SUPPLEMENTAL MATERIALS AND METHODS

S1 Patient Selection

Clinical charts of renal transplant recipients treated with rapamycin (2003-2010) at AMC were retrospectively reviewed. The inclusion criteria were: deceased-donor renal transplantation, daily rapamycin administration, occurrence of diarrhea while on rapamycin requiring hospitalization, and colonoscopy performed for evaluation of diarrhea. Exclusion criteria included: presence of enteric infection (e.g. *C. difficile*, CMV, etc.), graft-versus-host disease, and/or recent changes in medications or doses. Medical records were reviewed to obtain patient serum rapamycin levels at baseline and at the time of the diarrhea episode. Serum levels of concurrently administered tacrolimus were also noted. The research protocol was approved by the AMC Institutional Review Board.

S2 Antibodies

The antibodies to S6, phospho-S6, mTOR, Ezrin, p-Ezrin, GAPDH, CFTR, p62, and LC3 were purchased from Cell Signaling, Inc. (Danvers, MA). NHERF1 antibody was purchased from Sigma-Aldrich Corp (St. Louis, MO). Antibodies against NHE3 were as follows: NHE31-A (Alpha Diagnostic) for rat western blot; 19F5 (Millipore) used for rat immunofluorescence; sc-16103-R (Santa Cruz Biotechnology) for human immunohistochemistry; NBP1-46581 (Novus) for mouse western blot; and AB1380 (Mark Donowitz's lab)[1] for mouse immunofluorescence. EZ-Run™ Pre-staining Rec protein ladder (Fisher) was used in all western blot analyses.

S3 Human Tissue Selection and Immunostaining

Paraffin blocks containing tissue biopsies of the terminal ileum were obtained from 11 renal transplant patients on rapamycin referred for colonoscopy to evaluate diarrhea and from 10 control patients. The ileal control specimens were from subjects who had no history of diarrhea and underwent colonoscopy with terminal ileal biopsies for iron deficiency anemia or abdominal pain to exclude Crohn's disease. Pathological examination of control specimens revealed normal histology. All tissues were identically processed. Five-micron thick sample sections were deparaffinized and rehydrated and treated with 3% H2O2, followed by heating in saturated lead thiocyanate for 10 min each. Slides were rinsed twice in distilled water and placed in phosphate-buffered solution (PBS) for 5 min prior to immunohistochemical staining for NHE3 using the DAB (3, 3'-diaminobenzidine) Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). Sections were incubated with polyclonal anti-human NHE3 (sc-16103-R from Santa Cruz) (1:200) overnight, followed by washing with PBS twice for 5 min each. The slides were incubated with biotinylated goat anti-rabbit antibody (1:1000) for 1h and then incubated with streptavidin-conjugated horseradish peroxidase (HRP) for 30 min. Staining of NHE3 in ileum was quantified using ImageJ software. Scanned images were first converted to 8-bit grayscale. For each image, surface and cytosol NHE3 were measured from each cell to obtain the ratio of surface/surface+cytosol NHE3. As indicated in Fig 2a, the blue line represents a standardized, arbitrary membrane segment containing apical membrane and cytosol for NHE3 quantitation with the ImageJ program. The peak intensity at the membrane edge was used to represent apical NHE3, whereas average intensity in the cytosol was used to represent cytosolic NHE3 (Fig 2b-c). Background correction was performed using image analysis from an empty area on the same slide. Surface/surface plus cytosol ratios for each specimen were determined as the average from at least 5 individual cells on each slide (Fig 2d).

