

Article type : Original

HEP-19-0734

A molecular mechanism underlying genotype-specific intrahepatic cholestasis resulting from *MYO5B* mutations

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Key words: Hepatocyte polarity, bile canaliculus, myosin Vb, rab11, trafficking

Author contributions: AW, QL, FC, KK, JD, NH, CL, YQ performed experiments, AW, QL, JSW, DA, SIJ: study design, data interpretation, wrote manuscript

Conflicts of interest: Authors declare no conflicts of interest

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Abstract

background and rationale for the study: Progressive familial intrahepatic cholestasis (PFIC)6 has been associated with missense but not biallelic nonsense or frameshift mutations in *MYO5B*, encoding the motor protein myoVb. This genotype-phenotype correlation and the mechanism via which *MYO5B* mutations give rise to PFIC are not understood. The aim of this study was to determine whether the loss of myoVb or expression of patient-specific myoVb mutants can be causally related to defects in canalicular protein localization and, if so, via which mechanism.

Main results: We demonstrate that the cholestasis-associated P660L mutation in myoVb caused the intracellular accumulation of bile canalicular proteins in vesicular compartments. Remarkably, the knockout of *MYO5B* in vitro and in vivo produced no canalicular localization defects. In contrast, the expression of myoVb mutants consisting of only the tail domain phenocopied the effects of the Myo5b-P660L mutation. Using additional myoVb and rab11a mutants, we demonstrate that motor domain-deficient myoVb inhibited the formation of specialized apical recycling endosomes, and that its disrupting effect on the localization of canalicular proteins was dependent on its interaction with active rab11a and occurred at the *trans*-Golgi Network/recycling endosome interface.

Conclusions: Our results reveal a mechanism via which *MYO5B* motor domain mutations can cause the mislocalization of canalicular proteins in hepatocytes which, unexpectedly, does not involve myoVb loss-of-function but, as we propose, a rab11a-mediated gain-of-toxic function. The results explain why biallelic *MYO5B* mutations that affect the motor domain but not those that eliminate myoVb expression are associated with PFIC6.

Introduction

Hepatocytes are polarized epithelial cells with basolateral/ sinusoidal plasma membrane domains that face the blood circulation and apical/ canalicular plasma membranes that form the bile canaliculi via which bile is safely moved out of the liver. Tight junctions separate the sinusoidal and canalicular domains and prevent the mixing of bile and blood. Defects in the polarized distribution or function of cell surface proteins can cause severe liver diseases (1). Of these, progressive familial intrahepatic cholestasis (PFIC) is characterized by the inability of hepatocytes to secrete bile into the canaliculi resulting in the buildup of bile components and liver failure. PFIC can be caused by mutations in different genes (2), including *ATP8B1* (PFIC1), *ABCB11* (PFIC2), *ABCB4* (PFIC3), *TJP2* (PFIC4) and *NR1H4* (PFIC5). *ATP8B1*, *ABCB11* and *ABCB4* encode canalicular membrane transporters. Mutations in these proteins affect their expression, canalicular localization or function and consequently impair bile salt secretion (*ABCB11/BSEP*) or phospholipid dynamics in the canalicular membrane (*ATP8B1*, *MDR3*). *NR1H4* encodes the Farnesoid X receptor, a transcription factor that regulates the expression of *ABCB11/BSEP*. *TJP2* encodes the tight junction protein zona occludens (ZO)-2 and mutations in these presumably leads to the leaking of bile out of the canaliculi.

Recently, mutations in the *MYO5B* gene were reported in a group of PFIC patients who presented elevated bilirubin and bile acid levels with normal gamma-glutamyl transpeptidase (GGT) levels and did not have mutations in any of the other PFIC genes (3,4). Unique *MYO5B* mutations were associated with each affected family. *MYO5B* encodes the actin-filament based motor protein myoVb. MyoVb binds selected small GTPase rab proteins including the *trans*-Golgi Network (TGN)- and/ or recycling endosome-associated rab8 and rab11a, and has been implicated in apical plasma membrane protein trafficking. Mutations in *MYO5B* can also cause microvillus inclusion disease (MVID) (5–8), a congenital enteropathy characterized by intractable diarrhea and malabsorption and, at the cellular level, by the mislocalization of apical brush border proteins. Notably, many - but not all - MVID patients also develop cholestasis leading to liver failure (9).

How *MYO5B* mutations may lead to PFIC is not known. Given the effect of *MYO5B* mutations on the apical localization of brush border proteins in enterocytes in MVID, it is

possible that myoVb is similarly needed for the correct localization of bile canalicular proteins in hepatocytes and can cause cholestasis when mutated. *In vitro* studies in the hepatic WIF-B9 cell line, in which the ectopic expression of a rat myoVb tail fragment impaired canalicular protein trafficking (10), may support this hypothesis. *In situ* studies, however, showing no immunohistochemical abnormalities of canalicular transporters in liver biopsies of some patients with *MYO5B* mutations presenting severe cholestasis (3,11), challenge this hypothesis. Further, while missense, nonsense and frameshift *MYO5B* mutations all have been associated with MVID (reviewed in (7)), biallelic nonsense and frameshift mutations predicted to eliminate myoVb expression are noticeably absent in non-MVID cholestasis patients (3,12). Thus, not all pathogenic *MYO5B* mutations may lead to PFIC and/or canalicular protein localization defects.

