

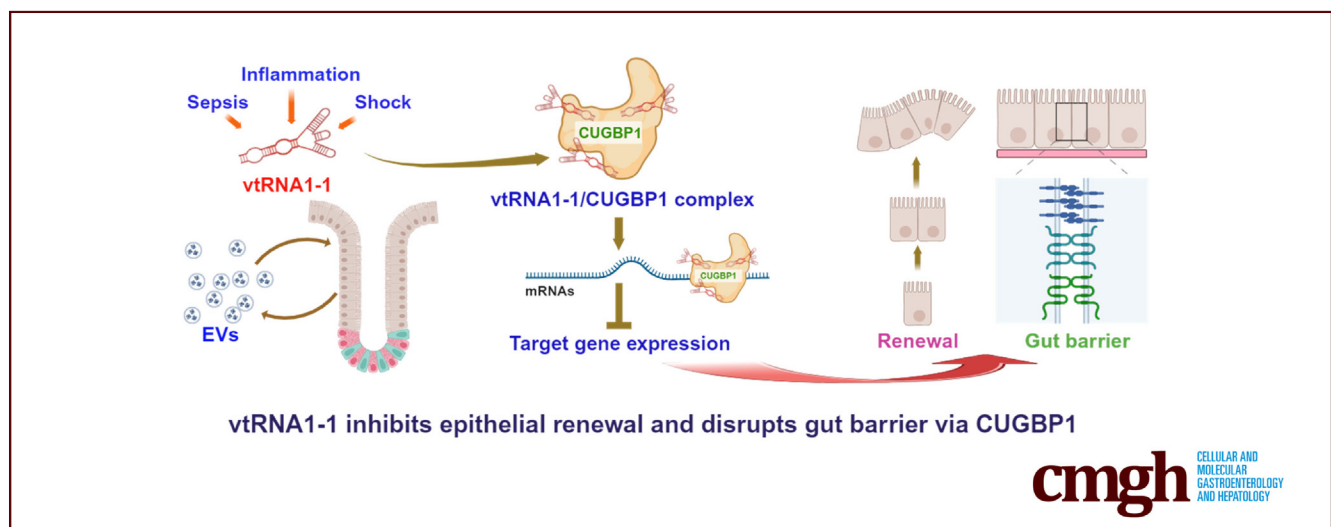
ORIGINAL RESEARCH

Noncoding Vault RNA1-1 Impairs Intestinal Epithelial Renewal and Barrier Function by Interacting With CUG-binding Protein 1



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SUMMARY

We investigated the role of noncoding vault RNAs in intestinal epithelium homeostasis. Our results demonstrate that increased levels of *vtRNA1-1*, either in extracellular vesicles of serum or mucosal tissues, disrupts epithelial renewal and barrier function via interaction with CUG-binding protein 1.

BACKGROUND & AIMS: Small noncoding vault RNAs (vtRNAs) are involved in many cell processes important for health and disease, but their pathobiological functions in the intestinal epithelium are underexplored. Here, we investigated the role of human *vtRNA1-1* in regulating intestinal epithelial renewal and barrier function.

METHODS: Studies were conducted in *vtRNA1-1* transgenic (*vtRNA1-1*Tg) mice, primary enterocytes, and Caco-2 cells. Extracellular vesicles (EVs) were isolated from the serum of shock patients and septic mice. Intestinal organoids (enteroids) were prepared from *vtRNA1-1*Tg and littermate mice. Mucosal growth was measured by Ki67 immunostaining or BrdU

incorporation, and gut permeability was assessed using the FITC-dextran assay.

RESULTS: Intestinal tissues recovered from shock patients and septic mice evidenced mucosal injury and gut barrier dysfunction; vtRNA levels were elevated in EVs isolated from their sera. In mice, intestinal epithelial-specific transgenic expression of *vtRNA1-1* inhibited mucosal growth, reduced Paneth cell numbers and intercellular junction (IJ) protein expression, and increased gut barrier vulnerability to lipopolysaccharide exposure. Conversely, *in vitro* silencing of *vtRNA1-1* increased IJ protein levels and enhanced epithelial barrier function. Exposing enteroids to *vtRNA1-1*-rich EVs augmented paracellular permeability. Mechanistically, *vtRNA1-1* interacted with CUG-binding protein 1 (CUGBP1) and increased CUGBP1 association with *claudin-1* and *occludin* mRNAs, thereby inhibiting their expression.

CONCLUSIONS: These findings indicate that elevated levels of *vtRNA1-1* in EVs and mucosal tissues repress intestinal epithelial renewal and barrier function. Notably, this work reveals a novel role for dysregulation of the *vtRNA1-1*/CUGBP1 axis in the pathogenesis of gut mucosal disruption in critical illness. (*Cell Mol Gastroenterol Hepatol* 2025;19:101410; <https://doi.org/10.1016/j.jcmgh.2024.101410>)

Keywords: Gut Permeability; Mucosal Growth; Paneth Cells; RNA-binding proteins; Small Noncoding RNAs.

The mammalian intestinal epithelium is a specialized domain that interacts with and protects sub-epithelial tissue against luminal noxious substances and the gut microbiome.^{1,2} The maintenance of intestinal epithelium homeostasis is a dynamic process that requires epithelial cells to alter gene expression patterns quickly to modulate apoptosis, proliferation, migration, differentiation, and cell-to-cell interactions.^{3,4} Disrupted intestinal epithelial integrity is common in critical disorders (eg, shock, sepsis, major trauma, thermal injury, and extensive surgery), resulting in epithelial barrier dysfunction.⁵ The resulting 'leaky' gut permits the translocation of luminal toxic substances and bacteria and their metabolites into the bloodstream and, in some instances, leads to multiple organ dysfunction syndrome and death.^{6,7} Effective therapies to preserve intestinal epithelial integrity and protect gut barrier function are limited, primarily because of incomplete understanding of the mechanisms underlying mucosal disruption in critical illness.

Emerging evidence indicates that control of noncoding RNAs (ncRNAs) and RNA-binding proteins (RBPs) plays an important role in maintaining intestinal epithelial homeostasis in stressful environments, whereas dysregulation of specific ncRNAs and RBPs impairs mucosal renewal, compromises epithelial host defenses, and thereby disrupts intestinal barrier function.^{4,8-10} However, little is known regarding how dysregulated ncRNAs and RBPs in intestinal epithelial cells (IECs) in the critically ill impact the function of neighboring and remote tissues. Intercellular communication directed by the secretion of extracellular vesicles (EVs) is gaining increasing attention.^{11,12} EVs released from many cell types, including IECs, can transfer a variety of bioactive molecules to neighboring or distant tissues, thereby playing previously unrecognized functional roles.^{13,14} The involvement of IEC-derived EVs and their cargo ncRNAs in regulating intestinal epithelial integrity is particularly important in critically ill surgical patients because they often exhibit systemic gut barrier dysfunction rather than only localized changes in paracellular permeability.^{6,8}

Vault RNAs (vtRNAs), small (~100 nucleotide) ncRNAs transcribed by RNA polymerase III, are highly conserved across mammalian genomes. Humans produce 4 vtRNA paralogs, *vtRNA1-1*, *vtRNA1-2*, *vtRNA1-3*, and *vtRNA2-1*, whereas mice express only 1 vtRNA.¹⁵ vtRNAs are associated with giant cytoplasmic ribonucleoprotein (RNP) particles termed vaults and commonly identified in EVs.^{12,16} Moreover, vtRNAs play important roles outside of vault RNPs and can function independently of vault particles.^{15,17} vtRNAs are implicated in many cellular processes such as mRNA splicing, nuclear transport, drug resistance, synaptogenesis, lysosome function, apoptosis, influenza virus replication, and tumorigenesis,^{15,18-23} although only about 5% of total cellular vtRNA is incorporated into vaults.¹⁸ The 4 human vtRNAs differ only slightly in their primary and second structures, but they have distinct biological functions via different targets and mechanisms.^{15,17} For example, *vtRNA1-1* selectively regulates autophagy by interacting directly with RBP p62,¹⁷ whereas *vtRNA2-1* acts as a tumor suppressor by associating with

protein kinase R in a wide range of cancer cells.^{23,24} Expression of *vtRNA1-1* is often upregulated in cancer cells due to dysregulation of oncogenic signals including PI3K/Akt and MAPK pathways and plays an important role in cancer progression by altering apoptosis, autophagy, and sensitivity to chemotherapy.²⁰ The levels of *vtRNA1-1* also increase after infection with the influenza A virus, which enhances viral replication by suppressing PKR-mediated innate immunity.²⁵ Although *vtRNA2-1* is involved in regulating gut barrier function via interaction with HuR,²⁶ the exact role of *vtRNA1-1* and specifically its presence in EVs remains unknown in the context of intestinal epithelial homeostasis and dysfunction.

In this study, we provide evidence that *vtRNA1-1* modulates intestinal epithelial homeostasis by interacting with CUG-binding protein 1 (CUGBP1). We found that EVs isolated from the serum of hemorrhagic shock patients exhibited increased vtRNAs, including *vtRNA1-1*, that were associated with disrupted gut epithelial integrity. Transgenic expression of human *vtRNA1-1* in mice inhibited small intestinal mucosal growth and increased gut barrier vulnerability to stress. Our results further show that exposing intestinal organoids (enteroids) to *vtRNA1-1*-rich EVs increased paracellular permeability *ex vivo*. Moreover, *vtRNA1-1* interacted with and enhanced CUGBP1 binding activity, thus inhibiting the expression of tight junction (TJ) proteins such as claudin-1 and occludin. These findings reveal that *vtRNA1-1* functions as a repressor of intestinal epithelial renewal and barrier function and identify the *vtRNA1-1*/CUGBP1 axis as a novel therapeutic target for interventions to promote intestinal mucosal regeneration and enhance gut barrier function in the critically ill.


