

Supplementary Materials and Methods

Reagents and Materials

BODIPY 577/618 maleimide, nigericin, 2',7'-Bis(carboxyethyl)-5-6-carboxyfluorescein-AM (BCECF), 1,4-(and 5)-benzenedicarboxylic acid, 2-[10-(dimethylamino)-4-fluoro-3-oxo-3H-benzo[c]xanthen-7-yl] (carboxy SNARF-4F), Alexa Fluor-conjugated streptavidin, Hoescht 33342, and Alexa Fluor-conjugated mouse monoclonal and rabbit polyclonal secondary antibodies were from Life Technologies. IRDye monoclonal and polyclonal secondary antibodies were from Rockland, Inc. Monoclonal anti-hemagglutinin (HA) affinity matrix was from Roche Diagnostics (Cat #: 1181501600). Rabbit polyclonal human NHE3 antibody was from Novus, Inc (Cat #: NBP1-82574). Hamster anti-murine CD3 monoclonal antibody (2C11) from Pharmingen was a gift from Dr Jerrold Turner (Brigham and Women's Hospital).

Nanoparticle Materials

The following monomers were purchased from Sigma-Aldrich: bisphenol A glycerolate diacrylate (B7) (CAS 4687-94-9), trimethyl propane triacrylate (B8) (CAS 15625-89-5), and 1,3-diaminopropane (E1) (CAS 109-76-2). 4-amino-1-butanol (S4) (CAS 13325-10-5) was purchased from Alfa Aesar. Bioreducible base monomer 2,20-disulfanediybis(ethane-2,1-diyl) diacrylate (BR6) was synthesized as described by Kozielski et al.^{e1} Carboxylate ligand C5 was synthesized as described by Rui et al.^{e2}

Cells

All immortalized cell lines and heterologous expression of NHE3 have been described previously.^{e3,e4} Briefly, PS120 cells (a fibroblast cell line lacking all endogenous plasma membrane NHEs) were stably transfected with triple HA-N-terminally tagged rabbit NHE3 (HA3-NHE3) constructs. Polarized Caco-2/BBe cells (a human colon cancer cell line with small-intestinal properties), which express NHERFs 1–4, but undetectable NHE3 activity, were transduced on day 12 after confluency with an adenovirus-triple HA-tagged NHE3 construct and studied 48 hours after infection. Human jejunal enteroid cultures were established from deidentified normal, healthy adult individuals, as previously described,^{e5–e8} with Johns Hopkins University Institutional Review Board approval (NA_0038329). Human enteroids, which express BB NHE3, were cultured in Matrigel (Corning Cat #: 356231) and studied as confluent monolayers on collagen IV coated Transwell inserts (Corning Cat #: 3470), as described.^{e9,e10}

Mice

Male and female C57Bl/6N mice (8–12 weeks old) were starved overnight and provided water ad libitum before experiments that consisted of (1) euthanasia and acute removal of jejunum for studies by multiphoton measurement of basal NHE3 activity, as previously reported,^{e11} (2) cannulation for measurement of basal in vivo net water transport by perfusion using the nonabsorbable marker,

Fe(CN)₆^{e12} or (3) formation of upper small-intestinal 3- to 4-cm loops for study of baseline fluid absorption or CTx or *Escherichia coli* heat-stable enterotoxin-induced fluid secretion and intestinal fluid secretion induced by intraperitoneal anti-CD3 monoclonal antibody injection.^{e12} All experimental protocols performed in mice were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (M019M118).

In Vitro Transport Assays Cells, Enteroids, and Mouse Intestine

NHE3 activity in PS120, Caco-2/BBe cells, and human jejunal enteroids was determined fluorometrically (Photon Technologies Inc) using the intracellular pH-sensitive dye BCECF, as reported.^{e4,e7,e13}

PS120/HA3-NHE3 cells were seeded on glass cover slips and studied at 70% to 80% confluency. Caco-2/BBe/HA3-NHE3 cells were grown to confluency on Transwell inserts (polyester, 0.4- μ m pore size) and studied ~14 to 16 days later. Human duodenal enteroid monolayers on Transwell inserts were differentiated for 5 days in the absence of Wnt3a, R-spondin, and noggin, as described.^{e9,e10}

Rates of Na⁺/H⁺ exchange were determined as Na⁺-dependent alkalization in the presence of 50 μ mol/L HOE-694 (to inhibit all other plasma membrane NHE isoforms) in HCO₃⁻-free solutions, with internal calibration using the nigericin/K⁺ method, as previously described.^{e7,e13} Kinetic analysis was used for PS120 cell studies (expressed as V_{max}) and initial rates (Δ pH/min) for Caco-2/BBe cells, mouse intestine in vitro, and human enteroids.

For in vitro mouse jejunal transport studies, NHE3 activity (in the presence of 50 μ mol/L HOE-694) was measured using SNARF-4F at 37°C in a perfusion chamber using a 2-photon microscope (Olympus FV1000) and 25 \times objective, as described.^{e11} In vitro determination of electrogenic active ion transport in mouse jejunum was performed by Ussing chamber/voltage clamp technique, as described.^{e14}

For the measurement of active anion secretion using the Ussing chamber/voltage clamp technique, mice were euthanized, and intact jejunum was mounted between 2 halves of an Ussing chamber for measurement of I_{sc}, potential difference (PD), and transepithelial electrical resistance. These measurements were performed at 37°C with tissue exposed to a solution (gassed with 5% CO₂/95% O₂) containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, 10 mmol/L HEPES, with 10 mmol/L D-glucose on the mucosal surface and 10 mmol/L mannitol on the serosal surface. Tissue was continually voltage clamped to zero PD except for short periods to determine the PD. Tissues were allowed to reach steady-state values, followed by sequential addition to the serosal surface of 10 μ mol/L FSK and 10 μ mol/L carbachol, with determination of peak increase in I_{sc} caused by each.