Notably, causality between patient *MYO5B* mutations and the mislocalization of bile canalicular proteins in hepatocytes has not been experimentally addressed. The need to decisively determine whether a causal relationship exists between patient-specific *MYO5B* mutations and canalicular protein localization defects is particularly relevant for PFIC presenting in MVID patients. Indeed, because MVID patients receive life-long total parenteral nutrition (TPN), which itself may induce cholestasis and liver failure (13,14), it is difficult to determine whether liver symptoms in these patients are *MYO5B* mutation- or TPN-induced.

The aim of this study was to address the causal relationship between patient *MYO5B* mutations and canalicular protein mislocalization and to clarify the PFIC disease mechanism in these patients. We demonstrate that myoVb is dispensable for the correct localization of bile canalicular proteins yet can cause cholestasis-associated defects in their localization when mutated via an unexpected mechanism involving the small GTPase rab11a.

Experimental Procedures

Cell culture

HepG2 cells (ATCC HB8065) were maintained in high-glucose DMEM with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 100IU/ml penicillin, and 100µg/ml streptomycin in a humidified atmosphere. For experiments, cells were plated on poly-L-lysine-coated coverslips and used 3d later. HUES9 cells were maintained on vitronectin in E8 (Thermo-Fisher). Cells were passaged every 4-5 days with 1% RevitaCell Supplement added to the cells overnight on day of passage. Differentiation of HUES9 cells to hepatocytes was as previously described (15).

Viral transduction

Lentiviral particles were produced using a second-generation system based on pCMVdr8.1 and pVSV-G. 1×10^6 HEK293T cells were transferred to a poly-L-lysine coated 9cm² plates in 1.3ml culture medium. 1200ng of lentiviral vector, 1000ng pCMVdr8.1 and 400ng pVSV-G were mixed with 7.8µl Fugene/HD in 200µl Opti-MEM, and added to suspension HEK293T. After overnight incubation, medium was refreshed, and after 48h viral particles were harvested and filtered (0.45µm PVDF membrane filter). 1d after plating, cells were incubated with viral particles for 16h (supplemented with 8µg/mL polybrene). Antibiotics (2.5µg/ml puromycin, 4µg/ml blasticidin) were added 24h after viral incubation.

CRISPR knockout

A lentiviral CRISPR construct targeting Exon3 of *MYO5B* was generated using the plentiCRISPR-V2 vector (Addgene#52961) following provided protocols (gRNA target sequence: tcttacggaatccagatatc). Cells were transduced and selected with puromycin as described. Cells were plated on poly-L-lysine coating at 18 cells/cm² with untreated cells/cm² as feeder layer. After 4d cells were selected with puromycin (2.5µg/ml) to kill feeder cells, and remaining colonies were isolated as separate lines. To deplete MyoVb

in HUES9 cells, the cells were incubated with *MYO5B*-targeting lentiCRISPR viral supernatant for 5h (in E8-medium supplemented with 8µg/mL polybrene). After 48h, cells were selected with puromycin (1µg/ml). Selected cells were then plated at 28cells/cm², and the resulting colonies were mechanically passaged after 3 weeks. Clones were checked for myoVb knockout via Western blot.

Plasmids

Full-length human myoVb-coding sequence was amplified from HepG2 cDNA through PCR, including a myc-encoding '5 overhang in the forward primer sequence. Amplified myc-myoVb was inserted into pENTR1a vectors. All described myoVb mutants were generated by modification of this construct using the Q5® Site-Directed Mutagenesis Kit (NEB), with primers designed in the NEBaseChanger™ tool. MyoVb and thereof derived mutant constructs were transferred to lentiviral vectors for mammalian expression through Gateway™ cloning, using LR-Clonase™-II (Thermo-Scientific) as per manufacturer's instruction. Full-length myc-myoVb constructs were transferred to pLenti-CMV-Blast-DEST (706-1) (Addgene#17451), and myc-myoVb tail domain constructs to pLenti-CMV-Puro-DEST (w118-1) (Addgene#17452). EGFP-rab11aWT and EGFP-rab11aS25N (16) were gifts from R.E. Pagano (Mayo Clinic, USA).

Western blotting

Cells were resuspended in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH8.0) containing protease inhibitors. Lysates were mixed with sample buffer (2% SDS, 5% β-mercaptoethanol, 0.125M Tris-HCl, pH 6.8, 40% glycerol, 0.01% bromophenolblue) and incubated at 70°C for 10min. Samples were resolved with SDS-PAGE and electro-transferred onto PVDF membranes. Membranes were blocked with Odyssey-blocking buffer and incubated overnight at 4°C with primary antibodies (supplemental Table T1). After incubation with secondary antibodies, immunoblots were scanned with the Odyssey (LI-COR Biosciences). Relative quantification was performed using the Odyssey software.

