Nedd4-2–dependent Ubiquitination Potentiates the Inhibition of Human NHE3 by Cholera Toxin and Enteropathogenic Escherichia coli

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SUMMARY

Inhibition of Na+/H+ exchanger NHE3 in the intestine is a major cause of diarrhea. Using humanized mice expressing human NHE3, we explore the unique properties of hNHE3 that contribute to diarrheal symptoms occurring in humans.

BACKGROUND & AIDS: Diarrhea is one of the most common illnesses and is often caused by bacterial infection. Recently, we have shown that human Na+/H+ exchanger NHE3 (hNHE3), but not non-human NHE3s, interacts with the E3 ubiquitin ligase Nedd4-2. We hypothesize that this property of hNHE3 contributes to the increased severity of diarrhea in humans.

METHODS: We used humanized mice expressing hNHE3 in the intestine (hNHE3int) to compare the contribution of hNHE3 and mouse NHE3 to diarrhea induced by cholera toxin (CTX) and enteropathogenic Escherichia coli (EPEC). We measured Na+/H+ exchange activity and fluid absorption. The role of Nedd4-2 on hNHE3 activity and ubiquitination was determined by knockdown in Caco-2bbe cells. The effects of protein kinase A (PKA), the primary mediator of CTX-induced diarrhea, on Nedd4-2 and hNHE3 phosphorylation and their interaction were determined.

RESULTS: The effects of CTX and EPEC were greater in hNHE3int mice than in control wild-type (WT) mice, resulting in greater inhibition of NHE3 activity and increased fluid accumulation in the intestine, the hallmark of diarrhea. Activation of PKA increased ubiquitination of hNHE3 and enhanced interaction of Nedd4-2 with hNHE3 via phosphorylation of Nedd4-2 at S342. S342A mutation mitigated the Nedd4-2–hNHE3 interaction and blocked PKA-induced inhibition of hNHE3. Unlike non-human NHE3s, inhibition of hNHE3 by PKA is independent of NHE3 phosphorylation, suggesting a distinct mechanism of hNHE3 regulation.

CONCLUSIONS: The effects of CTX and EPEC on hNHE3 are amplified, and the unique properties of hNHE3 may contribute to diarrheal symptoms occurring in humans. (Cell Mol Gastroenterol Hepatol 2022;13:695–716; https://doi.org/10.1016/j.jcmgh.2021.11.006)

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Infectious diarrhea caused by enteropathogenic bacteria is a major cause of mortality in young children worldwide and in the wider population within developing countries. The pathogenesis of diarrhea is multifactorial, but infectious diarrhea often results from altered intestinal
transport of electrolytes and fluid. The major Na⁺ absorptive mechanism in the intestine is electroneutral NaCl absorption mediated by the Na⁺/H⁺ exchanger 3 (NHE3), which is functionally linked to a Cl⁻/HCO₃⁻ exchanger. Inhibition of NHE3 activity in the intestine is associated with decreased fluid absorption, which can contribute to diarrheal symptoms. The direct evidence linking NHE3 to diarrhea has come from the studies of mice deficient in NHE3 that develop mild chronic diarrhea and intestinal distention, resulting from retention of alkaline fluid in all intestinal segments. In humans, recessive mutations in the NHE3 gene (Slc9A3) cause congenital diarrhea. Diarrhea is one of the most common symptoms in patients with inflammatory bowel disease, including Crohn’s disease and ulcerative colitis. Recent genome-wide association studies have established a strong association between ulcerative colitis and the Slc9A3 gene locus, whereas a single nucleotide polymorphism in Slc9A3 gene is linked to decreased NHE3 activity.

Inhibition of NHE3 and the resulting diarrhea can be caused from infection of the gastrointestinal tract by a variety of microorganisms, including *Vibrio cholerae*, enteropathogenic *Escherichia coli* (EPEC), and *Clostridium difficile*. These pathogens produce toxins that alter intestinal transport of electrolytes and fluid. Infection by *Vibrio cholerae* increases Cl⁻ secretion and decreases Na⁺ absorption due to the presence of cholera toxin (CTX) secreted by the bacteria. CTX activates adenylate cyclase, increases 3',5'-cyclic adenosine monophosphate, and activates protein kinase A (PKA). PKA phosphorylates and decreases NHE3 protein abundance in the plasma membrane via stimulation of NHE3 endocytosis, while also preventing delivery of NHE3 to the surface membrane. EPEC is a common cause of diarrhea in infants and young children, and the rapid onset of diarrhea in EPEC infections is a result of reduced absorption of ions and solutes. EPEC specifically inhibits NHE3 activity via a mechanism requiring the effector protein EspF of a type III secretion system, but the signaling cascade inhibiting NHE3 has not been elucidated.

Ubiquitination is a regulated, post-translational modification that conjugates ubiquitin (Ub) to Lys residues of target proteins and controls their intracellular fate. The covalent ligation of the 76-amino acid peptide Ub to substrate proteins is a highly conserved process that occurs via the sequential action of 3 enzymes: Ub-activating enzyme E1, Ub-conjugating enzyme E2, and Ub ligase E3. E3 ligases play a pivotal role in ubiquitination because these recognize the acceptor proteins and hence dictate the high specificity of the ubiquitination reaction. We have shown recently that NHE3s of human and non-human primates differ from NHE3s of other animals by having a PY (PPxY) motif. A PY motif is necessary for the interaction of a neural precursor cell expressed, developmentally down-regulated 4 (Nedd4) family of E3 Ub enzymes. The presence of a PY motif in human NHE3 (hNHE3) permits ubiquitination of NHE3 by Nedd4-2 in PS120 cells and has also been shown to increase the basal rate of internalization of hNHE3. Recently, ubiquitin-specific peptidase 7 (USP7), USP10, and USP48 have been identified as deubiquitinating enzymes (DUBs) targeting hNHE3. Therefore, ubiquitination of hNHE3 is coordinated by the on-reaction by Nedd4-2 and off-reaction by the DUBs.

The use of small animals such as mice and rats as surrogates for humans in scientific studies has advantages because of their small size, short reproductive cycle, and ease of genetic manipulation. However, several components of mouse biological systems are incongruent with those of humans. For instance, small animals such as mice and rats are not perceived to develop diarrhea as often, or as severely, as humans, although a direct comparison of free-living humans exposed to a variety of pathogens that may cause diarrhea and laboratory animals living in a controlled environment is difficult. To overcome such a limitation, we generated a humanized mouse strain expressing hNHE3 in the intestinal epithelial cells (IECs). The goal of this study is to test the hypothesis that ubiquitination of hNHE3 by Nedd4-2 renders hNHE3 more reactive to enteropathogenic agents, thus contributing to increased severity of diarrhea. We used an IEC line and the humanized mouse to establish that hNHE3 displays the unique biochemical property of ubiquitination-dependent regulation, which significantly amplifies the effects of CTX and EPEC, thereby contributing to increased intestinal water loss.

**Results**

**Inhibition of hNHE3 by Forskolin is Greater Than Non-Human NHE3**

To demonstrate that the extent of inhibition of hNHE3 is greater than of non-human NHE3, we compared the effect of the adenylate cyclase activator forskolin (FSK) on hNHE3 and rabbit NHE3 (rbNHE3) expressed in Caco-2bbe cells. Na⁺/H⁺ exchange activity of NHE3 was determined by the rate of initial Na⁺-dependent intracellular pH (pHi) recovery. All NHE3 activity measurements were conducted in the presence of 30 μmol/L Hoe-694 or 2 μmol/L dimethyl amiloride, which inhibit endogenous NHE1 and NHE2 activities. Cells expressing hNHE3 and treated with FSK for 30 minutes showed a concentration-dependent inhibition of NHE3 activity. The rate of Na⁺/H⁺ exchange by hNHE3 was reduced by 24% ± 4% and 55% ± 2% when treated with 1 μmol/L and 10 μmol/L FSK, respectively (Figure 1).

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Abbreviations used in this paper: ANOVA, analysis of variance; CFTR, cystic fibrosis transmembrane conductance regulator; CTX, cholera toxin; DRA, down-regulated in adenoma; DUB, deubiquitinating enzyme; ENaC, epithelial Na⁺ channel; EPEC, enteropathogenic E. coli; FSK, forskolin; HBSS, Hank’s balanced saline solution; hNHE3, human NHE3; IEC, intestinal epithelial cell; IF, immunofluorescence; Nedd4, neural precursor cell expressed, developmentally down-regulated 4; NHE, Na⁺/H⁺ exchanger; pH, intracellular pH; PCR, polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; SD, standard deviation; sh, short hairpin; Ub, ubiquitin; USP, ubiquitin-specific peptidase; WT, wild-type.

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Tukey post hoc test.

In comparison, the activity of rbNHE3 was inhibited by 17% ± 2% by 1 μmol/L FSK, and increasing FSK treatment concentration to 10 μmol/L did not result in further inhibition of rbNHE3 activity (18% ± 3%).

