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Chapter 6

Loss of intestinal Myosin Vb function impairs Claudin-1 trafficking but not barrier function in microvillus inclusion disease

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Abstract

Microvillus inclusion disease (MVID) is one of the most severe congenital diarrheal disorder affecting young children and is characterized by MYO5B mutations, impaired brush border development and chronic diarrhea. The aim of this study was to investigate the link between the loss of Myosin Vb function and intestinal epithelial barrier function. We demonstrate that loss of Myosin Vb expression or function in the epithelial cells of the duodenum and colon of individuals diagnosed with MVID, as well as in intestinal Caco-2 cells, impaired the trafficking of Claudin-1 to tight junctions, but not that of other examined cell-cell junction-associated proteins. Furthermore, MVID enterocytes showed a reduced expression of the apical polarity complex protein Crumbs3, and Claudin-1 was mislocalized in the enterocytes of Crb3 knock-out mice. Notably, the resultant intracellular accumulation of Claudin-1 was not accompanied by an decreased trans-epithelial electrical resistance or enhanced paracellular permeability of Caco-2 cell monolayers to macromolecules. MVID is the first human diseases in which Claudin-1 mislocalization is causally linked to a gene defect. We thus conclude that Myosin Vb controls the trafficking of Claudin-1, probably indirectly through Crumbs3, but an intestinal epithelial barrier defect is not likely to account for the chronic diarrhoea in MVID. Moreover, our results argue against a role for Claudin-1 in maintaining paracellular impermeability in the human intestine, and suggest that the frequently reported correlation between Claudin-1 redistribution and increased intestinal permeability and inflammation does not reflect a causal relationship.

Introduction

Mutations in the MYO5B gene, encoding the recycling endosome associated motor protein Myosin Vb, have recently been associated with Microvillus Inclusion Disease (MVID) 1–3. To date, 41 distinct homozygous
and (compound) heterozygous mutations have been identified in forty MVID patients. MVID patients develop nutrient malabsorption and intractable neonatal secretory diarrhea which is aggravated upon oral food intake. Patients depend on total parenteral nutrition for survival. At the cellular level, MVID is characterized by microvillus atrophy, and the intracellular retention of transmembrane apical brush border proteins including the main brush border anion transporter cystic fibrosis trans-membrane conductance regulator and the NHE-2 and -3 Na+/H+ exchangers and some basolateral proteins. MYO5B mutations have, at least in some patients, been correlated to reduced expression of the Myosin Vb protein in the MVID intestine. Despite the identification of MYO5B as the affected gene, the underlying disease mechanism, in particular the cause of the chronic diarrhea, remains unclear. So far, small bowel/colon transplantation is the only way to end the secretory diarrhea in MVID.

The intestinal epithelial cells form a tight monolayer along the crypt-villus axis of the intestine, and provide a physical barrier between gut lumen contents and the body tissue. Tight junctions form an intercellular and intramembranous barrier at the apex of the lateral (contacting) surfaces in epithelial cell monolayers that limit the paracellular transport of ions and water. In addition, tight junctions are tightly associated with apical-basal polarity and prevent the lateral diffusion of membrane proteins between the apical and basolateral plasma membrane domains. When tight junctions of intestinal epithelial cells are disrupted, uncontrolled paracellular leakage of solutes and water and, hence, diarrhoea occurs. Tight junctions consist of transmembrane and associated cytoplasmic proteins. Transmembrane proteins include occludin, junction-associated membrane protein A, and various members of the Claudin family. TJ-associated cytoplasmic proteins include...
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Zona Occludens (ZO)-1, -2 and -3, Cingulin, and others 12-14. The development and maintenance of tight junctions is linked at the molecular level to that of other cell-cell junctions 18,19, and Epcam 20,21, although in at least some cell types the formation of functional tight junctions is not strictly dependent on adherens junctions 22. Furthermore, the process of tight junction development is entwined with that of apical-basal cell polarity development via apical polarity complexes that include the apical polarity determinant transmembrane protein Crumbs3 23(p3),24,25.

