Chapter 2

AN OVERVIEW AND ONLINE REGISTRY OF MICROVILLUS INCLUSION DISEASE PATIENTS AND THEIR MYO5B MUTATIONS

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Published in Human Mutation (December 2013 Volume 34, Issue 12)
Abstract

Microvillus inclusion disease (MVID) is one of the most severe congenital intestinal disorders and is characterized by neonatal secretory diarrhea and the inability to absorb nutrients from the intestinal lumen. MVID is associated with patient-, family- and ancestry-unique mutations in the MYO5B gene, encoding the actin-based motor protein myosin Vb. Here we review the MYO5B gene and all currently known MYO5B mutations and for the first time methodologically categorize these with regard to functional protein domains and recurrence in MYO7A associated with Usher syndrome and other myosins. We also review animal models for MVID and the latest data on functional studies related to the myosin Vb protein. To congregate existing and future information on MVID geno-/phenotypes and facilitate its quick and easy sharing among clinicians and researchers, we have constructed an online MOLGENIS-based international patient registry (www.MVID-central.org). This easily accessible registry currently contains detailed information of 137 MVID patients together with reported clinical/phenotypic details and 41 unique MYO5B mutations, of which several unpublished. The future expansion and prospective nature of this registry is expected to improve disease diagnosis, prognosis and genetic counseling.

An introduction to microvillus inclusion disease

Microvillus inclusion disease (MVID; Online Mendelian Inheritance In Man 251850), previously known as familial protracted enteropathy (Davidson’s disease) or congenital microvillus atrophy, is a rare but life-threatening autosomal recessive enteropathy (Davidson et al, 1978; Cutz et al, 1989; Ruemmele et al, 2006). MVID is associated with patient- or family-specific mutations in the MYO5B gene, encoding the ubiquitously expressed myosin Vb protein (Erickson et al, 2008; Müller et al, 2008; Ruemmele et al, 2010; Chen et al, 2011; Szperl et al, 2011). The clinical presentation of MVID is characterized by intractable, severe, watery diarrhea beginning in early infancy, failure to absorb nutrients, and failure to thrive. The prognosis is generally poor and depends on total parenteral nutrition (TPN) and bowel transplantation. MVID is the leading cause of neonatal secretory diarrhea and accounts for ~7% of paediatric bowel transplantations worldwide. Most patients die at a young age as a consequence of total parenteral nutrition-induced liver failure or sepsis. Histological hallmarks of MVID small intestinal biopsies are hypoplastic atrophic or disorganized villi without crypt hypertrophy or immune cell infiltrate and, at the intestinal cellular level, microvillus atrophy, intracellular accumulation of brush border enzymes, and microvillus inclusions in the cytoplasm (Cutz et al, 1989; Ruemmele et al, 2006). Each of these hallmarks severely limits the absorptive capacity of the intestine. Diagnosis is established with light microscopy showing accumulation of periodic acid Schiff (PAS) reactivity and/or the brush border enzyme CD10 in the cytoplasm of the enterocytes (Groisman et al, 2002; Koepsell and Talmon, 2010) and, ultimately, with electron microscopy showing microvillus inclusions (Phillips et al, 1985).

Two forms of MVID are distinguished: an early-onset and late-onset form in which symptoms start within hours or 1-3 months after birth, respectively. Notably, considerable enterocyte-to-enterocyte variation exists with respect to the extent of microvillus atrophy, number of microvillus inclusions, and the accumulation of electron-dense ‘granules’,
without a clear correlation between these phenotypes (Iancu et al, 2007). It is also relevant to note that the percentage of enterocytes with microvillus inclusions varies greatly between patients and in single patients as a function of time. An atypical form of MVID, i.e., without detectable microvillus inclusions, has been proposed (Mierau et al, 2001; Weeks et al, 2003). However, because of the sparse distribution of microvillus inclusions and sampling issues, the absence of microvillus inclusions is difficult to confirm (Cutz et al, 1997). A useful marker for these inclusions at the light microscopic level is villin (Sherman et al, 2004). In one patient spontaneous passage of detached long bowel segments has been reported (Chiang et al, 2013). Furthermore, extra-intestinal manifestations have been reported in MVID patients, including obesity and cushingoid fat distribution without demonstrated endocrine disease (Gambarara et al, 2003), hematuria (Siahanidou et al, 2013), pneumocystis jiroveci pneumonia (Siahanidou et al, 2013), dihydropyrimidinase deficiency (Assmann et al, 1997), autosomal dominant hypochondroplasia (Heinz-Erian et al, 1999), microcephaly, renal Fanconi syndrome (Golachowska et al, 2012) or other renal problems (Halac et al, 2011), hypophosphatemic rickets (Kagitani et al, 1998), diabetes (Oatman et al, 2013), cardiac (Gathungu et al, 2008) and pulmonary (Halac et al, 2011) problems, and liver dysfunction (Halac et al, 2011; Siahanidou et al, 2013) including multiple hepatic adenomas (Burgis et al, 2013). Several children with MVID have been reported to require special education care or display psychiatric problems (Halac et al, 2011). It is unclear to what extent these extra-intestinal phenotypes may be iatrogenic or linked to the MYO5B mutations, and patient-specific disease model are eagerly awaited. In some patients non TPN-related liver dysfunction presented only after intestinal transplantation (Halac et al, 2011). It can thus be anticipated that more complex phenotypic manifestations will evolve with therapeutic interventions and, as improved health care will increase the life span of children with MVID, also with age.