**Generation of a Humanized Mouse Expressing Human NHE3**

The amplified effects of FSK on hNHE3 suggest that this may contribute to increased diarrheal symptoms. However, diarrhea that results from the loss of water in the intestine cannot be assessed using in vitro systems. To determine whether the presence of hNHE3 increases the severity of diarrhea in humans, we generated a transgenic mouse strain expressing hNHE3 in the IECs. We used the villin promoter to drive the expression of hNHE3 tagged with a VSVG tag at the C-terminus (Figure 2A). The transgenic mice were subsequently crossed with Nhe3−/− mice, resulting in a mouse strain that expresses hNHE3 in IECs in the absence of mouse NHE3 (Figure 2B). This mouse is called hNHE3int. Comparing transgenic expression of hNHE3 with mouse NHE3 by Western blotting showed that hNHE3 expression levels in the jejunum, ileum, and proximal colon are greater compared with mouse NHE3 expression in wild-type (WT) mice (Figure 2C). However, because the anti-NHE3 antibody (EM450) was raised against a peptide from hNHE3,22 it is likely that the amount of mouse NHE3 was underestimated. The mRNA expression levels of the cystic fibrosis transmembrane conductance regulator (CFTR) and the Cl−/HCO3− exchanger DRA (down-regulated in adenoma) were not altered in the IECs of hNHE3int mice (Figure 2D). However, NHE2 mRNA expression was decreased in hNHE3int mice (Figure 2D), which could be associated with the increased expression of the transgenic hNHE3.

Previous studies have shown that Nhe3+/− mice exhibit mild chronic diarrhea and intestinal distention caused by retention of fluid.4 hNHE3int mice did not show any sign of diarrhea or water accumulation in the intestine, and unlike Nhe3−/− mice, formed fecal pellets were present in hNHE3int mice (Figure 2E), demonstrating that the expression of hNHE3 has restored the Na+/H+ exchange activity in the intestine. In addition, Nhe3−/− and WT mice had similar histologic appearances of the intestinal tract with well-formed villi in the small intestine (Figure 2F, upper panels) and the absence of hyperplasia and increased immune cell infiltration in the colon that are observed in Nhe3−/− mouse colon (Figure 2F, lower panels).

It has been shown previously that NHE3 deficiency causes dysbiosis with reduced microbial diversity in the intestine.23 To determine whether the expression of hNHE3 alters the gut microbiota, we compared the cecal microbiota from WT, Nhe3−/−, and hNHE3int mice by profiling the major microbial taxonomic groups. As shown previously,23 there were increased levels of Bacteroidetes and Proteobacteria in Nhe3−/− mice, whereas Firmicutes expression was reduced compared with WT mice (Figure 3). This shift in the microbiota composition was largely reversed in hNHE3int mice, and this class level analysis of microbiota, although limited in depth, ascertains the importance of intestinal NHE3 expression for the homeostasis of gut microbiota. The absence of NHE3 is also associated with male infertility due to a defect in acidification of the epididymal fluid so that male Nhe3−/− mice are infertile.24 Although we did not specifically detect the presence of hNHE3 in the male reproductive system of hNHE3int mice, all the hNHE3int male mice that were used for breeding were reproductive, indicating that Na+/H+ exchange activity in the epididymal duct was restored in hNHE3int mice.

**Effects on Na+/H+ Exchange Activity and Na+/H+ -dependent Water Absorption by CTX Are Enhanced in hNHE3int Mice**

To determine whether the regulation of hNHE3 and mouse NHE3 differs in vivo, we assessed the effects of FSK on NHE3 activity by injecting FSK or dimethyl sulfoxide into a small segment of mouse ileum ligated on both ends to form a closed loop (Figure 4A). The mice were kept for 90 minutes under anesthesia on a warming bed, with the abdomen covered with a moist towel. Mice were then euthanized, and intestinal villi were isolated for the measurement of NHE3 activity as previously described.25 The basal activity of hNHE3 was higher than that of mouse NHE3 in WT mice, which correlates with increased expression of NHE3 than the endogenous level in control mice. However, FSK resulted in significantly greater inhibition of NHE3 activity in hNHE3int mice compared with that in WT mice (Δ82.3% for hNHE3int vs Δ43.4% for WT), so that hNHE3 activity in the presence of FSK was lower than mouse NHE3 activity (Figure 4A).

Na+ absorption by NHE3 in the IECs facilitates transepithelial water absorption across the intestinal epithelial barrier.3 Hence, NHE3 inhibition leads to decreased
Figure 2. Generation of humanized mice expressing hNHE3 in mouse intestine. (A) Schematic map of the transgenic construct consists of a villin gene promoter (P), a VSVG-tag, and hNHE3 gene. The drawing is not to scale. (B) NHE3 expression was determined in jejunal mucosal lysates of WT mice, NHE3 knockout (Nhe3⁻/⁻) mice, transgenic mice expressing both human and mouse NHE3 (TG hNHE3), and hNHE3int mice expressing only human NHE3 in intestinal tissue. Cell line Caco-2bbe transfected with human NHE3 was included as a positive control. Western blots were performed using P5D4 anti-VSVG antibody and EM450 polyclonal NHE3 antibody to detect hNHE3 and total NHE3, respectively. β-actin was used as loading control. (C) Western blot comparing mouse NHE3 in WT and hNHE3 in hNHE3int mouse intestine using EM450 anti-NHE3 antibody. Col, proximal colon; Ile, ileum; Jej, jejunum. (D) mRNA expression of CFTR, DRA, and NHE2 in the small intestinal mucosa was determined by quantitative real-time PCR of WT and hNHE3int mice. Relative expression is presented as fold changes relative to expression level in WT mice. Results are mean ± SD (n = 3). *P < .05 by unpaired t test. (E) Representative appearance of the intestinal tract of WT, Nhe3⁻/⁻, and hNHE3int mice. (F) Histology of proximal ileum and distal colon of WT, Nhe3⁻/⁻, and hNHE3int mice are shown. Bar, 100 mm. Results are representative of 3 independent experiments.
Na$^+$ absorption, which causes luminal water accumulation, a proxy for diarrhea. CTX treatment for 5 hours resulted in accumulation of fluid in the intestinal loop of WT mice as evidenced by increased weight-to-length ratios (Figure 4B). Although increased water accumulation in the intestinal loop was evident in both mouse strains, CTX treatment almost doubled the effect in hNHE3int mice compared with WT mice. However, CTX can cause both decreased absorption by NHE3 and increased secretion by CFTR, resulting in fluid accumulation. To evaluate whether the CFTR-dependent secretory activity was altered, fluid accumulation in an intestinal loop was determined in the presence of NHE3 and increased secretion by CFTR, resulting in fluid accumulation. To evaluate whether the CFTR-dependent secretory activity was altered, fluid accumulation in an intestinal loop was determined in the presence of NHE3 and increased secretion by CFTR, resulting in fluid accumulation.

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Figure 3. Microbial profiles of cecum contents of WT, Nhe3$^{-/-}$, and hNHE3int mice. Cecum contents of (A) WT (n = 5), (B) Nhe3$^{-/-}$ (n = 4), and (C) hNHE3int (n = 5) mice were collected under sterile conditions, and bacterial genomic DNA was extracted. Microbial profiles were determined by real-time PCR using specific primers (Table 1) as described previously. Data were analyzed by two-way ANOVA. *P < .01. Data are representative of 2 experiments. SEM, standard error of the mean.
Inhibition of hNHE3 Is Dependent on Nedd4-2

Our previous study showed that hNHE3 uniquely interacts with Nedd4-2, which ubiquitinates and internalizes hNHE3 under basal conditions.\textsuperscript{17} We therefore hypothesized that the increased reactivity of hNHE3 in response to CTX is a result of Nedd4-2–dependent regulation of hNHE3. To test this hypothesis, we examined the effect of loss of Nedd4-2 function by knocking down Nedd4-2 in Caco-2bbe cells. Figure 5A shows that Nedd4-2–specific lentiviral short hairpin (sh) RNA, shNedd4-2, led to decreased Nedd4-2 protein expression compared with cells treated with scrambled control shRNA (shCon). Importantly, silencing Nedd4-2 expression blocked FSK-induced inhibition of hNHE3 (Figure 5B). Our previous study demonstrated that the hNHE3–Nedd4-2 interaction requires the presence of \textsuperscript{723}PPNYDEE\textsuperscript{729} motif, called the extended PY motif (ePY), in hNHE3.\textsuperscript{17} To further assess the role of Nedd4-2, we expressed hNHE3 with the ePY motif mutated to Ala (ePYmut) in Caco-2bbe cells. Consistent with the effect of Nedd4-2 knockdown, Nedd4-2/ePYmut rendered hNHE3 nonresponsive to FSK (Figure 5C).

