Supplementary Methods

Patient tissue slides and IRB approval

Control pediatric sections were obtained from paraffin blocks at the Boston Children's Hospital by courtesy of Dr. Jay Thiagarajah. Paraffin sections of duodenal biopsies from the MVID patient were prepared at the Vanderbilt Children's Hospital (IRB No. 110835).

MxIF with patient biopsies

FFPE slides were deparaffinized and incubated in Trilogy® (Sigma) for antigen retrieval. The slides were cover-slipped with 50% glycerol in 0.1 M PBS containing 1 μM Hoechst 33342, and whole slide images were scanned using a Leica/Aperio Versa 200 with 20x objective (Leica Biosystems, Buffalo Grove, IL) for acquisition of autofluorescence signals of the tissues. Coverslips were gently removed, and the slides were pre-blocked with Dako serum-free protein blocking solution (X0909) for 1 hour at room temperature (r/t) or overnight at 4˚C. Primary conjugated antibodies listed in **Supplementary Table 1** were applied to the slides for 1 hour at r/t. Some antibodies were labeled with Zenon rabbit IgG labeling kits (Invitrogen) according to the manufacture's instruction. Fluorescence signals were quenched by carbonate buffer (pH 11) immediately after scanning of staining signals.[1] Background signals were imaged after each quenching step and subtracted from subsequent staining images. The MxIF data generated in this study are available at PediCODE (COngenital Diarrhea and Enteropathy) Consortium and Biorepository website.

IACUC approval

All animal studies were performed with approval from the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center (M2000104).

Mouse genome editing

C57BL/6J.Myo5b^{G519R} mice were produced by the Vanderbilt Genome Editing Resource (Vanderbilt University, Nashville, TN. USA). Ribonucleoprotein complexes (RNP) containing chemically modified ctRNA (crRNA + tracrRNA) (50 ng/ul) and SpCas9 protein (100 ng/ul) along with a 180 nt single stranded DNA (ssDNA) donor containing the *Myo5b^{G519R}* mutation (50 ng/ul) were diluted to the concentrations indicated in 10 mM Tris, 0.1 mM EDTA, pH 7.6 (Teknova) and delivered by pronuclear injection into one cell of a 2-cell C57BL/6J (Jackson Labs) embryo (**Figure 2A**) [2, 3]. crRNA sequence: 5'AGTTCTGATCAGTTCCTTTG. 180 nt ssDNA sequence: 5' CTGAACCCTCTGTCTACCAGGATGCTTCCCTGCCTTGTCCTGCTCCGTGAGACACTCTACC TGGTCTTGTGCTTATCCAGGTCCCTAAGCGGACCGACCAGAACTGGGCCCAGAAACTCTA CGAACGACACTCCAACAGCCAGCACTTCCAGAAACCACGCATGTCCAACACGGCCTTCA. 195 embryos were microinjected, and 7 of 52 pups produced contained the *Myo5b^{G519R}* allele (14.6%). Mosaic F0 animals were screened for the G519R point mutation by standard PCR spanning the full ssDNA sequence and into the flanking genomic DNA followed by restriction fragment length polymorphism assay. Animals carrying the desired mutation were confirmed by Sanger sequencing (**Supplementary Figure** 1B). F0 animals were bred one or two generations into the C57BL/6J strain to allow for segregation of possible off-target mutations prior to building strains for experimental analysis. One sequence-verified N1 male mouse, which carries *Myo5b$^{G519R/+}$* was crossbred with wild type C57BL/6J to maintain *Myo5b$^{G519R/+}$* and with *VilCre^{ERT2};Myo5b$^{flox/flox}$* mice to obtain *VilCre^{ERT2};Myo5b$^{G519R/flox}$* (Myo5b(G519R)) mice, respectively (N2). Four *Myo5b$^{G519R/+}$* mice of N2 generation were verified with Sanger sequencing and used to generate N3 generation of Myo5b(G519R) mice. Genotype of each mouse was confirmed by PCR (**Supplementary Figure** 1B). The following experiments were done with N2 and N3 generation mice.