Microscopy

Immunolabeling and fluorescence microscopy was performed essentially as described previously (3,8). Antibodies used are listed in supplemental Table T1). Fluorescent multiplex immunohistochemistry of paraffin embedded liver tissue of a previously reported PFIC6 patient (3) and an anonymous donor with cholangitis as control was performed with standard procedure of Tyramine Signal Amplification. Fluorescent images were analyzed using a combination of ImageJ and Adobe Photoshop. For electron microscopy, cells were fixed by adding dropwise an equal volume fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1M sodiumcacodylate buffer). After 10min this mixture was replaced by pure fixative at room temperature for 30min. After post-fixation in 1% osmiumtetroxide/1.5% potasiumferrocyanide (4°C;30min) cells were dehydrated using ethanol and embedded in EPON epoxy resin. 60nm sections were cut and contrasted using 2% uranyl acetate followed by Reynolds lead citrate. Images were captured with a Zeiss Supra55 in STEM mode at 26 KV.

qPCR

RNA was harvested using TRI reagent (Sigma). RNA was reverse transcribed in the presence of oligo(dT)12–18 (Invitrogen) and dNTPs (Invitrogen) with M-MLV reverse transcriptase (Invitrogen). Gene expression levels were measured by real-time quantitative RT-PCR using ABsolute QPCR SYBR Green Master Mix (Westburg) in a Step-One Plus Real-Time PCR machine (Applied Biosystems), and resulting data analyzed using the LinRegPCR method. The primers used to mutate *MYO5B* cDNA were:

forward –ACCAGCTGCCGTTCTTACGGA–

reverse –TGCGTTGTACATCAATTGGG–.

Statistics

For all phenotype quantifications, statistical significance of differences between triplicate experiments was determined using Student's *t*-test (two-tailed, unpaired, equal variance).

Replicates represent quantifications of >200 cells. P-values: *:p < 0.05, **:p <0.01,***
p<0.001.

Results

MyoVb deficiency does not disrupt canalicular protein localization

Displacement of bile canaliculi (BC) transporters to the cytoplasm of hepatocytes has been shown in liver biopsies of MVID patients presenting with cholestasis and homozygous missense mutations (c.1979C>T/p.P660L) (17) and a non-MVID PFIC patient with homozygous missense mutations (c.796T>C/p.C266R) (3) in the *MYO5B* gene. Here, we demonstrate the mislocalization of BC transporters to intracellular compartments in hepatocytes of an additional non-MVID PFIC6 patient carrying a missense *MYO5B* mutation (c.437C>T/p.S158F (3); Figure S1), thereby expanding the number of PFIC patients in which missense *MYO5B* mutations correlate with a cytoplasmic displacement of BC transporters. Intriguingly, in cholestatic MVID patients with nonsense *MYO5B* mutations a normal localization of BC transporters was reported (11), suggesting that loss of myoVb expression is not sufficient to induce BC transporter mislocalization. To address the requirement of *MYO5B* expression for the localization of bile canalicular transporters in hepatocytes, we examined the *in vivo* distribution of the Abcc2/Mrp2 and the structural BC protein radixin in the liver of whole-body *Myo5b* knockout mice (18). We observed their exclusive localization at bile canaliculi, indistinguishable from wild-type control mouse livers (Figure 1A-B). Human HepG2 cells (HepG2^{Par}) develop apical-basolateral polarity and bile canaliculus lumens (BC) between adjacent cells (19). In agreement with the observations in *Myo5b* KO mouse hepatocytes, HepG2 cells in which endogenous myoVb had been knocked out by CRISPR/Cas9 (Figure 1C; Figure S2A) (HepG2^{KO} cells) showed no defect in the canalicular localization of ABCC2/MRP2, which was indistinguishable from that in HepG2^{Par} cells (Figure 1D). Similar results were obtained with another canalicular protein, anoctamin (ANO)-6 (Figure S2B-C). We also generated a myoVb-deficient HUES9 human pluripotent stem cell line via CRISPR/Cas9 mediated gene knockout (HUES9^{KO}) (Figure 1E) and differentiated these to polarized hepatocyte-like cells (hiHeps). These hiHeps form *in vivo*-like multicellular bile canaliculi (Figure 1F) (15). Similar to the results in HepG2^{KO} cells, hiHeps derived from HUES9^{KO} cells formed multicellular bile canaliculi to which ABCC2/MRP2 exclusively localized, similar to HUES9^{Par}-derived hiHeps (Figure 1F).

These data show that missense myoVb mutations can correlate with a cytoplasmic displacement of BC transporters, while myoVb as such is dispensable for the correct localization of BC transporters at the canalicular membrane.

The MVID-associated myoVb-P660L mutation causes the intracellular accumulation of canalicular proteins

Causality between patient *MYO5B* mutations and the mislocalization of bile canalicular proteins in hepatocytes has not been experimentally addressed. Investigation of such causality is particularly relevant for PFIC presenting in MVID patients, as their long-term parenteral nutrition obscures the etiology of hepatic dysfunction. We therefore focused on the founding homozygous c.1979C>T mutation in the *MYO5B* gene of Navajo MVID patients (6). These patients display liver disease and liver biopsies from these patients have previously been shown to display cytoplasmic displacement of canalicular bile acid transporters and signs of perturbed polarity (17).