If hNHE3 inhibition is dependent on Nedd4-2, we postulated that ubiquitination of hNHE3 must be increased by CTX treatment. Therefore, we immunoprecipitated hNHE3 from Caco-2bbe cells before or after FSK treatment. FSK induced a significant increase in ubiquitination levels of hNHE3.
Figure 5. Inhibition of hNHE3 by FSK requires Nedd4-2. (A) Nedd4-2 expression was stably silenced in Caco-2bbe cells by using lentiviral shNedd4-2. Scrambled shRNA (shCon) was used as a control. Expression of endogenous Nedd4-2 was determined by Western blotting using anti-Nedd4-2 antibody. Representative blots from more than 3 independent experiments are shown. (B) Caco-2bbe/hNHE3 cells stably transduced with shNedd4-2 or shCon were treated with FSK or equal volume of dimethyl sulfoxide (Con) for 30 minutes. NHE3 activity was determined as the rate of Na\(^+\)-dependent pH\(_i\) recovery. Results are presented as mean ± SD. n = 17. ***P < .001. (C) Caco-2bbe/hNHE3 cells transfected with pcDNA control or pcDNA/Nedd4-2-ePY\text{mut} were treated with FSK as described above. n = 20. ***P < .001 by one-way ANOVA. (D) Caco-2bbe/rbNHE3 and Caco-2bbe/hNHE3 cells transduced with shNedd4-2 or shCon were treated with 10 \(\mu\)mol/L FSK for 30 minutes. NHE3 was immunoprecipitated with PSD4 antibody, and ubiquitination of NHE3 was determined by using P4D1 anti-Ub antibody. The panel below shows immunoprecipitated NHE3 blotted with EM450 antibody. Knockdown of Nedd4-2 is shown in the lower panel with \(\beta\)-actin as control. Representative blots from 3 independent experiments are shown. The lower graph shows the quantification of Ub normalized to NHE3 immunoprecipitated. *P < .05, ***P < .001 by two-way ANOVA Tukey post hoc test. (E) Mouse NHE3 and hNHE3 were immunoprecipitated from intestinal mucosal lysates of WT and hNHE3\text{int} mice, respectively, with EM450 anti-NHE3 antibody. Ubiquitination levels of NHE3 were determined and normalized to the amount of NHE3 immunoprecipitated. Quantification from 3 independent experiments is shown on the right. ***P < .001 by two-way ANOVA.
hNHE3, which was blocked by shNedd4-2 (Figure 5D). In comparison, FSK or Nedd4-2 knockdown did not alter rbNHE3 ubiquitination, consistent with the previous finding in PS120 cells that rbNHE3 or rat NHE3 is not ubiquitinated by Nedd4-2. To determine whether FSK-induced hNHE3 ubiquitination occurs in vivo, we immunoprecipitated hNHE3 and mouse NHE3 from hNHE3int and WT mouse intestine, respectively. Consistent with the results observed in Caco-2bbe cells, CTX significantly increased hNHE3 ubiquitination levels (Figure 5E). In comparison, the basal ubiquitination levels of mouse NHE3 were significantly lower and FSK had no effect.

Previous studies have shown that PKA-mediated inhibition of NHE3 involves internalization of NHE3 from the brush border membrane. To assess whether there is a difference in endocytic retrieval of hNHE3 and rbNHE3, we determined NHE3 abundance at the apical membrane of Caco-2bbe cells by surface biotinylation. FSK decreased surface membrane protein abundance of both rbNHE3 and hNHE3, but the effect was greater on hNHE3 than rbNHE3 (Figure 6A). Moreover, FSK-induced effect on hNHE3 surface abundance was ablated by shNedd4-2, whereas no difference was observed on rbNHE3 (Figure 6A), further demonstrating the importance of Nedd4-2 in hNHE3 regulation. The differential effects on NHE3 internalization by CTX in Caco-2 cells were confirmed by immunofluorescence (IF) confocal microscopic analysis of the ligated intestinal loops of mice. In WT mice, CTX treatment decreased brush border membrane expression of NHE3, causing the appearance of NHE3 in the subapical compartment (Figure 6B, left panels). In comparison, the retrieval of hNHE3 from the brush border membrane was significantly enhanced in the hNHE3int mice (Figure 6B, right panels), indicating that the increased inhibition of hNHE3 activity results in part from enhanced endocytosis of hNHE3 protein.

**Inhibition of hNHE3 by EPEC Is Nedd4-2 Dependent**

EPEC is a common cause of diarrhea in infants and young children, and the rapid onset of diarrhea in EPEC infections is a result of reduced absorption of ions and solutes. Specifically, EPEC significantly inhibits NHE3 activity via a mechanism requiring the effector protein EspF of a type III secretion system. To assess whether the Nedd4-2 dependence of hNHE3 extends beyond CTX-mediated inhibition, we compared the effects on EPEC on hNHE3 and rbNHE3 in Caco-2bbe cells. Cells were treated with Luria Broth or a commensal strain EFC-1, which did not alter the basal Na+/H+ exchange activities of hNHE3 or rbNHE3 (Figure 7A). However, when treated with the enteropathogenic strain EPEC, the extent of inhibition was significantly greater for hNHE3 compared with rbNHE3 (Figure 7B). In cells expressing hNHE3, the NHE3 activity was inhibited by 60.8% compared with 38.5% inhibition observed for rbNHE3. Moreover, EPEC-mediated inhibition of hNHE3 activity was abolished by knockdown of Nedd4-2 (Figure 7C), and EPEC failed to regulate hNHE3/ 

**Regulation of hNHE3 by PKA Is Dependent on Phosphorylation of Nedd4-2**

The results thus far have highlighted the importance of Nedd4-2 in enforcing hNHE3 inhibition. Nedd4-2 contains 4 WW domains (protein-protein interaction domains containing 2 conserved tryptophan residues) that bind PY motifs. To identify a WW domain interacting with hNHE3, each WW domain expressed as a glutathione S transferase fusion protein was used to pulldown hNHE3 from Caco-2bbe/hNHE3 cell lysate. Only the third WW (WW3) bound.
to hNHE3 (Figure 9A), indicating that hNHE3 interacts with Nedd4-2 via WW3.

We next determined whether FSK regulates the interaction between hNHE3 and Nedd4-2. We assessed the localization of hNHE3–Nedd4-2 interaction by confocal IF microscopy. Under basal conditions, hNHE3 was localized at the apical membrane (identified by WGA) with relatively weak colocalization with Nedd4-2 (Figure 9B). Treatment with FSK for 5 or 10 minutes decreased hNHE3 at the apical membrane, whereas colocalization of hNHE3 and Nedd4-2, quantified by determining Pearson’s coefficient, intensified. These results demonstrate that FSK increases the interaction of Nedd4-2 with hNHE3.

Previous studies have shown that Nedd4-2 is a substrate for phosphorylation by PKA at S342 and S448, and phosphorylation modulates Nedd4-2 interaction with its substrates.37,38 To determine phosphorylation of Nedd4-2, Caco-2bbe cells were treated with FSK, and phosphorylation of endogenous Nedd4-2 at S342 and S448 was detected using phospho-specific antibodies. FSK increased phosphorylation of Nedd4-2 at S342 as early as 10 minutes but did not affect phosphorylation at S448 (Figure 9C, left panels). Similar results were obtained in SK-CO15 cells (Figure 9C, right panels). To determine whether FSK increases the interaction of p-Nedd4-2 with hNHE3, we performed co-immunoprecipitation of hNHE3 and Nedd4-2 from Caco-2bbe cells treated with FSK. We observed increased amounts of pS342-HA-Nedd4-2 co-immunoprecipitated with hNHE3 after FSK treatment (Figure 9D, first row). Because the anti-pS342-Nedd4-2 antibody was not suitable for IF staining, we could not confirm the interaction of pS342-HA-Nedd4-2 with hNHE3 by IF microscopy. Surprisingly, we did not detect a change when the immunocomplexes were blotted using anti-Nedd4-2 antibody (Figure 8D, second row). However, this could be related to the limitation of co-immunoprecipitation that cannot detect region-specific interaction.

To determine whether Nedd4-2 phosphorylation is important for its interaction with hNHE3, we sought to express HA-Nedd4-2 with S342A mutation. Surprisingly, the expression levels of S342A-Nedd4-2 mutant were markedly lower than WT HA-Nedd4-2 in both Caco-2bbe and SK-CO15 cells (Figure 10A). Decreased expression levels were also observed for S448A and S342A/S448A mutation (Figure 10A). Therefore, we determined whether the S342A and S448A mutations alter protein stability by determining

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**Figure 6. Internalization of hNHE3 by PKA is dependent on Nedd4-2.** (A) NHE3 expression at the apical membrane of Caco-2bbe cells treated with FSK was determined by surface biotinylation. Surface NHE3 expression was normalized to total NHE3 expression. Relative surface-to-total NHE3 ratios with the result in untreated cells set at 100% (mean ± SD) are shown below the blots. n = 3. *P < .05, **P < .01 compared with corresponding controls by one-way ANOVA. (B) Cellular localization of NHE3 in CTX or control treated ileal tissues was determined by confocal IF microscopy. Representative immunolabeling of NHE3 (green) and DAPI (blue) are shown. Results are representative 2 independent experiments with 4 mice per group. Arrows indicate internalized NHE3. Scale bar: 10 μm.
the steady-state protein expression in the presence of translation inhibitor cycloheximide. The expression level of Nedd4-2 was unaltered over 4 hours as shown in Figure 10B. By contrast, the rates of decay of S342A and S448A mutants were more rapid, resulting in significant loss of the proteins in 4 hours. As a result, the expression level of S342A mutant was significantly lower than that of endogenous Nedd4-2 expression (Figure 10C) and could not be used as a dominant negative. As an alternative to the colonic epithelial cell lines, we used PS120 cells where the expression level of the S342A mutant was similar to that of WT HA-Nedd4-2 (Figure 10D). PS120 cells were co-transfected with hNHE3 and HA-Nedd4-2 variants, and co-immunoprecipitation was performed to determine whether the S342A mutation alters the hNHE3–Nedd4-2 interaction. Results in Figure 10E demonstrate that the interaction of the S342A mutant with hNHE3 was impaired compared with WT Nedd4-2, indicating that phosphorylation at S342 facilitates their interaction. To test for the functional importance of S342 phosphorylation, the effect of FSK on hNHE3 was determined. As expected, FSK inhibited hNHE3 activity in cells transfected with pcDNA or HA-Nedd4-2 (Figure 10F). In contrast, inhibition of hNHE3 by FSK was blocked by the S342A mutant, demonstrating that phosphorylation of Nedd4-2 at S342 is functionally important for hNHE3 regulation.

