Supplemental Methods:

Frozen Section Immunostaining

Fixed tissue was embedded in OCT and stored at -80 C prior to sectioning. Five-µm sections were cut using a cryotome. Slides were incubated in PBS for 15 minutes prior to staining to remove OCT. Sections were permeabilized with 0.3% Triton X-100 and blocked for 1 hour at room temperature in 10% donkey serum. Slides were then washed for 5 minutes in PBS. Primary antibodies were diluted in 1% donkey serum and 0.05% tween 20 in PBS overnight in a humidified chamber at 4℃. Slides were washed 3 times in PBS for 5 minutes prior to addition of secondary antibodies. Secondary antibodies along with fluorescently labeled Phalloidin were incubated for 1 hour at room temperature. Secondary antibodies were diluted 1:200 in PBS, Phalloidin was added 1:100 in PBS to visualize the actin rich brush border. Hoechst diluted 1:1000 in PBS was added to each section for 5 minutes to stain nuclei. Slides were washed 3 times for 5 minutes and coverslipped using Prolong Gold Antifade.

Imaging

All immunofluorescence images were acquired using a Zeiss Axio Imager microscope equipped with an Axiovision digital imaging system using a 20x or 40x objective or a Zeiss Confocal LSM 880 using 63x objective with Airyscan detector. The Axio 20x and 40x objective were respectively a Plan-apochromat with a numerical aperture of 0.8 M27 or 0.95 Korr M27. The Axio Imager microscope was equipped with an AxioCam HRm Rev.3. The LSM 880 63x objective had a numerical aperture of 1.4.

MYO5B Mean Fluorescence Intensity Quantification

Five 20x images at a fixed exposure were taken of MYO5B immunostained duodenum tissue from WT and MYO5B(P663L) pigs to determine whether levels of MYO5B protein were reduced in MYO5B(P663L) pigs compared to WT. Fiji (ImageJ) software was used to measure the mean fluorescence intensity of MYO5B in cells. The region above the nucleus, including the brush border, was measured in 100 cells per field of view for a total of 500 cells measured per pig.

Electron Microscopy

Small pieces of intestinal tissue were excised and prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) by fixing in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer at room temperature for 1 hour followed by overnight fixation at 4℃. Samples were washed a nd incubated with 1% osmium tetroxide for 1 hour followed by ethanol dehydration (30%, 50%, 70%, 95% and 100%). For TEM, samples were further dehydrated in propylene oxide. After dehydration, tissue pieces were infiltrated with and embedded in EPON 812 resin (Electron Microscopy Sciences, Cat# 14120). 60-70 nm ultrathin sections were placed on 300 mesh copper grids. Sections were stained with 2% uranyl acetate followed by Reynold's lead citrate. Images were acquired using a Philips/FEI T-12 Tecnai electron microscope. For SEM, the dehydrated samples were dried using hexamethyldisilazane (HMDS), mounted on stubs and sputter coated with gold/palladium. A FEI Quanta 250 scanning electron microscope was used to obtain SEM images.

Crypt Isolation and Enteroid Generation

Following excision, the duodenum was opened lengthwise, washed in PBS without Ca^{2+} and Mg^{2+} and cut into ~20 mm segments. The intestinal pieces were washed again until the supernatant was clear and then placed in 30 mL of advanced DMEM/F12 containing collagenase (1 mg/mL) and BSA (2 mg/mL) for 30 minutes at 37℃ with shaking. After 30 minutes, a 3-mL transfer pipette was used to gently remove villi and crypts from the pieces of intestine. Fifty µL of supernatant was examined using an inverted microscope to determine quality and quantity of crypts isolated. The supernatant containing villi and crypts was passed through a 70-µm filter to remove the villi. The flow through containing intestinal crypts was then centrifuged at 300 x g for 5 minutes. The resulting pellet was resuspended in Matrigel and 30 µL of Matrigel suspension containing crypts were aliquoted per well in a 48-well plate. The plate was incubated at 37℃ for 15 minutes to facilitate Matrigel polymerization and then overlaid with 300 µL of pre-warmed Human IntestiCult (Stem Cell Technologies, Cat# 06010) media containing penicillin/streptomycin. Media were changed every other day and resulting enteroids were passaged every 5-7 days. To passage enteroids medium was removed from wells and each well containing a Matrigel dome was gently resuspended in 1 mL of organoid harvesting solution (Trevigen #3700-100-01). Wells were placed on a shaker at 4℃ for 15-20 minutes to facilitate removal of Matrigel. Like wells were then pooled into a 15 mL tube and centrifuged at 300 x g for 5 minutes. Medium was aspirated and enteroids were resuspended in 1 mL of TrypLE Express (Thermo Fisher Scientific, Cat# 12604013) and incubated at 37℃ for 3 minutes. The TrypLE solution was triturated 10 times to reduce enteroids to small clumps of cells. Ten mL of DMEM were added to inactivate TrypLE prior to centrifugation at 300 x g for 5 minutes. TrypLE and DMEM