Tight junctions are dynamic structures and some tight junction proteins undergo endocytosis and recycling back to the plasma membrane 26-28. Interestingly, recycling endosomes have been shown to control the intracellular trafficking of key protein components of tight junctions as well as adherens junctions 27,29-32. Whether Myosin Vb is involved in the regulation of trafficking of tight junction proteins is not known. We hypothesized that defects in the trafficking of tight junction-associated proteins caused by loss of Myosin Vb function and, as a result, defects in tight junction function could help explain the chronic diarrhoea in MVID patients in the absence of brush border (an)ion transporters and oral food intake. Therefore, we investigated the involvement of Myosin Vb, as a key regulator of recycling endosome function 33, in the organization and function of tight junctions in intestinal epithelial cells in the context of MVID.

Materials and Methods

Cell culture. Caco-2 cells were cultured in DMEM with non-essential amino acids (Sigma) and 10% fetal calf serum (FCS) as described previously34.

shRNA treatment. The shRNA target sequence, region 2333-2351 of human MYO5B cDNA (target
sequence GGCTGCAGAAGGTGAAATA) was cloned into the shRNA expression vector described previously\(^3\). A target sequence in the Luciferase gene\(^3\) was used as a control. For the production of lentivirus, HEK293T cells were plated onto poly-L-Lysine-coated plates in DMEM supplemented with 10% FCS and 1% sodium pyruvate. Cells were transfected with CMVdR8.1, VSV-G, and pMID-i-2 using CaCl\(_2\) and Hank’s balanced salt solution. Medium was changed after 17 h. After 24 h virus-containing medium was collected, filtered and stored at -80°C. Caco-2 cells were transduced with lentivirus diluted in DMEM with 10% FCS. Expression of GFP in cells was indicative for successful transduction, as also evidenced by reduced Myosin Vb mRNA. In other experiments, Caco-2 cells/transwell filter (Corning, 0.4 micron pore size) were plated and 48 h later transduced with virus in the presence of polybrene (1:1000) for 16 h, and cultured for another 4 days.

**q-RT PCR.** RNA was extracted from cells according to the manufacturer’s instructions (Invisorb Spin Cell RNA mini kit, Westburg). cDNA was synthesized using poly dT primer and SuperscriptII reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. q-RT primers were designed by using Primer 3 (http://frodo.wi.mit.edu) (Table S1). Reactions were run on an ABI7500 (Applied Biosystems). Cycling conditions comprised 15 min polymerase activation at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 30 sec. Experiments were carried out in triplicate.

**Immunofluorescence labelling and microscopy.** Sections of formalin-fixed samples of human MVID and control intestines \(^3,3^4,3^5\) and of mouse \(Crb3^{+/+}\) and \(Crb3^{-/-}\) intestine (generous gift of B. Margolis of the University of Michigan Medical School (Ann Arbor, Michigan, USA) \(^3^6\)) were de-paraffinized, rehydrated, washed with PBS and subjected to epitope retrieval
with citric acid pH 6.0 in a microwave for 20 min. Non-specific binding sites were blocked with 5% FCS in PBS overnight. Primary antibodies were diluted in blocking solution with 0.05% Tween-20 at 37°C for 2h followed by incubation with AlexaFluor-488- or -543-conjugated secondary antibodies. Nuclei were stained with DRAQ5, and slides were mounted with DAKO mounting medium. Cultured cells were fixed with 3.7% PFA at room temperature for 20 min. Cells were incubated with 0.1 M glycine in PBS for 20 min, permeabilized with 0.2% TritonX-100 for 10 min and blocked with 3% FCS in PBS for 1 h. 30 and primary antibodies were added to the basolateral and apical side of the filter and cells were incubated at 37°C for 2 h. Cells were incubated with Cy5- or AlexaFluor-543-conjugated secondary antibodies and DRAQ5/DAPI at 37°C for 30 min. Filters were mounted in DAKO mounting medium. Specimens were examined and images were taken with a TCS SP8 CLSM (Leica). Claudin-1 (1:100 Invitrogen), Claudin-7 (1:100 Sigma), Cingulin (1:100 Novus Biologicals), ZO-1 (Invitrogen 1:200), Beta-catenin (1:100 Transduction lab). The antibody raised against the Crumbs3 protein was a generous gift of B. Margolis (University of Michigan Medical School, Ann Arbor, Michigan, USA).