Inhibition of hNHE3 by FSK Is Independent of NHE3 Phosphorylation at S552

A series of studies have demonstrated that phosphorylation at S552 and S605 of rat NHE3 is important for NHE3 endocytosis and inhibition of NHE3 activity. However, it is not known whether phosphorylation at S552 and S605 affects the interaction of Nedd4-2 with hNHE3 or influences phosphorylation of Nedd4-2 by PKA. To this end, we freshly expressed WT hNHE3 and hNHE3 carrying S552A or S605A mutation in Caco-2bbe cells. Cells were treated with FSK, and phosphorylation of hNHE3 was determined using phosho-specific NHE3 antibodies that recognize NHE3.
only if phosphorylated at S552 or S605. Phosphorylation of hNHE3 at S552 was increased by FSK (Figure 11A, lane 1 versus lane 2), but as expected, no signal was detected in hNHE3 with S552A mutation (Figure 11A, lanes 3 and 4). Because S552 was unaltered in the S605A mutant, increased phosphorylation at S552 was observed (Figure 11A, lane 5 versus lane 6). However, the anti-pS605 antibody failed to show any signal. It is not clear whether S605 of hNHE3 is not a site of phosphorylation, or the antibody does not cross-react with hNHE3. Phosphorylation at S552 by PKA was confirmed in hNHE3int mouse intestine exposed to CTX (Figure 11B). To determine the functional role of pS552 and pS605, the effect of FSK on the antiporter activity was determined. As shown earlier, the activity of WT hNHE3 was reduced by FSK, but surprisingly, FSK also inhibited the activities of S552A and S605A mutants (Figure 11C). These results suggested that PKA-induced regulation of hNHE3 is independent of phosphorylation at S552 and S605. To determine whether the mutations in hNHE3 alter the interaction with Nedd4-2, we performed co-immunoprecipitation of hNHE3 and Nedd4-2. In all cells, FSK increased phosphorylation of Nedd4-2 at S342 (Figure 11D, lower panels), indicating that hNHE3 phosphorylation at S552 and S605 does not alter Nedd4-2 phosphorylation. Moreover, co-immunoprecipitation of pS342-Nedd4-2 with hNHE3 proteins was increased by FSK in all cell lines (Figure 11D, upper panels). To determine whether hNHE3 mutants are still ubiquitinated, we transiently expressed HA-Ub and immunoprecipitated hNHE3. Results shown in Figure 11E depict that FSK similarly increased ubiquitination levels of hNHE3 variants, demonstrating that phosphorylation at S552 and S605 is not important for Nedd4-2 docking or ubiquitination of hNHE3. Because of the previous evidence demonstrating the importance of phosphorylation of non-human NHE3s, these data demonstrate a distinct...
regulatory mechanism of hNHE3 that differs from that of non-human NHE3.

Discussion
The cause of diarrhea is multifactorial, but secretory diarrhea results often from bacterial and viral infections that cause excessive secretion and impaired absorption of electrolytes and fluid across the intestinal epithelium. The current study was designed to determine whether the unique Ned4-2-dependent regulation of hNHE3 contributes to diarrhea in humans that is perceived to be more severe and frequent compared with laboratory animals. We report the results comparing the regulation of NHE3s of human and non-human by adenylyl cyclase activators and EPEC. A major finding is that the presence of hNHE3 in the intestine exacerbates diarrhea caused by CTX and EPEC. This conclusion was inferred from increased water accumulation in hNHE3int mouse intestine (a hallmark of diarrhea) compared with WT mouse intestine in response to CTX or EPEC.

Previously we reported that the basal internalization rate of hNHE3 in PS120 cells was almost twice the rate of rbNHE3 or ratNHE3. We now found that FSK has
heightened effects on hNHE3 than rbNHE3. There was a concentration-dependent inhibition of hNHE3 activity, reaching a greater level of inhibition of hNHE3 versus rbNHE3. This difference in hNHE3 activity was paralleled by increased fluid accumulation in the intestine of hNHE3<sup>int</sup> mice after CTX administration. CTX causes an accumulation of intestinal fluid that contributes to diarrhea through activation of Cl<sup>−</sup> secretion by CFTR and inhibition of Na<sup>+</sup> absorption by NHE3. The transgenic expression level of hNHE3 did not alter CFTR expression (mRNA) or activity, indicating that the difference in fluid absorption between hNHE3<sup>int</sup> and WT mice was a result of the presence of hNHE3 in the intestine of hNHE3<sup>int</sup> mice.

A growing body of evidence shows that imbalance of the gut microbiota increases the susceptibility to various pathogens, often resulting in diarrhea. Reduced microbial diversity predisposes Nhe3<sup>−/−</sup> mice to colitis. We found that transgenic hNHE3 expression largely reverses the dysbiosis caused by the absence of NHE3, although a more detailed microbial analysis is needed to compare the extent of the restoration.

The difference in intestinal fluid accumulation between hNHE3<sup>int</sup> and WT mice was reproduced by EPEC, suggesting that the effects are not limited to PKA-mediated regulation. However, EPEC administration to hNHE3<sup>int</sup> mice did not yield clear evidence for diarrhea, although increased stool water contents were observed intermittently. EPEC adhere...
to the surface of enterocytes via an “attaching and effacing” mechanism, but the efficacy of EPEC colonization in small animals such as mouse and rat is contentious. EPEC colonization in the intestine of 6- to 8-week-old C57Bl/6 mice has been demonstrated. On the other hand, no EPEC colonies were found in Swiss NIH mice, suggesting that EPEC colonization is influenced by the genetic background. hNHE3int mice are in the FVB/N genetic background, and inefficient EPEC colonization in these mice could have led to the lack of clear differences between hNHE3int and WT mice.

Consistent with our previous study, the regulation of hNHE3 by CTX and EPEC is Nedd4-2–dependent. Blockade of Nedd4-2 interaction by Nedd4-2 knockdown or mutations in the PY motif of hNHE3 ablated the effects of FSK and EPEC. In addition, CTX and EPEC increased ubiquitination levels of hNHE3. However, our current study investigating Nedd4-2 dependence was limited to in vitro assays because

Figure 11. Inhibition of hNHE3 by FSK is independent of phosphorylation of hNHE3 at S552. (A) Caco-2bbe cells expressing hNHE3 with S552A or S605A mutation were treated with FSK for 10 minutes. Phosphorylation of hNHE3 at S552 and S605 was determined using anti-pS552 and anti-pS605 antibodies. n = 3. (B) Closed ileal loops were treated with CTX or dimethyl sulfoxide (C) for 30 minutes, and lysates were made from scraped intestinal epithelial layers. Phosphorylation at S552 and hNHE3 expression in the lysates were determined by Western blotting. n = 3. (C) hNHE3 activities were determined in Caco-2bbe cells expressing hNHE3 variants that were treated with FSK (F) or dimethyl sulfoxide (C). n = 14–17. Results are presented as mean ± SD. ***P < .001 by multiple t test. (D) Caco-2bbe cells expressing hNHE3 variants were treated with FSK (F) or dimethyl sulfoxide (C) for 10 minutes. hNHE3 was immunoprecipitated (second row), and co-immunoprecipitated pS342-Nedd4-2 (top row) was determined. Bottom two lanes show immunoblots of pS342-Nedd4-2 and hNHE3 in cell lysates. Graph at the bottom shows quantification of p-Nedd4-2/hNHE3 ratios from 5 independent experiments. n = 5. ***P < .001. (E) Ubiquitination levels of hNHE3 variants were determined by immunoprecipituation of hNHE3, followed by immunoblotting with anti-Ub antibodies. Lower panel shows immunoprecipitated hNHE3. Ub levels normalized to hNHE3 immunoprecipitated are shown on the bottom. n = 4. **P < .01, ***P < .001 by multiple t test.
our efforts to generate hNHE3\textsuperscript{int} mice in the absence of Nedd4-2 were not successful. The best characterized substrate of Nedd4-2 is the epithelial Na\textsuperscript{+} channel (ENaC). Nedd4-2 deletion causes up-regulation of ENaC in the kidney and lung, causing electrolyte imbalance and respiratory stress.\textsuperscript{46,47} ENaC in the colon plays an important role in preserving sodium ions. However, colon-specific deletion of ENaC in mice does not cause diarrhea, despite sodium loss and aldosterone resistance,\textsuperscript{48} and it is not clear whether colonic ENaC function is altered in Nedd4-2 knockout mice. In comparison, intestine-specific deletion of NHE3 causes congenital sodium diarrhea in mice.\textsuperscript{19} Therefore, whether Nedd4-2 deficiency alters NHE3-dependent sodium and fluid absorption in vivo awaits future investigations.