Results

vtRNAs Are Enriched in EVs From Shock Patients and Septic Mice

To explore the involvement of EV vtRNAs in mediating the disrupted integrity of the intestinal epithelium in the critically ill, blood samples were collected from healthy

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Abbreviations used in this paper: AJ, adherens junction; ANOVA, analysis of variance; CLP, cecal ligation and puncture; C-siRNA, control small interfering RNA; CUGBP1, CUG-binding protein 1; ddPCR, droplet digital real-time PCR; EVs, extracellular vesicles; FITC, fluorescein isothiocyanate; H&E, hematoxylin and eosin; IECs, intestinal epithelial cells; IP, immunoprecipitation; lncRNA, long ncRNAs; LPS, lipopolysaccharide; miR, microRNA; mvRNA, mouse vtRNA; ncRNAs, noncoding RNAs; NSP, NanoSight particle; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Q, quantitative; RBPs, RNA-binding proteins; RIP, RNP immunoprecipitation; RNP, ribonucleoprotein; RT, reverse transcription; SDS, sodium dodecyl sulfate; SED, standard error of difference; SEM, standard error of the mean; siHuR, small interfering RNA targeting HuR; siRNA, small interfering RNA; si-vtR1-1, small interfering RNA targeting vtRNA1-1; TEER, transepithelial electrical resistance; TJ, tight junction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; vtRNAs, vault RNAs; vtRNA-Tg, vtRNA1-1 transgenic.

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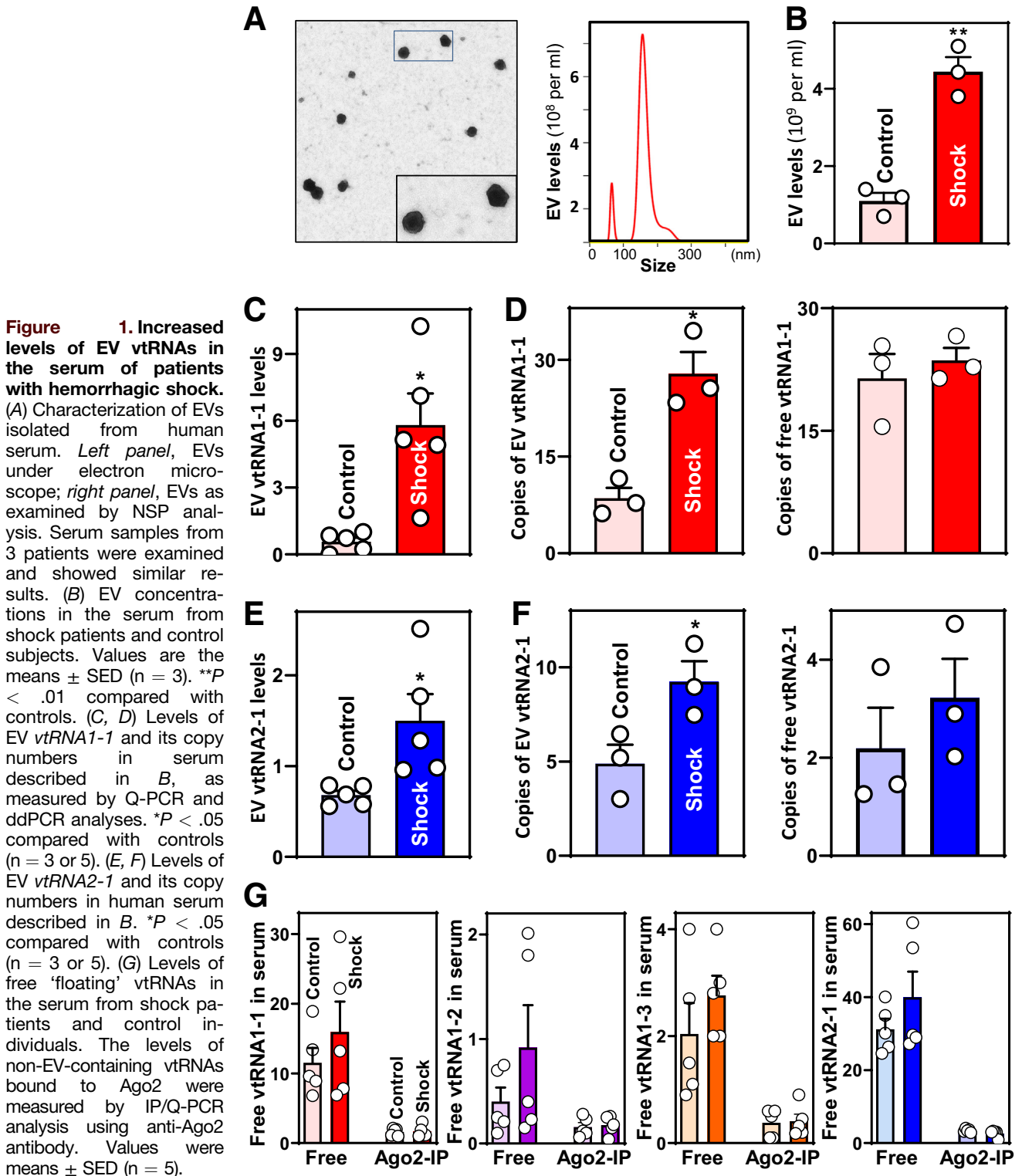
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<https://doi.org/10.1016/j.jcmgh.2024.101410>

subjects (controls) and patients with hemorrhagic shock (systolic blood pressure < 90 mmHg), a condition associated with intestinal mucosal injury and gut barrier dysfunction. EVs were isolated using protocols described previously^{27,28} and collected as pellets resuspended in

phosphate-buffered saline (PBS). The presence of EVs from human serum was confirmed by electron microscopy and NanoSight particle (NSP) analysis that revealed 50- to 200-nm diameter particles (Figure 1A). Interestingly, compared with control EVs, those isolated from the serum of shock



patients were both more concentrated (Figure 1B) and contained more *vtRNA1-1* and *vtRNA2-1* (Figure 1C, E). In contrast, the levels of EV *vtRNA1-2* and *vtRNA1-3* were low in EVs and exhibited no significant differences between the 2 groups. Droplet digital real-time PCR (ddPCR) analysis showed that copy numbers of EV *vtRNA1-1* and *vtRNA2-1* increased dramatically in subjects with hemorrhagic shock relative to controls (Figure 1D and F, left panels). In this experiment, for quantification, *vtRNA1-1* and *vtRNA2-1* levels were measured in at least 10,000 droplets/well of EV sample. We also examined changes in the levels of several microRNAs including (miR)-29b, miR-195, and miR-222 and long ncRNAs (lncRNAs) such as *uc.173*, *uc.230*, *H19*, and *SPRY4-IT1* in EVs isolated from serum of shock patients and controls, because these ncRNAs play an important role in the intestinal epithelium homeostasis and gut barrier function.^{4,29–35} Our results revealed no meaningful differences in the levels of EV miR-29b, miR-195, and miR-222 between shock and control subjects, and in EVs from both groups the levels of lncRNAs *uc.173*, *uc.230*, *H19*, and *SPRY4-IT1* were below the limits of detection.

We examined and compared ‘free floating’ (non-EV-containing) vtRNAs in serum from shock patients and control subjects and found no significant differences in the levels of any of the 4 human vtRNAs (Figure 1D and F, right panels; G). Non-EV-containing vtRNAs bound to Ago2 were also detectable in the serum, but the levels of these Ago2-bound vtRNAs in shock patients were indistinguishable from levels in control samples.

Consistent with observations in humans, mice exposed to septic stress induced by cecal ligation and puncture (CLP) for 24 h also exhibited increased serum levels of EV *vtRNA* (Figure 2A, left), associated with increased gut permeability; the latter had been reported previously.³⁶ CLP did not alter the abundance of free *vtRNA* in serum (Figure 2B) but did increase the levels of small intestinal mucosal *vtRNA*, as in our previous report.²⁶ Because targeted deletion of the *H19* gene in mice protects the gut barrier against CLP-induced injury,²⁹ we examined changes in EV *vtRNA* in *H19*-

deficient mice and found significantly reduced levels of EV *vtRNA* after CLP compared with littermate controls (Figure 2A, right). These findings strongly implicate vtRNA-containing EVs, but not ‘free floating’ serum vtRNAs, in gut mucosal pathology in critical surgical disorders.

vtRNA1-1 Inhibits Epithelial Renewal in Small Intestine and Compromises Gut Barrier Function

Although *vtRNA2-1* down-regulates gut epithelial barrier function,²⁶ the exact role of *vtRNA1-1* in intestinal epithelial homeostasis remains unknown. To examine the *in vivo* function of *vtRNA1-1* in the intestinal epithelium, we utilized a gain-of-function transgenic approach and generated mice that highly express human *vtRNA1-1* (*vtRNA1-1Tg*). The regulatory elements selected to drive *vtRNA1-1* expression were derived from the A33 *antigen* gene because of its temporally and spatially restricted expression pattern.³⁴ As described previously,^{37,38} we used the A33 promoter to drive intestinal epithelial tissue-specific expression of *vtRNA1-1*. The A33-*vtRNA1-1* transgene construct (Figure 3A) was microinjected into the male pronucleus of fertilized oocytes from mice on a pure C57BL/6 genetic background. A total of 6 transgenic founders was generated; however, only 2 (mouse #3 and #5) transmitted the transgene to their progeny. Male and female, 3- to 4-month-old *vtRNA1-1Tg* and littermate control mice were used for phenotype comparisons. As measured by quantitative real-time polymerase chain reaction (Q-PCR) analysis, *vtRNA1-1Tg* mice exhibited specific *vtRNA1-1* overexpression in intestinal mucosal tissues (Figure 3B), but not in tissues from heart, lung, liver, kidney, and spleen. Transgenic expression of human *vtRNA1-1* in mice did not alter the levels of endogenous mouse *vtRNA* (*mvRNA*) in the small intestinal mucosa (Figure 3C). *vtRNA1-1Tg* mice appeared grossly normal, and there were no significant differences in gastrointestinal gross morphology between *vtRNA1-1Tg* mice and littermates, although *vtRNA1-1Tg* mouse body weight was slightly less than that of control mice at 8 weeks after birth.