In Vivo Transport Assays

All in vivo studies were performed on 8- to 12-week-old C57Bl/6N mice anesthetized with isoflurane and kept at 37°C via heating lamps and rectal thermal probes.

BODIPY-Conjugated Peptide Studies

To measure peptide effects on basal net water transport, 3- to 4-cm loops were prepared with (1) inflow and outflow catheters. Loops were perfused (1 mL/min with a peristaltic pump) with 90 mmol/L NaCl, 25 mmol/L NaHCO₃, 30 mmol/L HEPES, 5 mmol/L KCl, 1.2 mmol/L CaCl₂, and 20 mmol/L glucose, pH 7.4, containing 2 mmol/L sodium ferrocyanide as a nonabsorbable marker with CP or N3SP. A 30-minute stabilization period was followed by three 20-minute perfusion periods. Ferrocyanide concentration was determined by colorimetric assay,^{e12} or (2) 150 μ L PBS (pH 7.4, 100 mmol/L NaCl, 25 mmol/L NaHCO₃, 5 mmol/L KCl, 1.2 mmol/L CaCl₂, 30 mmol/L HEPES) with control or test peptide (400 nmol/L) was incubated for 4 hours, the loops were drained, and 150 μ L PBS was added. After 30 minutes the animals were euthanized. The loops were removed and weighed, and wet weight/length was determined.

CTX-induced fluid secretion was determined on 3- to 4-cm small-intestinal ligated loops (starting ~2 cm distal to the ligament of Treitz) with instillation of 100 μ L of PBS, with or without 0.1 μ g purified CTx (Sigma-Aldrich) with 400 nmol/L of CP-1, N3SP-1, or PBS alone. The peptides were conjugated to BODIPY. After loop preparation, abdomens were closed, and mice were removed from anesthesia and recovered in separate cages for the next 6 hours. After this time, animals were euthanized by cervical dislocation, and intestinal segments were removed and weighed, and length was determined. Data are presented as loop weight/cm.

Similar studies were performed with instillation of *E coli* heat-stable enterotoxin (0.05 μ g) studied for 4 hours. In the anti-CD3 antibody experiments, jejunal loops were injected with 100 μ L of PBS, CP-1 (400 nmol/L per 100 μ L) or N3SP-1 (400 nmol/L per 100 μ L). Immediately after the abdomens were closed surgically, 200 μ g of anti-CD3 monoclonal antibody in 200 μ L PBS or 200 μ L PBS alone were injected intraperitoneally. The animals were euthanized 2.5 hours after the intraperitoneal injections, and loop weight/cm length was determined.^{e12}

Nanoparticle-Conjugated Peptide Studies

Effect on basal fluid transport and effect of tenapanor. Mice operated on as above had 3- to 4-cm closed ileal loops inoculated with 150 μ L PBS containing 400 nmol/L nanoparticle-CP-2 or -N3SP-1 Δ 7. After 4 hours, the loops were drained and inoculated with 150 μ L PBS. The animals were euthanized 30 minutes later, and loop weight/length was determined. In parallel studies, tenapanor (10 μ mol/L) was present in both incubation periods.

Similar to the BODIPY-conjugated peptide studies, the effects of nanoparticle-CP-2 or -N3SP-1 Δ 7 (4 μ mol/L) compared with PBS alone were determined on net fluid secretion produced by inoculation of 0.1 μ g CTx. The nanoparticle-peptides plus 0.1 μ g CTx in 150 μ L PBS were inoculated in 3- to 4-cm-long closed ileal loops. Additional controls included loops exposed only to 150 μ L PBS and those only exposed to the CTx/PBS. The animals were euthanized 4 hours later, and loop weight/cm length was determined.

Immunofluorescence/Confocal Microscopy

Mouse jejunum was fixed in 10% neutral buffered formalin, processed, and paraffin-embedded. Then, 5- μ m sections were washed in xylene and rehydrated in gradient ethanol. Sections were exposed to antigen retrieval in 10 mmol/L sodium citrate (pH 6.0) for 10 minutes, washed in double-distilled H₂O, and then blocked in buffer containing 2% bovine serum albumin, 15% fetal bovine serum, and 0.1% saponin for 30 minutes. Anti-NHE3 antibody (1:100) was incubated in blocking buffer overnight and then washed 3 times in PBS. Sections were then exposed to Alexa Fluor-conjugated secondary antibody (1:100) and Hoescht 33342 for 1 hour, washed in PBS, mounted, and coverslipped. Confocal images were obtained on a Zeiss 510 META confocal microscope or Olympus FlowView3000RS using 63 \times water or 40 \times oil immersion objectives (UPLSAPO 100xs 1.35 NA silicone objective), respectively.

Adenosine 3',5'-Cyclic Monophosphate Assay

Human jejunal enteroid monolayers on Transwell inserts (Corning Cat #: 3470) were differentiated for 5 days and then in serum-free media were exposed apically to nanoparticles containing 250 nmol/L CP-2 or N3SP-1 Δ 7. The peptide and polymer in PBS were prepared just before use and allowed to combine for 10 minutes before being added to the apical surface of enteroid monolayers at room temperature. Monolayers were incubated for 4 hours at 37°C in a 5% CO₂ incubator before the nanoparticles were removed; then, monolayers were washed 3 times with cold PBS and changed to differentiation media and moved to a 37°C incubator. Differentiation media was removed 24 hours later, and monolayers were washed twice with complete media with growth factor (advanced Dulbecco's modified Eagle medium/F12 with 10 mmol/L HEPES, 10 mmol/L GlutaMAX [Thermo Fisher Scientific], and 1000 U/mL penicillin and streptomycin). Next, 100 μ L of complete media with growth factor media containing 10 mmol/L theophylline, 50 μ mol/L cilostazol, and 50 μ mol/L rolipram was added to the apical and basolateral surfaces, followed by exposure to FSK (10 μ mol/L) at 37°C in 5% CO₂ for 45 minutes before apical and basolateral media were collected and cells were lysed in 200 μ L of 0.1 mol/L HCl for cAMP analysis. cAMP in apical, basolateral, and cells was determined using nonradioactive cAMP enzyme-linked immunosorbent assay kits from ENZO Life Sciences, using the acetylation protocol in which the yellow-colored product is inversely proportional to the amount of cAMP present in the sample.

