CHAPTER 3

Divergent effects of *MYO5B* mutations on apical brush border and apical recycling endosome organization in kidney and intestinal epithelial cells of Microvillus Inclusion Disease patients presenting with renal Fanconi syndrome

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Submitted
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ABSTRACT

Objectives: Microvillus inclusion disease (MVID) is a rare congenital enteropathy characterized by brush border atrophy and reduced expression of enzymes at the apical plasma membrane of enterocytes. MVID is caused by mutations in the MYO5B gene. MYO5B encodes the molecular motor protein myosin Vb which is expressed in all epithelial tissues. We report two MVID patients that also developed renal Fanconi syndrome, which has been correlated to apical plasma membrane defects in the proximal tubular epithelial cells of the kidney. The aim of this study was to determine whether MYO5B mutations in these patients correlate with similar apical plasma membrane defects in renal tubular epithelial cells as observed in the intestine.

Methods: Biopsies from kidney, duodenum, ileum, jejunum and colon of two MVID patients carrying MYO5B mutations and of age-matched controls were fixed in paraffin and analyzed with immunohistochemistry and transmission electron microscopy.

Results: MYO5B mutations in MVID patients with renal Fanconi syndrome do not correlate with aberrant apical plasma membrane morphology or altered apical recycling endosome organization in renal tubular epithelial cells. Structural defects of the brush border and apical recycling endosome organization is observed in enterocytes of all segments of the small intestine and colon.

Conclusions: MYO5B mutations have divergent effects on the apical membrane system in kidney and intestinal epithelial cells. Epithelial defects presented in MVID are therefore triggered by intestine-specific factors, the identification of which may provide new targets and open avenues for the development of alternative therapeutic strategies to combat this devastating disease.
INTRODUCTION

Microvillus Inclusion Disease (MVID, OMIM 251850) is a congenital intestinal malabsorption disorder that represents with intractable secretory diarrhea within few days (early onset) or weeks (late onset) of life, leading to total parenteral nutrition-dependency throughout life (Cutz et al., 1989, Davidson et al, 1978). MVID is characterized by villous atrophy, intracellular accumulation of the brush border-protein metallo-endopeptidase CD10 and periodic acid-Shiff (PAS)-positive material in the subapical cytoplasm of intestinal absorptive cells (Groisman et al., 2002; Ruemmele et al., 2004). Other brush border proteins including sucrase-isomaltase, alkaline phosphatase, sodium-proton exchanger protein 2 and -3 (NHE-2, NHE-3), cGMP-dependent protein kinase, cystic fibrosis trans-membrane conductance regulator (CFTR), and the sodium-glucose transporter 1 (SGLT1) have been reported to be present at reduced levels at the apical brush border membrane and to accumulate in the apical cytoplasm (Phillips et al., 1992). At the ultrastructural level a variable degree of microvillus atrophy, accumulation of secretory granules and the presence of microvillus inclusions are typically observed. It has been suggested that the diminished apical surface area, diminished surface expression of apical plasma membrane transporter systems and consequent abnormally low uptake of nutrient (Ameen and Salas, 2000) account for most of the clinical symptoms of MVID (Bijlsma et al., 2000).

