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Loss of MYO5B Leads to Reductions in Na⁺ Absorption with Maintenance of CFTR-dependent Cl⁻ Secretion in Enterocytes.

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Abstract

Background & Aims: Inactivating mutations in myosin VB (MYO5B) cause microvillus inclusion disease (MVID), but the physiological cause of the diarrhea associated with this disease is unclear. We investigated whether loss of MYO5B results in aberrant expression of apical enterocyte transporters.

Methods: We studied alterations in apical membrane transporters in MYO5B-knockout mice, as well as mice with tamoxifen-inducible, intestine-specific disruption of *Myo5b* (*VilCre^{ERT2};Myo5b^{flox/flox}* mice) or those not given tamoxifen (controls). Intestinal tissues were collected from mice and analyzed by immunoelectron microscopy or cultured enteroids were derived. Functions of brush border transporters in intestinal mucosa were measured in Ussing chambers. We obtained duodenal biopsy specimens from individuals with MVID and individuals without MVID (controls) and compared transporter distribution by immunocytochemistry.

Results: Compared to intestinal tissues from littermate controls, intestinal tissues from MYO5B-knockout mice had decreased apical localization of SLC9A3 (also called NHE3), SLC5A1 (also called SGLT1), AQP7, and sucrase isomaltase and subapical localization of intestinal alkaline phosphatase and CDC42. However, CFTR was present on apical membranes of enterocytes from MYO5B knockout and control mice. Intestinal biopsies from patients with MVID had subapical localization of NHE3, SGLT1, and AQP7, but maintained apical CFTR. Following tamoxifen administration, *VilCre^{ERT2};Myo5b^{flox/flox}* mice lost apical NHE3, SGLT1, DRA, and AQP7, similar to germline MYO5B KO mice. Intestinal tissues from *VilCre^{ERT2};Myo5b^{flox/flox}* mice had increased CFTR in crypts and CFTR localized to the apical membranes of enterocytes. Intestinal mucosa from *VilCre^{ERT2};Myo5b^{flox/flox}* mice given tamoxifen did not have an intestinal barrier defect, based on Ussing chamber analysis, but did have decreased SGLT1 activity and increased CFTR activity

Conclusions: Although trafficking of many apical transporters is regulated by MYO5B, trafficking of CFTR is largely independent of MYO5B. Decreased apical localization of NHE3, SGLT1, DRA, and AQP7 might be responsible for dysfunctional water absorption in enterocytes of patients with MVID. Maintenance of apical CFTR might exacerbate water loss by active secretion of chloride into the intestinal lumen.

Keywords

mouse model; mislocalization; sodium hydrogen exchanger; aquaporin

Introduction

Microvillus inclusion disease (MVID) is a rare congenital disorder that results in unremitting, life-threatening diarrhea in neonates^{1, 2}. Patients with MVID have inactivating mutations in the motor protein Myosin Vb (MYO5B)³⁻⁵. Newborns with MVID require life-long total parenteral nutrition (TPN), which carries a high risk for sepsis, or small bowel transplantation². As a result, the outcome for patients with MVID under the current treatment regimens remains poor. In an effort to understand the molecular mechanisms that underpin MVID, our lab⁷ and several other groups^{8, 9} have generated mouse models with loss of MYO5B that mimic the human disease.

Previous studies have identified the mislocalization of apical proteins, villus atrophy and intracellular inclusions that contain microvilli in the setting of MVID. However, the pathophysiology of MVID still remains unclear due to the complications that arise from prolonged TPN treatment in individuals with MVID. Prolonged TPN treatment is linked to intestinal atrophy and weakening of mechanical and immunological barriers in the intestine. Intestinal alterations that are observed in MVID patients that have been on TPN for months or years may not always solely result from loss of MYO5B^{10, 11}. Thus *in vivo* models of MVID are necessary to study intestinal abnormalities that arise from loss of MYO5B and are not confounded by TPN treatment. Early studies in patients with MVID showed abnormal glucose absorption and defective Na⁺ absorption^{1, 2} suggesting defects in ion transporters that promote net water absorption. In the small intestine, Na⁺ plays a pivotal role in the absorption of solutes and water across the apical brush border of enterocytes. The sodium hydrogen exchanger isoform 3 (NHE3, SLC9A3) and sodium glucose co-transporter (SGLT1, SLC5A1) are highly expressed in the brush border of the small intestine and play a major role in transepithelial absorption of Na⁺ and water. In addition to transporters, the aquaporin water channels (AQPs) are another component of the intestinal membrane that transports water and small-uncharged solutes across cellular membranes. The chloride bicarbonate exchanger, Down Regulated in Adenoma (DRA, SLC26A3), is a transporter that is essential for the proper absorption of chloride in the intestine¹³. Mutations in DRA give rise to congenital chloride diarrhea¹⁴. Excessive intestinal fluid secretion by activation of luminal Cl⁻ channels can also contribute to diarrhea. The cystic fibrosis transmembrane conductance regulator (CFTR) mediates Cl⁻ and HCO₃⁻ secretion in the intestine. CFTR can play a major role in net fluid secretion during secretory diarrhea through increased functional expression in the brush border of enterocytes¹⁵.

While much is known about the role of intestinal ion transporters in the settings of homeostasis and diarrheal disorders, further elucidation of the status of functional ion transport in the absence of MYO5B is needed. To address the mechanism of MVID induced diarrhea, we employed two mouse models of MVID, mouse-derived duodenal enteroids, an electrophysiological approach and human biopsies. Our work demonstrates that germline MYO5B knockout (MYO5B KO) mice and tamoxifen-induced intestinally targeted MYO5B knockout mice (VillinCre^{ERT2};MYO5B^{flox/flox}) exhibit improper localization of NHE3, SGLT1 and AQP7 in enterocytes. In contrast, CFTR was maintained on the apical membrane in MYO5B KO mice and tamoxifen-induced VillinCre^{ERT2};MYO5B^{flox/flox} mice.