Recombinant Protein Expression and Purification

As published previously, DNAs encoding maltose-binding protein (MBP) and glutathione S-transferase (GST) were cloned into pET28a (EMD Millipore) to make pMBP and pGST respectively.^{e15} DNA fragments encoding the C-terminus of rabbit NHE3 (aa 465–670 and 540–670) and full-length human NHERF2 were generated by polymerase chain reaction and inserted into pMBP or pGST, respectively, with expression of MBP-NHE3 465–670 and 540–670 or GST-NHERF2. These constructs were transformed

into BL21 (DE3) strain (EMD Millipore). Bacterial cultures were induced with 0.3 mmol/L isopropyl 1-thio- β -D-galactopyranoside at 37°C for 3 hours. MBP-tagged proteins were purified with a column packed with amylose resin by gravity flow following the New England Biolabs manuals of the pMAL system. GST-tagged proteins were purified in a gravity-flow column following the instructions from GE Healthcare. Purified protein was concentrated by Amicon Ultra-15 Centrifugal filter units (EMD Millipore) supplemented with 10% glycerol and 10 mmol/L dithiothreitol and stored at -80°C.

Overlay Experiment Methods

Recombinant proteins MBP-NHE3-465-670 and MBP-NHE3-540-670 and GST-NHERF2 were separated by 10% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gels and transferred onto a nitrocellulose membrane. All proteins were estimated to have a concentration of 0.067 μ mol/L. The blots were pre-overlaid with 0.33 μ mol/L N3SP-1 Δ 7 peptide or CP-2 for 1 hour at room temperature in blocking buffer containing 5% nonfat milk in PBS. GST-NHERF2 (0.067 μ mol/L) recombinant protein was then added and incubated for 16 to 20 hours at 4°C. The blots were washed 3 times, each for 4 minutes, with very gentle shaking in washing buffer (0.1% Tween-20 in PBS). The blots were then probed with primary antibody against NHERF2 (Sigma-Aldrich, Cat #: HPA001672) in blocking buffer overnight at 4°C and then washed as above. This was followed by addition of IRDye 680CW goat anti-rabbit secondary antibody (LI-COR Biosciences, Cat #: 926-68071) for 1 hour at room temperature. Blots were washed, and protein bands were visualized and quantitated using an Odyssey system and Image Studio software (LI-COR Biosciences).

BODIPY-Peptide Conjugation

BODIPY maleimides are hydrophobic and demonstrated by us and others to facilitate entry of small peptides into cells.^{e16,e17} Briefly, peptides were reconstituted in PBS and exposed to tris(2-carboxyethyl)phosphine (1 mmol/L) and BODIPY under inert conditions for 2 hours at room temperature with constant end-over-end rotation. Unconjugated BODIPY was removed from the solution using sulphagarose beads for 2 hours at room temperature with constant rotation. After beads were pelleted by gravity, the supernatant containing BODIPY-conjugated peptides was stored at 4°C and used within 1 week of preparation.

Nanoparticle Preparation

Polymer synthesis. Hyperbranched PBAE was synthesized as previously described by Rui et al.^{e2} Briefly, monomers BR6, B7, and B8 were combined at a 0.16 (BR6):0.64 (B7):0.2 (B8) molar ratio in anhydrous *N,N*-dimethylformamide. Monomer S4 was added at a 2.2:1 vinyl-to-amine ratio, with a final monomer concentration of 150 mg/mL. Polymerization proceeded overnight at 90°C with stirring. The resulting acrylate-terminated polymers were then end capped with monomer E1, purified by 2

diethyl ether washes, followed by a second round of end capping with monomer C5. Both reactions were performed at a final end cap concentration of 0.2 mol/L for 2 hours at room temperature. The resulting carboxylated polymers were precipitated by diethyl ether, and the remaining solvent was removed in a desiccation chamber under vacuum. The polymer was dissolved in anhydrous dimethyl sulfoxide to a final concentration of 100 mg/mL and stored with desiccant at -20°C.

Polymer characterization. Polymer structure was characterized by nuclear magnetic resonance (NMR) spectroscopy via 500 MHz ¹H-NMR in CDCl₃ (Bruker) and analyzed using TopSpin 3.5 software (Bruker). The presence of acrylate groups in the acrylate-terminated base polymer was confirmed by peaks in the 6 to 6.5 ppm range, which disappeared upon polymer end capping. Gel permeation chromatography (Waters) was used to characterize polymer molecular weight relative to polystyrene standards.

Nanoparticle synthesis and characterization. PBAE polymer and peptide were separately dissolved in pH 7.4 150 mmol/L PBS and combined at a 1:1 volume ratio. Final concentrations of the components were 6 ng/ μ L peptide and 180 ng/ μ L polymer (30:1 polymer-to-peptide weight ratio). Polymer and peptide nanoparticles were allowed to self-assemble at room temperature for 10 minutes. For characterization, nanoparticles were diluted 1:5 in PBS. Hydrodynamic diameter and zeta potential were measured using a Malvern Zetasizer Pro (Malvern Panalytical). Stability was assessed by incubating nanoparticles at room temperature up to 45 minutes and then measuring hydrodynamic diameter over 45 minutes. Nanoparticles were made immediately before each experiment.