**Calcium switch assay.** CaCo2 cells were plated on transwell filters and transduced with lentivirus on day 2 post seeding. The cells were allowed to grow for six days in total. The cells were washed with phosphate buffered saline (PBS) without calcium and magnesium three times before adding 2.5mM EDTA in PBS. The cells were incubated in EDTA for 8 min, washed twice with PBS and later fixed with 4% PFA in PBS at various time points.

**Electron microscopy.** For transmission electron microscopy, freshly obtained biopsy samples were processed for electron microscopy as described in 3.
For scanning electron microscopy, cells were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate overnight at 4°C and postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer at room temperature for 1 h. After dehydration samples were critical-point dried from carbon dioxide and sputter coated with 5 nm palladium/gold, and imaged at 2KV using a JEOL-JSM6301F scanning electron microscope.

Paracellular leakage assay. CaCo2 cells were plated on transwell filter support, transduced with the virus and allowed to grow to form a monolayer as mentioned above. On day 6, all transwell filer supports were washed with PBS twice. Add 1.5ml PBS (or 2.5 mM EDTA in PBS as control) on the basal side and 250 µg/ml FD4 or Lucifer Yellow in PBS (or 250 µg/ml PBS + 2.5mM EDTA as control). The cells were incubated with FD4 or Lucifer Yellow for 20min and the basal medium was collected in a tube and replaced with fresh PBS (or PBS with EDTA). Subsequent samples were collected every 20 min for 120 min. As a blank, an empty transwell filter was used to calculate the basal rate of flux between the apical and basal chambers. The sequentially collected basal solution were read on fluorometer with a standard curve of fluorescence intensity to FD4 or Lucifer Yellow concentration. The apparent permeability coefficient (P_{app}) was calculated using the following formula: \( P_{app}(\text{cm/s}) = \frac{dQ/dt \times 1}{A \times Co} \), where \( dQ/dt \) (µg/s) is the rate of appearance of FD4 or Lucifer Yellow on the receiver side from 20 to 120 min after application of the probe. \( Co \) (µg ml\(^{-1}\)) is the initial probe concentration on the donor side, and \( A \) (cm\(^2\)) is the effective surface area of the insert.

Measurement of trans-epithelial electrical resistance. Trans-epithelial electrical resistance (TEER) across monolayers of Caco-2 cells cultured on Transwell filter
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supports was measured with the EVOM2, epithelial Volthometer for TEER (Millipore), following the manufacturer’s instructions.

Results and Discussion

Loss of Myosin Vb function causes the redistribution of Claudin-1 but not of Claudin-7, JAM-A, ZO-1 and Cingulin in MVID enterocytes

We first determined the subcellular distribution of tight junction proteins (i.e., the sealing Claudin-1, the channel-forming Claudin-7, Cingulin, and ZO-1) in two patients diagnosed with MVID by confocal laser scanning microscopy. Patient 1 carried a homozygous c.4366C>T mutation in the *MYO5B* gene, and patient 2 carried compound heterozygous c.1540T>C and IVS33+3753G>C mutations in the *MYO5B* gene. Both patients presented persistent secretory diarrhoea from birth on and this disappeared after receiving a bowel transplantation. Immunolabeling was performed on both duodenal and colonic material of both MVID patients. Claudin-1 localized at the apical domain of epithelial cells in control duodenum (Figure 1A) and colon (supplementary Figure 1) and some intracellular staining was observed. In contrast, in epithelial cells in MVID duodenum (Figure 1A) and colon (supplementary Figure 1) Claudin-1 was absent from the apical domain and accumulated intracellular and close to the nucleus. A difference in the intracellular distribution of Claudin-1 was noted between the two patients, as Claudin-1 in MVID[c.1540T>C and IVS33+3753G>C] displayed a more condensed supranuclear appearance when compared to MVID[c.4255C>T]. In contrast to the striking redistribution of Claudin-1 in MVID enterocytes, Claudin-7, Claudin-3, Cingulin,
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**Figure 1. Loss of Myosin Vb causes redistribution of Claudin-1**