Recently, mutations in the \textit{MYO5B} gene coding for myosin Vb were reported in all but one MVID patients (Müller et al., 2008, Erickson et al., 2008, Ruemmele et al., 2010, Szperl et al., 2011). \textit{MYO5B} mutations were shown to correlate with a defective myosin Vb protein expression in MVID enterocytes (Szperl et al., 2011). Myosin Vb is an actin-based motor protein that binds to specific small GTPase rab proteins on recycling endosomes and transports these along actin filaments to the apical plasma membrane. Indeed, the knockdown of myosin Vb or the expression of a dominant-negative myosin Vb motorless tail domain in polarized Madin-Darby canine kidney
(MDCK) cells (Lapi erre and Golden ring, 2005), human epithelial colorectal adenocarcinoma Caco-2 cells (Ruemmele et al., 2010), human sub-bronchial gland Calu-3 cells (Swiatecka-Urban et al., 2007) and rat hepatoma/human fibroblast hybrid WIF-B9 cells (Wakabayashi et al., 2005) inhibit protein recycling to the apical plasma membrane and/or result in the accumulation of resident apical plasma membrane proteins in compartments in the subapical cytosol. It is therefore plausible that impaired apical brush border membrane development and maintenance in MVID enterocytes is caused by defects in the intracellular trafficking of resident apical plasma membrane proteins to the cell surface. This is in agreement with the reported defects in apical recycling endosome organization in MVID enterocytes (Szperl et al., 2011). Myosin Vb has been proposed to play a key role in apical brush border development (Wakabayashi et al., 2005; Müller et al., 2008; reviewed in Golachowska et al., 2010). Myosin Vb mRNA and protein is ubiquitously expressed in virtually all epithelial tissues. It is therefore conceivable that the pronounced structural and functional aberration as observed in the enterocytes of MVID patients also occur in other polarized epithelial cells. In support of this, the spectrum of symptoms in this syndrome has been suggested not to be exclusive for the small intestine, and microvillus inclusions in the stomach and colon, basolateral plasma membrane inclusions in gallbladder epithelium and poorly defined microvillus-bearing vesicles in renal tubular epithelial cells have been reported in some MVID patients (Cutz et al., 1997). Remarkably however, multi-organ clinical symptoms are not typically reported in MVID patients. In this study, we report two MYO5B mutation-carrying MVID patients that developed (intermittent) proximal tubular renal dysfunction characterized by aminoaciduria, reduced tubular reabsorption of phosphate, glucosuria, tubular acidosis and hypophosphatemic rickets, all characteristics of renal Fanconi syndrome.

Renal Fanconi syndrome can be caused by inborn errors of metabolism, secondary to primary Mendelian diseases such as cystinosis, or acquired through exposure to toxic agents. Renal Fanconi syndrome affects the apical reabsorption of various
substances and is considered to be a general defect in the function of the proximal tubules. Proximal tubular cells in the kidney contain a microvillus membrane, and loss of apical microvilli has been reported in a child with renal Fanconi syndrome associated with lysinuric protein intolerance (Benninga et al., 2007). The aim of this study was therefore to determine whether \textit{MYO5B} mutations in these patients correlate with similar apical plasma membrane defects in renal tubular epithelial cells as observed in the intestine.

**MATERIALS AND METHODS**

**Description of patients and clinical history**

Biopsy material from two patients diagnosed with MVID and age-matched non-MVID patients were included in this study.

**Patient 1** is a Dutch-Moroccan boy from consanguineous parents who was born at term and admitted at the hospital three days after birth because of excessive diarrhea and dehydration. Introduction of oral feeding failed due to progression of diarrhea and total parenteral nutrition support was started. Jejunum biopsy showed almost total villous atrophy and microvillus inclusions, confirming the diagnosis of MVID. \textit{MYO5B} gene sequencing revealed a homozygous stop codon in exon 33 (c.4366C>T, p.Gln1456X) (Szperl et al., 2011).

**Patient 2** is a Caucasian boy from non-consanguineous parents who received breast-feeding from birth on. At the age of two months he was admitted because of prolonged icterus. During the course of admittance he developed diarrhea, which progressed in severity. Enteral feeding failed as a result of progression of diarrhea upon introduction and total parenteral nutrition was started. Duodenum and colon biopsies showed villous atrophy and microvillus inclusions, confirming the diagnosis of MVID. The sequencing of the \textit{MYO5B} gene revealed compound heterozygous mutations including a de novo non-conservative substitution mutation in exon 12 (c.1540T>C, p.Cys514Arg) and a maternally derived mutation in intron 33 (c.4460-1G>C) (Szperl et al., 2011).
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Biopsy material

Kidney tissue was collected after presentation of renal Fanconi syndrome using a MAGNUM® biopsy gun with a 16G needle (Patient 1 at the age of 1 year and 11 months and from Patient 2 at the age of 2 years and 3 months). Intestine samples were taken after removal of the diseased organ during the transplantation procedure (Patient 1: age of 5 years and 6 months, Patient 2: age of 4 years and nine months) from duodenum, jejunum, jejunum/ileum, ileum and colon. Control material used this study is from age-matched non-MVID subjects. Biopsies were processed for electron and light microscopy using standard methods (Szperl et al., 2011).