solution was removed and enteroid fragments were resuspended in Matrigel. After Matrigel was polymerized at 37℃ (approximately 15 minutes) each dome of Matrigel were overlaid with 300 µL of pre-warmed human Intesticult media.

Enteroid Monolayers Cultured Using Air Liquid Interface

To generate 2D monolayers of pig enteroids, 3D enteroids were used approximately 5 days after passaging. 2.5 µL of Matrigel was added to 100 µL of ice cold PBS without Ca^{2+} and Mg^{2+} and the solution was added to the apical surface of each transwell. Transwells containing Matrigel in PBS were incubated at 37℃ for 1.5 hours. Generally, 2 wells containing 75 enteroids per well were used to generate one 6.5 mm transwell (Corning #3470). Four days after passaging, 3D enteroids were resuspended in 1 mL of organoid harvesting solution to de-polymerize the extracellular matrix and placed at 4℃ on a shaker for 15-20 minutes. After Matrigel de-polymerization replicate wells were pooled and centrifuged at 300 x g for 5 minutes. The organoid harvesting solution was removed and the pellet of enteroids was resuspended in 1 mL of TrypLE Express solution to dissociate enteroids. Enteroids were incubated for 5 minutes at 37℃ in TrypLE Express. Next, 1 mL of DMEM was added to the enteroids, followed by trituration with a 1 mL pipette, until a single cell suspension was achieved. 10 mL of DMEM were added to inactivate TrypLE. Cells were centrifuged at 300 x g for 5 minutes. During centrifugation, the transwell plate containing Matrigel in PBS was removed from the incubator and the solution was removed from each transwell. The plate was briefly dried in the hood with the lid removed. After centrifugation the pellet was resuspended in human Intesticult and 100 µL of the cell suspension were added to the top of each transwell. 600 µL of human Intesticult was added to the bottom of each

transwell and the plate incubated at 37℃. Human In testicult media was replaced after 2 days. For the air liquid interface, the following day (day 3 since 2D plating) the human Intesticult media from the apical domain was removed and the human Intesticult from the basolateral domain was replaced with differentiation media, lacking Wnt. The basolateral differentiation media was replaced on day 5 and any fluid that was present on the apical domain was also removed. On day 7, monolayers were fixed in either 4% PFA or 10% neutral buffered formalin for 30 minutes at room temperature. Monolayers were washed in PBS for 5 minutes. For cryoprotection, 30% sucrose in PBS solution was added to each transwell for 3 hours and then monolayers were embedded in OCT.

Forskolin Swelling Assay

Differentiated 3D enteroids from WT and MYO5B(P663L) pigs were analyzed to determine CFTR function *in vitro*. Forskolin increases the amount of intracellular cyclic AMP, which activates CFTR-mediated fluid secretion [1]. Thus, if CFTR is functional, enteroids will swell in response to forskolin administration [1, 2]. Enteroids were imaged before and 1 hour after administration of 5 μM forskolin (Tocris) to assess the change in diameter of each individual enteroid in response to cAMP stimulation. A JuLi stage microscope (NanoEnTek) was used to image the forskolin swelling assay, which was performed at 37℃ with 5% CO_2. Enteroid swelling was quantified using Fiji software (formerly known as ImageJ). The forskolin swelling assay data represent three independent experiments.

Fecal Chloride Measurement

Pig feces were collected and 0.1 gm of feces were resuspended in 500 µl MilliQ water. Samples were thoroughly mixed and centrifuged at 5,000 x g for 5 min at 4℃ to pellet intestinal solids. Cl^- concentrations were determined from the resulting supernatant using a micro-chloride ion electrode (Lazar, LIS-146CLCM) and normalized to mM per gram feces.

Human Tissue Approval

This study used archival paraffin tissue from the duodenum of healthy individuals and patients with MVID through approved IRBs at Phoenix Children's Hospital and Vanderbilt University Medical Center.