Importantly, *vtRNA1-1Tg* mice (the line generated from founder #3) displayed substantial inhibition of small intestinal mucosal growth, marked by histological features and the proliferating crypt cell population. As shown in Figure 3D, crypts and villi in the small intestinal mucosa of *vtRNA1-1Tg* mice were substantially diminished relative to those from control littermates. Quantification of these reductions indicated that the lengths of crypts and villi in the mucosa of *vtRNA1-1Tg* mice were decreased by ~25% and ~35%, respectively. Consistently, the numbers of Ki67-positive cells decreased by ~58% in *vtRNA1-1Tg* mice compared with those observed in control littermates (Figure 3E). Increasing *vtRNA1-1* levels in the intestinal epithelium also impacted Paneth cells (Figure 3D). Staining of whole mounts of the small intestine demonstrated that in control littermate mice, lysozyme-positive Paneth cells were located at crypt bases; compared with littermate controls, Paneth cell numbers were reduced by ~36% in the small intestinal mucosa of *vtRNA1-1Tg* mice. However, increased

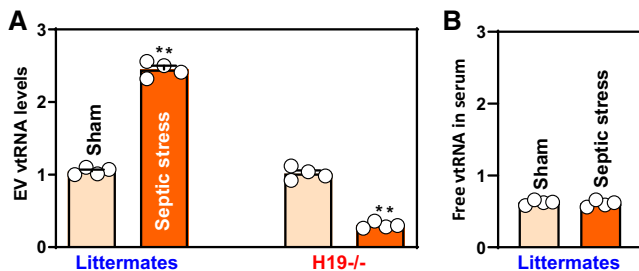


Figure 2. Effect of septic stress on the levels of EV *vtRNA* in mice. (A) Levels of EV *vtRNA* in littermate and *H19*-deficient (*H19*^{-/-}) mice after exposure to septic stress induced by CLP. Serum was harvested 24 hours after CLP, and EVs were isolated. Values are the means \pm SED ($n = 4$). $**P < .01$ compared with sham. (B) Levels of free ‘floating’ *vtRNA* in the serum of littermate mice described in A. In all experiments, statistical significance was analyzed using unpaired, 2-tailed Student’s *t*-test.

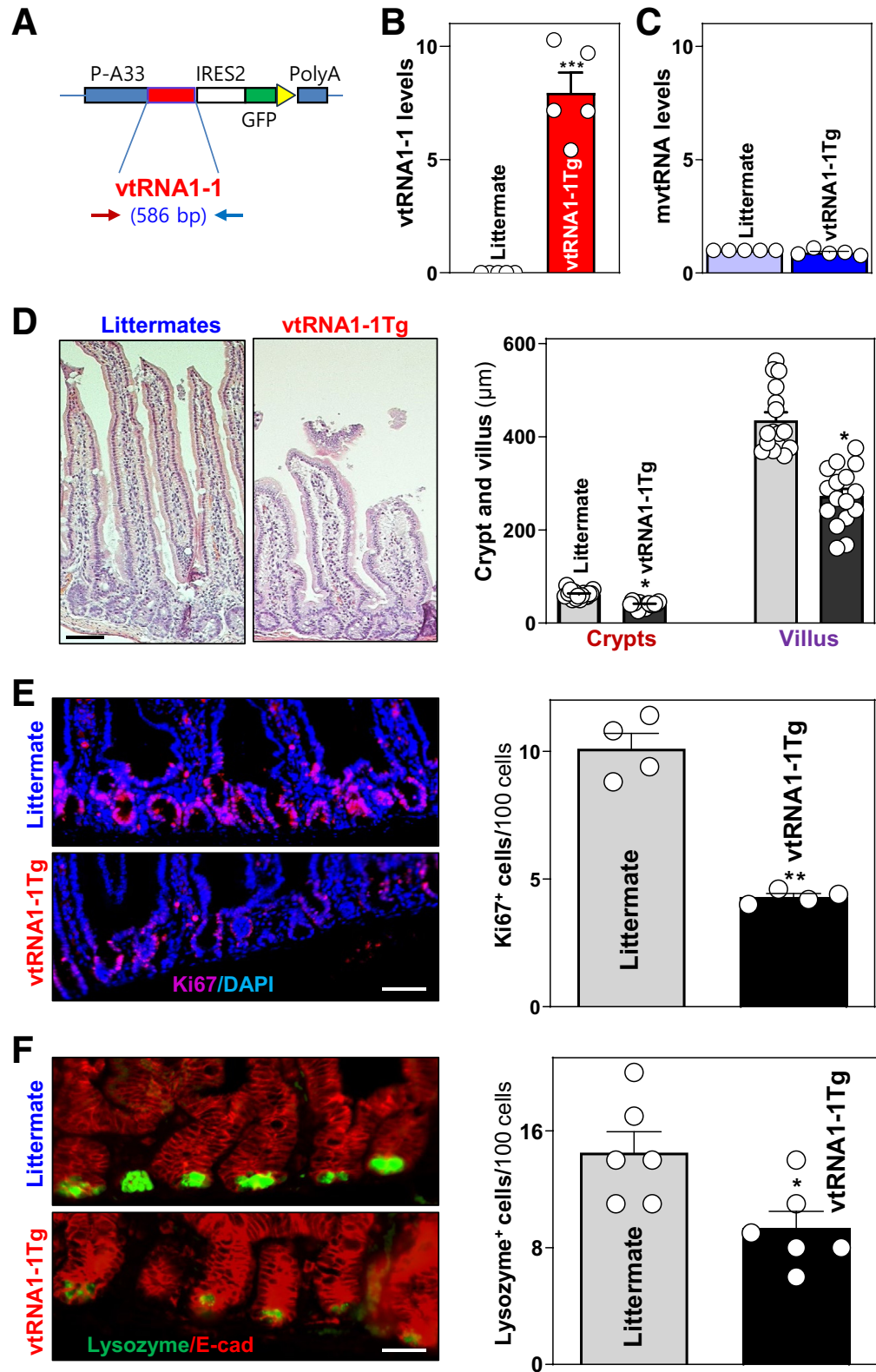


Figure 3. Transgenic expression of the *vtRNA1-1* inhibits mucosal renewal and impairs Paneth cell function in the small intestine of mice. (A) The A33/vtRNA1-1/GFP construct used to be injected into fertilized oocytes for generating vtRNA1-1Tg mice. (B) Levels of mucosal vtRNA1-1 in littermate control and vtRNA1-1Tg mice as measured Q-PCR analysis. Values are the means \pm SEM (n = 5). *** P < .001 compared with littermate controls. (C) Levels of mouse vtRNA (mvtRNA) in the small intestinal mucosa described in B. (D) Photomicrographs of hematoxylin and eosin (left) and changes in the length of villi and crypts (right) of the mucosa described in B. Scale bars: 25 μ m. * P < .05 compared with littermates. (E) Proliferating cells in small intestinal crypts as measured by Ki67 immunostaining (left). Red, Ki67; blue, DAPI. Right panel, quantitative data of Ki67-positive cells. ** P < .01 compared with littermates. (F) Changes in Paneth cells (lysozyme-positive cells) in the mucosa described in B. * P < .05 compared with littermates. Statistical significance was analyzed using unpaired, 2-tailed Student's t -test.

vtRNA1-1 levels failed to alter small intestinal enterocyte differentiation as determined by villin immunostaining analysis and did not alter growth of colonic mucosa.

Intestinal epithelium-specific transgenic expression of *vtRNA1-1* in mice reduced the expression of small intestinal mucosal TJ and adherens junction (AJ) proteins. As shown

in Figure 4A (left), the small intestinal mucosa of vtRNA1-1Tg mice exhibited decreased levels of several TJ proteins, including claudin-1, claudin-2, claudin-3, claudin-7, occludin, JAM-A, and ZO-2, and AJ protein E-cadherin. Locally increasing the levels of vtRNA1-1 in the intestinal epithelium also decreased the levels of the proliferation-associated protein PCNA (Figure 4A, right-top). Because autophagy is crucial for host intestinal epithelial defense against invasive pathogens and to maintain gut barrier function,^{39,40} we examined changes in the levels of autophagy-related proteins in the intestinal epithelium of vtRNA1-1Tg mice. Unexpectedly, transgenic expression of vtRNA1-1 in the intestinal epithelium increased the abundances of mucosal ATG-7, ATG-16L1, LC3B I/II, and beclin-1 proteins (Figure 4A, right). Although the exact roles of altered levels of mucosal autophagy-associated proteins in vtRNA1-1Tg mice remain unknown, this upregulation of autophagy likely represents a compensatory mechanism to maintain the intestinal epithelial barrier function following reduction in the levels of TJ/AJ proteins. In addition, transgenic expression of vtRNA1-1 failed to alter the content of HSC70 and p62 proteins in the intestinal mucosa. We examined another line of vtRNA1-1Tg mice generated from an independent founder (founder #5) and showed similar decreases in intestinal epithelial renewal and the levels of TJ and AJ proteins.

Transgenic expression of vtRNA1-1 in mice did not alter baseline gut permeability as measured by fluorescein isothiocyanate (FITC)-conjugated dextran assays (Figure 4B), but it increased vulnerability of the gut barrier in response to the bacterial product lipopolysaccharide (LPS). There was a significant increase in gut permeability in vtRNA1-1Tg mice compared with littermates after exposure to the same doses of LPS (Figure 4C). Treatment of littermate mice with a low dose of LPS (0.1 mg/kg) for 5 days failed to alter gut permeability but induced remarkable gut barrier dysfunction in vtRNA1-1Tg mice. In addition, transgenic vtRNA1-1 expression did not induce apoptosis in the intestinal epithelium, as no significant increase in cell death was observed in vtRNA1-1Tg mice. As measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, rates of apoptotic cell death were <2% in both littermate control and vtRNA1-1Tg mice.