Morphology of nanoparticle-peptides by transmission electron microscopy. PBAE nanoparticle-peptide (2 μ mol/L) in PBS was applied to a square copper 400 mesh grid (Electron Microscopy Sciences). Samples were air dried overnight at room temperature. Samples were then dipped in a 0.5 wt% uranyl acetate solution and air dried for 1 hour at room temperature. Once samples were fully dried, the mesh grid was imaged on a Hitachi 7600 transmission electron microscope (Hitachi High-Technologies).

Effects of pH on Nanoparticle Viability and Uptake

B16F10 cells were plated at 15,000 cells/well of a 96-well plate in RPMI 1640 medium with 5% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific). Media were changed 24 hours after plating and replaced with serum-free RPMI 1640. PBAE nanoparticles encapsulating fluorescent peptide or fluorescent peptide alone at 2 μ mol/L concentration in PBS were exposed to varying pH conditions (pH 1.2, pH 4, and pH 7) for 15 minutes. After 15 minutes, samples were neutralized to pH 7 and added to cells. Samples were incubated with cells at 37°C for 2 hours. After 2 hours, media was replaced. At 24 hours, cell viability was measured via CellTiter96 MTS assay (Promega). Samples were normalized to a cell-only control.

At 48 hours, cells were trypsinized, washed, and resuspended in fluorescence-activated cell sorter buffer (PBS with 1% fetal bovine serum). Cells were then measured for uptake via flow cytometry using an Attune NxT cytometer (Thermo Fisher Scientific). Uptake was measured as the percentage of cells positive for fluorescent peptide as well as the geometric mean fluorescence of cells. Gating was determined using a cell-only negative control.

Effects of pH on Nanoparticle-Peptide Sizing by Dynamic Light Scattering

Nanoparticle-peptide (2 $\mu\text{mol/L}$) in PBS was exposed to varying pH conditions (pH 1.2, pH 4, and pH7) for 15 minutes. After 15 minutes, samples were neutralized to pH 7. Particle size and distribution were then immediately measured via dynamic light scattering using a Zetasizer Pro (Malvern Panalytical).

Nanoparticle-Peptide Uptake in B16F10 Cells

B16F10 cells^{e1} were seeded at 10,000 cells/well in a 96-well plate. Biotinylated N3SP-1 Δ 7 was conjugated to streptavidin-Alexa Fluor 488 and used to synthesize nanoparticles as described. B16-F10 cells were treated with nanoparticles or free peptide at a concentration of 0.25–2 $\mu\text{mol/L}$ peptide dose for multiple times up to 4 h, then washed 3 times with PBS and replenished with fresh media. The following day, peptide uptake was quantified by measuring Alexa Fluor 488 fluorescence on a BD Accuri C6 Flow Cytometer (BD Biosciences) connected to a HyperCyt autosampler (IntelliCyt Corp). All flow cytometry data were analyzed using FlowJo software (BD Biosciences).

Nanoparticle-Peptide Uptake in Enteroids

BODIPY- and nanoparticle-conjugated peptides were loaded into cells, enteroids, and mouse intestine by conjugation with maleimide or with nanoparticles. BODIPY-conjugated peptides were exposed to the cells and apical surface of polarized cells and lumen of intestinal loops. The enteroid studies are described in most detail: 50 μL of nanoparticles were added to the apical side of enteroids in fresh serum-free media for a final concentration of 250 nmol/L peptide. After varying times from 15 minutes to 4 hours, enteroids were washed 3 times with PBS to remove extracellular nanoparticles, and wells were replenished with fresh serum-containing media. At varying times after nanoparticle addition up to overnight, enteroids were serum starved for 2 hours then fixed in 4% paraformaldehyde for 30 minutes. After enteroids were washed with PBS, formaldehyde was neutralized with 20 mmol/L glycine. Enteroids were then permeabilized in 0.1% saponin and 1% bovine serum albumin for 30 minutes. Fixed and permeabilized enteroids were stained for biotinylated peptide using streptavidin-conjugated Alexa Fluor 488 (1:100) and Hoechst stain (1:200). Enteroids were thoroughly washed with PBS and mounted onto microscope slides. Uptake was visualized using an Olympus FV3000RS confocal microscope and 40 \times objective (Olympus).

Structural Modeling of Human Sodium-Hydrogen Exchanger 3 C-terminus and Sodium-Hydrogen Exchanger 3 Peptides

Rosetta^{e18–e20} was used to predict the structure of the human NHE3 C-terminus and NHE peptides. Structural fragment libraries extracted from the Protein Data Bank for NHE3 sequence regions 585 to 605 (CP-1) and 568 to 605 (N3SP-1) and several N-terminal truncations, including 578 to 605 (N3SP-1 Δ 10) (Supplementary Figure 2A) were generated and used to perform a low-resolution conformational search followed by a full-atom model. Ten thousand models were generated for each peptide, and the top 10% of lowest-energy models were clustered, as previously described.^{e18} A similar approach was used to model single amino acid deletions from the N3SP-1 N-terminus. Models representing the most frequently sampled conformations for each peptide were selected for presentation.

Statistical Analysis

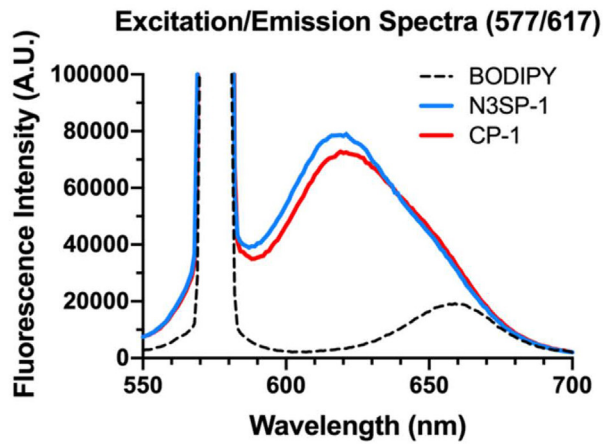
Statistical significances were calculated by paired or unpaired *t* tests (where indicated) or multiple comparisons analysis of variance and were assessed to compare groups including a minimum of $n = 3$ replicates. *P* values are indicated in figures and figure captions.