A) Sub-cellular distribution of Claudin-1, Claudin-7, Cingulin and ZO1 in Control and enterocytes of two MVID patient with distinct MYO5B mutations. Solid white arrows show TJ or lateral staining of these proteins. Yellow arrows show intracellular mislocalization of these proteins. Asterisk indicates the apical lumen in the picture. The dotted line indicates the apical surface in the tissue. Nuclei are stained with DAPI and shown in blue.

B) Sub-cellular distribution of Claudin-7, Cingulin, Zo1 and Claudin-1 in Caco2 cells is depicted in the x-z axis as side views in red. The cells transduced with lentiviral virus express GFP shown in green while non-transduced cells are controls and do not show GFP. White arrows indicate TJ or lateral junction and normal distribution. Yellow arrows indicate intracellular mis-localization of these proteins due to the knock down. Nuclei are stained blue.
ZO-1 and JAM-A predominantly localized at the lateral plasma membrane and/or apical aspect of the lateral plasma membrane domains in duodenal biopsies (Figure 1A and supplementary Figure 1) and colon biopsies (supplementary Figure 2) from both control and MVID patients, consistent with their distribution profiles reported in other studies. It was noted that a larger fraction of Claudin-7 appeared in vesicular structures in the cytoplasm of control duodenum when compared to both MVID samples, while in MVID[c.1540T>C and IVS33+3753G>C] duodenum Claudin-7 shifted more to the apical side of the lateral plasma membrane (Figure 1A). Furthermore, some intracellular Claudin-3-positive dots were observed in the enterocytes of one MVID individual, but these were not observed in another MVID individual (supplementary Figure 1, arrows).

To establish a causal relationship between Myosin Vb function and Claudin-1 distribution we employed human Caco-2 cells, which can be cultured as differentiated and polarized cells such that their phenotype resembles the enterocytes of the small intestine. The knockdown of Myosin Vb in Caco-2 cells using lentiviral transduction of shRNA against Myosin Vb, which has been shown to reproduce phenotypic hallmarks of MVID enterocytes, replicated our observations in the MVID intestine (Figure 1B). Thus, no overt differences were observed in the subcellular distribution of Claudin-7, Cingulin and ZO-1 in GFP-negative (uninfected/control) and GFP-positive (infected) Caco-2 cells. (Figure 1B, side view/x-z images, white arrows). In contrast, Claudin-1 was clearly redistributed from the lateral surface in control cells to the intracellular, supranuclear region in Myosin Vb knockdown cells (Figure 1B).

Together, these data demonstrate that loss of Myosin Vb function can be causally linked to the redistribution of Claudin-1 in MVID enterocytes.
Loss of Myosin Vb function impairs trafficking of Claudin-1 to tight junctions