Nedd4-2 interacts with its substrates, including hNHE3 and ENaC, at cell surface.\textsuperscript{17,50} IF microscopic analysis of hNHE3 and Nedd4-2 immediately after FSK treatment revealed significant overlaps of IF signals of hNHE3 and Nedd4-2, suggesting dynamic regulation of hNHE3–Nedd4-2 interaction at the apical region of the cells. Previous studies have shown phosphorylation of Nedd4-2 by PKA and serum and glucocorticoid regulated kinase 1 in COS-7 cells and Xenopus oocytes.\textsuperscript{37,38} Consistently, FSK induced phosphorylation of Nedd4-2 at S342 in colonic epithelial cells, and we observed increased presence of p-S342–Nedd4-2 in the NHE3-containing immunocomplex. Using PS120 cells, we demonstrated that the S342A mutation impaired Nedd4-2 binding to hNHE3. However, the assessment of the interaction between the S342A mutant and hNHE3 in Caco-2 cells was hindered by the low expression levels of S342A (and S448). We found that phosphorylation of Nedd4-2 is important for the maintenance of Nedd4-2 expression in colonic epithelial cells. Phosphorylation-dependent Nedd4-2 stabilization has previously been observed. Dephosphorylation at S448 by a phosphatase reduced Nedd4-2 abundance, whereas phosphorylation of Xenopus Nedd4-2 at S444 (equivalent to S448 in human Nedd4-2) increased its expression.\textsuperscript{51,52}

We demonstrated in this study that hNHE3 regulation by EPEC is PKC dependent. Previous studies have shown that EPEC rapidly activate host cell PKC and disrupt epithelial tight junctions through the activation of PKC-\(\epsilon\), and acute activation of NHE2 by EPEC has been shown to be dependent on PKC\(\alpha\) and PKC\(\epsilon\).\textsuperscript{30,53,54} Although whether EPEC-mediated NHE3 regulation is mediated by PKC has not been studied prior to the current study, there is an unmistakable similarity between the effects of PKC and EPEC on the different NHE isoforms. A study in PS120 cells has shown that the PKC activating phorbol 12-myristate 13-acetate inhibits NHE1 and NHE2 but activates NHE3.\textsuperscript{35} Interestingly, Nedd4-2 is activated by PKC to promote ubiquitination of membrane proteins, including cation amino acid transporter CAT-1, glutamate transport GLT-1, and organic anion transporter 1.\textsuperscript{15,56–58} Future studies are needed to investigate the mechanism of PKC-dependent regulation of Nedd4-2.

Activation of PKC by FSK, 8-bromo-cyclic adenosine monophosphate, parathyroid hormone, and dopamine results in phosphorylation of NHE3.\textsuperscript{11,12,29,59} Phosphopeptide mapping of NHE3 with mutational alteration of several serines has identified S552 and S605 as the primary sites of PKA-dependent phosphorylation in rat NHE3.\textsuperscript{11,39} Recently, NHE3 in human renal proximal tubule cells was shown to be phosphorylated at S552 by gastrin-mediated signaling, but interestingly this effect was PKC-dependent and independent of PKA.\textsuperscript{60} We showed here that FSK increased phosphorylation at S552 of hNHE3 in Caco-2bbe cells and hNHE3\textsuperscript{int} mouse intestine. One significant finding from the current study is that S552A and S605A mutations did not block PKA-mediated inhibition of hNHE3. These results contradict the previous studies on rat NHE3 that phosphorylation at these sites is a prerequisite for NHE3 regulation by PKA.\textsuperscript{31,39} In addition, the S552A and S605A mutants co-immunoprecipitated pS342–Nedd4-2, and the ubiquitination levels of S552A and S605A mutants were elevated in response to FSK, suggesting that phosphorylation of hNHE3 at these serines does not affect Nedd4-2-mediated ubiquitination of hNHE3. It is not clear how PKA is able to inhibit hNHE3 without altering hNHE3 phosphorylation. On the other hand, despite the importance of phosphorylation at S552 and S605 for the regulation of rat NHE3,\textsuperscript{11,39} how the phosphorylation impacts NHE3 is not clear. Previous studies using opossum kidney cells and rat kidney have indicated that phosphorylation of NHE3 does not directly impact the antiporter activity.\textsuperscript{61,62} These studies imply that NHE3 phosphorylation elicits endocytosis of NHE3. Although the mechanical link between NHE3 phosphorylation and endocytosis is not known, dynamin and clathrin are involved in this process.\textsuperscript{12,59,63} Since the initial description of ubiquitination as a mechanism of protein degradation, it has emerged as a major player in endocytosis of membrane proteins.\textsuperscript{12,64–66} Ubiquitinated cargoes can be internalized via clathrin-dependent as well as independent pathways.\textsuperscript{66,67} In addition, different clathrin adaptors may be involved in Ub-dependent versus Ub-independent internalization of cargoes.\textsuperscript{58,69} In the case of hNHE3, the internalization is not triggered by phosphorylation of hNHE3, but instead it is mediated by Nedd4-2–dependent ubiquitination. We postulate that inhibition of hNHE3 and non-human NHE3s is mediated via alternative mechanisms of clathrin-mediated endocytosis involving different adaptor proteins. In addition, we speculate that inhibition of hNHE3 is more efficient in part because Ub ligation occurs at multiple sites, enabling increased recruitment of clathrin-mediated endocytic machinery. Whether hNHE3 is regulated by other enteropathogenic bacteria via a similar mechanism remains to be determined.

The current study demonstrates that the effects of CTX and EPEC are greater in hNHE3\textsuperscript{int} mice compared with WT mice. However, we recognize a few potential limitations of the current hNHE3\textsuperscript{int} mouse model. First, because of the transgenic construct of hNHE3 gene, it is likely that more than 2 copies of hNHE3 gene are present, which result in higher basal expression levels and activity compared with WT mice. Second, hNHE3 is driven by the villin promoter so that any regulation at the transcription level is absent. This
limits the use of the model for a long-term study because Nhe3 expression can be transcriptionally suppressed by proinflammatory cytokines.\textsuperscript{70} Third, the villin promoter might have caused the expression of hNHE3 protein in lower crypt cells that are normally devoid of NHE3 expression.\textsuperscript{71} This could have contributed to the elevated basal activity of NHE3, although we have not specifically investigated the status of hNHE3 expression or activity in the lower part of the crypts. These limitations can be overcome with a knock-in mouse model that replaces mouse NHE3 with hNHE3 under the control of the endogenous NHE3 promoter.

With these limitations, our data demonstrate that CTX and EPEC augment the inhibition of hNHE3 via ubiquitination of hNHE3 that is distinct from the regulation of non-human NHE3. Inhibition of hNHE3 is dependent on Nedd4-2, which ubiquitinates and facilitates internalization of hNHE3. This unique biochemical feature of hNHE3 potentiates Na\textsuperscript{+} and water loss, and it may contribute to diarrheal symptoms occurring in humans.

### Materials and Methods

#### Reagents and Antibodies

CTX and FSK were purchased from Sigma-Aldrich (St Louis, MO). EPEC (E2348/69) and commensal \textit{E. coli} EFC-1 and EPEC were from Jan Klapproth (University of Pennsylvania, Philadelphia, PA).\textsuperscript{72,73} Rabbit polyclonal anti-NHE3 antibody EM450 and mouse monoclonal anti-VSVG P5D4 were previously described.\textsuperscript{22,74} Commercial antibodies used are listed in Table 1. All other chemicals were obtained from Sigma-Aldrich or EMD Millipore (Billerica, MA), unless otherwise specified.

#### Cell Culture and Plasmids

Caco-2bbe and SK-CO15 cells were cultured as previously described.\textsuperscript{22} pcDNA3.1 construct carrying hNHE3 (pcDNA-hNHE3V) or rbNHE3 (pcDNA-rbNHE3V) with a C-terminal VSVG epitope has been described.\textsuperscript{17} Caco-2bbe cells expression hNHE3 previously generated were used otherwise specified.\textsuperscript{26} Caco-2bbe cells were stably transfected with pcDNA-rbNHE3 and acid-selected as previously described.\textsuperscript{17} The lentiviral plasmid pLKO.1 containing shNedd4-2 or a scrambled control shRNA (shCon) was purchased from Sigma-Aldrich. pCI-HA-NEDD4L with or without a mutation in S342 or S448 was acquired from Dr Massague/Addgene (plasmid #27016, 27036, and 27037). The coding cDNA was subcloned into pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA). hNHE3 constructs with S552A and S605A mutation were generated using pCDNA/hNHE3V as a template by using a QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA). The oligonucleotide primers designed to introduce the mutations are as follows (codon changes are underlined): forward primer S552A, 5\textsuperscript{'}-GCT GAG GGA GAG CGC GGG GCC CTG GCC TTC ATC CGC TCC-3'; reverse primer S552A, 5\textsuperscript{'}-GGA GCG GAT GAA GCC CAG GGG CCC CTC TCC CTC AGC-3'; forward primer S605A, 5\textsuperscript{'}-TCT CTG CAG CAG CGA CGG GCC ATG-3'; reverse primer S605A, 5\textsuperscript{'}-CAT GTC CTC GCC CGT GAT GCC CGG CGC TCG CTG CTC AGA-3'. The presence of mutation was confirmed by nucleotide sequencing.

#### Bacterial Culture

EPEC and EFC-1 were grown overnight in LB broth. On the next day, 900 \muL of overnight culture were transferred into 30 mL of antibiotics-free Dulbecco modified Eagle medium and grown to an OD\textsubscript{600} of 0.4. Bacteria were pelleted and resuspended in 4 mL fresh medium. Caco-2bbe cells cultured to 10-14 days after confluence in 6-cm dishes were cultured overnight in the absence of penicillin/streptomycin. Caco-2 cells were then inoculated with 1 \times 10\textsuperscript{9} colony-forming unit of bacteria per well for up to 90 minutes. For in vivo perfusion assays, 1 mL of \textit{E. coli} was added to 10 mL perfusion buffer.