As another approach to gauge the impact of transgenic vtRNA1-1 expression on gut epithelial homeostasis, we examined the growth of enteroids generated from vtRNA1-1Tg and littermate control mice. Using methods described previously,⁴¹ enteroids were derived from proliferating crypts of the mucosa of the mouse small intestine. Enteroids derived from both littermate control and vtRNA1-1Tg mice consisted of multiple buds and cells on day 4 after initial culture. However, enteroids derived from vtRNA1-1Tg mice grew much slower than those from littermate mice, as evidenced by a significant decrease in enteroid surface area (Figure 5A) and BrdU incorporation (Figure 5B, top) in enteroids derived from vtRNA1-1Tg mice compared with those generated from control littermates. In agreement with this observation, enteroids derived from vtRNA1-1Tg mice also exhibited reduced numbers of Paneth cells (Figure 5B,

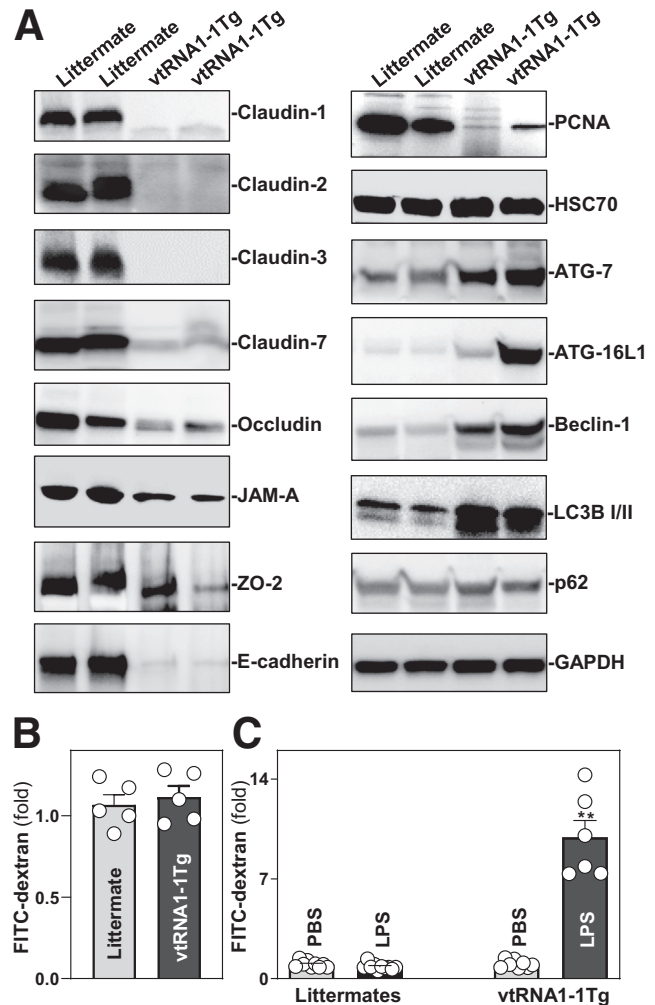


Figure 4. Transgenic expression of vtRNA1-1 decreases the levels of intercellular junction proteins and increases vulnerability of the gut barrier to LPS. (A) Changes in the levels of tight junctions claudin 1, occludin, ZO-2, and JAM-A, adherens junction E-cadherin, and autophagy-associated proteins in the small intestinal mucosa of littermate and vtRNA1-1Tg mice. GAPDH immunoblotting was performed as an internal control for equal loading. (B) Gut permeability in the mice described in A. FITC dextran was given orally, and blood samples were collected 4 hours thereafter for measurement. Values are the means \pm SEM ($n = 5$). (C) Increased vulnerability of the gut barrier in response to LPS. Mice were intraperitoneally injected with LPS (0.1 mg/kg) in 200 μ l PBS once daily for 5 days. Gut permeability was measured 24 hours after treatment with the last dose of LPS. Values are the means \pm SEM ($n = 6$ or 8). ** $P < .01$ compared with PBS or littermate controls exposed to LPS. In B and C, statistical significance was analyzed using unpaired, 2-tailed Student's t -test except for results in A. All experiments in A were repeated 3 times with similar results.

bottom). Because enteroids lack stromal, immune, and neural cells, these results indicate that the disrupted renewal of intestinal epithelial cells in vtRNA1-1Tg mice results primarily from an epithelial cell-autonomous regulatory effect of this vtRNA and not from the effects of secreted factors from other cell types in the gut. Together,

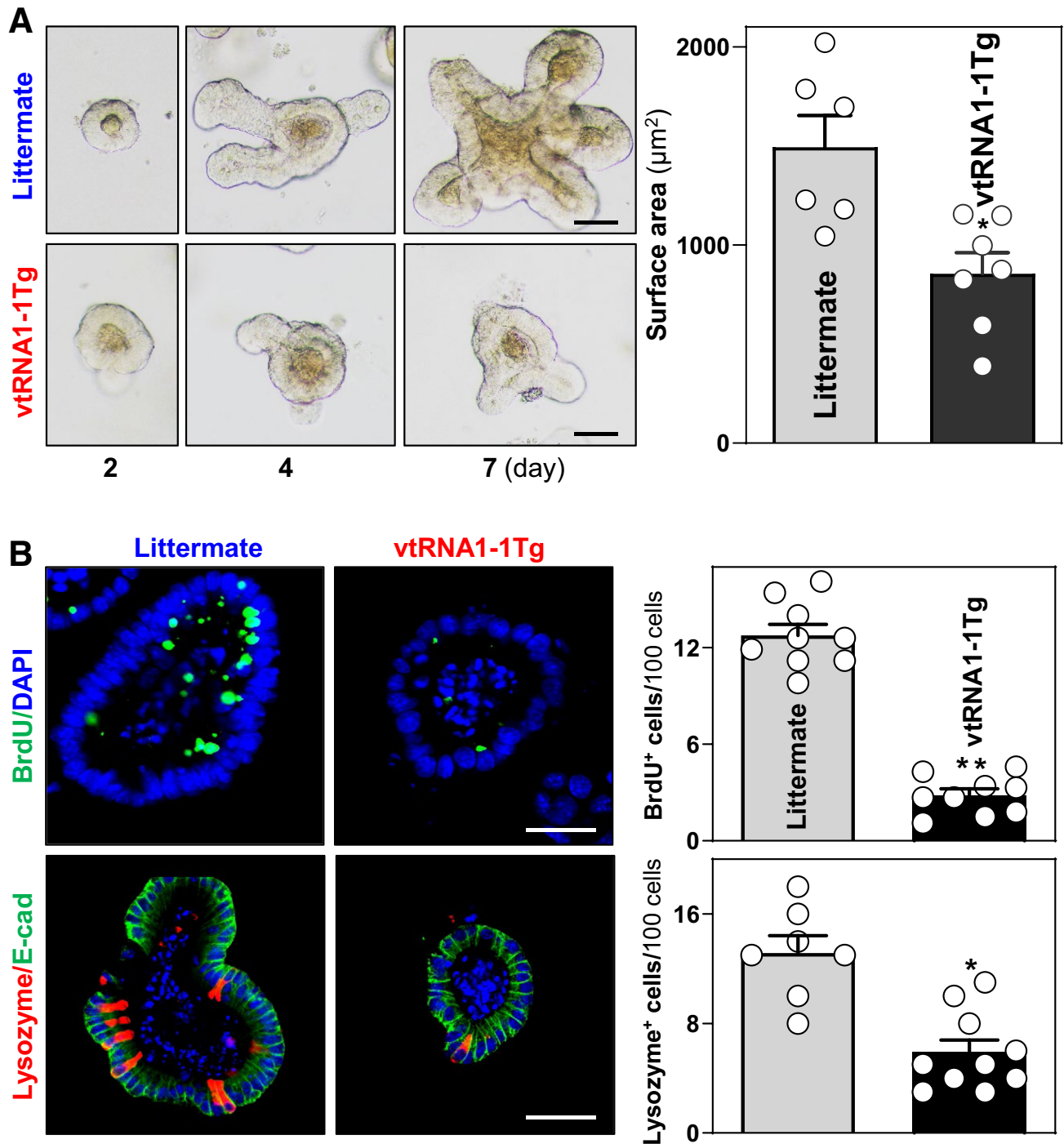


Figure 5. Increased *vtRNA1-1* inhibits the growth of enteroids and disrupts Paneth cell function *ex vivo*. (A) Growth of enteroids derived from the proximal small intestine of littermate control and *vtRNA1-1Tg* mice. Images were taken at different times after culture. Scale bars: 50 μ M. Values are the means \pm SEM ($n = 6$ or 7). $*P < .05$ compared with littermate controls. (B) Proliferating cells (*top*) and Paneth cells (*bottom*) in enteroids on day 3 after culture, as measured by BrdU labeling and immunostaining of lysozyme as marker of Paneth cells. $*P < 0.05$; $**P < .01$ compared with littermate controls. In all experiments, statistical significance was analyzed using unpaired, 2-tailed Student's *t*-test.

the results from these *in vivo* and *ex vivo* experiments strongly support the concept that *vtRNA1-1* is a negative regulator of intestinal epithelial renewal and gut barrier function.

vtRNA1-1 Decreases Intercellular Junctions and Impairs Epithelial Barrier Function In Vitro

To further define the role of *vtRNA1-1* in the regulation of gut barrier function, 2 sets of experiments were

performed in Caco-2 cells. First, we examined the effect of *vtRNA1-1* silencing on TJ/AJ protein expression and epithelial barrier function. The levels of cellular *vtRNA1-1* decreased specifically 48 hours after transfection with small interfering RNA (siRNA) targeting *vtRNA1-1* (si-*vtR1-1*); there were no significant differences in the levels of *vtRNA1-2*, *vtRNA1-3*, and *vtRNA2-1* between cells transfected with si-*vtR1-1* or control siRNA (Figure 6A). This decrease in *vtRNA1-1* levels by si-*vtR1-1* transfection increased the abundances of claudin-1, claudin-2, claudin-3, claudin-7, and occludin, although it failed to alter the levels of JAM-A, ZO-2, and E-cadherin (Figure 6B). Importantly, *vtRNA1-1* silencing enhanced epithelial barrier function, as revealed by an increase in transepithelial electrical resistance (TEER) (Figure 6C) and a decrease in the levels of paracellular flux of FITC-dextran (Figure 6D). Transfection with si-*vtR1-1* did not alter cell viability.