S4 Animals

$mTOR^{f/f}$ (B6.129S4-Mtortm1.2Koz/mTOR) and villin-cre (B6.SJL-Tg(Vil-cre)997Gum/J) mouse lines were obtained from the Jackson laboratory. $Atg7^{flox/flox}$ mice were acquired from Dr. Masaaki Komatsu [24]. Animals were housed in the AMC Animal Resource Facility. $mTOR^{f/f}$ and $Atg7^{f/f}$ mice were crossed with villin-cre mice to generate $mTOR^{f/f}$:villin-cre and $Atg7^{f/f}$:villlin-cre mice, respectively (referred to as $mTOR^{-/-}$ or $Atg7^{-/-}$ in the text) in which mTOR or Atg7 is restrictively deleted in intestinal epithelial cells. Genotyping to identify $Atg7^{-/-}$ mice was performed by PCR using the following primers: wild-type Atg7 sense 5'-GCATGTCTGTGGTTGCTTC-3', antisense 5'-AGAGGGGTACAGGGGCATAC-3', floxed Atg7 sense 5'-GGACTTGTGCCTCACCAGAT-3', antisense 5'-CTCGTCACTCATGTCCCAGA-3' and Cre sense 5'-GCATTTCTGGGGATTGTTA-3' and antisense 5'-CCCGGCAAAACAGGTAGTTA-3'. Primers used to identify $mTOR^{-/-}$ mice were: sense: 5'- TTATGTTTGATAATTGCAGTTTTGGCTAGCAGT -3'; –anti-sense: 5'-TTTAGGACTCCTTCTGTGACATACATTTCCT -3. All experiments were performed according to the guidelines set by the Animal Care and Use Committee of AMC and in accordance with NIH guidelines for animal care and use.

S5 Isolation of Intestinal Epithelial Cells

Intestinal epithelial cells were prepared according to the protocol developed by Evans GS et al [25] with a slight modification. Briefly, ileal tissues were obtained and rinsed in cold Hank's Balanced Salt Solution (HBSS) with 1g/L D-glucose for 3-4 times. Washed tissues were then diced using a scalpel and suspended in HBSS-2% glucose-0.1mg/ml dispase-300 μg/ml collagenase solution. Villus and crypt cells were then liberated by shaking at room temperature

for 25 min. Liberated cells were harvested by centrifugation at 300g for min at 4^{o}C. Cells were washed once in HBSS-glucose buffer prior to use for western blot analyses.

S6 Tissue Biotinylation

Ileal tissues were dissected from animals and placed on an ice-cold metal block. Two ileal patches were collected using a 5-mm biopsy punch. The tissues from the ileum were washed twice in ice-cold PBS and once in borate buffer (154 mM NaCl, 10 mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl2, pH 9.0). Plasma membrane proteins were biotinylated at 4^{o}C by gently shaking the tissue for 20 min with 600 μl of borate buffer containing 1.5 mg of sulfo-NHS-SS-biotin. An additional 400 μl of the same buffer was then added, and tissues were rocked for an additional 20 min. Tissues were then washed extensively with quenching buffer (120 mM NaCl and 20 mM Tris, pH 7.4) to remove excess sulfo-NHS-SS -biotin, followed by two PBS rinses. Tissues were then lysed in 1 ml of N1 buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM EDTA trisodium, 3 mM EGTA, 0.05% SDS and 1% Triton X-100) and sonicated on ice. An aliquot of 25 μg supernatant was saved for total NHE3 measurement, the rest of the supernatant was subjected to avidin precipitation for 2 h at 4°C. The neutravidin-agarose beads (Thermo Scientific) were washed five times in N1 buffer, and bound proteins were solubilized in sample buffer, yielding the surface fraction.

S7 Western Blot

Protein lysates prepared from ileal crypt samples were resolved in 8% Bis-Tris gel and nitrocellulose membranes were first blocked in 5% nonfat dry milk in TBST (25 mM Tris–HCl, pH 7.4, 1.5 M NaCl, 0.05% Tween-20) for 1h at room temperature and then incubated with rabbit anti-S6, anti-Ezrin, anti-phospho-S6, anti-phospho-Ezrin, anti-CFTR and anti-GAPDH

antibodies at 1:1000 dilution or anti-NHERF1 (1:500) at 4 °C overnight. Primary antibodies were removed by several washings in TBST, and membranes were incubated with HRP-conjugated secondary antibody (1:10,000) in 5% milk in TBST. Signals were visualized with ECL (Pierce) and quantified by densitometry. The intensities of signals were normalized to GAPDH as a loading control. We also performed western blot analysis to detect expression of E-Cadherin, a marker of intestinal epithelial cells. There was no difference when normalization was with GAPDH or E-Cadherin among all the groups that were examined in Suppl. Fig 3.