Previous studies have used the Caco2-BBE colonic cancer cell line to investigate molecular changes that arise from loss of MYO5B¹⁵. However, caveats exist with cancer-derived cell models. Herein we used mouse enteroids generated from neonatal MYO5B KO mice to advance our understanding of the role of MYO5B in maintenance of the apical brush border. Enteroids generated from germline MYO5B KO mice also showed apically expressed CFTR and responded to forskolin demonstrating functional CFTR. Electrophysiological studies of adult VillinCre^{ERT2};MYO5B^{flox/flox} mice after administration of tamoxifen demonstrated that CFTR is the major contributor to short circuit current (I_{sc}) in these mutant mice, while in control mice CFTR has no basal activity under stable conditions. Adult VillinCre^{ERT2};MYO5B^{flox/flox} mice also showed significantly less SGLT1 activity than control mice. Finally, immunostaining of human biopsies from patients with MVID showed decreased apical expression of NHE3 and SGLT1, but normal CFTR expression. Collectively, these results suggest that loss of transporters that absorb Na⁺ and thus facilitate water absorption and the maintenance of functional CFTR are the likely drivers of the diarrhea associated with MVID.

Methods

Animals

The Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University Medical Center approved all experimental procedures and animal care and maintenance. Germline MYO5B knockout mice and VillinCre^{ERT2}MYO5B^{flox/flox} mice were generated as previously reported⁷. Intestinal tissue was collected from the proximal small intestine (duodenum and jejunum) of germline MYO5B KO mice and control littermates within one week of life. To activate Cre recombinase, 2 mg of tamoxifen was administered via IP injection to 8-12 week old VillinCre^{ERT2};MYO5B^{flox/flox} and VillinCre^{ERT2} or MYO5B^{flox/flox} (control) mice. Adult mice were euthanized and samples collected 4 days after tamoxifen administration.

Human tissue

Paraffin-embedded tissues from normal intestine and from the duodenum of Navajo MVID patients were obtained as previously described¹⁶. The intestinal tissue was collected from individuals ranging from one day old to one year old.

Immunoelectron Microscopy

Duodenal tissue collected from 2 day old control and MYO5B KO mice were fixed in 4% buffered formaldehyde solution for 3 days. Thawed cryosections (~100 nm thick) were subjected to indirect immunogold labeling with antisera detecting NHE3, STX3, Rab11 and Rab8a as previously described in detail¹⁷. Bound primary antibodies were visualized by Nanogold®-Fab' goat anti-rabbit IgG (H+L) (Nanoprobes, 2004, diluted 1:150) followed by silver enhancement with HQ-Silver® (Nanoprobes, 2012).

Short-circuit current (I_{sc}) measurement in Ussing Chambers

Mucosal-submucosal preparations were obtained from murine jejunum and terminal ileum. The intestinal segments were opened along the mesenteric border, and the tunica muscularis

was stripped with fine forceps under a stereomicroscope in ice-cold Krebs buffer. Two preparations were prepared from each segment and then mounted in sliders with an aperture = 0.1 cm² (Physiologic Instruments, San Diego, CA). Removal of muscle layer was confirmed by histological examination of paraffin sections.

Luminal and serosal surfaces of tissue were bathed with 4 ml Krebs-Ringer solution, maintained at 37°C using a water-recirculating heating system. The bathing solution contained 117 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 11 mM glucose. To prevent prostaglandin synthesis the cyclooxygenase inhibitor, indomethacin (10 μM), was routinely added into the serosal bath. The solution was bubbled with a gas mixture of 95% O₂-5% CO₂ to maintain the pH at 7.4.

The tissues were short-circuited by a voltage clamp (Physiologic Instruments) at zero potential difference with compensation for solution resistance. Short-circuit current (I_{sc}) was continuously recorded by the DataQ system (Physiologic Instruments). To determine tissue conductance (G_t , mS/cm²) clamped voltage was changed automatically at ±3 mV for 20 ms every 2 sec. Transmucosal resistance (R_t , Ω•cm²) was calculated as inverse of G_t . An increase of I_{sc} indicates luminal-to-serosal current flow, e.g., anion secretion or cation absorption. The tissues were stabilized for 30 min before the effects of drugs were investigated. A potent CFTR inhibitor (R)-BPO-27 fully blocks Cl⁻ current in Ussing chambered murine jejunum at 1 μM¹⁹. CFTR activity was determined using a concentration of BPO-27 racemate that should completely abolish CFTR-dependent currents (10 μM; MedChem Express, Monmouth Junction, NJ). SGLT1 activity was represented by phlorizin (0.1 mM)-sensitive I_{sc} . This concentration of phlorizin blocks SGLT1-mediated 3-O-methylglucose transport up to 30 mM in rat jejunum²⁰, and we confirmed that I_{sc} increase by luminal glucose (10 mM) was fully reversed by 0.1 mM of phlorizin in murine jejunum (data not shown). DMSO < 0.3% in the bathing solution did not affect the I_{sc} or G_t .

Results

Germline MYO5B Loss Leads to Loss of Apical Ion Transporters

Despite recent work on the generation of mouse models that recapitulate the MVID phenotype, the underlying cause of the severe diarrhea remains unknown. To characterize the intestinal defects that give rise to persistent diarrhea in neonates with MVID, we examined key apical markers and ion transporters in the proximal small intestine of control and MYO5B-deficient neonatal mice. The proximal small intestine was chosen since the largest epithelial defects following loss of MYO5B manifest in this intestinal region⁷. To identify enterocyte morphology, the plasma membrane and actin cytoskeleton linker phosphorylated ezrin (P-ERM immunostaining) was examined (Supplemental Figure 1A). In control duodenum, antibodies against P-ERM immunostained the microvilli on the apical surface of enterocytes as expected. In MYO5B KO duodenum, P-ERM staining showed the presence of intracellular P-ERM positive inclusions along the length of the villi and diminished apical brush border staining consistent with the reduced length of microvilli previously reported⁷.