An online registry for MVID patients and their MYO5B mutations

To gain insight in the intestinal and extra-intestinal clinical manifestations of MVID and how these evolve with therapeutic interventions and age, a detailed understanding of the spectrum of MYO5B mutations, and the ability to establish genotype-phenotype correlations as a function of time is needed. Therefore, we have thoroughly reviewed the literature on MVID and MYO5B and constructed an international Microvillus Inclusion Disease (MVID) Patient Registry, accessible at http://www.MVID-central.org, to aid all clinicians and scientists working in the field of Microvillus Inclusion Disease and the MYO5B gene.

The registry, the initiative of which is supported by the International Transplantation Association, contains all MVID patients who have been published in the medical literature. Information has been collected with regard to 1) patient/family details (including gender, consanguinity, and ancestry), 2) genetic details (including MYO5B cDNA change, paternal origin, exon/intron, homozygous/(compound) heterozygous, type of mutation), 3) protein
details (including protein change, protein domain, predicted functional consequence), and
4) clinical details (including early/late onset, (age at) transplantation, cause of death, other
clinical manifestations). The MVID Registry can be searched for patients or mutations, and
each by several categories, and thus can be used as a central, quick reference for all who
work in the MVID field. The inclusion of unpublished patient information improves the
quality of the data and the use of the registry. Therefore, anyone can upload new data via
the “Submit data” button using a provided excel sheet in order to standardize the data.
Submitted data will be inserted in the registry after review by the data curator. User login
is required as this generates a unique user ID that is attached to each patient submitted,
enabling the user to come back at a later stage and edit or enter additional information
to the submitted data and, as such, thereby also offering longitudinal and prospective
studies in which patients and disease / phenotype progression can be followed in time.

The database has been build using the Molecular Genetics Information System
(MOLGENIS) software framework (Swertz et al, 2004; Swertz and Jansen, 2007; Swertz
et al, 2010) and the Observation Object Model (Observ-OM) data structure (Adamusiak
et al, 2012) which has been used before for patient registries (van den Akker et al, 2011;
Janssen et al, 2012) and can be downloaded as open source for any research group that
MOLGENIS comes with an easy to use text file format to upload data, and provides users
with search options and genome browser to navigate the data.

A first analysis of the MVID patient registry

Since its first description in 1978 (Davidson et al, 1978), we could retrieve 137 cases
world-wide from the literature (Medline database) and medical centres and collected
information about ancestry, consanguinity, and gender, coupled to genotype and
phenotype data. Of all patients with available information 95% and 5% display an early
and late onset of clinical symptoms, respectively. One patient with late-onset MVID
recovered from the disease (Croft et al, 2000). There is a 1.54 male/female ratio (77 males
(61% of patients with known sex) and 50 females (39%); the gender of 12 patients (9% of
total) is not reported). Consanguinity was reported in 38 patients (41% of all patients
with consanguinity information) with a gender preference. Thus, 76% of progeny from
reportedly consanguineous parents were male, while 71% of all female patients are
from reportedly non-consanguineous parents (Chi-squared test, p=0.026). The reason
for this unexpected gender-bias is unclear and the identification of more patients will
determine whether this holds true. Of 48 patients (34%) in the registry, consanguinity is
not known or reported. Geographically, there is a relatively high prevalence of MVID in
the Mediterranean region (49% of all patients with reported geographical information)
and in Navajo Indians in the US (14%), which fits well with the current global prevalence
of consanguineous marriages (Bittles and Black, 2010) (www.consang.net).