Statistics

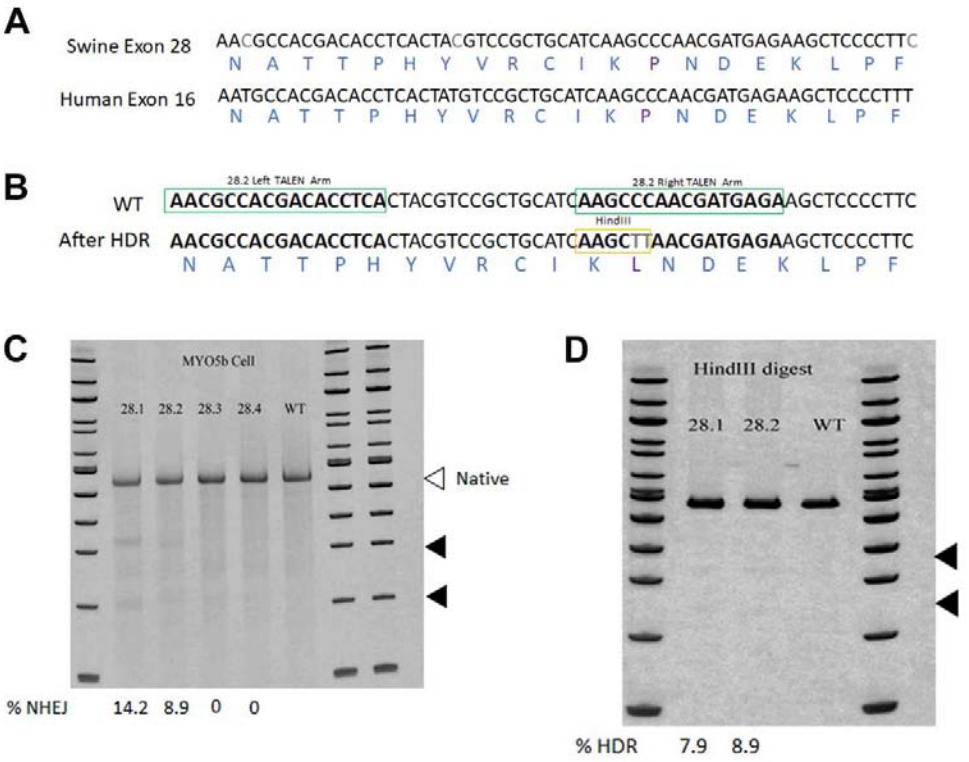
Values are reported as the mean ± SEM. Statistical significance for the forskolin swelling assay was determined using a two-tailed Student's t test using Prism Graphpad Software. A $P<0.05$ was considered significant.

Supplemental Table I: List of primary antibodies used for immunofluorescence staining.

Antibody	Company	Catalog	Dilution
Alkaline Phosphatase	Rockland	200-4135S	1:2000
BSEP	Kamiya Biomedical	PC-064	1:200
CD10	Abcam	Ab951	1:50
CFTR	Gift from Christopher	R3194 [3-5]	1:200

	Marino		
DPPIV	R&D Systems	AF1180	1:200
Gamma Actin	Santa Cruz Biotech	SC-65638 AF647	1:100
GM130	BD Biosciences	610822	1:200
MDR1	Abcam	Ab3366	1:200
MRP2	Enzo	ALX-801-016-C250	1:200
MYO5B	Novus Biologicals	NBP1-87746	1:200
NHE3	Novus Biologicals	NBP1-82574	1:100
p120	BD Biosciences	610133	1:200
P-ERM	Cell Signaling Tech	3726	1:200
Rab11a	US Biologicals	R009	1:200
SGLT1	Novus Biologicals	NBP 238748	1:100