Immunohistochemistry

Kidney and intestinal biopsies of MVID patients and age-matched controls were fixed in formalin, embedded in paraffin and cut in 3μm thick sections. Slides were dried overnight in 60°C and deparaffinised during xylol/ethanol washing steps. Epitopes were retrieved by protease K digestion (Sigma) or with citric acid pH 6.0 (autoclaved; 5 min., 120°C). Endogenous peroxidase was deactivated with 3.5% H2O2. Following blocking of nonspecific binding sites in 4% normal goat serum, slides were incubated with primary antibodies. These included monoclonal mouse antibodies against CD10 (clone 56C6, Monosan), polyclonal antibodies raised against a synthetic peptide derived from the C-terminal hyper-variable region of the human Rab11a sequence (Zymed Laboratories Inc), and polyclonal antibodies raised against a synthetic peptide corresponding to C- or N-terminal residues (amino acids 1093-1112 or 23-41, respectively) of human myosin Vb (Antagene Inc; 60B923). Samples were then washed and incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies (GE Healthcare). Diaminobenzidine was used as a substrate for peroxidase and the nuclei were stained with hematoxyline. Slides were dehydrated with ethanol, dried and mounted. Immunohistochemical labeling for CD10 was performed in an automated Ventana BenchMark Ultra system according to standard manufacturer protocol and PAS staining was routinely performed (Sakura
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Tissue-Tec) (Szperl et al., 2011).

Electron microscopy
Freshly obtained biopsy samples were processes for electron microscopy as described in Szperl et al. (2011). Briefly, samples were fixed in 2% glutaraldehyde in phosphate buffer, rinsed in 6.8% sucrose in phosphate buffer, and post-fixed in a solution of 1% osmium tetroxide in 0.1M sodium cacodylate buffer containing 11.2% potassium ferrocyanide. Samples were dehydrated with ethanol and processed according to standard procedures upon embedding in Epon. Coupes were contrasted with uranyl acetate and lead citrate.

Laboratory analyses
β2-microglobulin, creatinin and carnitin were measured using standard techniques. The glomerular filtration rate (GFR) was calculated from 24-hour urine samples and measured with iothalamate together with the renal plasma flow (ERPF) by para-aminohippurate and, in Patient 2, also by inulin clearance. Amino acid profiles in plasma and urine were measured by cation exchange column chromatography coupled to post-column ninhydrine derivatization and spectrophotometric detection on a Biochrome 20 amino acid analyser (Biochrome, Cambridge, UK) according to manufacturer’s protocols. Amino acid fractional resorbtions were calculated and normalized to creatinine.

RESULTS

Renal Fanconi syndrome in two patients with Microvillus Inclusion Disease
Two Dutch patients diagnosed with Microvillus Inclusion Disease and carrying MYO5B mutations (see Materials and Methods) have developed renal Fanconi syndrome.

Patient 1 was screened for renal tubular dysfunction during evaluation for
intestinal transplantation. Analysis of fractional excretion of sodium, tubular reabsorption of phosphate, and β2-microglobulin clearance indicated renal Fanconi syndrome (Fig. 1, Table 1). No disturbance in the glomerular function was measured (Table 1). Concomitant with a reduction of sodium, phosphate and bicarbonate in the total parenteral nutrition (TPN), tubular phosphate reabsorption (TRP) gradually returned to normal values (61% and 85% at 2.3 and 2.5 yrs, respectively) as did and fractional sodium excretion (FENa) (0.4% and 0.3% at 2.3 and 2.5 yrs, respectively). Also recovery from generalized aminoaciduria was observed. Following a year without symptoms, renal Fanconi syndrome symptoms temporarily appeared again. At the age of five, Patient 1 received a combined small intestine and colon transplant, and renal Fanconi syndrome spontaneously resolved after enteral feeding was fully restored.

Patient 2 was hospitalized for the evaluation of growth failure during which excessive renal losses of phosphate were observed. Further examination showed generalized aminoaciduria (Fig. 1, Table 1) and severe rickets (Fig. 1), which are characteristics of renal Fanconi syndrome. No disturbances in the glomerular function were measured (Table 1). Patient 2 received a multi-organ (small intestine, large intestine, pancreas and liver) transplant at the age of five, and the renal Fanconi syndrome spontaneously resolved after enteral feeding was fully restored.