In order to determine whether this *MYO5B* mutation, leading to a P660L substitution in the myoVb protein, could be causally linked to canalicular protein localization defects, a full-length human myc-tagged *MYO5B* gene with the c.1979C>T mutation was constructed via site-directed mutagenesis, and either the myc-tagged wild-type myoVb or myc-myoVb-P660L protein was expressed in HepG2^{KO} cells. The expression of myoVb-P660L in HepG2^{KO} cells caused the intracellular accumulation of the bile canalicular proteins ABCC2/MRP2 and anoctamin (ANO)6, when compared to HepG2^{KO} cells expressing the wild-type *MYO5B* gene (Figure 2A-C). This was accompanied by a reduction in the amount of BC (Figure S2D). Notably, HepG2^{Par} cells that expressed myoVb-P660L showed a less severe phenotype (more BCs with subapical localization of the mutant protein and less intracellular accumulation of the mutant protein and canalicular proteins) when compared to HepG2^{KO} cells that expressed myoVb-P660L (Figure S2E-H, *c.f.*, Figure 2). These data demonstrate that in the absence of wildtype myoVb, mutant myoVb-P660L caused the intracellular accumulation of BC-resident proteins, and provide evidence that this mutation is causally linked to the hepatic canalicular defects as observed in liver biopsies of the patients. Moreover, given the absence of a localization defect in myoVb-depleted cells, the results also indicate that the

disruptive effect of myoVb-P660L on the localization of canalicular proteins in hepatocytes cannot be explained by the mere loss of myoVb function.

The tail domain of myoVb is sufficient to cause the intracellular accumulation of vesicles and bile canalicular proteins

Because the mutated motor domain but not the absence of the myoVb protein produced a disease phenotype, we hypothesized that regions distal to the motor domain (IQ-domains, coiled-coil domains and/or the globular tail-domain) may have been instrumental for the disruptive effects on the localization of canalicular proteins. Therefore, we generated a human myoVb mutant that lacked the motor domain, IQ-domains and part of the coiled-coil domain (Figure 3A), which is similar to conventionally used dominant-negative myoVb tail domain constructs (hereafter referred to as MyoVb/ Δ 1-1195). Similar to myoVb-P660L, the expression of MyoVb/ Δ 1-1195 in HepG2^{KO} cells resulted in the intracellular accumulation of ABCC2/MRP2 and ANO6 and a reduction in the number of BCs (Figure S3A-E). Notably, the effect of myoVb/ Δ 1-1195 was more severe when compared to myoVb-P660L and was also observed when expressed in HepG2^{Par} cells (Figure 3B-E, Figure S4A). Also, the canalicular protein dipeptidyl-peptidase (DPP)IV accumulated inside the cells (Figure S4B-C). The myoVb/ Δ 1-1195 mutant itself colocalized with the intracellular clusters (Figure 3B). Electron microscopy of HepG2 cells expressing the myoVb mutant revealed the presence of large clusters of vesicles which were not observed in control HepG2 cells (Figure 3F). Finally, the expression of myoVb/ Δ 1-1195 in pluripotent HUES^{Par} stem cell-derived hepatocytes also caused the intracellular accumulation of ABCC2/MRP2 and ANO6 (Figure S4D), which indicated that the effects caused by this mutant are not specific for the HepG2 cell line. Together, these results indicate that in contrast to the loss of myoVb, the expression of the tail domain of myoVb mimicked the myoVb-P660L-induced intracellular accumulation of BC proteins.

Motor-less myoVb induces the accumulation of apical and basolateral proteins in clustered compartments with mixed recycling endosome and *trans*-Golgi Network identity

The intracellular clusters of BC proteins and the appearance of clusters of vesicles in myoVb mutant-expressing cells suggested that these clusters represented intracellular organelles. In order to determine the identity of the mutant myoVb-induced ABCC2/MRP2- and ANO6-containing intracellular clusters, we performed immunofluorescence microscopy in cells co-labeled with markers for different organelles. Proteins that make up BC microvilli, such as F-actin, the ABCC2/MRP2- and F-actin-binding protein radixin or other phosphorylated ERM proteins, did not co-localize with the BC protein-containing intracellular cluster in cells expressing myoVb/ Δ 1-1195 (Figure 4A, Figure S5A), indicating that these clusters did not represent microvillus inclusions, which represent a hallmark of enterocytes in MVID patients (20).