The primary function of the small intestine is the digestion and absorption of nutrients and water. While absorption under homeostatic conditions is well understood, little is known in regards to the movement of water and nutrients in the setting of MVID. To address the role of ion transport in MVID-associated diarrhea, neonatal murine proximal small intestine was examined for several ion transporters that facilitate nutrient and water absorption (NHE3, SGLT1, AQP7). NHE3 plays a major role in the transepithelial absorption of Na⁺ and water in the small and large intestine²¹. Immunostaining of NHE3 in control mice showed robust expression in the apical brush border of enterocytes with no subapical staining (Supplemental Figure 1B). Control mice also exhibited proper apical localization of the surface marker DPPIV. In contrast, MYO5B KO enterocytes showed diffuse subapical expression of NHE3 as well as NHE3 positive inclusions that were also positive for DPPIV as previously reported⁷ (Supplemental Figure 1B). In addition to the dual NHE3 and DPPIV positive inclusions, DPPIV was also mislocalized subapically in MYO5B KO mice. SGLT1, the sodium-glucose co-transporter that drives water and glucose absorption, labeled the apical surface of control enterocytes. SGLT1 immunostaining of MYO5B KO mouse duodenum showed a large amount of intracellular SGLT1. Additionally, we observed an overall decrease in expression of apical SGLT1 (Figure 1A). To determine whether SGLT1 was trafficked to lysosomes, tissue was co-immunostained with the lysosome marker, Lamp2. Control mice showed large Lamp2 positive lysosomes aligned below the apical membrane, in accordance with previously published reports²² (Figure 1A). In the MYO5B KO mice, Lamp2 positive lysosomes appeared less distinct and dispersed throughout the enterocyte cytoplasm, with some lysosomes surrounding inclusions containing SGLT1 (Figure 1A). Aquaporin 7 (AQP7), a water-selective membrane channel, localized to the apical surfaces of enterocyte microvilli of control duodenum. In contrast, MYO5B KO mice appeared to lose apical expression of AQP7 in duodenal enterocytes and instead showed exclusively cytoplasmic expression of AQP7 (Figure 1B). Similar to Lamp2 immunostaining, we observed a disorganized pattern of Lamp1 in MYO5B KO mice (Figure 1B). Collectively, the decreased apical expression of apical transporters that regulate sodium and water absorption may all individually contribute to the severe diarrhea in MYO5B KO mice.

Given the mis-localization of key intestinal ion transporters, other apical membrane markers were also examined by immunostaining. The apical brush border enzyme, intestinal alkaline phosphatase (IAP), is mislocalized in epithelial cells of patients with MVID and in an inducible mouse model of MVID^{9, 23}. As expected, control mice displayed exclusive apical staining for IAP, confirmed by colocalization with the apical marker DPPIV (Figure 1C). In contrast, MYO5B KO mice had decreased apical IAP expression with large amounts of cytoplasmic IAP, including IAP and DPPIV dual positive inclusions. Previous investigations have shown that apical Rho GTPase and CDC42 are lost from the apical cortex in patients with MVID²⁴. Consistent with these studies, control mice exhibited apical CDC42 staining in enterocytes, while MYO5B KO mice had no apical CDC42 expression (Supplemental Figure 2A). Dual staining with P-ERM clearly demonstrated that loss of CDC42 is not correlated with the presence of inclusions (Supplemental Figure 2A). Another component of the brush border, sucrase isomaltase (SI) was investigated to determine its localization following loss of MYO5B (Supplemental Figure 2B). As expected, SI was present in the

brush border of enterocytes of control mice. However, MYO5B KO mice showed no apical brush border expression of SI and altered Lamp1 localization.

Abnormal Subapical Vesicle Accumulation in Enterocytes of MYO5B KO Mice

Transmission electron microscopy (TEM) immunogold labeling was performed on control and MYO5B KO duodenal tissue to examine in greater detail the subcellular structures associated with the localization of NHE3 and cellular trafficking proteins Syntaxin 3, Rab8a and Rab11 following loss of MYO5B (Figure 2). In contrast to control enterocytes, MYO5B KO mice showed an accumulation of subapical vesicles/tubules in duodenal enterocytes similar to the electron microscopy pattern in the duodenum from patients with MVID^{17, 25}. Furthermore, NHE3 displayed a subapical distribution in these accumulated vesicles/tubules that contrasts with the NHE3 localization to the brush border microvilli found in control mouse tissue. Syntaxin 3 (STX3), an apical t-SNARE protein, participates with MYO5B, Rab8a and Rab11a in apical cargo trafficking^{17, 25}. As expected in control mice, immunogold labeling for these apical trafficking proteins demonstrated STX3 predominately localized to the brush border; while Rab8a and Rab11 were found in the subapical cytoplasm. In MYO5B KO mice immunogold labeling of STX3, Rab8a, and Rab11 showed localization throughout the cytoplasm as well as in association with the distinct accumulated subapical vesicles/tubules that form in enterocytes after the loss of MYO5B.

CFTR is Maintained at the Apical Membrane Following Loss of MYO5B

While defects in absorptive ion transporters are important factors in diarrhea, active secretion of Cl⁻ can also contribute to diarrhea. CFTR is the major cyclic adenosine monophosphate (cAMP)-regulated Cl⁻ channel activated in secretory diarrhea²⁶. Unlike the absorptive ion transporters, which were all decreased in MYO5B KO mice, both control and MYO5B KO mice showed the presence of apical CFTR along the brush border of enterocytes throughout the villi (Figure 1D). Although MYO5B KO mice also showed CFTR positive inclusions that were also positive for F-actin, the staining pattern of CFTR in MYO5B KO mice showed no diffuse subapical staining as was seen for SGLT1 and NHE3. To determine whether Cl⁻ was being secreted into the intestinal lumen of MYO5B KO mice, the luminal contents of each segment of the intestine were collected and analyzed for Cl⁻ ions. In the duodenum, MYO5B KO mice had higher levels of luminal Cl⁻ than littermate controls (control 8.13 ± 1.39 , MYO5B KO 18.22 ± 4.24 , $P=0.0066$) (Supplemental Figure 3A). The jejunum, ileum and colon showed comparable levels of luminal Cl⁻ between control and MYO5B KO mice (Supplemental Figure 3B-D).