S8 Immunohistochemistry

Rat and mouse ileal tissues were harvested and rinsed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS at 4°C for overnight. Fixed tissues were rinsed with PBS and transferred to 30% sucrose in PBS for overnight incubation. Tissue was embedded with tissue-freezing medium (TissueTec O.C.T., Sakura). Cryosectioning was performed with a microtome cryostat at -20°C and 20-μm sections were collected on microscope slides (SuperFrost Plus, Thermo Scientific, USA). For immunofluorescenct staining, the sections were rinsed three times in PBS followed by blocking in 5% normal goat serum and 1% bovine serum albumin in PBS for 1 h. Sections were then incubated in either anti-NHE3 antibody 19F5 (1:100) for rat ileum or Ab1380 (1:30) for mouse ileum at 4°C overnight. The sections were rinsed with PBS (pH 7.2) for 30 min and then incubated in a 1:200 dilution of Alexa 488-conjugated goat secondary antibody for 1h at room temperature. Finally, the sections were rinsed with several changes of PBS (pH 7.2). Sections were placed on coverslips and mounted in Fluoromount-G (SouthernBiotech) for visualization of NHE3.

S9 Measurement of Na^+/H^+ Exchange Activity

PS120 cells that were stably transfected with human NHE3 and NHERF2 were originally established in Dr. Mark Donowitz's laboratory at Johns Hopkins University. The cells were grown to a confluence of 70-80% on glass coverslips and placed in serum-free medium for 6 h prior to use. Cells were then treated with rapamycin (200 nM final concentration) for either 20 min or 30 min. Na^+/H^+ exchanger activity was measured fluorometrically using the intracellular pH-sensitive dye 2-,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester. The experiments were performed under basal conditions. PS120 cells were incubated with NH_4Cl medium containing 200 nM rapamycin for 20 min or 30 min at 37 °C before addition of NH_4Cl. Coverslips were then subjected to sequential perfusion with tetramethylammonium- and sodium-containing media as described previously[26]. Na^+/H^+ exchange rates (H^+ efflux) were calculated as the product of the buffering capacity at each pHi and the Na^+-dependent change in pHi. The results were analyzed using Origin software (Microcal) by fitting the data to a general allosteric model as described by the Hill equation. In all experiments, Vmax and Km(H)i values were calculated. Means and S.E. were calculated from kinetic parameters from at least three different experiments for each condition. Results from multiple coverslips for each experimental condition were averaged and considered as n=1.

S10 Measurement of Intestinal Water Absorption

This assay was performed essentially as described previously with slight modification[36]. Briefly, 4-week-old mice were fasted (but allowed water *ad libitum*) for 12 prior to each experiment. Mice were anesthetized under isoflurane (isoflurane vaporizer). An incision was made to open the abdomen along the midline. A 5-cm loop of jejunum/ileum was cannulated with 0.76-mm internal diameter polyethylene tubing. The abdomen cavity was covered with a damp cotton mesh for the duration of the experiment. Mice were kept warm during surgery via heat lamp or

heated pad. Mice were first perfused with flushing solution (140 mM NaCl, 10 mM HEPES, pH 7.4) (warmed to 37^{o}C) at 0.15 to 1 ml/min for 10 min to remove the luminal contents, followed by perfusion with hypotonic flushing solution (50 mM NaCl, 5 mM HEPES, 2.5 mM KCl, 20 mM glucose, pH 7.4, 37^{o}C) at 1 ml/min for 10 min. Mice were then perfused with 7 ml testing solution (hypotonic solution + 2 mM sodium ferrocyanide) at the same rate for 2 h. One-milliliter aliquots of perfusate were sampled after the first 15 min and at the end of the perfusion for evaluating the concentration of sodium ferrocyanide. Afterwards, the mice were sacrificed and the length of the perfused segment was measured. To measure the concentration of ferrocyanide, perfusates were centrifuged at 12000 rpm for 5 min at room temperature. After adding 8 μl of freshly made 3% H2O2 solution into 100 μl of perfusate, the mixtures were immediately placed on a shaker at room temperature for 10 min and then the O.D. was read at 420 nm at 15, 30, 45, 60, 90, and 120 min after the start of the reaction. The concentration in perfusates was determined based on the standard concentrations of ferrocyanide. Water absorption was calculated as: $(V_i-V_f)/(T\times L)$. V_i is the measured initial perfusate volumn; V_f is calculated as $V_i([\text{ferrocyanide}]_i/[\text{ferrocyanide}]_f)$; T is hours of perfusion; L is the length of the perfused intestinal section in cm.