Supplementary References

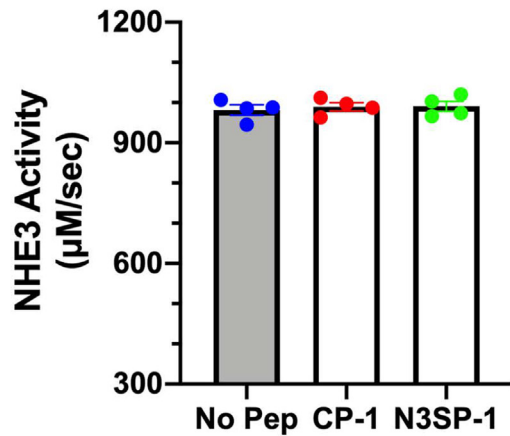
- e1. Kozielski KL, Tzeng SY, Green JJ. A bio-reducible linear poly(beta-amino ester) for siRNA delivery. *Chem Commun (Camb)* 2013;49:5319–5321.
- e2. Rui Y, Wilson DR, Choi J, et al. Carboxylated branched poly(beta-amino ester) nanoparticles enable robust cytosolic protein delivery and CRISPR-Cas9 gene editing. *Sci Adv* 2019;5:eaay3255.
- e3. Zachos NC, Tse M, Donowitz M. Molecular physiology of intestinal Na⁺/H⁺ exchange. *Annu Rev Physiol* 2005;67:411–443.
- e4. Levine SA, Montrose MH, Tse CM, et al. Kinetics and regulation of three cloned mammalian Na⁺/H⁺ exchangers stably expressed in a fibroblast cell line. *J Biol Chem* 1993;268:25527–25535.
- e5. Zachos NC, Kovbasnjuk O, Foulke-Abel J, et al. Human enteroids/colonoids and intestinal organoids functionally recapitulate normal intestinal physiology and pathophysiology. *J Biol Chem* 2016;291:3759–3766.
- e6. Sunuwar L, Yin J, Kasendra M, et al. Mechanical stimuli affect *Escherichia coli* heat-stable enterotoxin-cyclic GMP signaling in a human enteroid intestine-chip model. *Infect Immun* 2020;88:e00866–19.
- e7. Foulke-Abel J, Yu H, Sunuwar L, et al. Phosphodiesterase 5 (PDE5) restricts intracellular cGMP accumulation during enterotoxigenic *Escherichia coli* infection. *Gut Microbes* 2020;12:1752125.
- e8. Yin J, Sunuwar L, Kasendra M, et al. Fluid shear stress enhances differentiation of jejunal human enteroids in Intestine-Chip. *Am J Physiol Gastrointest Liver Physiol* 2021;320:G258–G271.

- e9. Noel G, Baetz NW, Staab JF, et al. A primary human macrophage-enteroid co-culture model to investigate mucosal gut physiology and host-pathogen interactions [published correction appears in *Sci Rep* 2017;7:46790]. *Sci Rep* 2017;7:45270.
- e10. Staab JF, Lemme-Dumit JM, Latanich R, et al. Co-culture system of human enteroids/colonoids with innate immune cells (i.e. macrophages and neutrophils). *Curr Protoc Immunol* 2020;131:e113.
- e11. Murtazina R, Kovbasnjuk O, Chen TE, et al. NHERF2 is necessary for basal activity, second messenger inhibition, and LPA stimulation of NHE3 in mouse distal ileum. *Am J Physiol Cell Physiol* 2011;301:C126–C136.
- e12. Clayburgh DR, Musch MW, Leitges M, et al. Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. *J Clin Invest* 2006;116:2682–2694.
- e13. Sarker R, Grønberg M, Cha B, et al. Casein kinase 2 binds to the C terminus of Na⁺/H⁺ exchanger 3 (NHE3) and stimulates NHE3 basal activity by phosphorylating a separate site in NHE3. *Mol Biol Cell* 2008;19:3859–3870.
- e14. Tse CM, In JG, Yin J, et al. Enterohemorrhagic *E. coli* (EHEC)-secreted serine protease EspP stimulates electrogenic ion transport in human colonoid monolayers. *Toxins (Basel)* 2018;10:351.
- e15. Yang J, Singh V, Chen TE, et al. NHERF2/NHERF3 protein heterodimerization and macrocomplex formation are required for the inhibition of NHE3 activity by carbachol. *J Biol Chem* 2014;289:20039–20053.
- e16. Zachos NC, van Rossum DB, Li X, et al. Phospholipase C-gamma binds directly to the Na⁺/H⁺ exchanger 3 and is required for calcium regulation of exchange activity. *J Biol Chem* 2009;284:19437–19444.
- e17. Boehning D, van Rossum DB, Patterson RL, et al. A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc Natl Acad Sci U S A* 2005;102:1466–1471.
- e18. Bender BJ, Cisneros A 3rd, Duran AM, et al. Protocols for molecular modeling with Rosetta3 and RosettaScripts. *Biochemistry* 2016;55:4748–4763.
- e19. Rohl CA, Strauss CE, Misura KM, et al. Protein structure prediction using Rosetta. *Methods Enzymol* 2004;383:66–93.
- e20. Bonneau R, Strauss CE, Rohl CA, et al. De novo prediction of three-dimensional structures for major protein families. *J Mol Biol* 2002;322:65–78.