Second, we examined the influence of increased *vtRNA1-1* levels on the expression of TJ and AJ proteins. Transfection of a plasmid expressing *vtRNA1-1* under control of the pCMV promoter (pcDNA3.1 backbone) increased the levels of cellular *vtRNA1-1* but did not alter the abundances of *vtRNA1-2*, *vtRNA1-3*, and *vtRNA2-1* (Figure 7A). Ectopically expressed *vtRNA1-1* decreased the levels of TJs claudin-1, claudin-2, claudin-3, claudin-7, occludin, JAM-A, and ZO-2 (Figure 7B) but did not alter E-cadherin expression levels. Moreover, elevating *vtRNA1-1* levels disrupted epithelial barrier function because it decreased TEER (Figure 7C) and increased paracellular flux of FITC-dextran (Figure 7D). These results indicate that *vtRNA1-1* impairs intestinal epithelial barrier function by lowering the levels of several TJ proteins.

vtRNA-rich EVs Increase Enteroid Paracellular Permeability

To elucidate the role of EV *vtRNA1-1* in regulating intestinal epithelial barrier function, we microinjected FITC-dextran into 3-dimensional enteroids as described previously.^{42,43} To harvest *vtRNA*-rich EVs from IECs, Caco-2 cells were transfected with the vector expressing *vtRNA1-1* or *H19* or with siRNA specifically targeting HuR (siHuR). Ectopically overexpressed *H19* and HuR silencing were also chosen to generate *vtRNA*-rich EVs from Caco-2 cells, because *H19* knockout decreased EV *vtRNA* in mice exposed to CLP (Figure 2A), and because targeted deletion of HuR from the intestinal epithelium inhibits autophagy that modulates EV release.^{2,10} The levels of *vtRNA1-1* increased dramatically in EVs isolated from conditional media incubated with cells overexpressing *vtRNA1-1* or *H19* and with HuR-silenced cells for 48 h (Figure 8A, left). The levels of *vtRNA2-1* increased only in EVs from the conditional media incubated with cells transfected with *H19* expression vector or siHuR but not from cells overexpressing *vtRNA1-1* (Figure 8A, right). The levels of *vtRNA1-2* and *vtRNA1-3* were low or undetectable in EVs isolated from all 3 conditional media incubated with cells overexpressing *vtRNA1-1* or *H19* and with HuR-silenced cells. On the other hand, EVs isolated from the medium

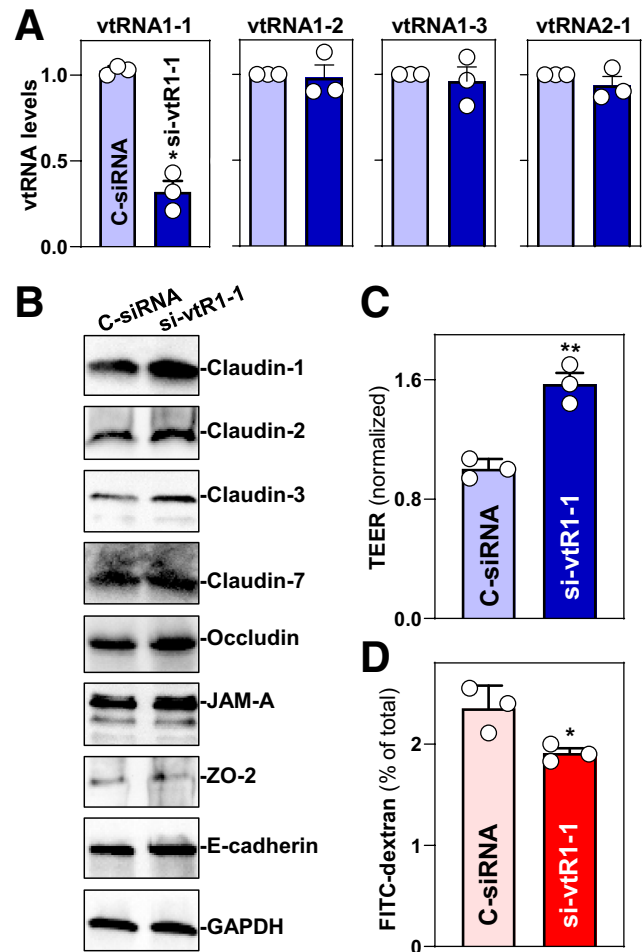


Figure 6. *vtRNA1-1* silencing improves intestinal epithelial barrier function *in vitro*. (A) Levels of *vtRNAs* in Caco-2 cells 48 hours after transfection with si-*vtR1-1*. Values are the means \pm SEM ($n = 3$). * $P < .05$ compared with C-siRNA. (B) Immunoblots of claudins, occludin, ZO-2, JAM-A, and E-cadherin in cells described in A. GAPDH immunoblotting was performed as an internal control for equal loading. Three separate experiments were performed and showed similar results. (C, D) Changes in TEER and FITC-dextran paracellular permeability in cells described in A. TEER assays were performed on 12-mm Transwell filters; paracellular permeability was assayed by adding the membrane-impermeable trace molecule FITC-dextran to the insert medium. * $P < .05$; ** $P < .01$ compared with C-siRNA. In A, C, and D, statistical significance was analyzed using unpaired, 2-tailed Student's *t*-tests.

incubated with cells transfected with control vector or control siRNA (C-siRNA) did not alter the levels of EV *vtRNA1-1* and served as controls.

To test the function of EV *vtRNAs* *ex vivo*, enteroids were seeded in a basement matrix, embedded in standard growth medium for 2 days, and then cultured in medium containing EVs at concentrations of 50 ng/ μ l protein, equivalent to the constant EV number 1×10^7 as measured by NSP analysis. Twenty-four hours after administering EVs, FITC-dextran in 2 μ l PBS was microinjected into the enteroid lumen (Figure 8B). Paracellular permeability was examined at

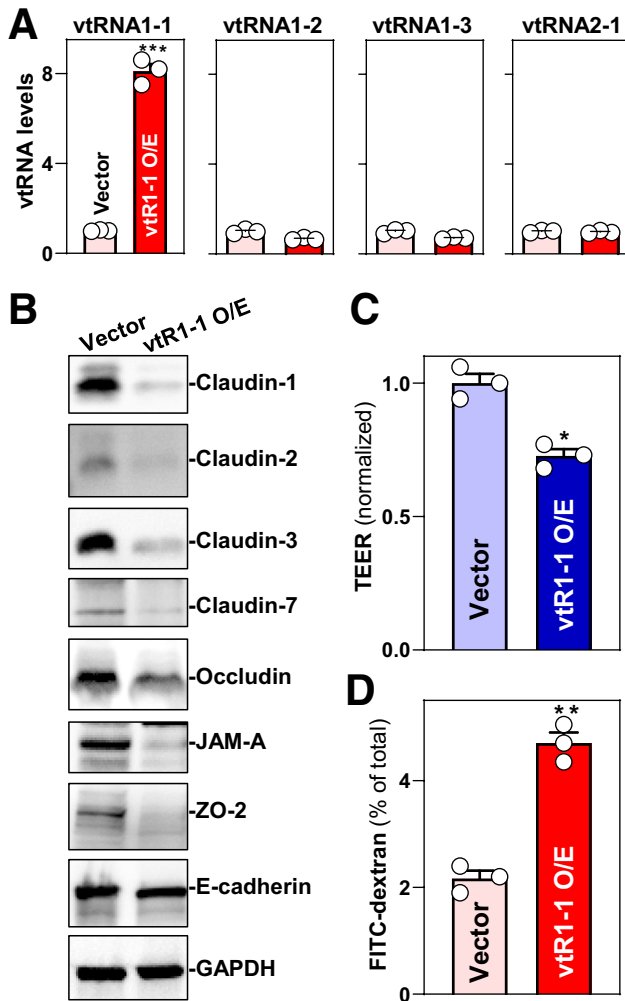


Figure 7. Ectopically expressed *vtRNA1-1* disrupts intestinal epithelial barrier function *in vitro*. (A) Levels of *vtRNAs* in Caco-2 cells 48 h after transfection with the *vtRNA1-1* expression vector (*vtR1-1*). Values are the means \pm SEM ($n = 3$). *** $P < .001$ compared with control vector. (B) Immunoblots of claudins, occludin, ZO-2, JAM-A, and E-cadherin in cells described in A. GAPDH immunoblotting was performed as an internal control for equal loading. Three separate experiments were performed and showed similar results. (C, D) Changes in TEER and FITC-dextran paracellular permeability in cells described in A. * $P < .05$; ** $P < .01$ compared with control vector. In A, C, and D, statistical significance was analyzed using unpaired, 2-tailed Student's *t*-tests.

different times after microinjection. As shown, treatment of enteroids with EVs from *vtRNA1-1* overexpressed cells increased paracellular permeability significantly, as evidenced by rapidly decreasing luminal FITC intensity in enteroids exposed to *vtRNA1-1*-rich EVs, compared with EVs from control cells (transfected with control vector) (Figure 8C, D). Exposure to EVs from cells overexpressing *H19* or *HuR*-silenced cells also increased paracellular permeability in enteroids, which exhibited a similar pattern in the decrease of luminal FITC intensity after microinjection of FITC-dextran (Figure 8E, F). These results suggest

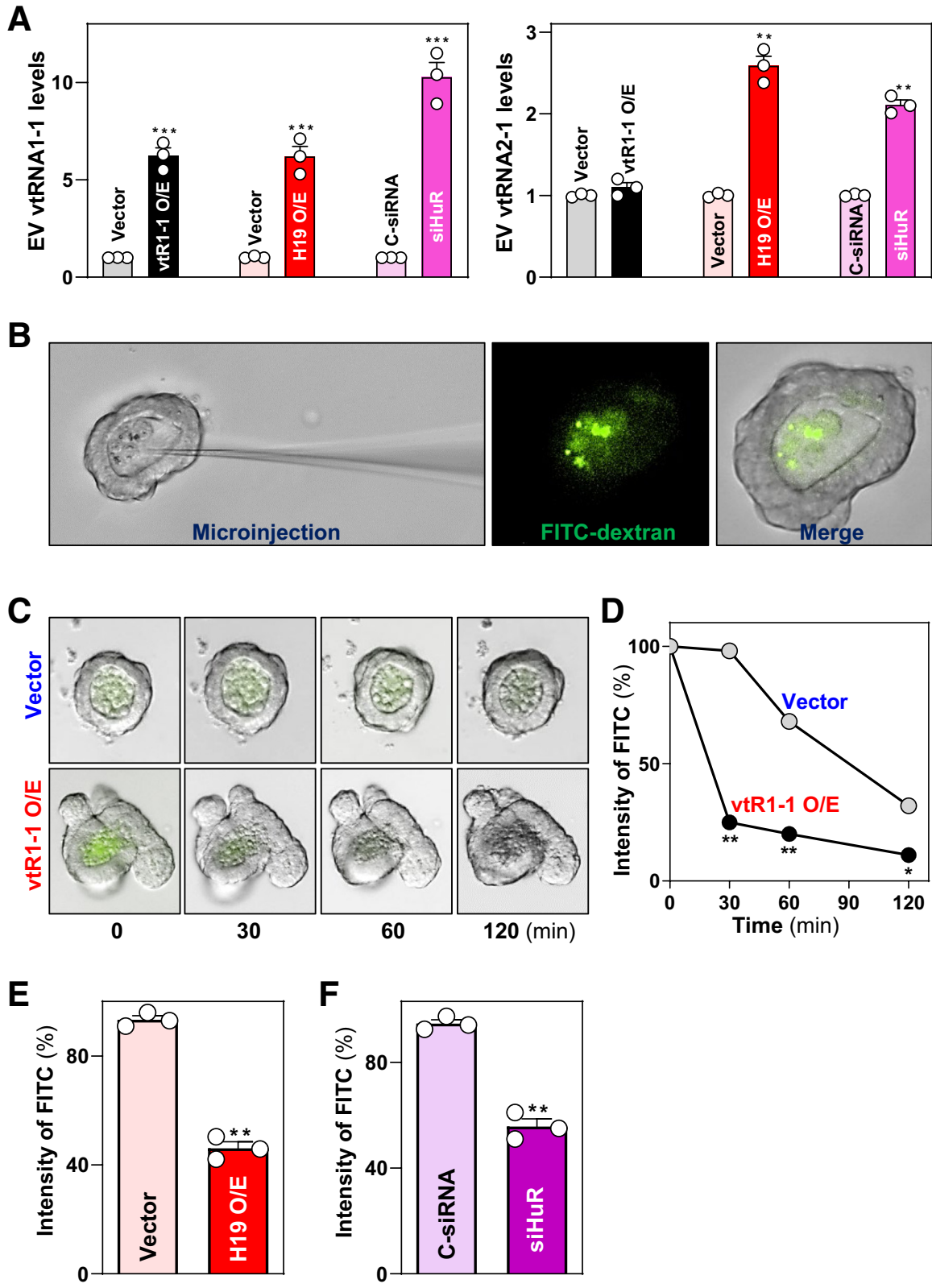
that EVs transfer *vtRNAs* generated from IECs, thus contributing to the increased levels of intracellular *vtRNA1-1* in neighboring and distant intestinal epithelium and subsequent systemic gut barrier dysfunction in shock patients.