Consanguinity allowed the identification of MYO5B as the mutated gene in MVID by
homozygosity mapping (Müller et al, 2008). Mutations in the MYO5B gene have been
associated with all but one (Müller et al, 2008) MVID patients. The \textit{MYO5B} gene (OMIM# 606540) is located at chromosome 18 (18q21.1) between 47,349,156 bp and 47,721,451 bp from the p-arm telomere. \textit{MYO5B} has a genomic size of 372,296 bases, consists of 42 exons, and encodes the myosin Vb protein (1,848 amino acids, 213,672 Da; pI 6.77; Figure 1). \textit{MYO5B} is ubiquitously expressed in various tissues (Rodriguez and Cheney, 2002). \textit{MYO5B} is epigenetically silenced in human gastric cancer (Dong et al, 2013) and GWAS studies have associated \textit{MYO5B} with bipolar disorder (Howrigan et al, 2011) and platelet function (Guerrero et al, 2011), but no gastric cancer nor symptoms of pediatric bipolar disorder or platelet dysfunction have (yet) been reported in children with MVID. Of the 137 MVID patients that are currently included in the registry, 40 patients have been linked to in total 41 unique \textit{MYO5B} mutations (Figure 1). Attempts to identify more \textit{MYO5B} mutations retrospectively are hampered by the fact that many children that were previously reported in the literature have died and by the limited availability of patient material. The known \textit{MYO5B} mutations include 16 unique homozygous mutations in 25 patients (all but 1 from reportedly consanguineous parents), and 25 heterozygous mutations in 15 patients (all from reportedly non-consanguineous parents) (Figure 1). In 6 out of these 15 patients (40%) the second mutation was not found and, as such, the contribution of additional genes giving rise to MVID in some of the patients cannot be formally excluded. One patient with compound heterozygous \textit{MYO5B} mutations developed one \textit{MYO5B} mutation de novo as a paternal inheritance was ruled out (Szperl et al, 2011). One unpublished patient inherited one maternal and two paternal \textit{MYO5B} mutations, and one patient carried one heterozygous \textit{MYO5B} single nucleotide polymorphism. The pathological authenticity of identified \textit{MYO5B} mutations is evidenced by the absence of these mutations in non-MVID controls (Müller et al, 2008; Szperl et al, 2011), the co-segregation of \textit{MYO5B} mutations and disease through family pedigrees (Erickson et al, 2008; Müller et al, 2008; Chen et al, 2011; Szperl et al, 2011) (our unpublished data), and the reproduction of some of the cellular phenotypes associated with MVID upon reduction of myosin Vb expression in cultured intestinal epithelial cell lines (Ruemmele et al, 2010). An important additional line of evidence is the location of the mutations in protein regions of known structural or functional importance. This has received little attention but likely has implications for the precise cellular processes that will be affected and thus on phenotypic variations, as will be discussed below. In order to appreciate the implications of \textit{MYO5B} mutations for protein function and cellular consequences, the latest data on structure-function relationship of the myosin Vb protein and on the role of myosin Vb in cell biological processes are briefly discussed.
Figure 1 A) Schematic representation of the myosin Vb protein, its functional domains, and the location of known homozygous (hom), heterozygous (het) and compound heterozygous (c-het) mutations (missense in black, nonsense in red and insertions/deletions/duplications in blue text color). Protein data are deduced from GenBank RefSeq-file accession number NG_012925.1 for the human MYO5B gene. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. The differently colored boxes in the domains represent the predicted consequences for the protein (see article text). B) Schematic representation of a myosin Vb dimer and the organization of the myosin motor domain in functional subdomains. Represented in colours are the four subdomains (N-terminal: grey, U50: marine blue, L50: sand, Converter: green), the central-β-sheet in cyan, the lever arm (pale cyan helix, with a calmodulin light chain (light pink). The nucleotide-binding elements (P-loop: pale green, Switch I: magenta and switch II: orange) surrounding the nucleotide (represented in sticks), the connectors (Relay: yellow, SH1 helix: red, Strut: hot pink) between subdomains and elements of the transducer (loop I: light purple) are also depicted. The 50 kDa cleft that separates elements of the actin-binding interface is also indicated. From the Myosin Va post-rigor structure with nucleotide bound (PDB: 1W7J). C-D) The missense mutations found in the motor domain of Myosin V are depicted with the following colour code: (Green: mutations in domains that are important for actin interactions, Red: mutations in the ATP binding site, Magenta: mutations in regions of importance for the allosteric rearrangements of the myosin head during the kinetic cycle, Orange: mutations that may lead to protein misfolding
Figure 2 A) MYO5B missense mutations recurring in other myosins. Protein data are deduced from GenBank RefSeq-file accession number NG_012925.1 for the human MYO5B gene, NG_009086.1 for the human MYO7A gene, NG_023444.1 for the human MYH6 gene, NG_011537.1 for the MYH3 gene, NG_007884.1 for the MYH7 gene, and NG_013015.1 for the MYH8 gene. B) Conserved residues in the MYO5B motor domain of which the equivalent residues in other myosins have been found to be mutated in relation to human disease.