To elucidate further enterocyte abnormalities that arise from loss of MYO5B, an enteroid culture system was utilized (Figure 3). Enteroids were generated from the crypts of control and MYO5B KO duodenum. Gross morphology of enteroids revealed no differences between control and MYO5B KO derived enteroids (Supplemental Figure 4A). However, immunostaining of enteroids from MYO5B KO mice revealed intracellular abnormalities similar to MYO5B KO tissue. Enteroids generated from MYO5B KO mice had characteristic F-actin and phospho-ERM-positive inclusions (Figure 3A and B). Staining for CFTR showed similar apical expression between control and MYO5B KO enteroids (Figure 3B). To determine whether CFTR was functional in control and MYO5B KO enteroids, we

generated germline MYO5B KO mice that express LifeAct-eGFP, which labels F-actin in live cells²⁷. LifeAct and LifeAct;MYO5B KO mouse derived enteroids showed robust GFP labeling of the brush border lining the lumen of enteroids. Further, LifeAct;MYO5B KO-derived enteroids showed GFP-positive inclusions (Supplemental Figure 4B). A forskolin swelling assay was used to determine whether CFTR was functional following loss of MYO5B²⁸. Forskolin increases intracellular cAMP, thereby activating CFTR and inducing apical fluid secretion into the enteroid lumen, resulting in swelling^{28, 29}. LifeAct and LifeAct;MYO5B KO mouse derived enteroids were differentiated by withdrawal of Wnt conditioned media and treated with 5 μ M forskolin. Differentiation of enteroids was determined by maturation or thickening of the brush border as identified by phalloidin (F-actin) staining and LifeAct fluorescence. Live imaging and quantification showed similar enteroid swelling of LifeAct and LifeAct;MYO5B KO-derived enteroids (Figure 3C and D). These findings suggest that loss of MYO5B results in inclusions *in vitro*, but does not interfere with the presence or functional capacity of CFTR on the apical membrane. Collectively, these results indicate that the decreased expression pattern of apical transporters that promote water absorption (NHE3, SGLT1 and AQP7) in MYO5B KO mice and the maintenance of apical CFTR and secretion of Cl⁻ into the intestinal lumen could both contribute to the severe diarrhea in MYO5B KO mice.

Human MVID Results in Loss of Apical Transporters and Maintenance of CFTR

Since the MYO5B mouse model revealed robust changes in apical proteins, we sought to determine if patients with MVID also exhibited similar mislocalization of key ion transporters. Duodenal biopsies were collected from healthy individuals and from patients with a Navajo-specific mutation that causes MVID (MYO5B-P660L). In the duodenal biopsies from healthy individuals, NHE3 was localized to the apical brush border of villus enterocytes (Figure 4A). Patients with MVID showed decreased apical NHE3 in the brush borders of enterocytes and diffuse subapical NHE3 especially in enterocytes at the tips of the villi. SGLT1 and DPPIV staining of healthy tissue showed completely apical expression, while MVID biopsies showed cytoplasmic SGLT1 and DPPIV (Figure 4B). AQP7 immunofluorescence in healthy patient duodenal biopsies showed localization in the brush border just below CD10 immunostaining. CD10 is a membrane associated neutral peptidase that is normally expressed in the brush border of the small intestine³⁰. In intestinal biopsies from patients with MVID, AQP7 was completely lost from the brush border and CD10 showed diffuse subapical staining (Figure 4C). In contrast, CFTR immunostaining showed apical expression in MVID biopsies similar to control tissue (Figure 4D). These findings closely correspond to findings in the MYO5B KO mice, suggesting that this mouse model of MVID closely recapitulates the human disease.

Loss of MYO5B in Adult Mice Induces Loss of Apical Transporters

To evaluate the possibility that the observed MYO5B KO intestinal phenotypes were the result of the inclusions that are present in the neonatal mice, we used a tamoxifen inducible VillinCre^{ERT2}MYO5B^{fllox/fllox} mouse model. This mouse model effectively deletes MYO5B in the intestine leading to severe diarrhea within 3–4 days in adult mice⁷. At 4 days post-induction, VillinCre^{ERT2};MYO5B^{fllox/fllox} mice exhibited loss of transporters that promote water absorption similar to MYO5B KO mice. As shown in Figure 5A P-ERM-positive

inclusions were rarely present in adult VillinCre^{ERT2};MYO5B^{flox/flox} mice following loss of MYO5B; however, DPPIV showed diffuse cytoplasmic localization. NHE3 immunostaining demonstrated loss of apical NHE3 in VillinCre^{ERT2};MYO5B^{flox/flox} mice (Figure 5B). Examination of SGLT1 also showed a dramatic loss of apical expression in the brush borders of enterocytes after tamoxifen induced MYO5B loss (Figure 5). Lamp2 positive lysosomes also displayed a different staining pattern than control mice. Lamp2 lysosomes were dispersed from the normal alignment below the apical membrane in VillinCre^{ERT2};MYO5B^{flox/flox} mouse enterocytes (Figure 5B and 5C). In addition to the decreased apical expression of NHE3 and SGLT1, VillinCre^{ERT2};MYO5B^{flox/flox} mice showed complete loss of apical expression of AQP7 (Figure 6A). SI staining was abundant along the brush border of villus enterocytes in control mice, but was lost from the brush border in VillinCre^{ERT2};MYO5B^{flox/flox} mice (Figure 6B). IAP also showed robust apical staining in control mice; however, VillinCre^{ERT2};MYO5B^{flox/flox} mice showed subapical expression of IAP in enterocytes (Figure 6B). Immunostaining of the chloride transporter DRA showed abundant apical staining predominantly along the brush border of the villus enterocytes (and not crypt region) in control mice. However, VillinCre^{ERT2};MYO5B^{flox/flox} mice showed loss of expression of DRA in the brush-border of enterocytes (Figure 6C). CFTR was observed in the brush border of enterocytes in the villi of control mice. Interestingly, CFTR was more highly expressed in the crypt and midway up the villi of tamoxifen-induced VillinCre^{ERT2};MYO5B^{flox/flox} mice compared to control mice (Figure 6D).