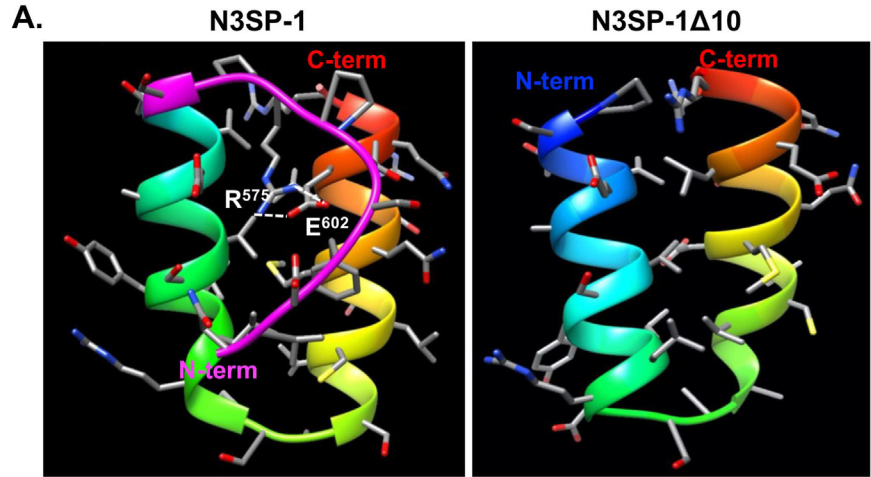
A.



B.



Supplementary Figure 1. (A) Fluorescence emission spectra of BODIPY alone vs BODIPY-conjugated to CP-1 and N3SP-1 showing increase in fluorescence in presence of peptide with an emission peak of ~617 nm. A.U., arbitrary units. (B) Failure of CP-1 and N3SP-1 (400 nmol/L) to alter basal NHE3 activity in PS120 cells in the absence of BODIPY conjugation. Exposure to CP-1 and N3SP-1 was performed as in studies shown in [Figure 2C](#). Neither CP-1 nor N3SP-1 altered basal NHE3 activity compared with otherwise untreated control PS120 cells ($n = 4$). Data are shown as the mean \pm standard error of the mean.

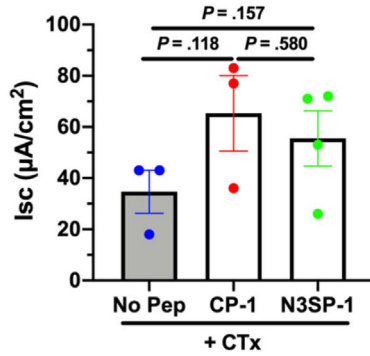


Supplementary Figure 2. Structural models of NHE peptides. (A) Structural model of N3SP-1 (residues rabbit NHE3 568–605) and N3SP-1-NΔ10 (residues 578–605) shown in ribbon diagram representation and colored using a rainbow scheme from N-terminal region (blue) to C-terminal region (red). All structural images were generated using the University of California San Francisco Rosetta Chimera package.^{e19} (B) Sequence alignment of human, rabbit, rat, and mouse NHE3 in the C-terminal area used to design the N3SP.

B.

P48764 SLC9A3_HUMAN	569	NVDFT-PRSSTVEASVSYLLRENVS	AVCLDMQSLQRR	605
P26432 SLC9A3_RABBIT	568	NVDFSTPRPSTVEASVSYLLRESAS	AVCLDMQSLQRR	605
P26433 SLC9A3_RAT	566	NVDFSTPRPSTVEASVSYFLRENVS	AVCLDMQSLQRR	603
G3X939 SLC9A3_MOUSE	564	NVDFNTPRPSTVEASVSYFLRENVS	AVCLDMQSLQRR	601
		****. ** *****.***.*****		

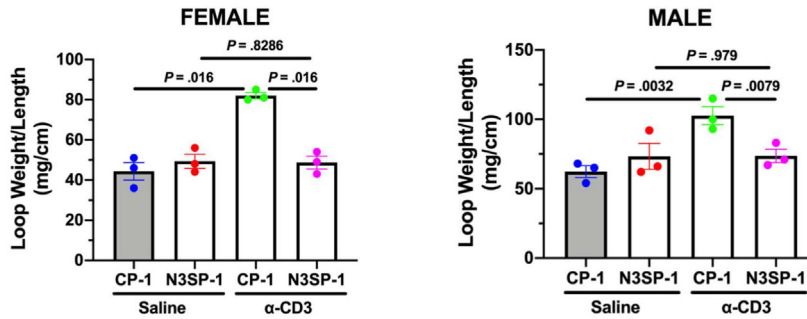
A.