vtRNA1-1 Regulates TJ Expression and Barrier Function Via Interaction With CUGBP1

To define the mechanism underlying the actions of *vtRNA1-1* in regulating intestinal epithelial barrier function, we tested the hypothesis that *vtRNA1-1* represses TJ expression by interacting with the RBP CUGBP1. It has been reported that CUGBP1 regulates expression of TJ proteins such as claudins and occludin by binding directly to the 3'-untranslated regions of their mRNAs.^{4,31,36} First, we examined the association of endogenous *vtRNA1-1* with endogenous CUGBP1 by RNP immunoprecipitation (RIP) assays using anti-CUGBP1 and control IgG antibodies, followed by isolation of bound RNA in both RIP reactions. After reverse transcription, Q-PCR analysis was used to measure the levels of *vtRNA* enrichment in the CUGBP1 IP relative to IgG IP, as described.³² As shown, *vtRNA1-1* and *vtRNA2-1* were highly enriched in CUGBP1 IP compared with control IgG IP (Figure 9A), although there were no significant changes in the levels of total input *vtRNAs* (Figure 9B). These results demonstrate the interaction between *vtRNA1-1* and CUGBP1 in IECs.

Second, we examined if association of *vtRNA1-1* with CUGBP1 altered the abundance of TJ proteins. As reported previously,^{36,44} CUGBP1 bound to the mRNAs encoding claudin-1 and occludin, but this association was increased by *vtRNA1-1* overexpression (Figure 9C). The levels of CUGBP1/*claudin-1* or CUGBP1/*occludin* mRNA complexes were greatly augmented in *vtRNA1-1* transfected cells relative to cells transfected with control vector. On the other hand, *vtRNA1-1* overexpression did not alter total CUGBP1 abundance or its subcellular distribution (Figure 9D). As expected, ectopically overexpressed CUGBP1 decreased the levels of claudin-1 and occludin proteins, but *vtRNA1-1* and CUGBP1 synergistically inhibited TJ protein expression, as evidenced by an additional decrease in the levels of claudin-1 and occludin in cells co-transfected with CUGBP1 and *vtRNA1-1* expression vectors when compared with cells transfected with CUGBP1 alone (Figure 9E).

Finally, we examined the role of *vtRNA1-1*/CUGBP1 interaction in the regulation of epithelial barrier function *in vitro*. Consistent with our previous study,⁴⁵ ectopically expressed CUGBP1 impaired epithelial barrier function, as shown by decreased TEER and increased paracellular flux of FITC-dextran in CUGBP1-transfected cells relative to cells transfected with control vector (Figure 9F). However, this disruption of epithelial barrier function by CUGBP1 was enhanced by *vtRNA1-1* overexpression, because decreased TEER and increased paracellular flux of FITC-dextran were enhanced in cells co-transfected with CUGBP1 and *vtRNA1-1* expression vectors compared with cells transfected with CUGBP1 alone. These findings indicate that *vtRNA1-1*



disrupts intestinal barrier function at least partially by interaction with CUGBP1.

Discussion

Constant epithelial renewal is essential to maintaining intestinal epithelial integrity and effective gut barrier function particularly in response to stress.^{46,47} Nonetheless, the precise mechanisms underlying this process remain largely unknown. In the present study, we provide strong evidence that *vtRNA1-1* plays an important role in regulating intestinal mucosal homeostasis and gut permeability. Tissue-specific transgenic expression of human *vtRNA1-1* in the intestinal epithelium inhibited mucosal renewal in the small intestine, resulting in the dysregulation of IJ protein expression and predisposing the gut barrier to pathological stress in mice. Experiments aimed at identifying the mechanisms underlying the actions of *vtRNA1-1* in this process revealed that *vtRNA1-1* interacts physically with CUGBP1; this association reduced expression of the TJ proteins claudin-1 and occludin by enhancing CUGBP1 binding to their mRNAs. The findings that *vtRNA1-1* is highly enriched in EVs from hemorrhagic shock patients *in vivo*, that silencing *vtRNA1-1* expression *in vitro* enhances epithelial barrier function, and that exposing murine enteroids to *vtRNA1-1*-rich EVs *ex vivo* increases paracellular permeability suggest that vtRNAs play an important role in maintaining gut epithelial homeostasis. These findings also position *vtRNA1-1*, expressed either in EVs or mucosal tissues, as a key player in intestinal epithelial pathology in patients with critical illness.

We identified a substantial increase in the levels of EV *vtRNA1-1* and *vtRNA2-1*, but not *vtRNA1-2* and *vtRNA1-3*, in serum obtained from hemorrhagic shock patients relative to that obtained from healthy individuals. Compared with those from control mice, EVs isolated from mice with CLP-induced sepsis also displayed increased levels of EV vtRNA. Consistently, levels of intestinal mucosal vtRNAs were markedly increased in tissues from critically ill patients and septic mice, conditions associated with mucosal injury and gut barrier dysfunction, as reported previously.²⁶ A murine genetic model with tissue-specific transgenic gene expression revealed that increasing the levels of human *vtRNA1-1* locally in the intestinal epithelium inhibited mucosal renewal of the small intestine and impaired epithelial defenses, highlighted by reduced Paneth cell numbers. Moreover, enteroids derived

from *vtRNA1-1*Tg mice grew much more slowly than those from littermate control mice and this inhibition of mucosal growth *ex vivo* was associated with defective Paneth cell function. In contrast, there were no significant differences in the rates of colonic mucosal growth between *vtRNA1-1*Tg and littermate control mice, even though *vtRNA1-1* levels were also increased in the colonic epithelium of *vtRNA1-1*Tg mice. Although the exact mechanism underlying the failure of *vtRNA1-1* overexpression to alter colonic mucosal growth remains unknown, it could be related to the fact that turnover of the colon mucosa is slower than that observed in the small intestine.^{33,46} Consistent with the current findings, we have reported that targeted deletion of the RBP HuR, ablation of the circular RNA *Cdr1as* locus, *H19* knockout, or decreasing the levels of *uc.173* in the intestinal epithelium only affects the growth of small intestinal mucosa without affecting renewal of colonic mucosa.^{29,41,47,48}

Ectopically expressed *vtRNA1-1* in the intestinal epithelium not only decreased levels of small intestinal intercellular TJ and AJ proteins but also increased gut barrier vulnerability to pathological stress such as low-dose LPS. Further studies in cultured cells and enteroids strongly support the view that *vtRNA1-1* functions as a repressor of intestinal epithelial barrier function. *vtRNA1-1* silencing by transfecting Caco-2 cells with si-*vtRNA1-1* enhanced epithelial barrier function, as demonstrated by an increase in the levels of several TJ proteins and TEER values and a decrease in paracellular flux of FITC-dextran. Conversely, *vtRNA1-1* overexpression disrupted epithelial barrier function by reducing TJ protein levels *in vitro*. Microinjecting FITC-dextran in 3-dimensional enteroids showed that EV vtRNAs negatively affect gut barrier function, because treating enteroids with vtRNA-enriched EVs increased paracellular permeability *ex vivo*. On the other hand, transgenic expression of *vtRNA1-1* in the intestinal epithelium did not directly cause gut barrier dysfunction in the absence of pathological stress, although it decreased basal TJ protein expression. This inconsistency might result from altered autophagy in the epithelium of *vtRNA1-1*Tg mice, because increasing the levels of mucosal *vtRNA1-1* induced the abundances of several autophagy-associated proteins. In support of this possibility, inducing autophagy improves epithelium-host defense and enhances gut barrier integrity.^{40,49}

Our results also suggest that *vtRNA1-1* regulates gut barrier function by interacting with CUGBP1, an evolutionarily conserved molecule that plays an essential role in

Figure 8. (See previous page). *vtRNA1-1*-rich EVs increase epithelial paracellular permeability in primarily cultured enteroids. (A) Levels of *vtRNA1-1* (left) and *vtRNA2-1* (right) in EVs isolated from the conditional media. Caco-2 cells were transfected with the vectors expressing *vtRNA1-1* or lncRNA *H19*, or with siHuR. Conditional media were collected 48 hours after transfection, and EVs were isolated. Values are the means \pm SEM ($n = 3$). $**P < .01$; $***P < .001$ compared with control vectors or C-siRNA. (B) Pictures of microinjection (left) and luminal FITC-dextran (right) in enteroids derived from the proximal small intestine of wild-type mice. (C) Images of enteroids 24 hours after incubation with EVs generated by cells overexpressing *vtRNA1-1* (*vtRNA1-1* O/E) or EVs from control group (Vector). Pictures were taken at different times after microinjection with FITC-dextran. (D) Changes in paracellular permeability in enteroids described in C. Values are the means \pm SEM ($n = 3$). $*P < .05$; $**P < .01$ compared with controls. (E, F) Changes in epithelial permeability in enteroids incubated with EVs generated from cells overexpressing *H19* (*H19* O/E) or siHuR-transfected cells. Paracellular permeability was examined 30 minutes after microinjection with FITC-dextran as described in B. $**P < .01$ compared with vector or C-siRNA. In A, E, and F, statistical significance was analyzed using unpaired, 2-tailed Student's *t*-test. In D, statistical comparison between time-course curves was by 1-way ANOVA with Bonferroni's hoc test.