In vitro induction of Cre recombinase using 4-hydroxytamoxifen (4-OHT) in VillinCre^{ERT2};MYO5B^{flox/flox} mouse derived enteroids resulted in the formation of inclusions that were P-ERM positive, but did not result in any gross morphological changes in the cultured enteroids (Supplemental Figure 5A&B). Immunostaining of enteroids treated with vehicle (ETOH) showed that normally enteroids express sucrase isomaltase (SI) and NHE3 on the apical membrane (Supplemental Figure 5C). However, *in vitro* induced loss of MYO5B with 4-OHT treatment resulted in decreased apical expression of SI and NHE3 as well as intracellular accumulation of SI and NHE3 (Supplemental Figure 5A&B). We observed a similar mislocalization of other brush border components, DPPIV and intestinal alkaline phosphatase, following 4-OHT treatment of VillinCre^{ERT2};MYO5B^{flox/flox} derived enteroids (Supplemental Figure 6A). However, stimulation of CFTR through administration of forskolin resulted in similar degrees of swelling between ETOH or 4-OHT treated VillinCre^{ERT2};MYO5B^{flox/flox} enteroids (Supplemental Figure 6B&C). These data closely mirror our *in vivo* findings and emphasize the advantages of enteroids as an experimental tool.

Increased Contribution of CFTR in VillinCre^{ERT2};MYO5B^{flox/flox} Mice

Measurement of Cl⁻ in the intestinal luminal contents showed similar levels between control mice and VillinCre^{ERT2};MYO5B^{flox/flox} mice following tamoxifen induced MYO5B loss (Supplemental Figure 7). These results suggest that, following tamoxifen treatment, VillinCre^{ERT2};MYO5B^{flox/flox} mice may maintain the ability to secrete Cl⁻ into the intestinal lumen via CFTR or a calcium dependent Cl⁻ channel. While the Cl⁻ measurement of luminal contents showed no statistical difference in VillinCre^{ERT2};MYO5B^{flox/flox} mice

treated with tamoxifen, the true degree of Cl^- secretion or inability to absorb intestinal chloride may not be fully represented in the samples due to the profound diarrhea that is present following loss of MYO5B. The watery content of the collected luminal samples in the tamoxifen treated VillinCre^{ERT2};MYO5B^{flox/flox} mice may result in a greater degree of dilution compared to control mice. Thus, to better delineate the functional capacity of ion transport Ussing chambers were used to measure epithelial membrane properties in intestinal mucosa of tamoxifen treated VillinCre^{ERT2};MYO5B^{flox/flox} and control mice. Tissue resistance measurements showed that the jejunum and ileum mucosa of tamoxifen-treated VillinCre^{ERT2};MYO5B^{flox/flox} mice did not have a barrier defect (Figure 7A&B). These results ruled out the possibility that induced VillinCre^{ERT2};MYO5B^{flox/flox} mice have a “leaky” gut and suggested that the diarrhea following loss of MYO5B was not the result of loss of water through junction defects. Measurement of I_{sc} demonstrated that, in the jejunum and ileum of tamoxifen-induced VillinCre^{ERT2};MYO5B^{flox/flox} mice, CFTR was the greatest contributor to electrogenic ion transport under stable conditions (Figure 7C&D). In contrast, in control mice, SGLT1 contributed a larger proportion of the I_{sc} . Furthermore, induced VillinCre^{ERT2};MYO5B^{flox/flox} mice had significantly less SGLT1 activity than control mice (Figure 7C&D). These findings closely coincide with immunostaining suggesting that tamoxifen-treated VillinCre^{ERT2};MYO5B^{flox/flox} mice had loss of apical SGLT1, but maintained functionally active CFTR. Although the remaining I_{sc} in control tissues could not be fully characterized, this positive I_{sc} might represent the summation of sodium- or proton-coupled nutrient absorptive currents³¹ that were decreased in induced VillinCre^{ERT2};MYO5B^{flox/flox} mice, contributing to malabsorption. Finally, induced VillinCre^{ERT2};MYO5B^{flox/flox} mice exhibited a greater dependence on CFTR for alterations in I_{sc} than control mice after forskolin stimulation (Figure 7E and F). Collectively, these data support the concept that MVID diarrhea manifests through a loss of apical proteins that drive water absorption coupled to the preservation of functional CFTR to further promote water loss.

Discussion

Our group previously reported the development and characterization of three mouse models of MYO5B deletion. These genetic models of MVID included germline, constitutive intestinally targeted, and inducible intestinally targeted deletion of MYO5B⁷. This previous investigation reported that the diarrheal pathology that develops from MYO5B loss is not the result of microvillus inclusions, but postulated that diarrhea resulted from deficits in transporter presentation at the apical membrane in duodenal enterocytes. The results presented here seek to further our understanding of MVID by providing a comprehensive analysis of transporter deficits that result from loss of functional MYO5B using two of our previously reported mouse models. Germline and inducible intestinally targeted MYO5B KO mice both displayed prominent loss of the apical transporters NHE3, SGLT1 and AQP7. While our neonatal mice (control and MYO5B KO) did not express DRA, likely due to the developmental stage at which they were analyzed, we found that adult VillinCre^{ERT2};MYO5B^{flox/flox} mice treated with tamoxifen lost apical expression of DRA in enterocytes. Biopsies from patients with MVID showed similar losses in expression of apical membrane proteins. Our lab has reported decreased apical expression of SGLT1 in MVID

enterocytes¹⁶. Caco2 cells with MYO5B knockdown also showed decreased levels of NHE3 and MVID patient tissue demonstrated decreased NHE3 expression in the brush border of enterocytes^{7, 15}. Moreover, Vogel and colleagues published work showing that MYO5B promotes delivery of NHE3 to the apical membrane²⁵. Our findings in the germline and induced VillinCre^{ERT2}MYO5B^{flox/flox} mice and in MVID tissue corroborate these previous studies and show that, in two *in vivo* models of MYO5B deletion, NHE3 and SGLT1 expression is decreased at the apical brush border of enterocytes.