Mouse tissues

At the 8-9 week of age, Myo5b(G519R) mice and littermate controls (*VilCre^{ERT2};Myo5b$^{flox/+}$* and *Myo5b$^{G519R/+}$*) received a single dose of tamoxifen citrate (100 mg/kg) by intraperitoneal (IP) injection (day 0). For a comparison with MYO5B deletion, *VilCre^{ERT2};Myo5b$^{flox/flox}$* (Myo5bΔIEC) mice[4] received the same tamoxifen solution in parallel. Body weight changes and diarrhea symptoms were investigated daily. Some mice received EdU (50 mg/kg) by IP injection 24 hours before euthanizing. On day 4, mice were euthanized and the duodenum (0–8 cm from the pyloric ring), jejunum (8 cm following the duodenum), ileum (distal 8 cm from the ileocecal junction), and colon were collected. Each segment was fixed and embedded in paraffin as a Swiss-roll shape[5]. A different set of Myo5b(G519R) and control mice were used for electrophysiological experiments on day 4. The proximal 4 cm of duodenum was taken, and mucosa-submucosal sheets were prepared in cold Krebs Buffer Ringer (KBR) solution[6], and mounted in Üssing chambers with 0.5-cm^2-window sliders (Physiologic Instruments, Leno, NV). The serosal bath contained KRB bubbled with 95% O_2-5% CO_2, and the luminal bath contained unbuffered Krebs solution with 100% O_2[7, 8]. Short-circuit current (I_{sc}) was continuously measured under the voltage clump mode, and luminal pH was maintained at pH 7.0 by titration with 0.005 M HCl.

Mouse tissue sections for immunohistological assessment

For phalloidin staining, OCT-embedded frozen sections were cut 15-µm thick. Phalloidin-AF568 conjugated (1:4000) and Hoechst diluted in 1xPBS containing 0.1% Triton X-100 were incubated with the cryosections. For immunostaining, 4-µm thick paraffin sections were used according to the protocol previously described.[6] Fluorescence signals were visualized using a Nikon Ti-E microscope with an A1R laser scanning confocal system or Zeiss Axio Imager M2 with Apotome.

Electron Microscopy

Tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate, followed by sequential post-fixation in 1% tannic acid, 1% OsO_4, and en bloc stained in 1% uranyl acetate. All samples were dehydrated using a graded ethanol series.

For TEM, samples were subsequently infiltrated with Epon-812 using propylene oxide as the transition solvent, followed by polymerization at 60°C for 48 hours. Samples were sectioned at a nominal thickness of 70 nm using a Leica UC7 ultramicrotome. TEM imaging was performed using a Tecnai T12 operating at 100 keV using an AMT nanosprint CMOS camera.

For SEM samples, after dehydration, the samples were critical point dried using a Tousimis Samdri-PVT-3D critical point dryer. Afterwards, part of each sample was fractured using a dulled scalpel followed by depositing a thin layer of platinum using a Cressington 108 sputter coater. Samples were imaged on a Quanta 250 SEM operating at 5 keV.

Chemicals

USP grade tamoxifen citrate, 5-ethynyl-2'-deoxyuridine (EdU), and 10x PBS were purchased from Thermo Fisher Scientific (Hanover Park, IL), and others were from Sigma Aldrich. Tamoxifen citrate was dissolved in 10% DMSO and 90% corn oil. EdU was dissolved in sterile saline and warmed in a water bath at 60˚C.

Statistics

Results in graphs are expressed as Mean $\pm$ S.D. Statistical differences were determined with a significant *P* value of <0.05 using GraphPad Prism 9.

Supplementary Figure 1. Myo5b(G519R) position and genomic editing strategy in mice.

A. Homology modeling of mouse Myo5b protein. G519R point mutation on a flexible loop between alpha helix is indicated in color. The model with a mutation prediction was visualized using UCSF-Chimera.[9]

B. Sanger sequence-confirmed expected mutation in a N3 *Myo5b*$^{G519R/+}$ mouse DNA.

C. Genotyping strategies and PCR examples for *Myo5b*$^{G519R/+}$. (Upper) The primers (Myo5BG519RFwd: CCTAAGCGGACCGACCAGAAC and Myo5BG519RRev1: GTGCCTCGTCTTCTCAACAGTCTG) detect mutant sequence with 307 bp product. (Lower) Three of 5 pups in a N3 litter show G519R allele.