By contrast, intracellular clusters containing BC proteins co-localized with the apical recycling endosome markers rab11a and its interacting protein rab11a-FIP5/rab-interacting protein (rip11) (Figure 4B-C). This subcellular distribution of rab11a and its rip11 was markedly distinct from their exclusive subapical distribution in HepG2 cells that did not express the mutant protein. However, the subcellular distribution of rab11a and rip11 in HepG2^{KO} cells and HepG2^{Par} cells was indistinguishable, supporting that myoVb expression is dispensable for canalicular polarity (Figure S5B-C). Also, the recycling endosome-associated rab8, which in control HepG2 cells showed a relatively dispersed cytoplasmic staining pattern, colocalized with the clusters in cells expressing the myoVb mutants (Figures 4D). LAMP1, a marker of late endosomes/lysosomes, did not co-localize with the canalicular protein-containing clusters and its subcellular distribution pattern was not visibly altered (Figure S5D). In addition to BC proteins, the sinusoidal transferrin receptor and its ligand transferrin, which upon its endocytosis is recycled to the sinusoidal surface via recycling endosomes was found to co-localize with the BC protein-containing clusters (Figure 4E, Figure S5E). Moreover, when fluorescently-labeled transferrin was allowed to be endocytosed in control HepG2 cells or HepG2 cells expressing mutant myoVb, its subsequent recycling to the cell surface was inhibited in

cells expressing the myoVb mutant as evidenced by its persistent accumulation in the ANO6- and transferrin receptor-containing clusters (Figure 4E).

While the *cis*-Golgi protein giantin did not co-localize with the BC protein-containing clusters (Figure 5A), we found that three markers of the *trans*-Golgi Network (TGN) partly colocalized with the BC protein-containing clusters (Figure 5B-D). These included TGN46 and Golgin-97. With the exception of AP1y, which in addition to its typical TGN-like distribution pattern was also observed in the subapical region of control HepG2 cells, TGN46 and golgin-97 did not show a subapical localization in control HepG2 cells (Figure 5B-D). Together, these results indicate that the BC protein-containing clusters in cells expressing the myoVb mutant represented trafficking-incompetent compartments with a mixed apical recycling endosome and TGN identity.

The disrupting effect of motor-less myoVb on canalicular protein localization requires active rab11a

We hypothesized that the myoVb tail domain induced canalicular defects through rab8 or rab11a function rather than via competition with endogenous myoVb. To test whether the observed defects are mediated through rab8 or rab11a, we generated a myovb mutant which comprised only the globular tail domain (the last 383 amino acids) and did not contain the binding site for rab8 in ExonC/Exon30 (referred to as myoVb/ Δ 1-1460) (Figure 6A). Like myoVb/ Δ 1-1195, myoVb/ Δ 1-1460 mutant led to the intracellular accumulation of BC proteins and a reduction in the number of BCs albeit to a lesser extent than the myoVb/ Δ 1-1195 mutant, suggesting that rab8 binding may contribute but is not essential to induce the effect (Figure 6B-C, Figure S5A). Indeed, substitution of glycine at position 1300 in myoVb/ Δ 1-1195 to a leucine, a mutation known to abolish the interaction between myoVb and rab8 (21), partially ameliorated its disrupting effects to the levels seen with the myoVb/ Δ 1-1460 mutant (Figure 6B,D). By contrast, when tyrosine at position 1714 in myoVb/ Δ 1-1460 was mutated to glutamic acid (Y1741E) (Figure 6E-G), a mutation that abolishes myoVb binding to rab11a (21), the intracellular accumulation of the canalicular proteins was completely abolished. Similarly, the introduction of this Y1714E mutation in the patient myoVb-P660L mutant reduced the intracellular accumulation of the canalicular proteins (Figure 7A-C). Notably, the

introduction of Y1714E in myoVb/ Δ 1-1460 or myoVb-P660L did not lead to an increase in the number of BC.

Moreover, the myoVb/ Δ 1-1195 mutant, as well as the patient myoVb-P660L mutant, failed to cause the intracellular clustering of ABCC2/MRP2 when expressed in HepG2 cells that also expressed the EGFP-tagged mutant rab11aS25N (Figure 7D), which is expected to shift the equilibrium of rab11a towards the GDP/nucleotide free state and exert dominant-negative effects on endogenous rab11a by occupying the endogenous guanine nucleotide exchange factors (GEFs). Consistent with reports in other cells (22) and the previously reported location of two rab11 GEFs, REI-1 and Crag at the TGN, EGFP-rab11aS25N colocalized with the TGN in HepG2 cells (Figure 7E). While the expression of EGFP-tagged mutant rab11a-S25N in HepG2 cells, as such, inhibited polarity development, it did not cause the intracellular clustering of BC proteins (Figure 7F-H). Cells expressing wildtype EGFP-rab11a showed normal BC formation and a subapical distribution of the EGFP-rab11a, similar as wild type cells (Figure 7F-G). Thus, the intracellular clustering of BC proteins in cells expressing myoVb-P660L or the myoVb tail domain is not phenocopied by loss of rab11a function. Together, we conclude that the interaction of the myoVb-P660L or myoVb/ Δ 1-1195 mutant with active rab11a is required for the myoVb-P660L- or myoVb/ Δ 1-1195-induced intracellular accumulation of BC proteins but not inhibition of polarity development, and that loss of rab11a function inhibited polarity development independent of myoVb.