S11 NHE3 Activity Determined by Two-photon Microscopy

Na^+/H^+ exchange activity assays in intact mouse ileum were conducted using the ratiometric pH-sensitive dye SNARF-4F acetoxymethyl ester (SNARF-4FAM) as previously reported [20, 27]. The experiment was performed using a 510 Meta two-photon confocal microscope (Zeiss) (Imaging Core at Albany Medical College) with an inverter (LSM Technology) along with a water immersion **objective (W Plan-Apochromat 20×/1.0 DIC M27 75mm) (Zeiss), with an**

optical field depth up to 2.4 mm). After 12 hours of fasting (*ad libitum* access to water), mice were sacrificed by an overdose of isoflurane. The abdomens were immediately opened by a midline incision and the proximal ileum was excised and further opened along the mesenteric border. Tissue segments were flattened by fixing to a glass coverslip with Krazy Glue (Elmer's Products Inc., Columbus, OH) with the mucosal surface facing up. During the entire course of dissection, the ileum tissues were placed in cold 'Na^+ buffer' (138 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 25 mM mannose, and 20 mM HEPES, pH 7.4). Tissue preparations were loaded with 20 μM SNARF-4FAM in Na^+ buffer at 37°C for 25 min in a tissue culture incubator with 5% CO_2. Then the tissue was mounted into a perfusion chamber (RC-26 with P-1 platform, Warner Instruments) with the temperature controlled at 25°C. After sequential acidification with an NH4Cl prepulse (40 mM NH4Cl in 78 mM Na^+ solution) for 15 min followed by incubation in *N*-methyl-D-glucamine ('NMDA') (Sigma) buffer (same as Na^+ buffer with NMDA replacing Na^+) for 15 min, the ileum preparation was perfused with Na^+ buffer to evaluate Na^+/H^+ exchange activity as the initial rate of pH recovery. Both NMDA and Na^+ buffer contain 50 μM HOE694 (Sonafi-Aventis, Bridgewater, NJ) to inhibit NHE1 and NHE2. Following Na^+ perfusion, the pHi for each tissue was calibrated using the K^+/nigericin (10 μM) (Life Technologies) method. K^+ clamp buffer contained 20 mM HEPES, 20 mM MES, 75 mM KCl, 35 mM K gluconate, 14 mM Na gluconate, 1 mM CaCl2, 1 mM MgSO4, 2 mM TMA-Cl (Sigma), adjusted to pH 6.2, 6.8, 7.4. All buffers contained 1 mM probenecid (Sigma P8761, St. Louis, MO) to prevent leakage of SNARF-4FAM. Images were acquired from optical files 10-40 μm from the villus tip. Acquired images were subjected to analysis of fluorescence intensity at emissions of 640 nm and 580 nm. Regions of interest (ROI) were randomly chosen in 2–3 individual villi and fluorescence intensities in each ROI were acquired using the ZEN

2011 program (Zeiss). An area outside the villi was also selected for the measurement of background. The intensity of background was subtracted from each chosen ROI prior to calculating the 640/580 ratio. The 640/580 ratios were then converted into pH values with internal pH standards derived from the K^+ /nigericin clamp. The Na^+/H^+ exchange activity of NHE3 was determined as the initial rate in pH*i* change by calculating the pH*i* change within 5 min after perfusion of Na^+ buffer.

SUPPLEMENTAL FIGURE LEGENDS

Suppl. Figure 1. Genotyping to verify $mTOR^{f/f}$ and $mTOR^{-/-}$ mice

Suppl. Figure 2. A portion of rats chronically treated with a high dose (20 mg/kg) of rapamycin developed diarrhea.