Supplementary Figure 2. Loss of apical sodium transporters and disrupted terminal web structure in both proximal and distal small intestine of Myo5b(G519R) mice.

A. Immunostaining for SGLT1, LAMP1, and villin in the duodenum of control and Myo5b(G519R) mice. Active sodium absorption through SGLT1 is an important driving force for water absorption in proximal intestine.[10, 11] Control tissues demonstrate strict apical localization of SGLT1 (magenta), indicated by colocalization with brush border marker, villin (green). SGLT1 is internalized in most enterocytes along Myo5b(G519R) villi. Lysosome marker, LAMP1 (yellow), staining in Myo5b(G519R) tissue is expanded in the cytoplasm compared to control.

B. Immunostaining for the apical sodium-dependent bile acid transporter (ASBT) in the ileum. ASBT is electrogenic, coupled with sodium in a 2:1 sodium:bile acid stoichiometry,[12] suggesting that ASBT contributes to active water absorption in the ileum. In control enterocytes, ASBT (magenta) localizes to the apical membranes, as indicated by ACTG1 (green) brush border staining. Villus enterocytes in Myo5b(G519R) mice presented increased overall expression and

strong sub-apical staining of ASBT. Scale bars: 50 μm in merged images and 10 μm in magnified images.

C. Immunostaining for sodium-hydrogen exchanger 3 (NHE3, magenta), CFTR (green), and ACTG1 (white) in the duodenum. The apical NHE3 is mis-localized in MVID model animals and in previously reported MVID patients.[13-15] NHE3 deficient mice display secretory diarrhea, indicating its importance in sodium and water absorption.[16] In contrast to the apical localization of NHE3 in control villi, NHE3 expression in MYO5B(G519R) villi is internalized and below the apical membrane (marked by ACTG1). Localization of CFTR in the crypt is similar between control and MYO5B(G519R) tissues, but apical CFTR expression in control villi is diminished and is instead strongly expressed subapically in MYO5B(G519R) tissues. Scale bars: 10 μm.

D. Ion transport measurements in control and Myo5b(G519R) mouse duodenum in an Üssing chamber system with pH titration. NHE3 inhibitor (Tenapanol) had no effect on I_{sc} or HCO_3^- secretion, and forskolin increased both I_{sc} and HCO_3^- secretion in control and Myo5b(G519R) mice. CFTR inhibitor (R-BPO-27) decreased I_{sc} in Myo5b(G519R) duodenum greater than in control duodenum, indicating that CFTR-dependent chloride secretion is upregulated in Myo5b(G519R) mice.

E. Average HCO_3^- secretion rate during 15 min after each chemical administration. *$P < 0.05$ and ***$P < 0.005$ by two-way ANOVA with Bonferroni's multiple comparisons correction. Each datapoint represents each value of individual mouse. $n = 3$ mice in each genotype.

F. Immunostaining for DPP4 (red), alpha-actinin-4 (green), and AKAP350 (blue) in jejunal villus epithelia. ACTG1 (white) in separate images indicate microvillus structures. Control enterocytes show AKAP350 expression in $DPP4^+$ brush border and $actinin\text{-}4^+$ terminal web regions. The enterocytes of Myo5b(G519R) mouse duodenum have no DPP4 signals and decreased

expressions of Actinin-4 and AKAP350, as well as ACTG1 on the apical domain. Arrows indicate a microvillus inclusion. Scale bars: 10 μm.

Supplementary Figure 3. Expanded epithelial proliferation in Myo5b(G519R) mouse intestine.

A. Immunostaining for proliferative marker PCNA (yellow), stem cell marker OLFM4 (magenta), and ACTG1 (white) in the duodenum of control and Myo5b(G519R) mice. Neonatal Myo5b deficient and Myo5bΔIEC mouse intestines contain elongated crypts.[4, 5] The $PCNA^+$ region is expanded in Myo5b(G519R) mouse intestine. Subapical OLFM4 signals remain in upper crypt and bottom villus cells in Myo5b(G519R) tissues (arrows), suggesting disrupted cell differentiation signaling pathways. Scale bars: 50 μm (left panels), and 20 μm (right panels).