The apical brush border membrane and recycling endosome organization are unaffected in kidney epithelial cells of MVID patients presenting with renal proximal tubular dysfunction (Fanconi syndrome)

When evaluating overall renal tubular histology, dilated tubules were observed in Patient 2 when compared to those of age-matched non-MVID control patients and Patient 1 (Fig. 2A). PAS staining of kidney biopsies from age-matched non-MVID control patients showed a clear staining of the apical brush border membrane (Fig. 2A, black arrows) which appeared fuzzy because of the microvilli-rich and therefore extensively folded brush border membrane. In addition a sharp outline of the tubular
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Figure 1. A, B) Fractional resorption of amino acids in MVID Patient 1 (A) and Patient 2 (B) (dark grey) and reference values (light gray). Three groups of amino acids were distinguished on the basis of their fractional resorption. A first group of amino acids for which the resorption was negative, a second group with the values intermediate, and a third group with values almost normal. For both patients the amino acid groups were very similar. (For Patient 2 the calculated fractional resorption of C was -417%). (C) X-ray shows typical signs of rickets (Patient 2).

structure reflecting the basement membrane is observed (Fig. 2A, arrowheads). A very similar staining pattern is observed in the renal tubules of both MVID patients (P1 and P2). No intracellular PAS staining is detected in the proximal tubular epithelial cells of either MVID patient.

At the ultrastructural level, proximal tubular epithelial cells of both MVID patients revealed a well-developed apical brush border (Fig. 2B1 and 2B2, black arrows). No microvillus atrophy or presence of microvillus inclusions was observed. It should be noted that many electron dense deposits of unknown origin (Fig. 2B3; arrowhead)
were observed in Patient 1, a feature also frequently observed in MVID enterocytes.

Also, the mitochondria in the tubular cells of Patient 1 appeared swollen (Fig. 2B1; white arrow). These data demonstrate that the apical brush border is not visibly affected in MVID patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>GFR Creatinine Iothalamate (ml/min/1.73m²) (N:80-120)</th>
<th>FENa (%) (N&lt;2)</th>
<th>TRP (%) (N&gt;80)</th>
<th>β2-microglobulin clearance (%) (N&lt;1)</th>
</tr>
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<tr>
<td>1.9 yrs</td>
<td>40</td>
<td>nd</td>
<td>4.2</td>
<td>34</td>
</tr>
<tr>
<td>2.1 yrs</td>
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</tr>
<tr>
<td>2.25 yrs</td>
<td>52</td>
<td>190</td>
<td>3.17</td>
<td>0</td>
</tr>
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Table 1. Glomerular filtration rate (GFR), fractional excretion of sodium (FENa), tubular reabsorption of phosphate (TRP) and β2-microglobulin clearance of two MVID patients at two different time points during the course of their disease.

We next analyzed the distribution of the apical recycling endosomal system. The apical recycling endosome marker Rab11a localizes predominantly in the subapical area of the cells of control samples (Fig. 2C, ctrl, black arrow), similar as observed in the epithelial cells of the control intestine (c.f. Szperl et al., 2011). However, whereas in the enterocytes of MVID patients Rab11a labeling is dispersed or can not be detected (Szperl et al., 2011), Rab11a displays a normal subapical localization in the proximal tubular epithelial cells of both MVID patients, indistinguishable from control patients (Fig. 2C, arrows). These data reveal that in contrast to the strikingly impaired organization of the apical brush border and apical recycling endosomes distribution in intestinal epithelial cells, no obvious abnormalities are seen in the tubular epithelial cells of the kidney.
The apical brush border membrane and recycling endosome organization in intestinal epithelial cells is affected along the entire horizontal axis of the MVID intestine

The absence of morphological apical plasma membrane defects in proximal tubular epithelial cells in MVID patients may suggest that the apical plasma membrane defects as observed in the enterocytes of these patients are due to intestine-specific factors. In order to obtain further support for this we examined the intestinal epithelial cells from all segments of the MVID intestine. For this, samples from explanted MVID intestines were taken from the duodenum, jejunum, ileum and colon of both patients, fixed in paraffin and prepared for PAS, CD10, myosin Vb and Rab11a labeling. In the control intestine, a typically abundant PAS staining (Fig. 3) and CD10 (Fig. 4) staining was observed at the intestinal brush border membrane. Significant CD10 staining was also observed in the supranuclear area, possibly reflecting newly synthesized CD10 passing through the Golgi apparatus (Fig. 4).