Ussing chamber studies were used to determine the functional impact of loss of MYO5B on intestinal apical transporters. Importantly these experiments demonstrated that VillinCre^{ERT2}MYO5B^{flox/flox} mice do not have an intestinal barrier defect or “leaky gut.” These data are significant in light of the complications that arise in analyzing human MVID. Since the majority of neonates with MVID present with severe diarrhea shortly after birth, in most instances total parenteral nutrition (TPN) is immediately implemented with suspension of enteral feeding. As a result, defects in the intestinal epithelium of MVID patients may not necessarily represent the direct effects of inactivating mutations of MYO5B, but instead may reflect the impact of prolonged TPN exposure and a lack of luminal nutrition in the context of MYO5B loss¹⁰. Thus, changes in barrier integrity that have been previously reported may be the result of protracted TPN, since tamoxifen treated VillinCre^{ERT2}MYO5B^{flox/flox} mice did not manifest any changes in tissue resistance compared to control mice. Schneeberger *et al.* reported enlarged intercellular spaces between enterocytes based on TEM in their inducible intestinally targeted MYO5B knockout mouse model⁹. In our previous publication, we found no large intercellular spaces between enterocytes in our VillinCre^{ERT2}MYO5B^{flox/flox} mice⁷. This discrepancy may be due to differences in mouse models and mouse facilities.

Analysis of inducible intestinally targeted MYO5B KO mice provides novel insights into intestinal epithelial alterations that are the direct result of inactivation of MYO5B and independent of TPN. We observed that induced VillinCre^{ERT2}MYO5B^{flox/flox} mice had significantly reduced SGLT1-mediated I_{sc} when measured in Ussing chambers. These results strongly support our immunostaining findings that SGLT1 expression is decreased on the apical membrane of neonatal germline MYO5B KO mice and induced VillinCre^{ERT2}MYO5B^{flox/flox} mice.

Previous investigations in Caco2 cells with over-expression of CFTR had suggested that MYO5B may be a regulator of apical trafficking of CFTR²⁵. In our analysis of apical transporters, we observed maintenance of apical CFTR in germline MYO5B KO mice and up-regulation of CFTR in the crypts of induced VillinCre^{ERT2}MYO5B^{flox/flox} mice. Likewise, biopsies from patients with MVID also showed maintenance of CFTR on the enterocyte apical membrane. Analysis of functional ion transport using Ussing chambers showed that CFTR contributes to basal and stimulated I_{sc} to a greater degree in tamoxifen treated VillinCre^{ERT2}MYO5B^{flox/flox} mice than in control mice. Moreover, analysis of luminal contents in both germline and induced VillinCre^{ERT2}MYO5B^{flox/flox} mice showed Cl^- present at similar concentrations in control and KO mice. Forskolin swelling experiments in germline MYO5B KO-derived enteroids that expressed a fluorescent F-actin reporter also supported the finding that CFTR was present and functional on the apical

membrane of enterocytes. Thus, the findings with CFTR over-expression in cultured immortalized cells may not fully recapitulate the trafficking of the endogenous transporter in enterocytes.

An early study of jejunal tissue from MVID patients reported Cl^- secretion in MVID tissue that was close to the maximal rate of Cl^- secretion in normal tissue³². More recent studies have reported preservation of CFTR in the brush border of Caco2 cells with MYO5B knockdown and on the apical membrane of enterocytes in MVID tissue^{15, 23}. Furthermore, knockdown of MYO5B in T84 cells did not affect CFTR ion transport, suggesting that CFTR trafficking to the apical membrane is independent of MYO5B¹⁵. Trafficking of CFTR to the brush border of enterocytes is dependent on Myosin 1a³³. Even in the absence of MYO5B, the myosin motors, Myosin 1a and Myosin 6, are postulated to deliver CFTR to the brush border³³. We speculate that this results in maintenance of CFTR in the brush border despite loss of MYO5B largely contributing to the severe diarrhea in MVID. The proper trafficking of CFTR once endocytosed is likely partially dependent on MYO5B since inclusions in the germline MYO5B KO mice contain CFTR. Despite the presence of inclusions containing CFTR, we observed maintenance of CFTR on the apical membrane indicating that proper delivery of CFTR to the brush border is not MYO5B dependent, which supports the findings that Myosin 1a is responsible for CFTR movement into the brush border³³. Therefore, we postulate that while CFTR is trafficked independent of MYO5B, the proper trafficking of other transporters involved in water absorption (SGLT1, NHE3, AQP7 and DRA) are MYO5B dependent. Immunostaining of germline, induced VillinCre^{ERT2}MYO5B^{flox/flox} mice and MVID patient tissue strongly implicate different trafficking patterns of apical membrane proteins and enzymes.