MVID-associated nonsense *MYO5B* mutations producing truncated myoVb mutants do not disrupt hepatocyte polarity and canalicular protein localization

We reasoned that if the disrupting effect of motor-deficient myoVb mutants on the localization of canalicular proteins required their interaction with rab11a via the distal C-terminal binding Y1714 residue, nonsense *MYO5B* mutations that cause a premature translation termination codon and the resultant synthesis of truncated myoVb proteins should not lead to polarity defects. We therefore generated different *MYO5B* nonsense mutation previously reported in MVID patients and listed in the MVID registry (www.mvid-central.org): myoVb-R363X (c.1087C>T), myoVb-R1016X (c.5382C>T) and myoVb-R1795X (c.5383C>T) (Figure 8A) and expressed these in HepG2^{KO} cells. Note that in

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contrast to the myoVb-R363X and myoVb-R1016X mutants the myoVb-R1795X mutant contains the rab11a-binding site. Western blot analyses confirmed that these mutants led to the expression of truncated myoVb proteins at their predicted molecular weights (Figure 8B). Fluorescence microscopy showed that the mutants failed to cause the intracellular accumulation of ABCC2/MRP2 (Figure 8C). These results demonstrate that nonsense MVID-associated *MYO5B* mutations and the expression of resultant truncated myoVb mutants that lack the globular tail-domain do not cause defects in BC formation and canalicular protein localization, and support our observations that the C-terminal region and a conserved interaction with rab11a is required for myoVb mutants to disrupt the localization of canalicular proteins.

Discussion

Determination of causality between patient *MYO5B* mutations and mislocalization of bile canicular proteins in cholestatic patients is essential to understand the etiology of the disease, but thus far had not been experimentally addressed. In this study we demonstrated that the founding Navajo myoVb-P660L mutant (6) when expressed in hepatic HepG2 cells caused the aberrant localization of canicular proteins as well as the sinusoidal transferrin receptor to intracellular clusters. These data are in agreement with observations in hepatocytes in liver biopsies of patients with this mutation and, therefore, show that the liver symptoms and hepatocyte defects observed in Navajo MVID patients (17) are likely a direct consequence of their *MYO5B* mutation rather than a sole consequence of intestinal failure- or TPN-induced liver damage. The causality between myoVb-P660L and the mislocalization of canicular transporters suggest a similar mechanisms for PFIC6 in patients carrying other missense *MYO5B* mutations. Although different missense mutations may affect the myoVb protein differently (7,12), the clear mislocalization of canicular proteins to intracellular compartments as shown in this study for another PFIC6 patient with a different missense *MYO5B* mutation supports this.

Insight in the mechanism via which (mutant) myoVb (de)regulates hepatocyte function is necessary to understand the pathogenesis of the disease. We demonstrated that the loss of myoVb in human or mouse hepatocytes, as well as the expression of patients' nonsense *MYO5B* mutations, did not cause the aberrant localization of canicular proteins, indicating that myoVb function as such is not required for the correct localization of bile canicular proteins. Instead, the hepatocyte polarity phenotype as observed in myoVb-P660L expressing cells was faithfully phenocopied by the expression of myoVb mutants that lacked the entire motor domain and consisted of only the tail domains of the myoVb protein.

These results indicated that the effects of myoVb-P660L on the localization of canicular proteins could not be explained by a mere loss of myoVb motor function. Indeed, additional mutagenesis experiments showed that the disrupting effect of myoVb motor domain mutants on the localization of canicular proteins was critically dependent on their ability to interact with active rab11a. Further, the absence of intracellular clusters of

BC proteins upon inhibition of rab11a activation indicated that the mechanism via which myoVb mutants exerted their effects on the distribution of canalicular proteins involved active rather than inhibited rab11a function.

A recent study demonstrated that the globular tail domain of myoVb induced the clustering of rab11a-decorated lipid vesicles (liposomes) in a chemically defined *in vitro* reconstitution system by stimulating homotypic rab11-rab11 interactions (23). While this effect has thus far not been demonstrated in living cells, it would fit with the myoVb-Y1714- and rab11a-dependent clustering of rab11a and associated cargo and the appearance of clusters of vesicles that we observed in cells expressing myoVb-P660L or only the myoVb tail domain. Conceivably, the C-terminal tail domain of mutant myoVb, upon its displacement from its normal subapical location, in this way induced the ectopic clustering of TGN- and/ or recycling endosomes-derived transport vesicles via rab11a and thereby perturbed the correct distribution of bile canalicular proteins.

The results from this study are relevant for understanding the unexplained genotype-genotype correlations that have been reported for PFIC6. Indeed, PFIC in non-MVID patients has been associated with only biallelic missense mutations and, in contrast to the enteropathy in MVID, has not been associated with biallelic *MYO5B* mutations that are predicted to result in the loss of myoVb protein expression, such as nonsense or frame-shift mutations (3,12). In agreement with these clinical findings, we found that the expression of truncated myoVb resulting from MVID-associated nonsense *MYO5B* mutations did not cause a canalicular protein localization defect. Together with the findings that myoVb as such is not required for the correct localization of canalicular proteins *in vitro* and *in vivo*, and that myoVb mutants required active rab11a for their disruptive effect on canalicular protein localization, this study thus provides a direct and simple explanation for this genotype-phenotype correlation in non-MVID PFIC6 patients. It may also lead us to speculate that intrahepatic cholestasis in patients with MVID (9,14) is less likely to be caused by their *MYO5B* mutations when these involve nonsense mutations than when these involve missense mutations. In support of this, a MVID patient was reported with only nonsense *MYO5B* mutations who presented with cholestasis with normal GGT levels for 9 months but liver biopsies showed normal canalicular protein localization. Cholestasis in this patient later spontaneously resolved (11).