The intracellular accumulation of Claudin-1 in Myosin Vb knockdown Caco-2 cells and MVID enterocytes suggested that Myosin Vb regulated the trafficking of Claudin-1 to the tight junctions. In order to investigate this further we used an experimental approach in which in a confluent monolayer of cells, the endocytosis of tight junction proteins is first triggered by treatment with the Ca\(^{2+}\)-chelator ethylenediaminetetraacetic acid (EDTA) \(^{41}\). Then, after subsequent washout of the EDTA the trafficking of these endocytosed proteins to the newly formed tight junctions can be followed as a function of time. Treatment of control and Myosin Vb knockdown Caco-2 cell monolayers with EDTA resulted in the redistribution of ZO-1 and of Claudin-1 from the apex of the lateral plasma membrane to vesicular structures in the apical cytosol (Figure 2). In EDTA-treated Myosin Vb knockdown cells, intracellular Claudin-1 displayed a more condensed accumulation in the apical cytoplasm (Figure 2). Residual plasma membrane associated Claudin-1 disappeared (Figure 2), suggesting that Myosin Vb was not required for the EDTA-induced endocytosis of Claudin-1. 120 min after EDTA washout, ZO-1 had reappeared at the apex of the lateral plasma membrane, showing a distribution pattern that was indistinguishable from the situation before EDTA treatment, in both control and Myosin Vb knockdown cells (Figure 2). At this time, a fraction of Claudin-1 had also reappeared at the lateral surface of control cells although a significant fraction of intracellular Claudin-1 was still observed (Figure 2). In Myosin Vb knockdown cells, in contrast, Claudin-1 was predominantly intracellular and no Claudin-1 had appeared at the cell surface.
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Figure 2. Myosin Vb impairs trafficking of Claudin-1. A) Localization of Claudin-1 (red) and Zo1 (blue) in confluent monolayer of Caco2 cells which are un-infected lentivirus for Myosin Vb knock down. B) Localization Claudin-1 in cells transduced with lentiviral for Myosin Vb knock down and expressing GFP (green). C-D) Cells treated with calcium chelator EDTA and then allowed to recover for time points 5 min to 240 min post EDTA treatment and stained for Claudin-1 (red). Non-green cells are control cells without viral transduction while green cells are lentivirus transduced cells with GFP (green). Yellow arrows indicate the enlarged intracellular accumulation while white arrow indicate normal distribution of Claudin-1 and ZO-1. White arrows indicate normal localization of Claudin-1 at the cell periphery. Yellow arrows indicate abnormal intracellular accumulation of Claudin-1.
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(Figure 2). After 240 min in control cells, Claudin-1 localized at the lateral surface indistinguishable from the situation before EDTA treatment, whereas in Myosin Vb knockdown cells Claudin-1 remained intracellular (Figure 2). Together these data demonstrate that Myosin Vb regulates the trafficking of Claudin-1 to the tight junctions, and that Claudin-1 at tight junctions is dispensable for the recruitment of ZO-1 and Cingulin at tight junctions in human enterocytes.

Mislocalization of Claudin-1 in MVID enterocytes is correlated with reduced expression of the apical polarity protein Crumbs3

The mammalian apical polarity protein Crumbs3, encoded by the CRB3 gene, has been demonstrated to play a pivotal role in the formation to tight junctions and of the apical plasma membrane. Moreover, Crb3 knock-out mice show apical microvillus atrophy and villus fusion, both of which are also observed in the MVID small intestine. Examination of Crumbs3 expression and localization showed a predominant apical distribution of the Crumbs3 protein in control enterocytes (Figure 3A, arrow), consistent with the endogenous distribution of Crumbs3 in mouse enterocytes and of ectopically expressed Crumbs3::GFP in the intestine of the nematode Caenorhabditis elegans. In contrast, Crumbs3 expression was reduced in the enterocytes of two unrelated MVID patients that carry distinct MYO5B mutations, and clearly no predominant Crumbs3 distribution was observed at the apical surface of the enterocytes (Figure 3B, arrow).

In order to investigate whether loss of Crumbs3 expression at the apical domain of the enterocytes could be responsible for the loss of Claudin-1 from tight junctions, we next examined the expression and distribution of Claudin-1 in the enterocytes of wild-
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Claudin-1 was predominantly localized at the apical domain of the enterocytes of control mice (Figure 3C, arrows). In contrast, this predominant apical localization was absent and Claudin-1 was mislocalized to intracellular puncta in the enterocytes of Crb3 knock-out mice (Figure 3D, arrows).