Myosin Vb (Fig. 5) and Rab11a (Fig. 6) displayed a typical subapical staining pattern, consistent with the location of apical recycling endosomes in the control samples. In striking contrast, in both MVID patients the PAS-positive material accumulated in the apical cytoplasm in enterocytes and this was observed in all segments of the small intestine and colon (Fig. 3). Of interest, residual PAS staining at the brush-border was observed in enterocytes of Patient 2. In addition, MVID patients show no (Patient 1; early onset) or severely reduced (Patient 2; late onset) labeling of CD10 at the brush border and extensive intracellular subapical deposits (Fig. 4) were observed. These intracellular deposits likely reflect microvillus inclusions observed with electron microscopy. The intensity of CD10 labeling decreases in the most distal part of the small intestine and is absent in colon, in both MVID patients and in the control (Fig. 4). The stainings for myosin Vb (Fig. 5) and Rab11a (Fig. 6) was below detection level in both MVID patients in all parts of intestine and colon tested. These data reveal that defects in apical plasma membrane apical recycling endosome organization appear along the entire horizontal axis of the MVID intestine.
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Figure 2. Immunohistological and ultrastructural characterization of renal tubular epithelial polarity features. A) PAS staining of age-matched control and MVID Patients P1 and P2. PAS reactivity is predominantly observed at the basal lamina (white arrowhead) and at the apical brush border (black arrow). Note the dilated tubules in P2. B) Electron micrographs of renal proximal tubules of MVID patients. Note the presence of well-defined microvilli (black arrows) in both patients, and the appearance of electron dense structures and swollen.
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mitochondria in Fig. 1. C) Subcellular distribution of Rab11a. Note that Rab11a localizes predominantly in the subapical domain of the tubular epithelial cells (black arrows) of MVID patients and age-matched controls. Scale bar 10µm.

DISCUSSION

In this study we describe two MVID patients which carry MYO5B mutations that have developed (intermittent) renal Fanconi syndrome with no disturbances in glomerular function. Renal Fanconi syndrome is characterized by aminoaciduria, glucosuria, tubular acidosis, reduced tubular reabsorption of phosphate and resultant hypophosphatemic rickets. A common cause of renal Fanconi syndrome in children, cystinosis, was not observed in these patients.

Mutations in the MYO5B gene cause MVID and loss of expression or function of the myosin Vb protein has been proposed to impair apical brush border membrane formation and cell polarity (Wakabayashi et al., 2005; Müller et al., 2008). At the cellular level, MVID and renal Fanconi syndrome both have been attributed to defects in apical protein trafficking, in particular the recycling of brush border proteins via endosomes in the apical cytoplasm in kidney epithelial (Marshansky et al., 2002) and intestinal epithelial cells (Ameen & Salas, 2000; Szperl et al., 2011), resulting in (re)absorption defects. Apical recycling plays also an important role in apical surface development (reviewed in Golachowska et al., 2010). Apical microvillus atrophy is a prominent feature of MVID, and loss of the apical brush border has been reported in a child with renal Fanconi syndrome associated with lysinuric protein intolerance (Benninga et al., 2007). Kidney biopsies taken from these MVID patients allowed us to compare for the first time the organization of the apical plasma membrane and apical recycling endosomes in kidney versus intestinal epithelial cells.