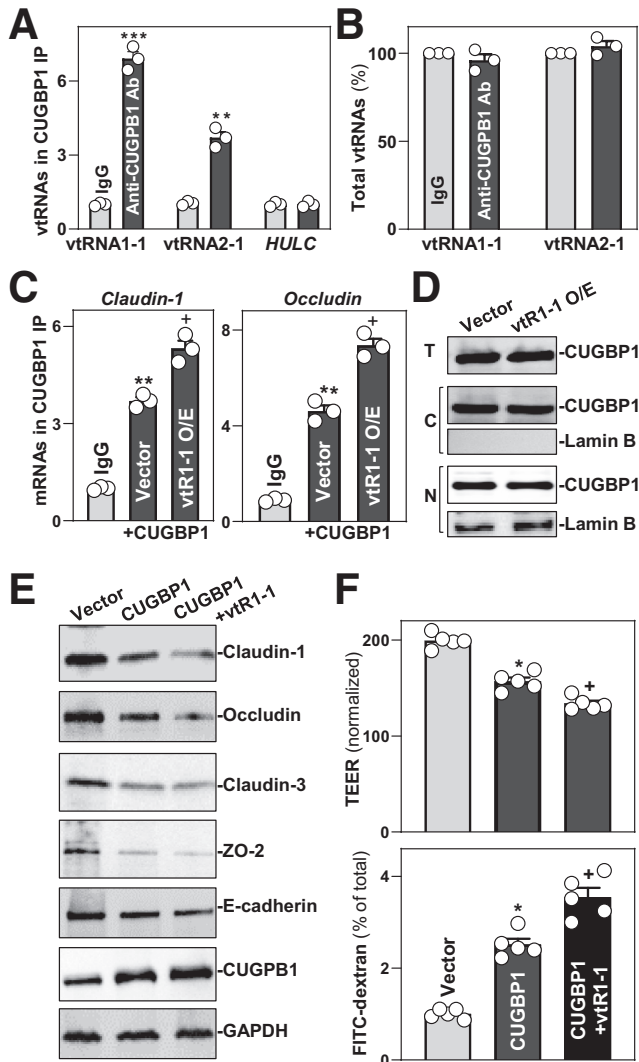


Figure 9. *vtRNA1-1* regulates TJ protein expression and epithelial barrier function via interaction with CUGBP1. (A) *vtRNA1-1* binds to CUGBP1. Association of endogenous CUGBP1 with endogenous *vtRNA1-1* and *vtRNA2-1* in Caco-2 cells was measured by RIP/Q-PCR analysis using anti-CUGBP1 antibody (Ab). Values are means \pm SEM ($n = 3$). $**P < .01$; $***P < .001$ compared with IgG. (B) Total input vtRNAs in cells described in A. (C) Levels of TJ protein mRNAs in the materials pulled down by anti-CUGBP1 Ab in cells transfected with a *vtRNA1-1* expression vector. Interactions of the mRNAs with CUGBP1 were examined 48 hours after *vtRNA1-1* overexpression. $**P < .01$ compared with IgG. $+P < .05$ compared with Vector. (D) Levels and cellular distribution of CUGBP1 48 hours after cells were transfected with *vtRNA1-1* expression vector (*vtR1-1*). Total (T), cytoplasmic (C), and nuclear (N) proteins were isolated and used for Western blotting analysis. Lamin B (a nuclear protein) was examined to monitor the quality of the nuclear and cytoplasmic fractions. (E) Immunoblots of TJ and CUGBP1 proteins in cells 48 hours after transfection with CUGBP1 alone or co-transfected with CUGBP1 expression vector and *vtR1-1*. GAPDH immunoblotting was performed as an internal control for equal loading. (F) Changes in the levels of TEER and FITC-dextran paracellular permeability in cells treated as described in C. $*+P < .05$ compared with control vector or CUGBP1 alone, respectively. In A, B, and C,

post-transcriptional gene regulation.⁵⁰ CUGBP1 contains 3 RNA recognition motifs through which it associates with specific mRNAs that often contain GU-rich elements in their 3'-untranslated regions or coding regions.^{44,50} Our previous studies demonstrated that CUGBP1 acts as a master regulator of intestinal epithelium homeostasis and that increasing CUGBP1 levels causes gut barrier dysfunction and inhibits mucosal growth *in vitro* and *in vivo* by inhibiting the translation of TJ proteins such as occludin and claudin-1 and proliferation-associated proteins including c-Myc.^{36,45,51} CUGBP1 also interacts with miR-222³⁵ and miR-195,⁵² long ncRNA *uc230*,⁴ and HuR⁴⁵ to jointly regulate target transcripts in the intestinal epithelium antagonistically or synergistically. *vtRNA1-1* bound to CUGBP1 and its overexpression enhanced CUGBP1 association with *claudin-1* and *occludin* mRNAs, thus enhancing CUGBP1-mediated repression of TJ protein expression. In addition, CUGBP1 inhibits intestinal epithelial renewal by post-transcriptionally suppressing expression of c-Myc, cyclin-dependent kinase 4, and insulin-like growth factor receptor.^{31,51,52} Nonetheless, the role of the interaction of *vtRNA1-1* with CUGBP1 in *vtRNA1-1*-induced inhibition of small intestinal mucosal growth remains to be fully elucidated. Because CUGBP1 is highly expressed in the intestinal epithelium and its cellular levels and binding affinity for target mRNAs are tightly regulated by RBPs and ncRNAs including *vtRNA1-1*,^{4,36,31,52} it is likely that *vtRNA1-1* affects gene regulatory programs governing intestinal epithelium homeostasis and barrier function at least partially by interacting with CUGBP1 or other RBPs.

Our results may have important clinical implications because the levels of *vtRNA1-1* and *vtRNA2-1* are markedly increased in EVs from patients with hemorrhagic shock. Abnormalities in the abundances of tissue vtRNAs also occur in patients with inflammatory bowel disease, viral infection, cancer, drug resistance, and preterm birth.^{24-26,53} This dysregulation of vtRNA expression contributes to the pathogenesis of different human disorders by altering autophagy, proliferation, apoptosis, cell-to-cell interaction, and host defense. In the present study, we further found that increasing the levels of *vtRNA1-1* inhibited intestinal mucosal growth and compromised gut barrier function, suggesting the potential of controlling *vtRNA1-1* expression to maintain intestinal epithelial homeostasis in pathological conditions. *vtRNA1-1* interacted with and enhanced CUGBP1-mediated inhibitory effect on TJ protein expression. In this regard, *vtRNA2-1* associates with HuR but prevents HuR binding to its target mRNAs in IECs.²⁶ These exciting findings showing the interaction between vtRNAs and RBPs advance our understanding of vtRNAs and their function in regulating intestinal epithelium homeostasis. It is possible that the regulation of RBP activity by vtRNAs

statistical significance was analyzed using unpaired, 2-tailed Student's *t*-tests, whereas comparison of means between more than 2 groups were performed by 1-way ANOVA with Bonferroni's hoc test for *F*. All experiments in *D* and *E* were repeated 3 times with similar results.

could represent a general mechanism underlying their biological functions in the gut epithelium.

In summary, the results of the present study indicate that by regulating intestinal epithelial renewal and barrier function, *vtRNA1-1* plays an essential role in altering intestinal epithelial homeostasis. Because EVs isolated from the serum of shock patients contained more *vtRNA1-1* and treatment of enteroids with *vtRNA1-1*-rich EVs increased paracellular permeability *ex vivo*, these findings suggest that EVs can transfer *vtRNA1-1*, thus contributing to the increased intracellular *vtRNA1-1* in neighboring and distant IECs of the epithelium and subsequent systemic gut barrier dysfunction in shock patients. Our results also suggest that the *vtRNA1-1*/CUGBP1 axis is a potential therapeutic target to preserve or reestablish intestinal epithelial integrity in patients with critical illness.

Materials and Methods

Chemicals and Cell Cultures

Biochemicals were purchased from Sigma, and culture medium and fetal bovine serum were from Invitrogen. Antibodies recognizing claudin-1 (CST-13255), claudin-2 (CST-48120), claudin-3 (CST-83607), claudin-7 (Inv-34-9100), occludin (sc-133256), JAM-A (sc-56323), ZO-2 (sc-33725), E-cadherin (sc-8426), lysozyme (sc-518012), CUGBP1 (sc-5261), p62 (sc-28359), HSC70 (MABE1120), and GAPDH (G9545) were obtained from Cell Signaling Technology, Invitrogen, Santa Cruz Biotechnology, and BD Biosciences. The secondary antibody conjugated to horseradish peroxidase was purchased from Sigma. All antibodies utilized in this study were validated for species specificity. Antibody dilutions used for Western blots of claudins, occludin, JAM-A, ZO-2, E-cadherin, CUGBP1, p62, HSP70, and GAPDH were 1:800 or 1000 (first Ab) and 1:2000 (second Ab), respectively, whereas antibody dilutions for immunostaining were 1:200 (first) and 1:2000 (second). Relative protein levels were analyzed by using Biorad Chemidoc and XRS system equipped with Image lab software (version 4.1). We also utilized "Quantity tool" to determine the band intensity volume; the values were normalized with internal loading control GAPDH. Caco-2 cells were purchased from the American Type Culture Collection and were maintained under standard culture conditions.⁴¹

Generation of *vtRNA1-1* Transgenic Mice and Animal Studies

To create a gain-of-function mouse model for *vtRNA1-1*, transgenic mice that specifically overexpressed the human *vtRNA1-1* in the intestinal epithelium were generated using A33-promoter as described in our previous studies.^{30,34} Briefly, the 586-bp fragment including the human *vtRNA1-1* locus and β -globin intron (222-bp 5' upstream sequence and 259-bp 3' sequence) were cloned into the pIRES-AcGFP1-Nuc vector, and the final *vtRNA1-1* expression vector, A33-*vtRNA1-1*/GFP, was used for microinjection into fertilized eggs.^{30,34,37} Transgenic founders on a pure C57BL/6J genetic background were established by pronuclear injection through a commercial service provided by the

Genome Modification Facility at Harvard University. Genotyping was performed by PCR in DNA extracted from tail clippings to identify the first generation of recombinant mice with human *vtRNA1-1*/GFP bicistronic RNA. Two founders were further characterized for the transmission to subsequently establish transgenic colonies. Both male and female and age-matched (6 or 8-week-old) *vtRNA1-1*Tg and control littermate mice were housed in a specific pathogen-free breeding barrier and cared for by trained technicians and veterinarians.