The results of this study are relevant for exploring new treatment strategies, for example for those PFIC6 patients who are nonresponsive to routine treatment (3,9). Our results suggest that the specific inhibition of the interaction between mutant myoVb and rab11a in the patients' hepatocytes may ameliorate the harmful effects of the myoVb mutant on canalicular protein localization and thereby the PFIC in patients. This study thus paves the way for the discovery of small molecule inhibitors of this interaction and the exploration of their potential beneficial effects.

Finally, the ectopic expression of the globular tail domain of myoVb has been widely used to implicate the involvement of myoVb in intracellular trafficking of a variety of proteins in a variety of cell types (10,24–27). The results from our study, demonstrating that the effects of the myoVb tail domain are not necessarily mimicked by the loss of myoVb expression or loss of rab11a activity, yet depend on their interaction with active rab11a, suggest that the need to recheck the interpretation of some of the studies using this myoVb mutant is warranted.

Acknowledgements

We thank R. Prekeris for his kind gift of anti-Rip11 antibodies. This work was supported by grants from the Nederlandse vereniging voor Gastroenterologie (to SIJ) and the Natural Science Foundation of China, No. 81873543, 81570468 (to JSW).

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Figure legends

Figure 1. MyoVb deficiency does not disrupt canalicular protein localization.

(A-B) Immunofluorescent staining of ABCC2 and radixin reveals their canalicular localization in both wildtype and *Myo5b* KO mouse liver sections. HNF4 α co-staining marks hepatocytes. (C) Knock-out of *MYO5B* in HepG2^{KO} cells (treated with *MYO5B*-targeting pLentiCRISPR V2) was confirmed by western blot (compared to parental line, HepG2^{PAR}). (D) In HepG2 cells, localization of ABCC2 and F-actin is unaffected by *MYO5B* knock-out (HepG2^{KO}), compared to HepG2^{PAR} control. Yellow arrowheads indicate BCs. (E) Western blot for myoVb in HUES9^{KO} cells confirmed *MYO5B* knock-out (compared to parental line, HUES9^{Par}). (F) HiHeps generated from HUES9^{KO} cells exhibit bile canaliculi formation comparable to HUES9^{Par}-derived hiHeps, with exclusive canalicular labeling of ABCC2. Scale bars: 10 μ m.

Figure 2. The MVID-associated myoVb-P660L mutation causes the intracellular accumulation of canalicular proteins.

(A,B): Immunofluorescent microscopy images of myc-tagged myoVb proteins, ABCC2 and ANO6 in HepG2^{KO} expressing myc-myoVb and -myoVb-P660L. HepG2^{KO} cells expressing myoVb-P660L show intracellular colocalization of myc and ABCC2 (white arrows). Yellow arrowheads indicate BCs. Scale bars: 10 μ m. (C). Quantification of the percentage of myc-positive cells that show intracellular clusters/accumulations of myc localized with ANO6. (D) Quantification of the percentage of myc-positive cells that show subapical localization of myc-tagged myoVb proteins.

Figure 3. The tail domain of myoVb is sufficient to cause the intracellular accumulation of vesicles and bile canalicular proteins.

(A) Schematic depiction of myoVb constructs. (B) Labeling of ANO6 and myc in HepG2^{Par} cells expressing myc-myoVb/ Δ 1-1195 (white arrows indicates intracellular colocalization of both markers). (C) Quantification of the percentage of HepG2^{Par} cells showing accumulation of ABCC2 (as shown in figure 4C) upon expression of myc-myoVb/ Δ 1-1195 compared to untreated cells, and cells transduced with an empty pLenti-Puro construct (control). (D,E) Labeling

of ABCC2 and F-actin or ANO6 in HepG2^{Par} cells expressing myc-myoVb/ Δ 1-1195, compared to untreated control. White arrows indicate intracellular accumulation of ABCC2 (and ANO6 in figure D). Scale bars: 10 μ m unless labeled otherwise. Yellow arrowheads indicate BC. (F) Electron microscopy images of HepG2^{Par} cells expressing myc-myoVb/ Δ 1-1195. Cells displayed large collections of vesicles (enlarged areas) which were not observed in control cells.

Figure 4. Motor-less myoVb induces the accumulation of apical and basolateral proteins in clustered compartments that display recycling endosome identity. (A) Phospho-ERM proteins are not present in myc-myoVb/ Δ 1-1195 clusters (white arrows). Yellow arrowhead indicate BC. (B-D) Labeling of rip11, rab11a and rab8 with ANO6 or ABCC2 in HepG2 expressing myc-myoVb/ Δ 1-1195 (white arrows), compared to control. White arrows indicate colocalization of endosomal proteins with BC-resident protein. Yellow arrowhead indicates juxta-nuclear staining of rip11 in non-polarized control cells. (E) Labeling of myc in control and myc-myoVb/ Δ 1-1195 expressing HepG2, fixed after 30 min incubation (t=0h) with fluorescently labeled transferrin (388Tf), and after a 2h chase period. 488-Transferrin localized with myc-myoVb/ Δ 1-1195 clusters (white arrows), at t=0h and at t=2h. Scale bars: 10 μ m.