#### Generation of hNHE3\textsuperscript{int} Mice

The hNHE3 cDNA with a carboxyl-terminal VSVG tag was amplified by polymerase chain reaction (PCR) using the primer pair: 5\textsuperscript{'}-AGCGTACGCCACCAGT4GGGACT CGGGCGCCG -3' (forward) and 5\textsuperscript{'}-GAGCGGTGTTCCTGGGC- CAGCCCGTTTCAT -3' (reverse). The PCR product was cloned into the pBS-KS/Villin MES vector using BsiWI and MluI sites.\textsuperscript{14} The sequence of hNHE3 in pBS-KS was verified by sequencing. The transgene was excised with Sall, purified, and injected into the pronuclei of fertilized eggs of...
FVB/N mice at the Mouse Transgenic and Gene Targeting Core at Emory University (Atlanta, GA). The hNHE3 transgene was identified by PCR analysis of tail genomic DNA using the following primers: 5'-AGCAGTACCTCTGA-CAGCGG-3' (forward) and 5'-CTCCATGGGCGAGCTTC-CATTGG-3' (reverse) for hNHE3 transgene. Murine NHE3 genotyping was determined by using primers previously described.4 Transgenic expression of hNHE3 in the intestine was confirmed by Western blotting using anti-VSVG antibody. Slc9a-deficient (Nhe3/-) mice on the mixed 129/Black Swiss genetic background were previously described.5 Nhe3/- mice regeneraten from Drs Ghishan and Kiera at the University of Arizona (Tucson, AZ). Two hNHE3 transgenic male mice expressing both human Slc9a gene and transgenic human Slc9a gene. Resulting male mice from the initial breeding were crossed with female Nhe3/- mice to obtain offspring expressing one allele of mouse Slc9a gene and transgenic human Slc9a gene. These mice were labeled hNHE3int mouse. Because the initial estimation of hNHE3 expression in the intestinal epithelium by Western blotting was high, hNHE3int male mice were backcrossed with female Nhe3/- mice for 3 generations to reduce the expression levels of transgenic hNHE3. All animal experiments were conducted under approval by the Institutional Animal Care and Use Committee of Emory University and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Co-Immunoprecipitation and Western Blot

Co-immunoprecipitation experiments were performed using lysates from Caco-2bbe cells or mouse intestinal mucosa prepared using lysis buffer (Cell Signaling, Danvers, MA) containing 150 mmol/L NaCl, 1 mmol/L β-glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na2 EDTA, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, 1% Triton X-100, and protease inhibitors cocktail tablets (Roche, Indianapolis, IN). Lysate (500 μg) was pre-cleared by incubation with 30 μl of protein A-Sepharose beads for 1 hour, and the supernatant was then incubated overnight with a primary antibody (anti-HA, anti-VSVG, or anti-NHE3 antibody). Immunocomplex was purified by incubating with 50 μl of protein A-Sepharose beads for 1 hour, followed by 3 washes in lysis buffer and 2 washes in phosphate-buffered saline. All of the above steps were performed at 4°C or on ice. Beads eluted with 2.5× Laemmli buffer. Cell lysates and eluted samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western immunoblotting and quantification were performed as described previously.17,25

Histologic and IF Analysis

Ileal and colonic sections from WT, hNHE3int, and NHE3/- mice were harvested, rinsed in Hank's buffered saline solution (HBSS), opened lengthwise, and fixed in 10% neutral buffered formalin (Electron Microscopy Sciences, Hatfield, PA). Fixed tissues were embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin-eosin for light microscopic examination. IF staining of hNHE3 and mouse NHE3 with anti-NHE3 antibody (Alpha Diagnostics, Burlington, NC) was performed as previously described.25

Quantitative Real-Time PCR

Total RNA was extracted from intestinal mucosal scrapes or cultured cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). One μg of total RNA was used for cDNA synthesis using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instruction. Quantitative PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a Mastercycler Realplex (Eppendorf, Hamburg, Germany). PCR primer sequences are listed in Table 2.

| Table 2. Primer Sequences for Real-Time PCR Analysis |

| Target | Forward primer | Reverse primer |
|--------|----------------|----------------|
| CFTR   | AGAGCGATTTCCTGGACGC | CCAGCGAAGGCTTGTAG |
| DRA    | AATGCTGATGGACCTTGTGCT | TGCCTCTCCACGTTAG |
| NHE2   | ACTGGGGTCACAACTTGTGG | CTCACAGGTGACCTAGA |
| β-actin | TACAGTGTACACACACACG | AAGGAACGGCTGAAAAGAG |
| Universal | AAATCTCAAA(G/T)GAATGACCG | CTCAGC(G/A)(G/A)ACGACG |
| Bacteroidetes | C(G/A)AAACAGGATTAGATACCT | GTGAGGGTCTCGGGAT |
| Firmicutes | TGAAGCTT(C/T)AAGAGAAAGC | ACCATGCACCCAGTGC |
| Actinobacteria | TACGCGGGGCAAGGTCA | TCG(A/G)TCGCCACCCCTC |
| α-Proteobacteria | CA(C/A)GATGTTAGAGTAAATT | CCCGCTAATCTCTTTGAT |
| γ-Proteobacteria | TCCTAGCTGCTGTAT(T/G)GTA | TCGTAGCTGCTGTGTA |
| Clostridium leptum (IV) | CTTTCTGAGGCGG(G/C)AGTTA | GAATGCCGACAGACTT |
| Clostridium cocoides | AAATGACGGATCCGACTAA | CTTGAGTTCTGCTGGAA |

Primer sequences were adapted from reference 25 and reference 23.
Microbiota Analysis

Mice were anesthetized with isoflurane and euthanized by cervical dislocation. The intestine was removed under sterile conditions, and the contents of the cecum were collected, snap frozen in liquid nitrogen, and stored at −80°C until needed. Bacterial total DNA (genomic DNA) was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Frederick, MD). Specific primer sets were used in the amplification of microbial 16S rRNA gene on a Real Plex4 RealTime System (Eppendorf, Hauppauge, NY) as previously published. The sequences of the primers are listed in Table 2.

Fluid Accumulation in Ileal Closed Loop

Mice were anesthetized using a ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine), and a small incision was made in the abdomen. Two sections of ileum approximately 3.5–6 cm were tied off using surgical string. One loop was injected with 100 μL of HBSS control buffer, and the second loop was injected with 10 μg CTX (diluted in 100 μL HBSS). To inhibit NHE3 activity, NHE3 inhibitor S3226 (0.5 mg in 100 μL HBSS) was added to each isolated intestinal loop. The incision in the abdominal cavity was sutured, and mice were allowed to recover for 5 hours after injection. Mice were euthanized using isoflurane and cervical dislocation. Loops were excised, photographed, measured for length, and weighed. The weight to length ratio was calculated and used as a measure of fluid accumulation over the 5-hour treatment period.

Intestinal Water Flux Measurement

Intestinal water flux was measured as previously reported. Briefly, a mouse anesthetized with sodium pentobarbital was placed on a 37°C heating block, and a small incision was made in the abdomen. An approximately 5-cm loop of the ileum (between 5 and 10 cm upstream of cecum) cannulated at the proximal and distal ends was flushed with saline for 10 minutes. This was followed by perfusion of pre-warmed perfusion solution (mmol/L: 118.4 NaCl, 4.7 KCl, 2.52 CaCl2, 1.18 MgSO4, 25 Na gluconate, 1.18 KH2PO4, pH 7.4) at 1 mL/min for 2 hours. When needed, EPEC or control EFC-1 bacteria (multiplicity of infection: 200) were added to the perfusion buffer. CFinh-172 (250 μg/kg) was administered by intraperitoneal injection to each mouse 1 hour before the start of perfusion. CTX (0.5 μg/mL) was added to the perfusate. Intestinal water flux was determined using the ratio fluorometric, pH-sensitive dye 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester as previously described. Caco-2bbe cells grown for 10–14 days after confluence were treated with 10 μmol/L FSK for 30 minutes during dye loading. For experiments using EPEC, cells were infected at a multiplicity of infection of 20 for 90 minutes. For the measurement of NHE3 activity in mouse intestine, isolation of villi from mice ileum was performed as previously described. In brief, mice were euthanized with isoflurane, and the ileum was flushed with cold phosphate-buffered saline to remove food particles. An equivalent segment of the proximal ileum (approximately 10 cm upstream of the cecum) was opened longitudinally and stabilized on a cooled stage. The villi were dissected under stereomicroscope using sharpened microdissection scissors. Isolated villi were mounted on coverslips and covered with light and solution penetrable polycarbonate membrane (GE, Minnetonka, MN). Coverslips were mounted on a perfusion chamber, placed on an inverted microscope, superfused with NH4+ buffer, followed by sequential perfusion with tetramethylammonium (130 mmol/L TMA-Cl, 20 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L TMA-PO4, 2 mmol/L CaCl2, 1 mmol/L MgSO4, and 25 mmol/L glucose) and Na+ buffer that drives Na+−dependent pH recovery. Na+ buffer was supplemented with 30 μmol/L HOE694 or 2 μmol/L dimethyl amiloride to inhibit NHE1 and NHE2 activities. The microfluorometry was performed using the Metafluor software (Molecular Devices, Sunnyvale, CA), and 2–3 traces of Na+−dependent pH recovery were captured from each coverslip, each trace originating from an independent group of cells. For each experiment, a minimum of 4 coverslips per group were studied. Na+−H+ exchange rate was described by the initial rate of pH1 recovery, which was calculated by determining slopes along the pH1 recovery by linear least-squares analysis over a minimum of 7 seconds.