B. The length of $PCNA^+$ cells was measured from the base of the crypt to the basolateral side of the highest $PCNA^+$ epithelial cell. The sum of $PCNA^+$ and $PCNA^-$ epithelial cell lengths were calculated as Crypt+Villus length. More than 10 regions were measured and averaged as a value of each mouse. $n = 5$ mice in each genotype. $^{***}P < 0.005$ by Two-way ANOVA with Bonferroni's multiple comparisons correction.

C. EdU (magenta) injected 24 hours before sampling tissues reached more distal regions of villi in Myo5b(G519R) mice compared to control mouse intestine. Tissue morphology and villus enterocytes were visualized with ACTG1 (green) and SGLT1 (white), respectively. Nearly all epithelial cells are EdU^+ in the Myo5B(G519R) small intestine. Scale bars: 50 μm.

D. The migration length of EdU^+ cell was measured from the base of crypt and compared to total mucosal height. Each datapoint represents an average value of at least 10 regions in each mouse. $^{*}P < 0.05$ by Mann-Whitney test. $n = 3$ mice in each genotype.

E. Scanning electron microscopy (SEM) images of mouse duodenum. (Left panels for each mouse) Compared to packed microvilli in control tissues, abnormal spaces between short and wide microvilli are identified in Myo5b(G519R) tissues. (Right panels for each mouse) Fractured tissues illustrate Myo5b(G519R) enterocytes possess short microvilli along the apical surface. Similar microvillus phenotype has been shown in Myo5bΔIEC mouse intestine together with disorganized intermicrovillar complex, which may prevent proper elongation of microvilli.[17] Scale bars: 1 µm.

1. **Supplementary Table 1. Antibodies for immunofluorescence staining.** Asterisks (*) indicate validated antibody for MxIF in human tissues.

Target	**Catalogue No.**	**AB_Resistry ID or clone**	**Dilution**	**Reactivity**
ACTG1	sc-65638 AF790	AB_2890622	1:50	*Hu, Mu
ACTG1	sc-65638 AF647	AB_2890621	1:50	*Hu, Mu
ACTG1	sc-65638 AF488	AB_2890619	1:100	*Hu, Mu
AKAP350	Own[18]	14G2	1:200	Hu, Mu
Alpha-actinin-4	ab198610	EPR2533(2)	1:50	Hu, Mu
ASBT (SLC10A2)	PA5-18990	AB_10981375	1:100	Mu
Beta-catenin	NBP1-54467IR	12F7	1:50	*Hu, Mu
CD10	sc-46656 AF488	AB_2890648	1:100	*Hu
CFTR (mouse)	Own[6]	NA	1 µg/ml	Mu
CHGA	NBP2-47850IR	CGA/493	1:2000	*Hu
Defensin 5A	NB110-60002IR	8c8	1:200	*Hu
DPP4 (mouse)	AF954	AB_355739	1:100	Mu
DPP4 (human)	NBP2-70588C	OTI11D7	1:200	*Hu
EGFR pY1068	ab205828	AB_2890267	1:200	*Hu, Mu
Ep-CAM	ab275122	EPR677(2)	1:100	*Hu
Ezrin	sc-32759-AF488	AB_2890903	1:50	*Hu

GLUT2	NBP2-22218AF647	AB_2890913	1:50	*Hu
LAMP1	sc-19992	AB_2134495	1:200	Mu
LAMP2A	ab282009	EPR4207(2)	1:50	*Hu
MYO5B	NBP1-87746	AB_11034537	5 µg/ml (Zenon labeled)	*Hu, Mu
NHE3	NBP1-82574	AB_11038394	1:100	Hu, Mu
PCNA	sc-56 AF647	AB_628110	1:50	*Hu, Mu
pNHE3	NB110-81529R	14D5	1:50	*Hu
PEPT1	sc-373742 AF488	E-3	1:50	*Hu, Mu
SGLT1	NBP2-38748	AB_2890609	5 µg/ml (Zenon labeled)	*Hu
SGLT1 (mouse)	Own[6]	NA	0.5 µg/ml	Mu
RAB7	sc-376362 AF594	B-3	1:100	*Hu, Mu
RAB11A	sc-166912 AF546	A-6	1:100	*Hu, Mu
RAB11A	71-5300	AB_2533987	5 µg/ml (Zenon labeled)	Hu, Mu
Villin	sc-58897 AF488	1D2C3	1:50	*Hu, Mu

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