**The Myosin V motor protein**

All myosins share a common organization in functional regions (Figure 1). The N-terminal motor domain contains conserved regions able to bind the nucleotide and more variable structural elements defining the actin-binding interface (Sweeney and Houdusse 2010). The C-terminal part of the motor domain is constituted by a subdomain named converter, which rotates in response to conformational changes linked to the motor cycle. The converter swing is transmitted to the adjacent elongated region and they both constitute the lever arm of the motor, which amplifies the structural rearrangements of the motor domain and produces nanometre displacements along the actin filament. Myosin V has a particular long lever arm that contains six consecutive IQ motifs to which calmodulin can bind. It is followed by a tail region, which is highly variable, both in length and sequence, among different classes of myosins. For myosin V, a long coil-coiled region dimerizes the motor after the lever arm and is followed by a globular C-terminal tail domain known as the cargo-binding domain.
The motor domain

The myosin motor domain undergoes a characteristic cyclic interaction with actin by exploring a series of different conformations characterized by either weak or strong actin-binding states. The key steps of the kinetic cycle include the detachment of nucleotide-free actin-bound myosin (rigor state) from the track upon ATP binding, the hydrolysis of ATP and the steps of the working stroke that occur upon reattachment to F-actin which promotes the sequential release of phosphate (Pi) and ADP, leading to the rigor state. The next cycle can begin with ATP rebinding. The rates of phosphate (Pi) and ADP release determine the duty ratio (fractional occupancy of strong actin-binding states) and ultimately dictate the kinetic properties of a specific class of myosin, and thus its particular motility function (Bloemink and Geeves, 2011). Myosin Vb has been characterized as a high duty-ratio, two-headed processive motor, similarly to Myosin Va (Watanabe et al, 2006). Myosin Va has been extensively studied and shown to walk with 36 nm steps upon multiple sequential interactions with actin without detaching (Mehta et al, 1999; Yildiz et al, 2003). Fluorescence imaging at one nanometer accuracy (FIONA) showed that the two heads step using a hand-over-hand mechanism (Yildiz et al, 2003; Snyder et al, 2004). The similarity in the kinetics of Myosin Va and Vb (Watanabe et al, 2006) and the fact that the length of the lever arm is conserved for these myosin isoforms allows to predict that Myosin Vb also walks with a hand over hand processive mechanism.

The motor domain, highly conserved in all classes of myosins, is made up of four major subdomains: the N-terminal subdomain, the Upper 50 (U50), the Lower 50 (L50) and the Converter (Figure 2B). These subdomains are linked together by highly conserved linkers that can rapidly change their conformation in concert with the movements of the subdomains that are at the basis of the allosteric rearrangements of the entire head upon the motor cycle. A large cleft named 50 kDa cleft separates the U50 and L50 subdomains, which both participate in the actin binding interface. The inner part of the cleft is adjacent to the position where the ATP γ-phosphate is bound and is close to the Switch II connector. The residues of the P-loop (part of the N-terminal subdomain), switch I (part of the U50) and Switch II compose the ATP-binding site.