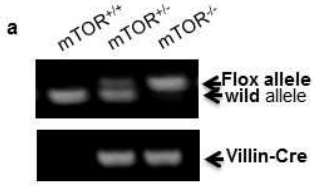
(a) Representative images of exposed intestines from controls and rapamycin-treated rats. (b) Quantification of the diameter of intestines. Sixteen rats (4/group) were treated with either vehicle or rapamycin, i.p. at 20 mg/kg/day for 14 days. A total of 3 rats displayed dilated intestine filled with a large amount of liquid stool.

Suppl. Figure 3. Western blot analysis of the levels of E-Cadherin in the lysates prepared from small intestine from rats (a), $Atg^{F/F}$ and $Atg^{-/-}$ mice (b), and $mTOR^{F/F}$ and $mTOR^{-/-}$ mice (c) treated wither either vehicle or rapamycin. n=3, * $p<0.05$.

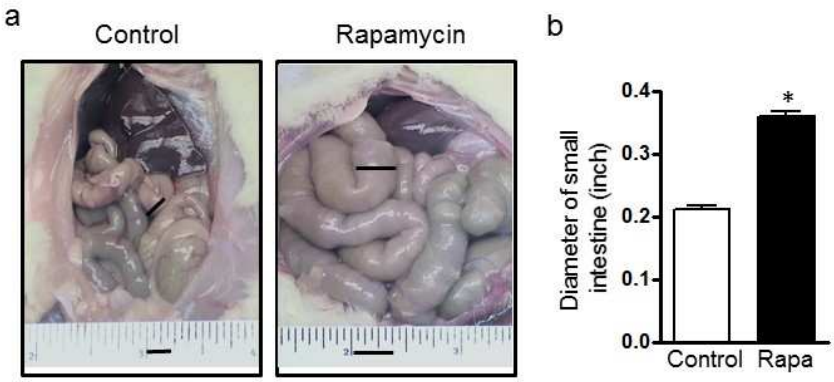
Suppl. Figure 4. Traces showing the change in ratio of fluorescent intensity at 640 and 580 over the course of acidification (ammonia and TMA) as well as during the uptake of Na^+ in ileal villi from $mTOR^{F/F}$ (a) and $mTOR^{-/-}$ (b) mice Each data point is the average from three different

regions. Representative images are from pre- (at 10 min) and post- (at 15 min) Na^+ buffer perfusion.