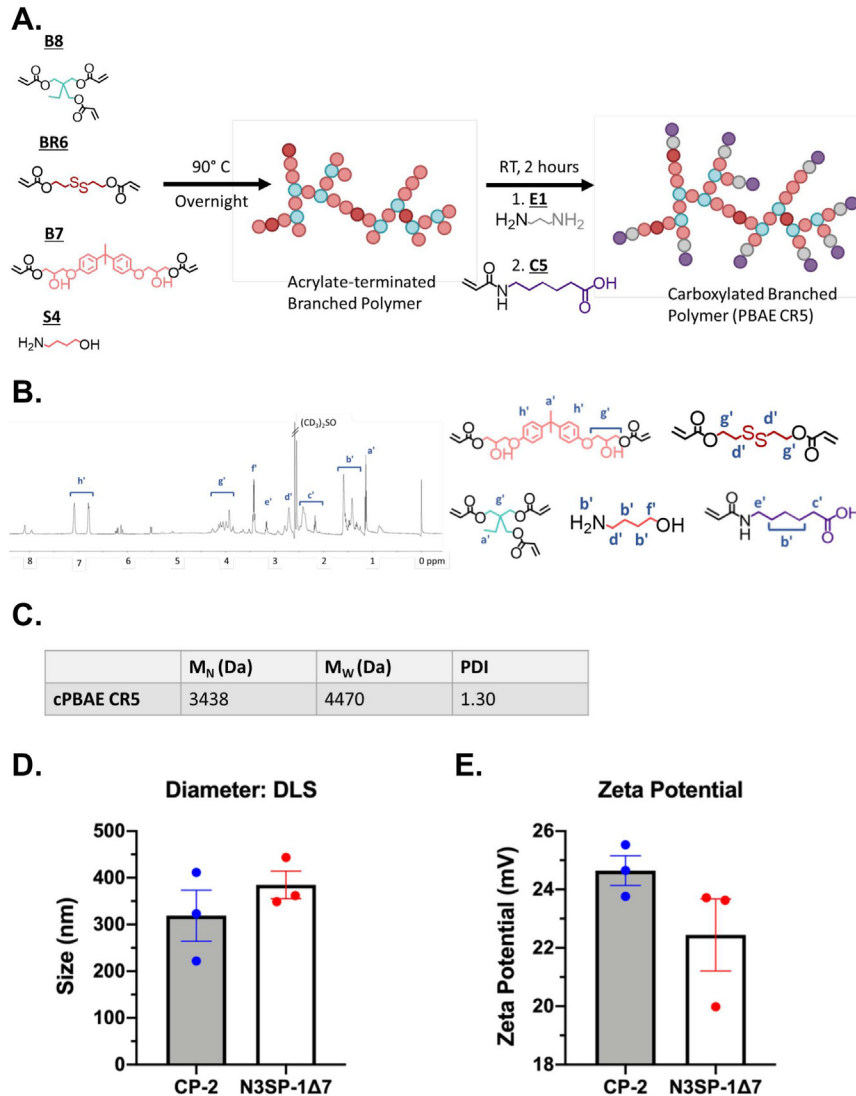
B.

	FSK	CCH
CTx + CP-1	100 ± 40 µA/cm ² (n=3)	97 ± 31 µA/cm ² (n=3)
CTx + N3SP-1	86 ± 31 µA/cm ² (n=4)	71 ± 30 µA/cm ² (n=4)

C.

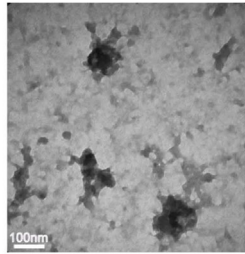


Supplementary Figure 3. (A) CTx-induced electrogenic anion secretion is not altered by N3SP-1 exposure for 6 hours. Mouse jejunal loops were exposed to 1 µg CTx in the presence of 400 nmol/L CP-1 or N3SP-1 or PBS for 6 hours. After animals were euthanized, the full-thickness loops were mounted in Ussing chambers, gassed with 5% CO₂/95% O₂ in Ringer's-HCO₃, and after stabilization, basal Isc (µA/cm²) was determined. Shown are Isc in a CTx-treated loop with no further addition of secretagogues. *P* values by paired *t* tests compare Isc in CTx-treated loops also inoculated with PBS, CP-1 (400 nmol/L) or N3SP-1 (400 nmol/L; n = 3). The same tissues were further studied by addition of 10 µmol/L FSK to the serosal surface, and peak increase in Isc determined. Then after stabilization of the Isc, 10 µmol/L carbachol was added to the same jejunal serosal surface and peak Isc determined. (B) The peak increases in Isc in mouse jejunal loops exposed to CTx as in panel A caused sequentially by basolateral exposure to FSK (10 µmol/L) and carbachol (CCH; 10 µmol/L). (C) Results from Figure 3D showing effect of CP-1 vs N3SP-1 on anti-CD3 monoclonal antibody-induced mouse jejunal fluid secretion separated based on sex. Similar results occurred with studies of both male and female mice (n = 3 for all conditions in both male and female mice). *P* values are by paired *t* tests. Data are shown as mean ± standard error of the mean.