All animal experiments were performed in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of University Maryland School of Medicine and Baltimore VA hospital. Animals were deprived of food but allowed free access to tap water for 24 hours. Two portions of the small intestine taken from 0.5 cm distal to the ligation of Trietz were removed, one for histological examination and the other for extraction of protein and RNA. Intestinal mucosal growth was examined by immunostaining of Ki67, whereas the mucosa was scraped with a glass slide for various measurements of RNAs and proteins as described previously.³³ Representative results from 2 independent founders were reported here and compared with those obtained from control littermate mice. To generate septic stress model induced by CLP, age-matched male and female mice were anesthetized by Nembutal, and CLP was performed as described.⁵⁴

Human Samples and EV Isolation

Blood was collected from shock patients and healthy control subjects in Shock Trauma Center, University of Maryland Health Science Center. The study was approved by the University Maryland Institutional Review Board. Blood samples were stored in pyrogen-free tubes and centrifuged at 4 °C and 2000 rpm for 10 minutes, then divided into sterile cryotubes and stored at -80 °C until analysis. EVs were isolated from the serum by the procedures with multiple steps of centrifugation as described.^{27,28} EV quality control was performed following Methodological Guidelines to Study EVs recommended by the American Heart Association.²⁸ The final pellets containing EVs were resuspended in PBS and characterized by NSP analysis and immunoblotting analysis of EV markers Alix and CD63. In addition, *vtRNA*-rich EVs were also isolated from serum-free conditional media collected 48 hours after cells were transfected with the *vtRNA1-1* expression vector or others. To isolate EVs from mice, C57BL/6J mice were exposed to CLP, and blood samples were collected 24 hours afterwards.

Enteroid Isolation and Culture

Isolation and culture of primary enterocytes were conducted following the method provided by Stem Cell Technologies, with minor modifications as described previously.^{41,55}

Briefly, primary crypts were released from the small intestinal mucosa in mice; then, the isolated crypts were mixed with Matrigel and cultured in Advanced Dulbecco's

modified Eagle medium/F12 medium. The growth of enteroids was examined under phase-contrast microscopy as described.⁴⁸

Plasmid Construction and RNA Interference

An expression vector containing *vtRNA1-1* cDNA under control of pCMV-promoter was constructed and used to increase *vtRNA1-1* in Caco-2 cells, whereas a vector containing a scrambled sequence of *vtRNA1-1* was used as control. Transient transfections were performed using the Lipofectamine reagent following the manufacturer's recommendations (Invitrogen). Forty-eight hours after transfection using LipofectAMINE, cells were harvested for analysis. Expression of *vtRNA1-1* was silenced by transfection with specific si-vtR1-1 as described.^{26,56} The si-vtR1-1 and C-siRNA (a scrambled version of si-vt1-1) were purchased from Santa Cruz Biotechnologies. For each 60-mm cell culture dish, 15 μ l of the 20 μ M stock duplex si-vtR1-1 or C-siRNA was used. Forty-eight hours after transfection using LipofectAMINE (Invitrogen 116668019), cells were harvested for analysis. An expression vector containing the human CUGBP1 cDNA under the control of the pCMV promoter was purchased from Origene and used to increase cellular CUGBP1 levels as described previously.⁵²

Histology and Immunofluorescence Staining

Dissected and opened intestines were mounted onto a solid surface and fixed in formalin and paraffin. Sections of 5- μ m thickness were stained with hematoxylin and eosin (H&E) for general histology. Using a grade micrometer eyepiece, the overall length of villus and crypts of each section was measured, as reported previously.⁴ The immunofluorescence staining procedure was carried out according to the method described previously.^{41,57} For experiments using mucosal tissue samples from mice, more than 5 slides (5- μ m thickness section) in each tissue sample were prepared for immunofluorescence staining. For studies in cultured enteroids, slides were fixed in 3.7% formaldehyde in PBS and rehydrated. All slides were incubated with a primary antibody recognizing lysozyme, or E-cadherin in blocking buffer overnight and then incubated with secondary antibody conjugated with Alexa Fluor-594 (Cat#: A32754, Molecular Probes) for 2 hours at room temperature. After rinsing 3 times, the slides were incubated with DAPI (Cat#: D1306, Molecular Probes) at a concentration of 1 μ M for 10 minutes to stain cell nuclei. Finally, the slides were washed, mounted, and viewed through a Zeiss confocal microscope (model LSM700). Slides were examined in a blinded, coded fashion, and they were decoded only after examination was completed. Images were processed using Photoshop software (Adobe).

Measurement of Gut Permeability

Epithelial barrier function *in vitro* was examined by using the 12-mm Transwell plate as described.^{45,35} FITC-dextran (70 kDa; Sigma-Aldrich), a membrane-impermeable molecule, served as the paracellular tracer and was added at a final concentration of 0.25 mM to the

apical bath wells that contained 0.5 mL of medium. The basal bath well had no added tracers and contained 1.5 mL of the same flux assay medium as the apical compartment. All flux assays were performed at 37 °C, and the basal medium was collected at different times after addition of FITC-dextran. The concentration of FITC-dextran in the basal medium was determined using a fluorescence plate reader with an excitation wavelength at 490 nm and an emission wavelength of 530 nm. TEER was measured with an epithelial voltmeter under open-circuit conditions (WPI) as described,³⁵ and the TEER of all monolayers was normalized to that of control monolayers in the same experiment.

Gut permeability *in vivo* was determined by examining the appearance in blood of FITC-dextran administered by gavage as described.³⁶ Briefly, mice were gavaged with FITC-dextran at a dose of 60 mg/100-g weight 4 hours before harvest. Blood sample was collected by cardiac puncture. The serum concentration of FITC-dextran was determined using a fluorescence plate reader as described above. In experiments in 3-dimensional enteroids, epithelial permeability *ex vivo* was assessed by microinjecting FITC-dextran. On day 3 after primary culture, FITC-dextran was injected into the lumen of enteroids using a micromanipulator and microinjector (Eppendorf Femtojet 4X) under a stereomicroscope (Nikon Ti TE2000) in a biosafety cabinet, as described.^{42,43} Rates of epithelial permeability were monitored by examining the diffusion speed of luminal FITC-dextran as analyzed with software NIS-Elements AR5.30.05.

Q-PCR and ddPCR Analyses

Total RNA was isolated by using the RNeasy mini kit (Qiagen) and used in reverse transcription (RT) and PCR amplification reactions as described.⁴⁸ Q-PCR analysis was performed using Step-one-plus Systems with specific primers, probes, and software (Applied Biosystems). To measure copy numbers of vtRNAs, ddPCR analysis was performed by using QX200 Droplet Digital PCR System (Bio-Rad) as described.^{56,58} Briefly, PCR reaction mixture containing cDNA was partitioned into aqueous droplets in oil via the QX100 Droplet Generator, and then transferred to a 96-well PCR plate. A 2-step thermocycling protocol (95 °C \times 10 minutes; 40 cycles of [94 °C \times 30 seconds, 60 °C \times 60 seconds], 98 °C \times 10 minutes) was undertaken in a Bio-Rad C1000 (Bio-Rad). The PCR plate was then transferred to the QX100 Droplet Reader for automatic reading of samples in all wells. Copy number of each vtRNA per μ l PCR reaction was directly determined. QuantaSoft 1.7.4 analysis software (Bio-Rad) and Poisson statistics were used to compute droplet concentrations (copies/ng RNA).

Western Immunoblotting and RIP Pull-down Assays

Whole-cell lysates were prepared by using the RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl) containing 1% sodium dodecyl sulfate (SDS), sonicated, and centrifuged at 4°C for 15 min. The supernatants were boiled and size-

fractionated by SDS-PAGE. After the blots were incubated with primary antibody and then secondary antibodies, immunocomplexes were developed by using chemiluminescence.

IP of RNP complexes was carried out to assess the association of endogenous CUGBP1 with endogenous vtRNAs or mRNAs encoding intercellular junction proteins as described.⁵¹ Twenty million cells were collected per sample, and lysates were used for IP for 4 hours at room temperature in the presence of excess (30 μ g) IP antibody (IgG, or anti-CUGBP1). RNA in IP materials was used in RT reactions followed by Q-PCR analysis. The amplification of *Gapdh* mRNA, found in all samples as low-level contaminating housekeeping transcripts (not CUGBP1 target), served to monitor the evenness of sample input, as reported previously.^{45,51}

Statistical Analysis

All values were expressed as the means \pm standard error of the mean (SEM) or standard error of difference (SED). Unpaired, 2-tailed Student's *t*-test was used when indicated, with $P < .05$ considered significant. When assessing multiple groups, 1-way analysis of variance (ANOVA) was utilized with Tukey's post hoc test.⁵⁹ The statistical software used was GraphPad InStat Prism 9.0. For non-parametric analysis rank comparison, we used the Kruskal-Wallis test.

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Received April 10, 2024. Accepted September 20, 2024.

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Conflicts of interest

This author discloses the following: Jian-Ying Wang is a Senior Research Career Scientist at the Biomedical Laboratory Research and Development Service (US Department of Veterans Affairs). The remaining authors disclose no conflicts.

Funding

This work was supported by Merit Review Awards (BX000332 to Jian-Ying Wang; BX004890 to Jean-Pierre Raufman) from the United States Department of Veterans Affairs; and grants from the National Institutes of Health (DK57819, DK61972, DK68491 to Jian-Ying Wang; GM140983 to Rosemary Kozar).