Supplemental Figure Legends



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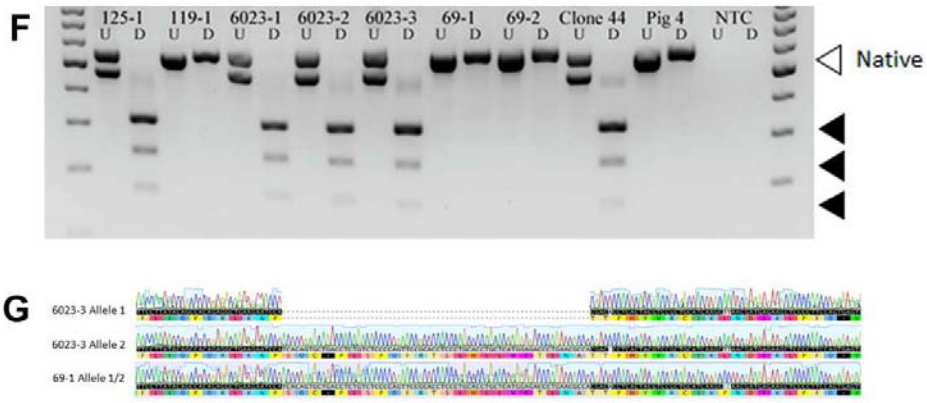
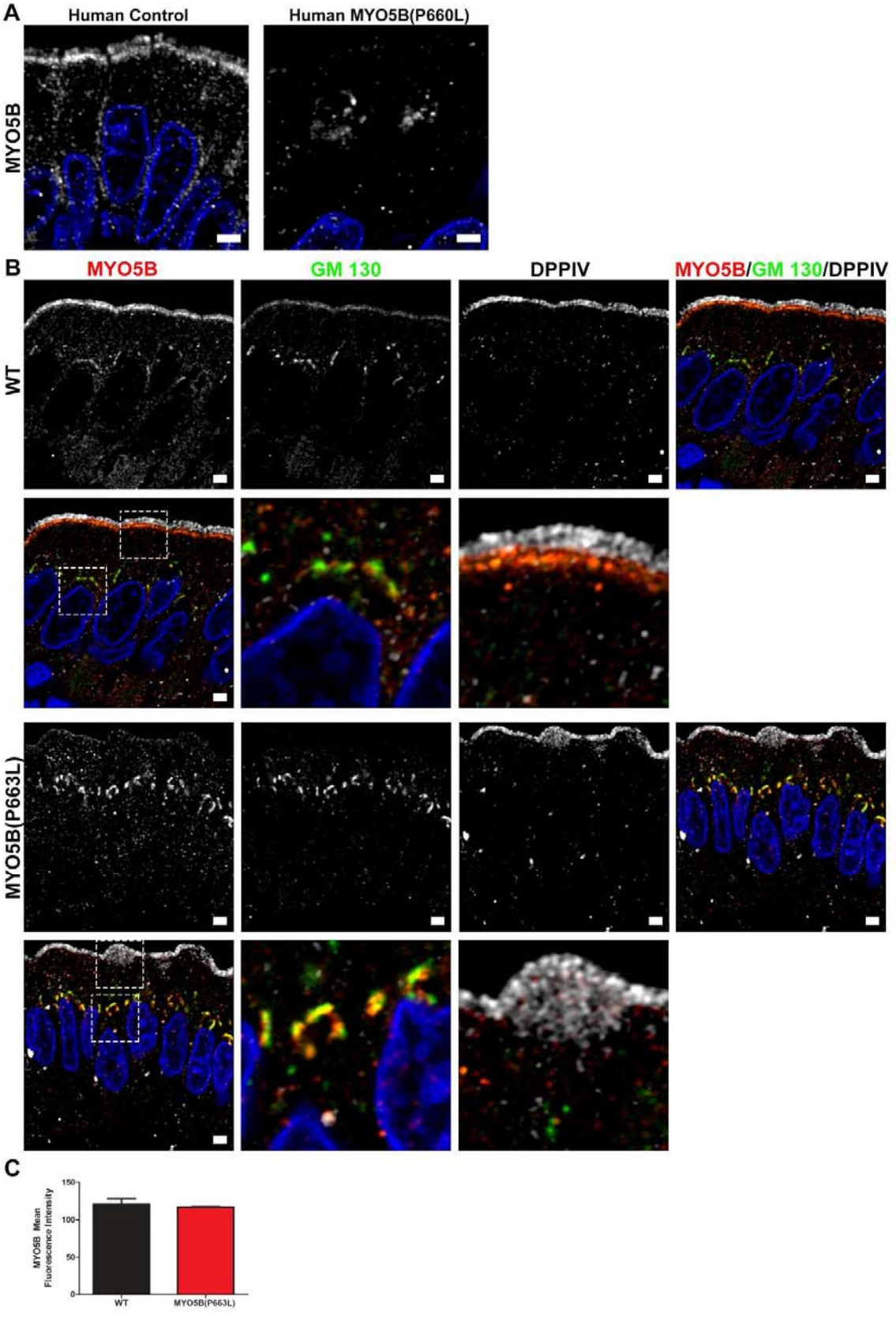
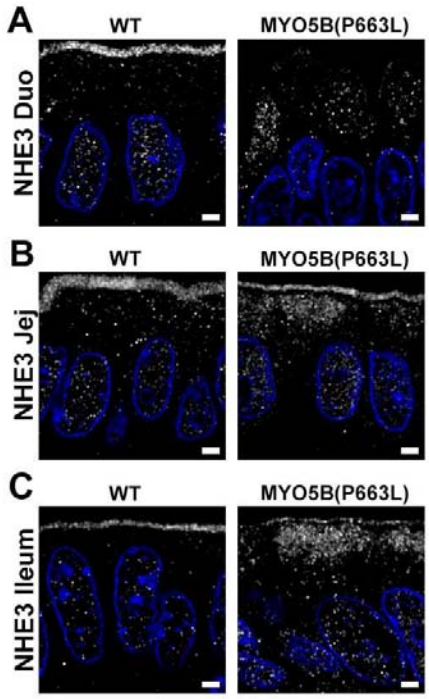
	Transfers	Pregnant	Live Piglets	Stillborn or Failure to Thrive
RCI1076 (Pig 4, Male, MYO5B Ex28 $^{P663L/KO}$)	3	1	1	2
6023-3 (Pig 4, Male, MYO5B Ex28 $^{P663L/KO}$)	3	0	0	0
6023-3 (Pig 4, Male, MYO5B Ex28 $^{P663L/KO}$)	2	1	1	0