As opposed to the pronounced intestinal epithelial brush border atrophy in the studied MVID patients, we found that MYO5B mutations did not affect apical brush border morphology and the PAS staining pattern in renal tubular epithelial cells. In
Figure 3. PAS staining along the horizontal axis of the intestine of two MVID patients and an age-matching duodenal control. PAS reactivity is mainly observed at the brush border (black arrow) and in the Goblet cells (white arrowhead). Note the absence (P1) and reduced (P2) PAS reactivity in the brush border of MVID patients, and the prominent subapical deposits (black arrows in P1 and P2). Scale bar 10µm.
Figure 4. CD10 staining along the horizontal axis of the intestine of two MVID patients and an age-matching duodenal control and appendix. CD10 is abundantly present at the brush-border (black arrow) and is also observed in a supranuclear compartment (white arrowhead). Note the absence (P1) and reduced (P2) staining of CD10 at the brush border and the prominent subapical deposits in the intestinal epithelial cells (P1 and P2) of the MVID patients. Scale bar 10µm.
Figure 5. Myosin Vb staining along the horizontal axis of the intestine of two MVID patients and an age-matching duodenal control. Myosin Vb is mainly localized in the apical cytoplasm (black arrow). Note the absence or severely reduced immunoreactivity towards the myosin Vb antibody in the intestinal epithelial cells of both MVID patients. Scale bar 10µm.
Figure 6. Rab11a staining along the horizontal axis of the intestine of two MVID patients and an age-matching duodenal control. Rab11a is mainly localized in the apical cytoplasm (black arrow). Note the reduced and patchy immunoreactivity towards the Rab11a antibody in the intestinal epithelial cells of both MVID patients. Scale bar 10µm.
addition, in contrast to the pronounced dispersal of the apical recycling endosome system marked by Rab11a in the intestinal epithelial cells, the spatial organization of the apical recycling endosome system was unaffected in renal tubular epithelial cells of MVID patients. Careful analysis of the brush border morphology and apical recycling endosome distribution along the intestinal horizontal axis (i.e., from duodenum to colon) revealed that the entire intestine is affected in MVID patients. Temporal kidney dysfunction has previously been reported in MVID patients (Gambarara et al. 2003; Kagitani et al. 1998). Long-term TPN has been reported to cause renal complications including glomerular changes which were partially related to infections and nephrotoxic medications or to dehydration (Buchman 1993; Lauverjat et al. 2006). However, tubular disturbances as measured by β2-microglobulinuria have not been associated with TPN (Buchman et al., 1993), and disturbances in glomerular function were not observed in the MVID patients (Table 1). It is unclear whether hypophosphatemic rickets and renal insufficiency are a result of complications from stool losses in MVID as proposed by Kagitani et al. (1998). The observations that 1) renal Fanconi syndrome in Patient 1 was intermittent and correlated with changes in TPN formulation, 2) the renal Fanconi syndrome disappeared in both patients following a bowel transplant, and 3) the apical brush border and apical recycling endosome organization appear unaffected in the proximal tubular epithelial cells, strongly suggest that the renal Fanconi syndrome is not a direct consequence of the MYO5B mutations. We can, however, not formally exclude that MYO5B mutations render the proximal tubular cells of some MVID patients more susceptible for changes in electrolyte- or fluid homeostasis, subsequently leading to failure of tubular transporters.

An important finding in this study is that MYO5B mutations thus exert divergent effects on the apical membrane system and structural polarity of kidney and intestinal epithelial cells. The typical apical brush border defects presented in MVID are therefore likely to be triggered by intestine-specific factors. We propose that these factors may be intrinsic (e.g., gene expression profile/ cell turnover rate) and/ or
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external (e.g., the gut lumen environment). It is conceivable that these factors preclude the intestinal epithelial cells from adjusting or mending the effects of \textit{MYO5B} mutations on the apical membrane system and structural polarity. Brush border atrophy in the intestine is believed to diminish the absorptive surface and expression of proteins involved in digestion and absorption at the apical plasma membrane and is, in this way, responsible for the malabsorption in MVID and consequent lifelong dependency on TPN. With intestine transplantation as the current standard treatment for MVID, which is sub-optimal at best, the identification of intestine-specific factors that trigger brush border defects in enterocytes carrying \textit{MYO5B} mutations may provide new targets and open new avenues for the development of alternative therapeutic strategies to combat this devastating disease.

ACKNOWLEDGEMENTS

We thank the patients, their parents and siblings, and the transplantation teams of the UMC Groningen. We thank the Dutch Digestive Diseases Foundation (MLDS) for supporting the national collaboration for patients with intestinal failure. We thank Dirk-Jan Reijngoud and Julius Baller for expert laboratory analyses and technical assistance and Marcory van Dijk for discussions. This research was sponsored by the Dutch Digestive Disease Foundation (MLDS (WO 08-17)), the Jan Kornelis de Cock Foundation, and De Drie Lichten Foundation. MG was supported by the Ubbo Emmius Foundation. SvIJ and ER were supported by the Royal Dutch Academy of Arts and Sciences.