Together these data demonstrate that Crumbs expression is severely reduced in MYO5B mutation-carrying MVID enterocytes and that loss of Crumbs3 in enterocytes results in the mislocalization of Claudin-1.

Figure 3. Claudin-1 mislocalization in MVID enterocytes is correlated with loss of Crumbs3 expression. A) Crumbs3 expression and distribution in human duodenum enterocytes of control and MVID individuals. B) Claudin-1 expression and distribution in mouse duodenum enterocytes of wild type control (Crb3+/+) and Crb3 knock-out (Crb3−/−) mice. Bars: 10 μm.
Loss of Myosin Vb function does not affect the lateral distribution of beta-catenin and EpCam or the monolayer organization of intestinal epithelial cells

The establishment and maintenance of tight junctions has been shown to be linked to that of adherens junctions \(^ {18-21}\). Nonetheless, adherens junctions and the epithelial monolayer arrangement appear normal in the small intestine epithelial cells of Crb3 knock-out mice \(^ {36}\). We found that the key adherens junction-associated protein beta-catenin displayed a normal distribution along the lateral plasma membrane in MVID enterocytes (Figure 4A) and/or shRNA-treated Caco-2 cells (data not shown). Also the lateral plasma membrane localization of the cell-cell adhesion protein EpCam \(^ {44}\), mutants of which in congenital tufting enteropathy \(^ {45}\) fail to reach the lateral plasma membrane \(^ {46}\) and give rise to chronic diarrhoea \(^ {47}\), was maintained in shRNA-treated Caco-2 cells (Supplementary Figure 3; the EpCam antibody did not work properly on our paraffin coupes). It was noted that in control Caco-2 cells EpCam showed an additional localization at the apical surface, and this apical (but not the lateral) fraction of EpCam was depleted in Myosin Vb knockdown cells (supplementary Figure 3).

In agreement with the localization of most cell-cell adhesion proteins at the lateral surfaces, the intestinal epithelial cell monolayer arrangement in MVID, despite the fusion of villi, appeared normal by haematoxylin and eosin staining (Figure 4B), and also Caco-2 cells that were treated with shRNA against Myosin Vb displayed a normal monolayer arrangement (c.f., Figure 1B and 2). Moreover, cell-cell junctions in MVID intestinal epithelial cells displayed a normal morphology when evaluated by transmission electron microscopy (Figure 4C). Indeed, typical electron-dense desmosomes (blue arrows), and the for tight junctions characteristic
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Figure 4. Loss of Myosin Vb does not inhibit cell-cell adhesion, monolayer organization and permeability. A) Beta-catenin (green) localization in control and MVID patient enterocytes. Nuclei stained with DAPI (blue). Asterisk indicates the apical lumen and dotted line is the apical plasma membrane. B) MVID patient intestinal tissue
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stained with hematoxylin indicates the organization of the tissue and enterocyte monolayer. C) Transmission electron micrographs indicate microvillus inclusions (MI) in MVID enterocyte. Black dotted box is blown up on right. Black arrows indicate the electron dense tight junctions. Blue arrows indicate cell-cell adhesions and red arrows indicate desmosome junctions. D) Cartoon depicting the arrangement for the flux assay to establish paracellular permeability coefficient in confluent Caco2 cells. Green is FITC-Dextran 4kda, red show tight junctions. Cells are grown on porous polycarbonate filter. Bottom chamber is the receiving chamber for diffusing FD4 gradient. Apparent coefficient is calculated by the given formula. E) Relative permeability coefficient plotted for control cells, knock down cells, EDTA treated cells and empty filter. F) Relative permeability coefficient plotted for control cells, knock down cells, EDTA treated cells and empty filter. G) Trans-epithelial electrical resistance (TEER) in control and knock-down cells. Student’s t-test was performed between two samples. p<0.05 was considered significant. The graph was plotted as an inverted plot with Y-axis on log scale to the base 10.

electron-dense areas of closely opposing plasma membranes between cells at the apex of the lateral membrane (double black arrow), were easily distinguished (Figure 4C; red arrows indicate areas with visible space between the opposing lateral plasma membranes).