Our results coincide with these previous reports, but differ from data published by Schneeberger *et al*⁹. Schneeberger and colleagues reported that administration of 4-OHT to enteroids derived from an inducible intestinally targeted MYO5B deletion mouse given had diminished swelling in response to forskolin⁹. We found that induction of MYO5B deletion with 4-OHT did not result in decreased swelling of our VillinCre^{ERT2};MYO5B^{flox/flox} derived enteroids. The discrepancy between our findings and the ones reported by Schneeberger *et al*. may result from the different mouse models used to derive the enteroids as well as the conditions used to grow the enteroids. Activation of Cre recombinase in the enteroids generated by Schneeberger *et al*. resulted in fewer budding structures and more cell death⁹. This phenomenon differs from our germline mutation-derived enteroids and our adult VillinCre^{ERT2};MYO5B^{flox/flox} derived enteroids in which we observed no difference in enteroid size, budding structures or increased cell death. As a result, we speculate that increased apoptosis in the *in vitro* activated enteroid model may decrease the number of viable enterocytes containing CFTR and may yield lower CFTR activity. Our *in vitro* forskolin swelling data is strongly supported by the presence of Cl^- in the intestinal lumen of MYO5B KO mice and immunostaining for CFTR in neonatal germline MYO5B KO mice and in enteroids from these mice. Moreover, our Ussing chamber data in adult induced VillinCre^{ERT2}MYO5B^{flox/flox} mice also support active apical CFTR, as does the immunostaining in this mouse model.

While differences exist between our forskolin induced enteroid swelling data and that reported by Schneeberger and colleagues, we did observe similar accumulation of intracellular alkaline phosphatase (Supplemental Figure 6A) in our 4-OHT treated VillinCre^{ERT2};MYO5B^{flox/flox} enteroids. Additionally, we also demonstrated the presence of P-ERM positive intracellular inclusions in 4-OHT-treated VillinCre^{ERT2};MYO5B^{flox/flox} enteroids. The presence of inclusions corroborates Schneeberger's TEM results showing subapical microvilli-lined inclusions in their 4-OHT-treated enteroids. These results suggest that, while induced deletion of MYO5B in adult VillinCre^{ERT2};MYO5B^{flox/flox} mice elicits few inclusions⁷, the enteroids prepared from these mice may recapitulate a more neonatal phenotype leading to increased inclusion formation after MYO5B deletion *in vitro*.

Mutated NHE3 has been associated with severe chronic diarrhea known as congenital sodium diarrhea, a phenotype similar to MVID^{34, 35}, while mutated DRA has been associated with congenital chloride diarrhea. Abnormal CFTR intestinal secretory pathways have been implicated in other forms of intractable secretory diarrheas presenting in newborns³⁵. To the best of our knowledge our results are likely specific to MVID diarrhea and may not be broadly applicable to other congenital diarrheas. Unlike individuals with congenital chloride diarrhea, patients with MVID require lifelong TPN and do not respond to current treatment regimens for other diarrhea diseases³⁶. We postulate that MVID is unique in causing altered trafficking of more than one transporter, since we see loss of apical NHE3, SGLT1, AQP7 and DRA. Our study also demonstrates a functional deficit in SGLT1 that occurs following loss of MYO5B. Moreover, we report that synchronous with loss of apical transporters, there is maintenance of CFTR and functional chloride secretion. Therefore, we suspect that MVID poses a unique challenge to physicians since it requires treatment that can both promote water absorption despite loss of transporters that drive hydration and simultaneously decrease CFTR mediated chloride secretion.

This is the first study to test functional ion transport deficits in a mouse model of MYO5B loss. Collectively, the data presented here suggest that loss of MYO5B results in altered trafficking of critical transporters to the apical membrane. These trafficking patterns are apparent in the preservation of apical CFTR, decreased expression of apical NHE3 coincident with increased subapical expression, loss of apical SGLT1 and AQP7, and absence of SI in the brush border of MYO5B deficient mice. Therefore, we postulate that MVID-associated diarrhea is due to improper apical expression of primary Na⁺ transporters, NHE3 and SGLT1 and chloride transporter DRA, which promotes malabsorptive diarrhea, and the maintenance of apical CFTR, which drives secretory diarrhea. Thus, MVID patients may benefit to some extent from inhibition of CFTR driven Cl⁻ secretion. With the advent of several potent CFTR inhibitors³⁷, studies involving CFTR inhibition in *in vivo* (mouse) and *in vitro* (mouse and human enteroids) models will likely point to new avenues for MVID therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: MYO5B KO mice exhibit loss of SGLT1, apical alkaline phosphatase and AQP7 but maintain apical CFTR expression.

(A) Lamp2 positive lysosomes (green) were present below the brush border labeled by SGLT1 (red) in control mice. MYO5B KO mice showed disorganized Lamp2 lysosomes as well as decreased apical SGLT1 and increased intracellular SGLT1. Arrows indicate several SGLT1 positive inclusions surrounded by Lamp2 lysosomes. (B) AQP7, is normally expressed in the brush border of duodenal enterocytes. Apical AQP7 (red) is absent in MYO5B KO mice. Lamp1 lysosomes (green) were aligned below the apical membrane in control mice. MYO5B KO mice had dispersed Lamp1 lysosomes. (C) Immunofluorescence

staining for IAP (red) in control mice showed clear apical expression. MYO5B KO mice exhibited subapical accumulation of IAP below the apical membrane. DPPIV (green) was present in numerous intracellular inclusions in MYO5B KO mice. (D) CFTR (red) and F-actin (green) are normally expressed in the brush border of enterocytes. Following loss of MYO5B F-actin positive inclusions were observed. CFTR was present in F-actin positive inclusions in MYO5B KO mice. However, CFTR was still present on the apical membrane of MYO5B KO mice. n=4-6 mice per group, scale bars=50 μ m.

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Figure 2: Localization of NHE3, Rab8a, Rab11 and STX3 by immunogold Transmission Electron Microscopy.

NHE3 immunogold labeled microvilli of control mice but was additionally present in subapical vesicles in MYO5B KO mice. Rab8a and Rab11 showed normal subapical distribution in control mice and in MYO5B KO mice a certain accumulation in the aberrant subapical vesicle clusters. STX3 was found on the brush border of control mice but was preferentially located in subapical vesicles in MYO5B KO mice. n=3-4 mice per group, scale bars=500 nm.