Figure 5. Motor-less myoVb induces the accumulation of apical and basolateral proteins in clustered compartments that also display *trans*-Golgi Network identity. (A) Giantin labeling in HepG2 cells expressing myc-myoVb/ Δ 1-1195 compared to control. White arrows indicate lack of colocalization. (B,C) Golgin-97 and TGN46 showed colocalization with intracellular cluster of ANO6 in HepG2 expressing myc-myoVb/ Δ 1-1195 (white arrows). (D) AP1y localized with ANO6 in intracellular clusters in HepG2 expressing myc-myoVb/ Δ 1-1195 (white arrows). Scale bars: 10 μ m.

Figure 6. The role of rab8 and rab11a binding sites in the disrupting effect of motor-less myoVb on canalicular protein localization. (A) Schematic depiction of the amino acid sequences of myoVb mutants. (B) Quantification of the percentage of HepG2

cells showing intracellular accumulation of ABCC2 upon expression of myoVb tail domain mutants. (C) HepG2 cells expressing myc-myoVb/ Δ 1-1460 showed intracellular ABCC2 accumulation (white arrows). Myc labeling showed myc-myoVb/ Δ 1-1460 localized diffusely in the cytoplasm. (D) Labeling of ABCC2, F-actin and myc in HepG2 expressing myc-myoVb/ Δ 1-1195-Q1300L and untreated control. White arrows indicate intracellular ABCC2 accumulation. (E) In HepG2 cells expressing myc-myoVb/ Δ 1-1460-Y1714E ABCC2 localized at the BC with F-actin (yellow arrowheads). Labeling for myc confirmed expression of the construct. (F) Quantification of the percentage of cells with intracellular MDR1-GFP accumulations (depicted in Figure 8G), upon expression of myc-myoVb/ Δ 1-1460 or its Y1714E mutant variant. (G) HepG2 cells expressing myc-myoVb/ Δ 1-1460 showed intracellular accumulation of the co-expressed BC marker MDR1-GFP (white arrows), but not when the Y1714E mutation was introduced in myc-myoVb/ Δ 1-1460.

Figure 7. The disrupting effect of motor-less myoVb on canalicular protein localization requires active rab11a. (A) ANO6 and myc labeling in HepG2^{KO} expressing myc-myoVb-P660L or myc-myoVb-P660L-Y1714E. Myc-myoVb-P660L frequently accumulated intracellularly with ANO6 (white arrows), whereas myc-myoVb-P660L-Y1714E appeared diffuse in the cytoplasm or subapical (yellow arrowheads). (B) Quantification of the percentage of myc-positive cells that show intracellular clusters/accumulations of myc localized with ANO6, in HepG2^{KO} cells expressing myc-myoVb-P660L or myc-myoVb-P660L-Y1714E. (C) Quantification of the percentage of myc-positive cells that show subapical localization of myc in HepG2^{KO} cells expressing myc-myoVb-P660L or myc-myoVb-P660L/Y1714E. (D) HepG2 co-expressing EGFP-rab11aS25N and myc-myoVb/ Δ 1-1195 in HepG2^{Par} cells (left side), EGFP-rab11aS25N and myc-myoVb-P660L in HepG2^{Par} cells (middle), and EGFP-rab11aS25N and myc-myoVb-P660L in HepG2^{KO} HepG2 (right side). In all three conditions, the presence of EGFP-rab11aS25N prevented the clustering of the respective myoVb mutants. (E) EGFP-rab11aS25N colocalized with golgin-97 in HepG2 cells. (F) ABCC2 labeling in HepG2 overexpressing wildtype EGFP-rab11a or EGFP-rab11aS25N. In EGFP-rab11aS25N transduced cells, BC formation was only seen in cells with no or very low expression of the construct (right side, box). (G) Quantification of BC formation

(expressed as BC/100 cells) in HepG2 cells expressing wildtype EGFP-rab11a or EGFP-rab11aS25N. (H) Quantification of the percentage of HepG2 cells showing accumulation of ABCC2 upon expression of wildtype EGFP-rab11a or EGFP-rab11aS25N.

Figure 8. MVID-associated nonsense *MYO5B* mutations producing truncated myoVb mutants do not disrupt hepatocyte polarity and canalicular protein localization. (A) Schematic depiction of the amino acid sequences of nonsense myoVb mutants. (B) Western blot showing the truncated myoVb mutant proteins expressed in HEK293 cells. (C) Immunofluorescence microscopy images showing the subcellular distribution of the truncated myoVb mutant proteins and the canalicular protein ABCC2. Scale bars: 10µm

Abbreviations

PFIC: progressive familial intrahepatic cholestasis

myoVb: myosin Vb

GGT: gamma glutamyltransferase

MVID: microvillus inclusion disease

TGN: trans-Golgi Network

TPN: total parenteral nutrition

BC: bile canaliculi

ANO6: anoctamin-6

WT: wild type

KO: knockout

GEF: guanine nucleotide exchange factor

EGFP: enhanced green fluorescent protein