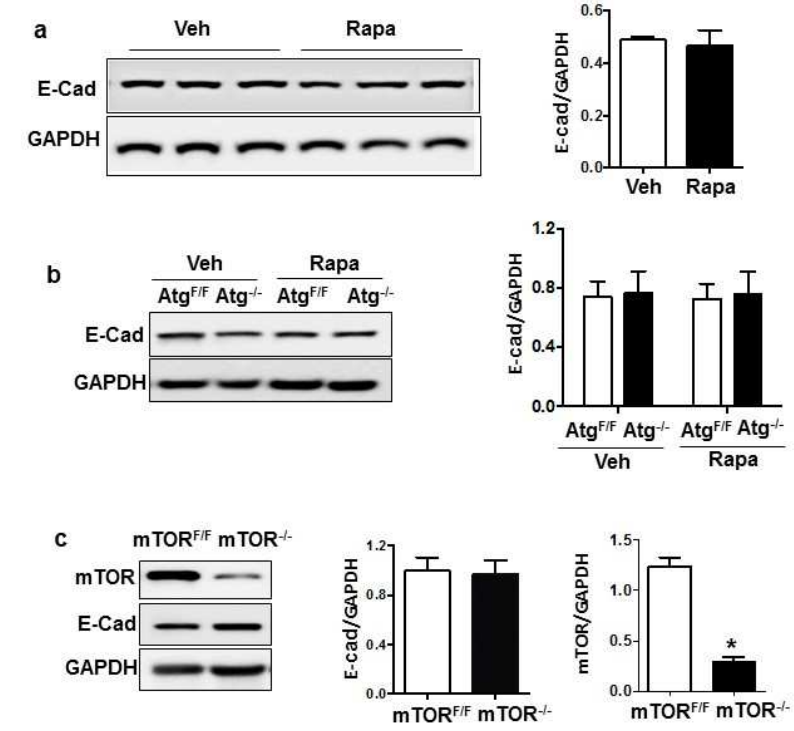
Suppl. Fig. 1



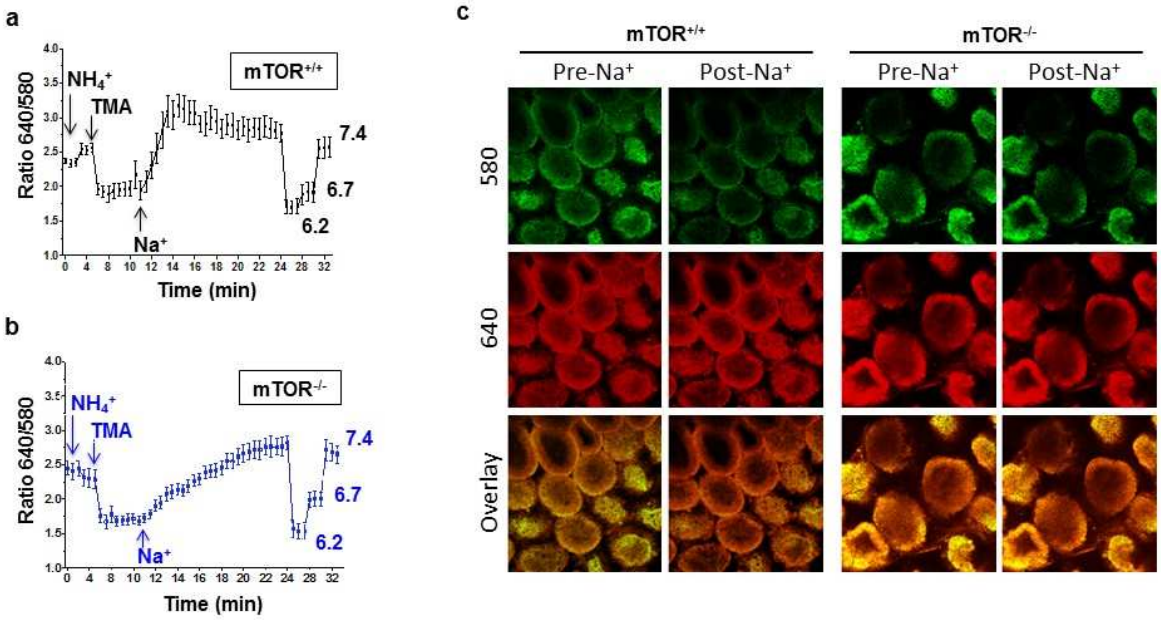
Suppl. Fig. 2


a
Control
Rapamycin
b
Diameter of small intestine (inch)
0.4
0.3
0.2
0.1
0.0
*
Control
Rapa

Suppl. Fig. 3


a
Veh
Rapa
E-Cad
GAPDH
E-cad/GAPDH
0.6
0.4
0.2
0.0
Veh
Rapa
b
Veh
Rapa
AtgF/F Atg-/-
AtgF/F
Atg-/-
E-Cad
GAPDH
E-cad/GAPDH
1.2
0.8
0.4
0.0
AtgF/F Atg-/-
AtgF/F Atg-/-
Veh
Rapa
c
mTORF/F mTOR-/-
mTOR
E-Cad
GAPDH
E-cad/GAPDH
1.2
0.8
0.4
0.0
mTORF/F mTOR-/-
mTOR/GAPDH
1.5
1.0
0.5
0.0
*
mTORF/F mTOR-/-

Suppl. Fig. 4


a
mTOR+/+
NH4+
TMA
Na+
7.4
6.7
6.2
Ratio 640/580
Time (min)
b
mTOR-/-
NH4+
TMA
Na+
7.4
6.7
6.2
Ratio 640/580
Time (min)
c
mTOR+/+
mTOR-/-
Pre-Na+
Post-Na+
Pre-Na+
Post-Na+
580
640
Overlay