Figure 3: MYO5B KO derived enteroids display intracellular inclusions and have functional CFTR.

(A) Differentiated enteroids from control and MYO5B KO mice immunostained for the basolateral marker p120 (green) and apical protein P-ERM (red). MYO5B KO enteroids have P-ERM intracellular inclusions. (B) CFTR (red) immunostaining showed comparable apical CFTR in control and MYO5B KO enteroids. Whole mount CFTR immunostaining of enteroids showed some expression in cells that may be Paneth cells which have been reported to take up primary and secondary antibodies¹⁸. (C) A forskolin swelling assay showed that lifeact and lifeact;MYO5B KO derived enteroids swelled to similar degrees in

response to 5 μ M forskolin. (D) Quantification of percentage increase in enteroid diameter measured in lifeact and lifeact;MYO5B KO enteroids. n=3 mice per group, forskolin swelling assay was repeated in at least three separate experiments. Scale bars=50 μ m.

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Figure 4: MVID small intestine shows loss of apical transporters but maintenance of CFTR. (A) Immunostaining for NHE3 (red) showed apical expression in enterocytes from healthy individuals. In contrast, MVID patient tissue showed diffuse subapical expression of NHE3. The basolateral membrane, as marked by p120 (green), appeared similar in control and MVID tissue. (B) SGLT1 (red) and DPPIV (green) immunofluorescence was observed in the brush border in healthy tissue. In MVID intestinal tissue SGLT1 and DPPIV was largely cytoplasmic. (C) The brush border protein CD10 (green) was present on the apical membrane of enterocytes in healthy tissue. In MVID tissue, CD10 showed diffuse subapical staining. AQP7 (red) immunostaining showed AQP7 present in the brush border of healthy biopsies. In contrast, apical AQP7 was decreased in MVID and instead was located in the

subapical domain. (D) Immunostaining for CFTR (red) showed CFTR present on the apical membrane of healthy individuals. In MVID biopsies CFTR immunofluorescence appeared to be increased and to remain on the apical membrane. n=3 per group. 3 biopsies were analyzed from healthy individuals aged 1 day to 6 weeks, 2 of the 3 individuals received TPN. From MVID patients, the biopsies were from individuals ranging from 6 weeks to one year, all biopsies (3) from MVID patients received TPN, scale bars=50 μ m. The entire biopsy was assessed for representative images, approximately 4 fields of view.

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Figure 5: Adult inducible intestinally targeted MYO5B KO mice show loss of apical transporters without inclusion formation.

(A) P-Ezrin (P-ERM) (red) and DPPIV (green) immunofluorescence staining showed apical expression in tamoxifen treated control mice. Induced VillinCre^{ERT2}MYO5B^{flox/flox} mice showed internalization of DPPIV but rare P-ERM positive inclusions. (B) Proximal small intestine from control and VillinCre^{ERT2}MYO5B^{flox/flox} were immunostained for NHE3 (red) and Lamp2 (green). NHE3 in induced VillinCre^{ERT2}MYO5B^{flox/flox} mice showed significant loss of apical NHE3 and increased subapical staining of NHE3 as well as enlarged Lamp2 lysosomes compared to control mice. (C) SGLT1 (red) was found along the

length of the villi in control mice. Apical SGLT1 was almost completely lost in VillinCre^{ERT2}MYO5B^{flox/flox} mice. n=4-6 mice per group, scale bars=50 μ m.

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Figure 6: Loss of MYO5B in adult mice leads to decreased apical expression of SI, AQP7 and DRA, but preservation of CFTR.
 (A) Immunostaining for p120 (green) and AQP7 (red) in control mice showed proper apical localization of AQP7. Tamoxifen-treated VillinCre^{ERT2}MYO5B^{flox/flox} mice lost apical expression of AQP7. (B) In control mice IAP (red) and SI (green) were both expressed on the apical membrane of enterocytes. In VillinCre^{ERT2}MYO5B^{flox/flox} tamoxifen-treated mice SI was greatly reduced on the apical membrane and most SI appeared to be intracellular. IAP remained at the tips of the villi in VillinCre^{ERT2}MYO5B^{flox/flox} but also showed a subapical staining pattern through much of the villi (C) Immunostaining for DRA

showed loss of apical DRA in tamoxifen-treated VillinCre^{ERT2};MYO5B^{flox/flox} mice. (D) CFTR (red) and DPPIV (green) immunostaining in control mice demonstrated apical expression. In VillinCre^{ERT2};MYO5B^{flox/flox} CFTR was present below the apical membrane in the villi, however CFTR was also present on the apical membrane and appeared to be highly expressed in the crypts. n=4-6 mice per group, scale bars=50 μ m.

Figure 7: Short circuit current (I_{sc}) demonstrated loss of SGLT1 function and maintenance of functional CFTR.

(A and B) Basal electrical resistance (R_t) (A) and I_{sc} (B) measurements in the jejunum and ileum of tamoxifen treated control and tamoxifen induced VillinCre^{ERT2};MYO5B^{flox/flox} mice from jejunum. Total values of R_t or I_{sc} demonstrated no difference between groups. (C and D) Based on inhibitor effects (SGLT1 inhibition by phlorizin and CFTR inhibition by BPO-27) basal I_{sc} in jejunum (C) and ileum (D) resulted in significantly lower SGLT1 activity and higher CFTR activity in VillinCre^{ERT2};MYO5B^{flox/flox} mice than control littermates. Contribution of CFTR to forskolin (10 μM) stimulated I_{sc} in jejunum (E) and ileum (F) of control and VillinCre^{ERT2};MYO5B^{flox/flox} mice. VillinCre^{ERT2};MYO5B^{flox/flox} mice had significantly increased CFTR dependence compared to control littermates. n=4-5 mice per group, data expressed as mean $\pm$ SEM. Significant differences are indicated by * in key, *P<0.05, unpaired t-test.