Changes in the closure of the cleft coordinate the actin and nucleotide binding sites. In the rigor state, full closure of the cleft creates an interface of strong affinity for actin but separates the different elements of the nucleotide-binding site, which cannot bind strongly the nucleotide. Upon ATP binding, the active site rearranges and the cleft opens, weakening the affinity of the motor for actin and allowing its detachment from the track. The transducer at the centre of the motor domain close to the ATP binding site is central for these rearrangements. It includes the last three strands of the central beta sheet as well as associated structural elements such as loop1, the beta-bulge (at the end of the last two strands) and the HO linker (that connects, via the HO helix, the actin binding interface and the nucleotide binding site). The transducer undergoes distortion to allow movements of the N-terminal and U50 subdomains, which are essential for track detachment, but it also plays key roles upon the force production events (Coureux et al, 2003; Coureux et al,
2004). These events occur on actin upon release of the hydrolysis products and control the swing of the lever arm and the duration of the force bearing states of the motor on actin. The lever arm swing is controlled by rearrangements of two connectors, the Relay and the SH1 helix, that connect the converter to the L50 and the N-terminal subdomains, respectively, substantially allowing direct communication between the nucleotide binding site, the actin binding interface and the lever arm.

**The tail domain**

In contrast to the conserved motor domain, the myosin tail domain is highly variable within different myosins of the superfamily and determines to a large extent the precise function of the motor protein in cells. Indeed, the tail domain contains targeting sites for different cellular partners that allow the motor to be recruited specifically and to carry cargoes. More precisely, via its tail domain myosin Vb dimers are recruited to a subpopulation of endosomes called recycling endosomes (RE) by directly binding to specific RE receptors including GTP-loaded (activated) small GTPases rab11a, rab11b, rab25, rab8a, rab10, and rab11-familily of interacting proteins 2 (rab11-FIP2) (Lapierre et al, 2001). An alternatively spliced exon D/exon 31 in myosin Vb is required for binding to rab10 (Roland et al, 2009; 2011). Notably, exon D is less ubiquitously expressed across tissues and, importantly, it does not appear in the intestine (Roland et al, 2009) suggesting that myosin Vb-rab10 interactions are not relevant for MVID. Binding sites in myosin Vb for rab11a have been mapped to residues Y1714 and Q1748 which are highly conserved in human MYO5A and MYO5B but not in MYO5C (Roland et al, 2011). Binding sites in myosin Vb for rab8a have been mapped to Q1300L and Y1307C in exon C/ exon 30 that abolishes binding to rab8a but not Rab11a (Roland et al, 2011). Not only myosin Vb but also myosin Va and myosin Vc have been reported to bind to rab8a, while myosin Va but not myosin Vc can bind to rab11a (Roland et al, 2011). Myosin Va and myosin Vc mRNAs are also expressed in the human small intestine (Rodriguez and Cheney, 2002). At the moment, the temporal and spatial regulation of these interactions are not yet understood, and may involve phosphorylation of myosin Vb, as has been shown for other myosins (Karcher et al, 2001).

**MVID-associated MYO5B mutations in relation to myosin Vb structure and function**

Forty-one distinct MYO5B mutations have been reported in 40 patients to date including 16 missense mutations, 20 nonsense mutations or splicing type, 4 deletions/insertions and 1 duplication (Erickson et al, 2008; Müller et al, 2008; Ruemmele et al, 2010; Chen et al, 2011; Szperl et al, 2011) (Figure 1A). Interestingly while nonsense, splice and deletions/insertions can be found either in the head or in the tail domain, all reported missense mutations – with the exception of one – are segregated to the myosin Vb motor domain. This is in striking contrast to the randomly distributed missense mutations identified in other myosins (www hgmd.com). There is no direct experimental evidence to this date that demonstrates the consequence of MVID-associated MYO5B mutations for the functioning
of the motor protein. Such data is eagerly awaited as understanding whether the motor function is totally impaired or whether the motor is just less efficient might be important to understand and describe how the phenotype, impact or onset of the disease might differ depending on the mutation. The current knowledge of myosin structures in the motor cycle and the role of specific domains and residues allows for some of the point mutations to be localized to domains with established roles in myosin protein function. Based on the crystal structures of the motor domain of chicken Myosin Va in the nucleotide-free ((Coureux et al, 2003); PDB code: 1OE9) or the Mg-ATP bound states ((Coureux et al, 2004); PDB 1W7J), we have modelled the reported mutations in the three-dimensional protein structure and categorized these in relation to the myosin Vb structural/functional domains (Figure 1C, D). We can distinguish five categories: 1) missense mutations in domains that are important for actin interactions, 2) missense mutations in the ATP-binding site, 3) missense mutations in domains of importance to allosteric rearrangements of the motor, 4) mutations that may lead to protein misfolding, and 5) mutations leading to a Premature Terminated Protein (PMT). The categorized MYO5B mutations, described according to the latest checklist provided by the Human Genome Variation Society (www.hgvs.org) (den Dunnen and Antonarakis, 2000), are briefly discussed below.