Taken together, these data demonstrate that of the cell-cell junction proteins examined, loss of Myosin Vb expression or function causes the redistribution of only Claudin-1 in intestinal epithelial cells, without causing overt changes in cell-cell adhesion junction morphology or cell monolayer arrangement.

**Loss of Myosin Vb function does not increase paracellular permeability to macromolecules**

Because loss of Myosin Vb function in vivo and in vitro led to the redistribution of Claudin-1, we next addressed the consequences of Myosin Vb loss of function for the epithelial barrier function. For this, control and Myosin Vb knockdown Caco-2 cells were cultured as confluent monolayers on semi-permeable filters. Fluorescein isothiocyanate -labelled 4 kDa dextran (FD4) was then added to the apical side of the
monolayer and the amount of FD4 that leaked to the basolateral compartment was calculated as a function of time (Figure 4D). As a positive control, cells were treated with EDTA to disrupt the tight junctions (c.f., Figure 2). Whereas treatment with EDTA caused a significant increase in paracellular leakage of the FD4 (Figure 4E), no significant difference in FD4 leakage was observed between control and Myosin Vb knockdown cells (Figure 4E). Similar results were obtained when, instead of FD4, the significantly smaller dye Lucifer yellow (453 Da), was used (Figure 4F). Furthermore, Measurement of the trans-epithelial electrical resistance revealed no differences between control cells and Myosin Vb knock-down cells (Figure 4G). These data demonstrated that in Caco-2 cells, the reduced expression of Myosin Vb and resultant redistribution of Claudin-1 did not lead to an impaired tight junction and barrier integrity.

Our data demonstrate that loss of Myosin-Vb expression/function causes impaired trafficking and intracellular retention of Claudin-1, but is not paralleled by an increase in the paracellular leakage of macromolecules. The chronic diarrhoea in MVID patients is therefore not likely due to a substantial intestinal epithelial barrier defect by means of a macromolecular leak flux mechanism, but rather due to perturbed salt/water transport across the cell surface. In support, MVID is not associated with intestinal inflammation, which is expected if the intestinal epithelial barrier function was compromised. In light of the reported mislocalization of TJ proteins including, Claudin-1, that accompanies intestinal inflammation and other pathophysiological conditions of intestines, our data indicate there is no causative role of Claudin-1 mislocalization in increased intestinal permeability. Finally, the presence of functional TJ in MVID enterocytes, in agreement with the absence of a lateral mixing of proteins between apical and basolateral surfaces of
MVID enterocytes, indicates that MVID is not caused by a defect in the core epithelial polarity program.

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**Supplementary figure 1. Loss of Myosin Vb does not cause redistribution of Claudin-3 or JAM-A.** Subcellular distribution of Claudin-3, and JAM-A in biopsies of duodenum in MVID patient samples with distinct mutations and a control sample. Solid white arrows show intracellular accumulation of Claudin-3 in one of the MVID duodenum stainings. Nuclei are stained with DAPI (blue).

**Supplementary figure 3. Loss of Myosin Vb does not cause a redistribution of Epcam.** Subcellular distribution of Epcam (Red) in control and Myosin Vb-knockdown Caco-2 cells. (scalebar 10 uM)
Supplementary figure 2. Loss of Myosin Vb causes redistribution of Claudin-1. Subcellular distribution of Claudin-1, Claudin-7, Cingulin and Zo1 in biopsies of colon in MVID patient samples with distinct mutations and a control sample. Solid white arrows show intracellular accumulation of Claudin-1 in MVID colon staining. Asterisk indicates the apical lumen in the picture. The dotted line indicates the apical surface in the tissue. Nuclei are stained with DAPI (blue).