Detection of NHE3 Ubiquitination

Cells were lysed in cold lysis buffer supplemented with 10 μmol/L MG132 to inhibit proteasomal degradation. Equal amounts of cell lysates (typically 0.5 mg) were incubated overnight with EM450 antibody, followed by incubation with protein A-Sepharose beads for 1 hour. Immunocomplex was washed twice in lysis buffer and once in phosphate-buffered saline. NHE3 was eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with P4D1 antibody.

Surface Biotinylation

Surface biotinylation of NHE3 was performed as previously described. Briefly, cells grown in Transwells 2 weeks after confluence were treated with or without FSK for the indicated times, followed by rinsing with cold phosphate-buffered saline. Then 0.5 mg/mL NHS-SS-biotin in borate buffer was added to the apical chamber for 10 minutes, rinsed, and lysed. An aliquot was retained as the total fraction representing the total cellular NHE3. One mg of lysate was incubated with streptavidin-agarose beads (Thermo Fisher Scientific), and biotinylated surface proteins were then eluted representing surface NHE3. Densitometric analysis was performed using Scion Image software (National Institutes of Health, Bethesda, MD).
**Statistical Analysis**

Statistical analysis was performed using independent samples two-tailed unpaired Student t test or analysis of variance (ANOVA), followed by the Tukey post hoc analysis. Results are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). A value of $P < .05$ was considered significant.

**References**

1. Cheng AC, McDonald JR, Thielman NM. Infectious diarrhea in developed and developing countries. J Clin Gastroenterol 2005;39:757–773.
2. Viswanathan VK, Hodges K, Hecht G. Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. Nat Rev Microbiol 2009;7:110–119.
3. Zachos NC, Tse M, Donowitz M. Molecular physiology of intestinal Na+/H+ exchanger. Annu Rev Physiol 2005;67:411–443.
4. Schultheis PJ, Clarke LL, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, Giebisch G, Aronson PS, Lorenz JN, Shull GE. Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. Nat Genet 1998;19:282–285.
5. Janecke AR, Heinz-Erian P, Yin J, Petersen BS, Franke A, Lechner S, Fuchs I, Melancon C, Uhlig HH, Travis S, Marinier E, Peric V, Ristic N, Gerner P, Booth IW, Wedenoja S, Baumgartner N, Vodopiutz J, Frechette-Duval MC, De Laffolie J, Persad R, Werner N, Tse CM, Kud K, Zachos NC, Sarker R, Zhu X, Muise AM, Zimmer KP, Witt H, Zoller H, Donowitz M, Muller T. Reduced sodium/proton exchanger NHE3 activity causes congenital sodium diarrhea. Hum Mol Genet 2015;24:6614–6623.
6. Seidler U, Lenzen H, Cinar A, Tessema T, Bleich A, Riederer B. Molecular mechanisms of disturbed electrolyte transport in intestinal inflammation. Ann N Y Acad Sci 2006;1072:262–275.
7. Anderson CA, Boucher G, Lees CW, Franke A, D’Amato M, Taylor KD, Lee JC, Goyette P, Imielinski M, Latiano A, Lagace C, Scott R, Amininejad L, Bumpstead S, Baidoo L, Baldassano RN, Barclay M, Bayless TM, Brand S, Buning C, Colombel JF, Denson LA, De Vos M, Dubinsky M, Edwards C, Ellinghaus D, Fehrmann RS, Floyd JA, Florin T, Fehrmann RS, Goyette P, Hecht G. The enteropathogenic Escherichia coli effector protein EspF decreases sodium hydrogen exchanger 3 activity. Cell Microbiol 2008;10:1735–1745.
8. Zhou H, Wiederkehr MR, Fan L, Collazo RL, Thielman NM. Infectious diarrhea in developed and developing countries. J Clin Invest 2003;111:931–943.
9. Field M. Intestinal ion transport and the pathophysiology of diarrhoea. J Clin Invest 2003;111:931–943.
10. No YR, He P, Yang J, Cole R, Tse M, Berman DE, Small SA, Petsko G, Donowitz M. Cholera toxin inhibits NHE3 by cAMP: role of protein kinase a and NHE3 phosphoserines 552 and 605. J Biol Chem 1999;274:3978–3987.
11. Hu MC, Fan L, Breton LA, Karin-Jimenez Z, Murer H, Moe OW. Dopamine acutely stimulates Na/H exchanger NHE3 endocytosis via clathrin-coated vesicles: dependence on protein kinase A-mediated NHE3 phosphorylation. J Biol Chem 2001;276:26906–26915.
12. Singh V, Yang J, Yin J, Cole R, Tse M, Berman DE, Small SA, Petsko G, Donowitz M. Cholera toxin inhibits NHE3 by cAMP: role of protein kinase a and NHE3 phosphoserines 552 and 605. J Biol Chem 1999;274:3978–3987.
13. Chen HD, Frankel G. Enteropathogenic Escherichia coli: unravelling pathogenesis. FEMS Microbiol Rev 2005;29:83–98.
14. Hodges K, Alto NM, Ramoswamy K, Dudeja PK, Hecht G. The enteropathogenic Escherichia coli effector protein EspF decreases sodium hydrogen exchanger 3 activity. Cell Microbiol 2008;10:1735–1745.
15. Piper RC, Lehner PJ. Endosomal transport via ubiquitination. Trends Cell Biol 2011;21:647–655.
16. No YR, He P, Yao BK, Yun CC. Unique regulation of human Na+/H+ exchanger 3 (NHE3) by Nedd4-2 ligase that differs from non-primate NHE3s. J Biol Chem 2014;289:18360–18372.
17. Armando I, Villar VA, Jones JE, Lee H, Wang X, Asico LD, Yu P, Yang J, Escano CS Jr, Pascua-Crusan AM, Felder RA, Jose PA. Dopamine D3 receptor inhibits the ubiquitin-specific peptidase 48 to promote NHE3 degradation. FASEB J 2014;28:1422–1434.
18. Han Y, Yun CC. Ubiquitin-specific peptidase 7 (USP7) and USP10 mediate deubiquitination of human NHE3 regulating its expression and activity. FASEB J 2020;34:16476–16488.
models of clinical disease. Annual Review of Pathology 2017;12:187–215.

22. Yoo BK, Yanda MK, No YR, Yun CC. Human intestinal epithelial cell line SK-CO15 is a new model system to study Na(+)/H(+) exchanger 3. Am J Physiol Gastrointest Liver Physiol 2012;303:G180–G188.

23. Larmonier CB, Laubitz D, Hill FM, Shehab KW, Lipinski L, Midura-Kiela MT, McFadden RM, Ramalingam R, Hassan KA, Golebiewski M, Besselsen DG, Ghishan FK, Kiela PR. Reduced colonic microbial diversity is associated with colitis in NHE3-deficient mice. Am J Physiol Gastrointest Liver Physiol 2013;305:G667–G677.

24. Zhou Q, Clarke L, Nie R, Cames K, Lai LW, Lien YH, Verkman AS. Mouse model of enteropathogenic Escherichia coli infection. Infect Immun 2005;73:1161–1170.

25. Hecht G, Koutsouris A. Enteropathogenic E coli attenuates secretagogue-induced net intestinal ion transport but not Cl- secretion. Am J Physiol 1999;276:G781–G788.

26. Staub O, Rotin D. Role of ubiquitylation in cellular membrane transport. Physiol Rev 2006;86:669–707.

27. Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Lepetit J, Staub O. Phosphorylation of Nedd4-2 by Skg1 regulates epithelial Na(+) channel surface expression. EMBO J 2001;20:7052–7059.

28. Snyder PM, Olson DR, Kabra R, Zhou R, Steines JC. cAMP and serum and glucocorticoid-inducible kinase (SGK) regulate the epithelial Na(+) channel through neuronal nitric oxide synthase. J Biol Chem 2004;279:45753–45758.

29. Kurashima K, Yu FH, Capadagbo AG, Szabo EZ, Grinstein S, Orlowski J. Identification of sites required for down-regulation of Na/H exchanger NHE3 activity by cAMP-dependent protein kinase. J Biol Chem 1997;272:28672–28679.

30. Kocinsky HS, Girardi ACC, Biesesderfer D, Nguyen T, Mentone S, Orlowski J, Aronson PS. Use of phospho-specific antibodies to determine the phosphorylation of endogenous Na(+)/H(+) exchanger NHE3 at PKA consensus sites. Am J Physiol Renal Physiol 2005;289:F249–F258.

31. Thiagarajah JR, Donowitz M, Verkman AS. Secretory diarrhoea: mechanisms and emerging therapies. Nat Rev Gastroenterol Hepatol 2015;12:446–457.

32. Li Y, Xia S, Jiang X, Wang G, Ma J, Fang Z, Yin J, Yin Y. Gut microbiota and diarrhea: an updated review. Frontiers in Cellular and Infection Microbiology 2021:11.