Missense mutations in domains important for actin interactions

In the motor domain, three currently identified Myosin Vb missense mutations are located near or in the actin-binding interface: c.1540C>T (p.Cys514Arg) (Szperl et al, 2011), and two that we report in this article: namely c.1591C>T (p.Arg531Trp) and c.1856C>T (p.Pro619Leu), from a Dutch male and Dutch female patient, respectively). Cys514 is an internal residue of L50, close to the actin-binding interface. Transitions in this region are necessary for the myosin to change its affinity for actin along the motor cycle. Arg531 in the L50 is part of the actin interface, near the activation loop which plays a major role in the early transitions of force production (Várkuti et al, 2012). Pro619 is located in Loop2, which is of primary importance for actin binding, notably upon re-association of the motor to its track. Of interest the p.Arg531Trp and p.Pro619Leu mutations were found in a single MYO5B gene.

Missense mutations in the ATP-binding site

Missense mutations c.502G>A (p.Gly168Arg) and c.656G>A (p.Arg219His) are substituting conserved residues in the nucleotide-binding site which, like GTPases, comprises three nucleotide binding elements: P-loop, Switch I and Switch II. Gly168 lies in the P-loop, which is an essential element of the ATP-binding site, strictly conserved in the sequence of all myosins. The equivalent missense mutation, c.487G>C (p.Gly163Arg) in Myosin VIIa, has also been described to account for Usher 1B syndrome leading to the retinal pigment epithelium disease in humans (Jacobson et al, 2008). Arg219 is a very conserved residue belonging to Switch I. It is involved in a salt bridge with Switch II in the structural state that promotes ATP hydrolysis. Interestingly, mutations for the equivalent residue in Myosin
VIIa, c.635G>A (p.Arg212His) and c.634C>T (p.Arg212Cys), have also been reported in Usher 1B syndrome patients (Weston et al, 1996).

**Missense mutations in domains that are important for allosteric rearrangements of the motor**

Five missense mutations (c.323T>G (p.Val108Gly), c.946G>A (p.Arg401His), c.1367A>G (p.Arg401His), c.966C>T (p.Arg656Cys)) affect residues localized in critical regions of the motor domain, important for allosteric rearrangements. The insertion (c.1355_1363dupAGTTCTGTA (p.Cys454insKFC)) is located in the helix that follows switch II, which is important for the coupling of the rearrangements between the nucleotide binding site, the actin interface and the lever arm. p.Gly316Arg substitutes a small aliphatic residue with a large charged arginine in a loop between two helices of the U50 interacting with the transducer. p.Arg401 is located in the HO helix and the aliphatic part of the side chain contributes to the buried interactions that stabilize the U50. These interactions are important for the communication between the nucleotide- and the actin-binding site that are essential for the force bearing states of the motor. Interestingly, the equivalent mutation c.1184G>A (p.Arg395His) in Myosin VIIa has been recently described in a Pakistani DFNB2 family affected by non-syndromic severe low frequency hearing loss (Borck et al, 2011). Asn456 is strictly conserved in the myosin superfamily and it lies in the L50 close to Switch II and to the nucleotide-binding site. It has a key role in transferring the allosteric changes of Switch II to the L50. The missense mutation p.Arg656Cys affects an important conserved residue belonging to the central beta-sheet, at the interface with both the SH1 helix and the Relay, which is important for the allosteric conformational changes of the motor. The late-onset homozygous MVID-associated p.Val108Gly, located in the middle of the second strand of the central beta sheet, corresponds to a region that is near the SH1 helix whose rearrangement contributes to the lever arm swing and regulates the ability of the motor to bear force.

**Mutations that may lead to protein misfolding**

The occurrence of three missense mutations (c.428C>A (p.Ala143Glu), c.1303G>A (p.Gly435Arg), c.1979C>T (p.Pro660Leu)) that affect conserved residues localized in the motor domain might lead to protein instability and misfolding, thus affecting myosin Vb function. The p.Ala143Glu mutation introduces a polar and charged Glutamate in the N-terminal subdomain and involves a buried residue of this subdomain. The p.Gly435Arg mutation introduces a bulky charged side chain in a buried position at the core of the transducer, this would at the minimum perturb the transitions that the transducer undergo and may even prevent correct protein folding.