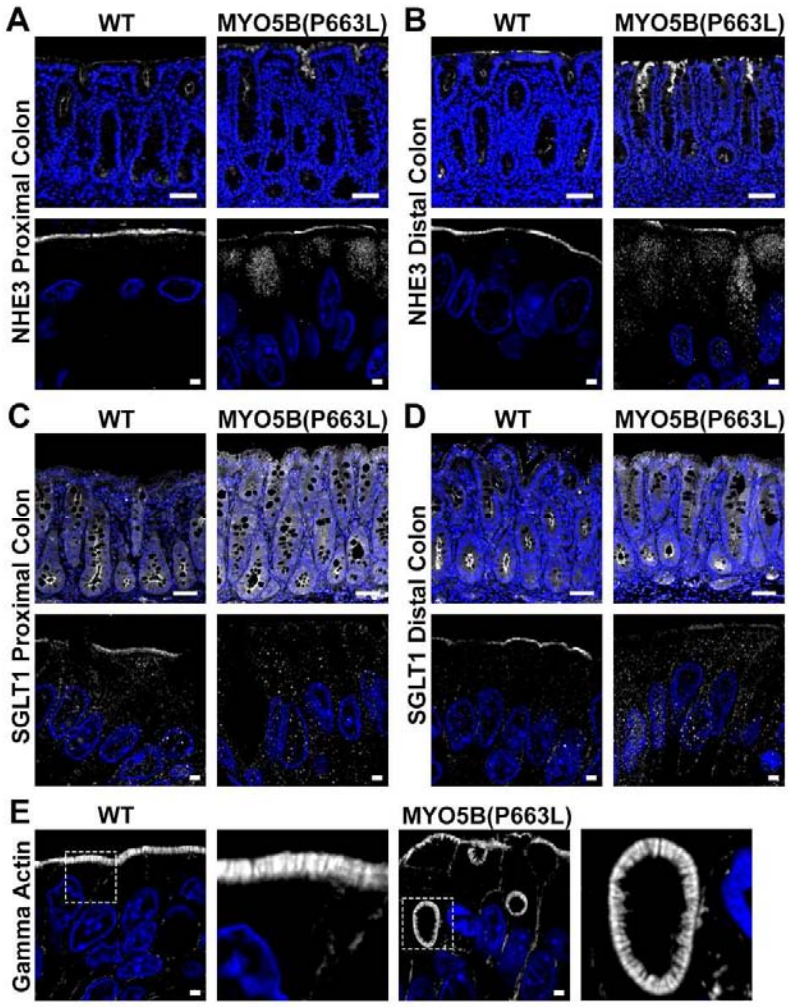
Supplemental Figure 1: Development of MYO5B P663L gene edited Landrace swine model of MVID. A) A portion of exon 28 and exon 16 of swine and human *MYO5B* gene respectively shows a nucleotide and protein alignment. This gene is well conserved between both species at an amino acid (AA) level of 83%. The mutation corresponds to a substitution of the amino acid Proline at position 660 and 663 in humans and swine, respectively. Gray letters indicate, differences in nucleotide sequence in comparison to human; blue, AA sequence; purple letters, Proline 660 or 663. B) The MYO5B^{P663L} homologous recombination (HR) template was designed around the chosen TALEN containing the P663L mutation, a unique HindIII site for RFLP to facilitate screening of HR-positive cells and animals, was introduced due to the intended mutation and provide mutations to eliminate TALEN re-binding to the targeted allele once HR occurred. Gray letters, differences in nucleotide sequence after HR occurred; bold letters, TALEN binding site; purple letter, L663. C) Gel image of Surveyor assay on transfected Landrace fetal fibroblast with 1 µg of TALEN mRNA. Cells were recovered for 3 days at 30^{o}C prior to quantification. The open arrowhead denotes the wild-type allele and the closed arrowhead denotes the RFLP or cut allele. D) Shows a gel image of a RFLP assay on male Landrace fetal fibroblast that were transfected with 1 µg of TALEN mRNA and 0.2 nMol of the HR template. These cells were recovered for three days at 30^{o}C prior to quantification. E) Table of the selected gene-edited pooled male fetal fibroblast used for somatic cell nuclear transfer and the outcomes. F) Gel image of a RFLP assay on the mutant and wild type control animals. G) Sequencing results of a semi-homozygous (MYO5B$^{P663L/KO}$) cloned (6023-3) animal and a wild type control animal (69-1).