33. Savkovic SD, Hecht G. Mouse model of enteropathogenic Escherichia coli infection. Infect Immun 2005;73:1161–1170.

34. Ghanekar Y, Chandrashaker A, Visweswariah SS. Human intestinal epithelial cell line SK-CO15 is a new model system to study Na(+)/H(+) exchanger 3. Am J Physiol Gastrointest Liver Physiol 2012;303:G180–G188.

35. Hecht G, Koutsouris A. Enteropathogenic E coli attenuates secretagogue-induced net intestinal ion transport but not Cl- secretion. Am J Physiol 1999;276:G781–G788.

36. Staub O, Rotin D. Role of ubiquitylation in cellular membrane transport. Physiol Rev 2006;86:669–707.

37. Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Lepetit J, Staub O. Phosphorylation of Nedd4-2 by Skg1 regulates epithelial Na(+) channel surface expression. EMBO J 2001;20:7052–7059.

38. Snyder PM, Olson DR, Kabra R, Zhou R, Steines JC. cAMP and serum and glucocorticoid-inducible kinase (SGK) regulate the epithelial Na(+) channel through neuronal nitric oxide synthase. J Biol Chem 2004;279:45753–45758.

39. Kurashima K, Yu FH, Capadagbo AG, Szabo EZ, Grinstein S, Orlowski J. Identification of sites required for down-regulation of Na/H exchanger NHE3 activity by cAMP-dependent protein kinase. J Biol Chem 1997;272:28672–28679.

40. Kocinsky HS, Girardi ACC, Biesesderfer D, Nguyen T, Mentone S, Orlowski J, Aronson PS. Use of phospho-specific antibodies to determine the phosphorylation of endogenous Na(+)/H(+) exchanger NHE3 at PKA consensus sites. Am J Physiol Renal Physiol 2005;289:F249–F258.

41. Thiagarajah JR, Donowitz M, Verkman AS. Secretory diarrhoea: mechanisms and emerging therapies. Nat Rev Gastroenterol Hepatol 2015;12:446–457.

42. Li Y, Xia S, Jiang X, Wang G, Ma J, Fang Z, Yin J, Yin Y. Gut microbiota and diarrhea: an updated review. Frontiers in Cellular and Infection Microbiology 2021:11.

43. Kocinsky HS, Girardi ACC, Biesesderfer D, Nguyen T, Mentone S, Orlowski J, Aronson PS. Use of phospho-specific antibodies to determine the phosphorylation of endogenous Na(+)/H(+) exchanger NHE3 at PKA consensus sites. Am J Physiol Renal Physiol 2005;289:F249–F258.

44. Dupont A, Sommer F, Zhang K, Repnik U, Basic M, Bleich A, Kuhnzel M, Backhed F, Litvak Y, Fulde M, Rosenshine I, Hornef MW. Age-dependent susceptibility to enteropathogenic Escherichia coli (EPEC) infection in mice. PLoS Pathog 2016;12:e1005616.

45. Frankel G, Phillips AD, Novakova M, Field H, Candy DC, Schauer DB, Douce G, Dougan G. Intimin from enteropathogenic Escherichia coli restores murine virulence to a Citrobacter rodentium eaeA mutant: induction of an immunoglobulin A response to intimin and EspB. Infect Immun 1996;64:5315–5325.

46. Shi PP, Cao XR, Sweezer EM, Kinney TS, Williams NR, Husted RF, Nair R, Weiss RM, Williamson RA, Sigmund CD, Snyder PM, Staub O, Stokes JB, Yang B.
Salt-sensitive hypertension and cardiac hypertrophy in mice deficient in the ubiquitin ligase Nedd4-2. Am J Physiol Renal Physiol 2008;295:F462–F470.

47. Boase NA, Rychkov GY, Townley SL, Dinudom A, Candi E, Voss AK, Tsoutsman T, Semsarian C, Melino G, Koentgen F, Cook DJ, Kumar S. Respiratory distress and perinatal lethality in Nedd4-2-deficient mice. Nat Commun 2011;2:287.

48. Malsure S, Wang Q, Charles RP, Sergi C, Perrier R, Christensen BM, Maillard M, Rossier BC, Hummeler E. Colon-specific deletion of epithelial sodium channel causes sodium loss and aldosterone resistance. J Am Soc Nephrol 2014;25:1453–1464.

49. Crane JK, Oh JS. Activation of host cell protein kinase C beta1Pix exchange factor stabilizes the ubiquitin ligase Nedd4-2 and plays a critical role in ENaC regulation by AMPK in kidney epithelial cells. Eur J Pharmacol 2014;732:32.

50. Zhou R, Patel SV, Snyder PM. Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC. J Biol Chem 2007;282:20207–20212.

51. Ho PY, Li H, Pavlov TS, Tuerk RD, Tabares D, Hodges K, Gill R, Ramaswamy K, Dudeja PK, Hecht G. Chronic regulation of the renal Na(+)/H(+) exchanger NHE3 by dopamine: translational and posttranslational mechanisms. Am J Physiol Renal Physiol 2013;304:F1169–F1180.

52. Kocinski HS, Dynia DW, Wang T, Aronson PS. NHE3 phosphorylation at serines 552 and 605 does not directly affect NHE3 activity. Am J Physiol Renal Physiol 2007;293:F212–F218.

53. Crane JK, Oh JS. Activation of host cell protein kinase C beta1Pix exchange factor stabilizes the ubiquitin ligase Nedd4-2 and plays a critical role in ENaC regulation by AMPK in kidney epithelial cells. Eur J Pharmacol 2014;732:32.

54. Xue J, Thomas L, Tahmasbi M, Valdez A, Dominguez Collazo R, Fan L, Hu MC, Zhao H, Wiederkehr MR, Moe OW. Acute regulation of Na(+)/H(+) exchanger NHE3 by parathyroid hormone via NHE3 phosphorylation and dynamin-dependent endocytosis. J Biol Chem 2000;275:31601–31608.

55. Liu T, Jose PA. Gastrin induces sodium-hydrogen exchanger 3 phosphorylation and mTOR activation via a phosphoinositide 3-kinase-/protein kinase C-dependent but AKT-independent pathway in renal proximal tubule cells derived from a normotensive male human. Endocrinology 2013;154:865–875.

56. Hu MC, Di Sole F, Zhang J, McLeroy P, Moe OW. Chronic regulation of the renal Na(+)/H(+) exchanger NHE3 by dopamine: translational and posttranslational mechanisms. Am J Physiol Renal Physiol 2013;304:F1169–F1180.

57. Chow CW, Khurana S, Woods M, Grinstein S, Orlowski J. The epithelial Na(+)/H(+) exchanger, NHE3, is internalized through a clathrin-mediated pathway. J Biol Chem 1999;274:37551–37558.

58. Mayers JR, Wang L, Pramanik J, Johnson A, Sarkeshik A, Wang YJ, Saengsawang W, Yates JR, Audhya A. Regulation of ubiquitin-dependent cargo sorting by multiple endocytic adaptors at the plasma membrane. Proc Natl Acad Sci U S A 2013;110:11857–11862.

59. Piper RC, Dikic I, Lukacs GL. Ubiquitin-dependent sorting in endocytosis. Cold Spring Harb Perspect Biol 2014;6.

60. Sigismund S, Woelk T, Puri C, Maspéro E, Tacchetti C, Transidico P, Di Fiore PP, Polo S. Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci U S A 2005;102:2760–2765.

61. Henry AG, Hislop JN, Grove J, Thorn K, Marsh M, von Zastrow M. Regulation of endocytic clathrin dynamics by cargo ubiquitination. Dev Cell 2012;23:519–532.

62. Wolfe BL, Marchese A, Trejo J. Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. J Cell Biol 2007;177:905–916.

63. Traub LM, Lukacs GL. Decoding ubiquitin sorting signals for clathrin-dependent endocytosis by CLASPs. J Cell Sci 2012;125:543–553.

64. Amin MR, Malakooti J, Sandoval R, Dudeja PK, Ramaswamy K. IFN-(gamma) and TNF-(alpha) regulate human NHE3 gene expression by modulating the Sp family transcription factors in human intestinal epithelial cell line C2BBer1. Am J Physiol 2006;291:C887–C896.

65. Pinto D, Robine S, Jaisser F, El Marjou FE, Louvard D. Regulatory sequences of the mouse villin gene that efficiently drive transgenic expression in immature and differentiated epithelial cells of small and large intestines. J Biol Chem 1999;274:6476–6482.

66. Babbin BA, Sasaki M, Gerner-Schmidt KW, Nusrat A, Klaproth JM. The bacterial virulence factor lysphostatin compromises intestinal epithelial barrier function by modulating rho GTPases. Am J Pathol 2009;174:1347–1357.
73. Liang Z, Zhan W, Zhu A, Yoon Y, Lin S, Sasaki M, Klapproth J-MA, Yang H, Grossniklaus HE, Xu J, Rojas M, Voll RJ, Goodman MM, Arrendale RF, Liu J, Yun CC, Snyder JP, Liotta DC, Shim H. Development of a unique small molecule modulator of CXCR4. PLoS ONE 2012;7:e34038.

74. Kreis TE. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. EMBO J 1986; 5:931–941.

75. He P, Lee SJ, Lin S, Seidler U, Lang F, Fejes-Toth G, Naray-Fejes-Toth A, Yun CC. Serum- and glucocorticoid-induced kinase 3 in recycling endosomes mediates acute activation of Na⁺/H⁺ exchanger NHE3 by glucocorticoids. Mol Biol Cell 2011;22:3812–3825.

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