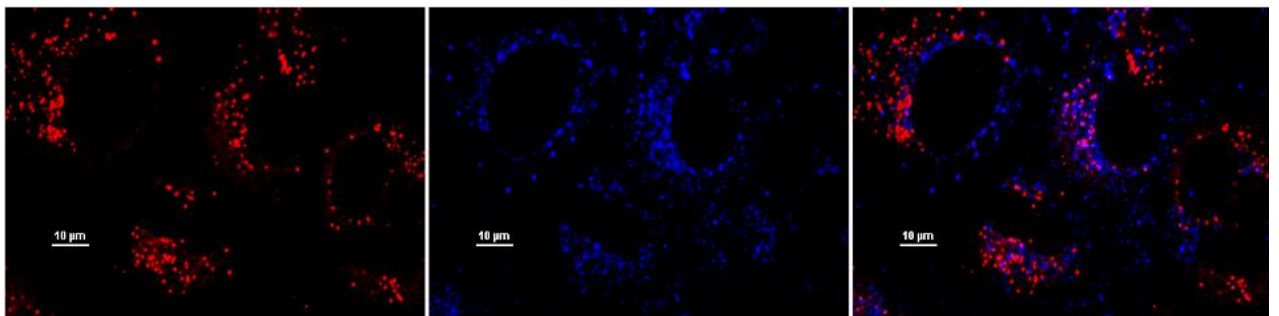


Fig. S4. Viroplasm-associated neutral lipid accumulates in cells prior to infection. MA104 cells were metabolically labelled with BODIPY™ 558/568 C12 (red) (A) 1 hour prior to RV infection and imaged 3 hpi (asterisks indicate infected cells) or (B) 2-3 hpi (all cells are in the confocal images are infected). Cells were fixed, permeabilized and imaged at 3 hpi. Viroplasms were detected using guinea pig antibody against NSP5 (blue). Bar, 10 µm.

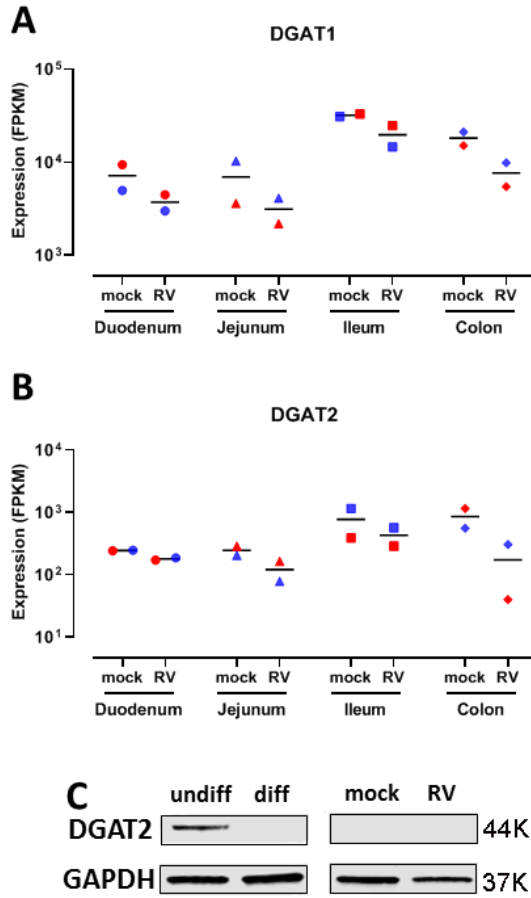


Fig. S5. RNAseq and protein expression levels of DGAT1 and DGAT2 in human intestinal enteroids. Enteroids derived from different intestinal segments (duodenum, circles; jejunum, triangles; ileum, squares; colon, diamonds) from two different individuals indicated in red and blue were mock- or human RV-infected and examined by RNAseq. FPKM (fragments per kilobase of exon per million mapped fragments) values for (A) DGAT1 and (B) DGAT2 at 24 hpi were plotted for each condition and HIE cultures derived from each segment of the small or large intestine. (C) Western blot analysis of DGAT2 expression in undifferentiated (undiff) and differentiated (diff) HIEs (left panel), and mock- and RV-infected differentiated HIEs (right panel). GAPDH is detected as a loading control. The primary antibody was detected with secondary antibody conjugated to IRDye680RD and IRDye800CW. Infrared images of the immunoblot were acquired using the Odyssey CLx and analyzed using Image Studio Lite software. Molecular weights are indicated to the right of the blot.

SI References

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