A
Human Control
Human MYO5B(P660L)
MYO5B
B
MYO5B
GM 130
DPPIV
MYO5B/GM 130/DPPIV
WT
MYO5B(P663L)
C
MYO5B Mean Fluorescence Intensity
150
100
50
0
WT
MYO5B(P663L)

Supplemental Figure 2: Expression of MYO5B in humans and pigs with Navajo mutation in MYO5B. (A) Immunostaining of biopsies from healthy (control) and MVID duodenum for MYO5B (white). Control biopsy demonstrated MYO5B expression primarily at the base of the apical brush border of enterocytes. Human MVID biopsies showed internalized MYO5B that was not closely aligned with the brush border. n=3 biopsies per group. (B) Immunofluorescence micrograph showing MYO5B (red), the Golgi apparatus marker GM130 (green), and the brush border enzyme DPPIV (white) in WT and MYO5B(P663L) swine enterocytes. MYO5B was localized to the base of the brush border in WT duodenum while in MYO5B(P663L) duodenum MYO5B was associated with the Golgi apparatus. DPPIV identified the brush border and was observed subapically in MYO5B(P663L) pigs. (C) Quantification of mean fluorescence intensity of MYO5B in WT and MYO5B(P663L) pig duodenum. Data reported as mean ± SEM. Scale bars=2 μm, n=2 MYO5B(P663L) pigs and 3 WT pigs.



Supplemental Figure 3: NHE3 is mislocalized in the jejunum and ileum of MYO5B(P663L) pigs. Immunofluorescence images of NHE3 in WT and MYO5B(P663L) enterocytes along the length of the small intestine. NHE3 was present in the apical membrane of enterocytes in WT pigs. In MYO5B(P663L) NHE3 was mislocalized, similar to the duodenum, in the jejunum and the ileum. Scale bars= 50 µm in low magnification, scale bars= 2 µm in high magnification, n=2 MYO5B(P663L) pigs and 3 WT pigs.



Supplemental Figure 4: Changes in apical localization of sodium transporters are present in the proximal and distal colon of MYO5B(P663L) pigs. (A & B) Micrographs of NHE3 immunostaining in the proximal and distal colon of WT and MYO5B(P663L) pigs. MYO5B(P663L) pigs showed subapical localization of NHE3 in the large intestine compared to WT pigs. (C & D) Immunostaining of SGLT1 in the proximal and distal colon of WT and MYO5B(P663L) pigs. MYO5B(P663L) pigs had decreased apical SGLT1 compared to WT pigs. (E) Gamma actin staining demonstrated the presence of inclusions lined by microvilli in the colon of

MYO5B(P663L) pigs. No inclusions were observed in WT pigs. Scale bars = 50 µm in low magnification, scale bars = 2 µm in high magnification.

Bibliography

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