**Mutations leading to a Premature Terminated Protein (PMT)**

A number of nonsense mutations, deletion and splicing type mutations (Trp14Ter, Gln149Ter, Ser186Ter, Ser289Ter, Gln341Ter, Arg363Ter, Ser370ArgfsTer27, Trp375Ter, Arg749Ter,
Gly777AsnfsTer6, Gln891Ter) correspond to premature truncated head fragments that would not be able to incorporate in dimers since the coiled-coil starts at residue 910. Other mutations (Arg1016Ter, Gln1456Ter, Asp1586Ter, Gln1614Ter, Arg1795Ter) would generate truncated molecules without a cargo-binding tail domain, which is necessary for the binding to RE-associated small GTPases and other putative partners. Indeed, consistent with the reported requirement for myosin Vb to apical RE-associated rab11a and rab8 for concentrating apical RE below the apical surface in MDCK cells, the subapical accumulation of rab11a is lost in the enterocytes - from duodenum to colon - of MYO5B mutation-carrying MVID patients (Szperl et al, 2011; Golachowska et al, 2012; Talmon et al, 2012). Of interest, the Gln1456Ter, Asp1586Ter and Gln1614Ter mutations are located distal to the rab8a-binding residues but proximal to the rab11a-binding residues (Roland et al, 2011). Due to the limited structural data and folding characteristics of the myosin Vb tail domain currently available, it is not easy to predict how these truncations would affect the general folding of the tail domain (Wei et al, 2013). However these different mutant forms of Myosin Vb could lead to partial functionality if some of the interactions with other the partners binding in these regions are conserved while others are affected. This may provide a basis for the phenotypic variations in MVID. In addition, mutations that delete the tail domain or mutations that result in reduced protein expression, both of which leave the recycling endosome-associated GTPase receptors free to interact with other proteins, may cause distinct cellular phenotypes in comparison to motor domain mutations which would occupy the recycling endosome-associated GTPase receptors and thus may produce dominant-negative effects on other GTPase-binding proteins. It should be noted that, as nonsense-mediated mRNA decay in mammalian cells generally degrades mRNAs that terminate translation more than 50-55 nucleotides upstream of the next exon (Nagy and Maquat, 1998; Maquat, 2004), it is predicted that of the MYO5B mutations leading to PMT only Gln149Ter, Gln341Ter, Arg1015Ter, Gln1614Ter and Arg1795Ter produce a (truncated) protein.

**MYO5B mutations recurring in other myosin genes associated with human disease**

When examining the mutated residues in MYO5B, we found that of ~30% of the conserved residues that are substituted in the myosin Vb motor domain, mutations in the equivalent residues or position have been found in other myosins spread over different phylogenetic classes (i.e., MYO7A, MYH7, MYH6, MYH3) in patients suffering from other rare diseases (Figure 2A). Of these, the equivalent residue of the mutated Arg656 in MYO5B is mutated in three other myosins. Interestingly, myosin Vb and these other myosins have in common that they play a role in actin-rich cellular protrusions, i.e. microvilli in epithelial cells and stereocilia in the hair cells of the inner ear, or play a role in endomembrane organization (Hartman and Spudich, 2012). The comparison of clinical and experimental information of these myosins and diseases may expedite the thus far limited understanding of the pathogenesis of these diseases, and lead to a better understanding of how the different myosins cooperate to organize cellular function (Hartman and Spudich, 2012).
Given the high conservation of the motor domain in the myosin family it can be expected that more of these recurrent mutations will be found. To facilitate their identification, we have retrieved from literature 361 mutated residues in 18 different myosins associated with human diseases. 165 of these residues were found to be conserved in MYO5B and the equivalent residues in the MYO5B gene were annotated for future reference (Figure 2B).