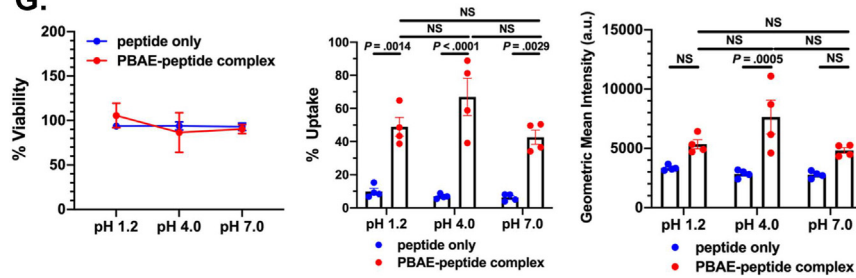


Supplementary Figure 4. Characterization of cPBAE CR5 polymer and nanoparticles. (A) Synthesis scheme for carboxylated branched PBAE CR5 polymer. RT, room temperature. (B) ¹H-nuclear magnetic resonance (NMR) spectrum confirming the chemical structure of cPBAE polymer CR5. Peaks correspond to protons labeled in the chemical structures shown. (C) The CR5 polymer was characterized by gel permeation chromatography demonstrating an average molecular weight of 3438 Da and a polydispersity (PDI) of 1.30. (D) Normalized diameter and (E) zeta potential of nanoparticles with CP-2 and N3SP-1Δ7. To form nanoparticles, CR5 was combined with CP-2/N3SP-1Δ7 in aqueous conditions at a 30:1 polymer-to-peptide weight ratio. The resulting self-assembled nanoparticles had a diameter of 319 ± 55 nm (CP-2) and 385 ± 30 nm (N3SP-1Δ7), as measured by dynamic light scattering, with a surface zeta potential of +24.6 ± 0.5 mV (CP-2) and +22.4 ± 1.2 mV (N3SP-1Δ7) measured by electrophoretic light scattering. Nanoparticles incubated in PBS for 45 minutes at room temperature remained stable, with no significant increase in size. (F) Morphology of PBAE-peptide (N3SP-1Δ7 shown) complexes by transmission electron microscopy. Scale bars = 100 nm. The expected loosely formed complexes are demonstrated. (G) Effect of pH (pH 1.2, 4.0, and 7.0) on nanoparticle-peptide compared with peptide alone on viability and uptake in B16F10 cells. *Left*: percentage viability; *Middle*: percentage uptake; and *Right*: geometric mean intensity (size) (n = 4). (H) Effects of pH (pH 1.2, 4.0, and 7.0) on nanoparticle diameter by dynamic light scattering (DLS). *Left*: average particle diameter measured by DLS after nanoparticle-peptide or peptide alone exposure to pH 1.2, 4.0, and 7.0; *Middle*: particle polydispersity after exposure to varying pH conditions; *Right*: peak one mean measured via DLS of particles exposed to varying pH conditions (n = 6). Significance in G and H determined in GraphPad Prism (GraphPad Prism Software) by 1-way analysis of variance with Tukey's multiple correction test. ns (not significant) = P > .05. (I) Time and concentration dependence of nanoparticle-peptide uptake in vitro in B16-F10 cancer cells (% positive). When nanoparticles were added to B16-F10 cells in vitro at concentrations from 0.25 to 2 μmol/L, dose-dependent cellular uptake was observed, with significant, time-dependent uptake over 4 hours. Time and concentration dependent uptake of nanoparticles formulated with fluorescently labeled CP-1 (*left*) and N3SP-1 (*right*) (percent positive shown). Data are shown as mean ± standard error of the mean.

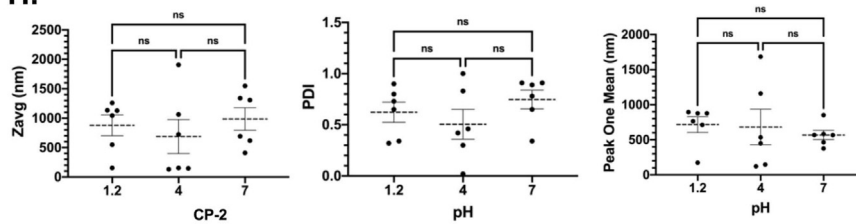
F.



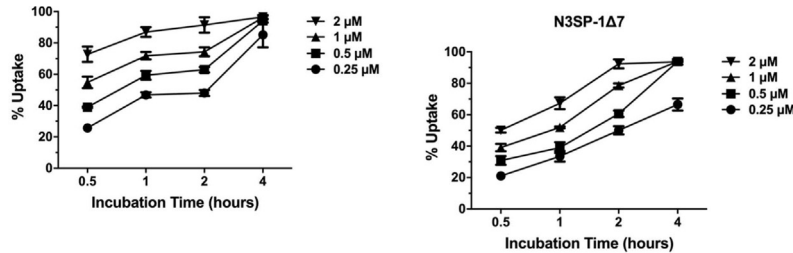
G.



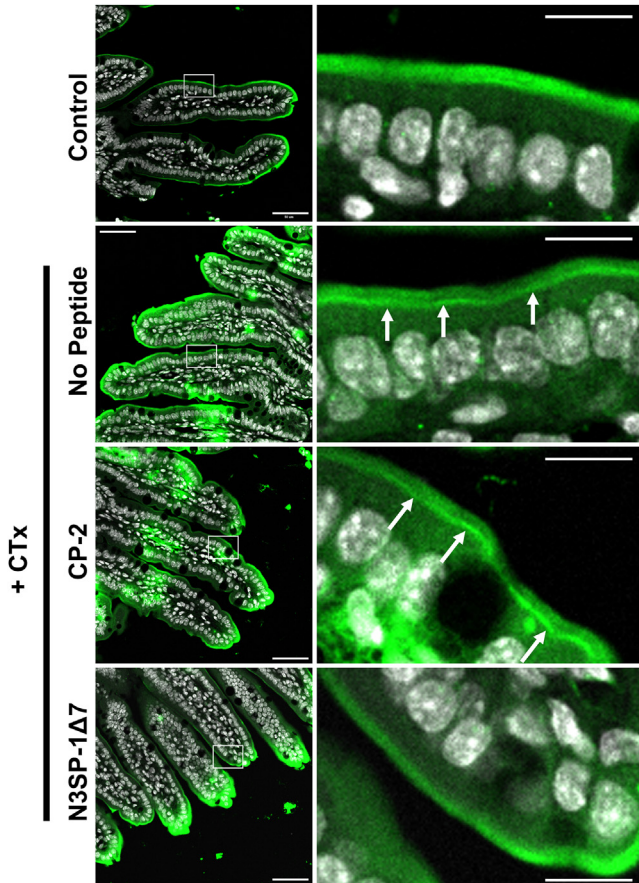
H.



I.



Supplementary Figure 4. (Continued)



Supplementary Figure 5. N3SP-1 Δ 7 prevents NHE3 trafficking after CTx exposure in murine ileal enterocytes. Ileal loops from similar experiments as those performed in [Figure 3B](#) were fixed, stained with anti-NHE3 antibody, and imaged by immunofluorescent confocal microscopy. Shown are results from a single experiment with similar results observed in 3 independent experiments. Arrows indicate localization of NHE3 at the base of the BB. White boxes in *left panel* images denote magnified areas displayed in corresponding *right panel* images. Scale bar = 50 μ m in *left images* and 10 μ m in *right images*.