MVID-associated MYO5B mutations in relation to morphological changes in MVID enterocytes

The identification of mutations in the MYO5B gene strongly support the earlier suggestions that defects in intracellular trafficking underlie the disease and are responsible for the morphological changes in MVID enterocytes (Cutz et al, 1989; Ameen and Salas, 2000; Golachowska et al, 2010). Indeed, RNA!-mediated knockdown of myosin Vb or overexpression of a dominant-negative MYO5B tail fragment has demonstrated the involvement of myosin Vb in the plasma membrane recycling or exocytosis of many proteins in a variety of cultured primary cells and cell lines, including kidney-derived epithelial cell (Hales et al, 2002; Nedvetsky et al, 2007; Roland et al, 2011), human airway epithelial cells (Swiatecka-Urban et al, 2007), lacrimal gland acinar cells (Xu et al, 2011), bladder umbrella cells (Khandelwal et al, 2013), muscle cells (Ishikura and Klip, 2008), hepatocytes (Wakabayashi et al, 2005), neurons (Lisé et al, 2006; Wang et al, 2008), and intestinal epithelial cells (Ruemmele et al, 2010). Directed mutagenesis and analysis in Madin-Darby canine kidney (MDCK) epithelial cells revealed that different myosin Vb/rab GTPase combinations control distinct protein trafficking steps between RE and the apical plasma membrane (Roland et al, 2011). In agreement with the only partially overlapping distribution patterns of rab11a and rab8a in epithelial cells, myosin Vb likely acts at multiple protein trafficking steps (Xu et al, 2011; Gidon et al, 2012). Based on these results it is likely that myosin Vb loss-of-function in MVID enterocytes impairs apical recycling and consequently results in a reduced expression of apical proteins at the brush border membrane. Microvillus inclusions may in a stochastic or mutation-dependent manner arise from the homotypic fusion of transport vesicles containing newly synthesized brush border proteins that have accumulated in the cytoplasm (Cutz et al, 1989; Gilbert and Rodriguez-Boulan, 1991), or arise when cell polarity is lost (Low et al, 2000). Of interest, microvillus inclusions have been reported in myosin Vb-depleted intestinal epithelial Caco-2 cells (Ruemmele et al, 2010) but are not typically observed following the perturbation of myosin Vb in cultured kidney-derived epithelial cell lines (Roland et al, 2011). This suggests that loss of myosin Vb function differentially affects epithelial cells of different organs (Golachowska et al, 2012), and underscores the need for specific model systems to study MVID pathogenesis.

Cellular and animal models for the study of MVID

Knockdown of myosin Vb expression in human intestinal epithelial Caco-2 cells using short- hairpin (sh)RNA technology was shown to recapitulate several of the morphological
hallmarks of MVID (Ruemmele et al, 2010). This will thus be a valuable cell model system to investigate the cellular mechanism underlying MVID and, by reintroducing shRNA-resistant MYO5B mutants, investigate the functional consequence of specific MVID-associated myosin Vb mutations. A MYO5B knockout mouse has not yet been reported and is eagerly awaited. Interestingly, two alternative mouse models for MVID have been reported. RAB8A knock-out (KO) mice exhibit the key symptoms and cellular defects observed in human MVID shortly after birth (Sato et al, 2007), and this seems consistent with the known interaction between rab8a-GTP and myosin Vb in epithelial cells (Roland et al, 2011). Microvilli in RAB8A KO enterocytes are shortened but not as much disorganized when compared to MVID enterocytes. RAB8A mutations have not been reported in MVID patients, although some MVID patients show reduced Rab8a mRNA expression (Sato et al, 2007). RAB8A KO mice do not develop defects in the kidney (Sato et al, 2007), which is in agreement the normal brush border morphology and Rab11a-positive RE distribution in kidney epithelial cells in at least two MVID patients with known MYO5B mutations (Golachowska et al, 2012). In addition to RAB8A knockout mice, intestine-specific CDC42 knockout mice also develop signs of MVID (Sakamori et al, 2012; Melendez et al, 2013), i.e., nutrient malabsorption, intracellular retention of the brush border protein alkaline phosphatase, and appearance of microvillus inclusions, albeit with very late onset (Sakamori et al, 2012). Rab8a activation is reduced in CDC42 KO enterocytes (Sakamori et al, 2012), suggesting that cdc42 can act upstream of rab8a. It is important to note that CDC42 KO intestines show prominent additional features, including loss of Paneth cells and mislocalization of the basolateral sodium-potassium ATPase (Sakamori et al, 2012; Melendez et al, 2013) and the adhesion proteins E-cadherin and beta-catenin (Sakamori et al, 2012), that are not typically seen in MVID patients (Ameen and Salas, 2000), and there is no evidence that cdc42 plays a role in the pathogenesis of MVID patients. It is also important to realize that microvillus inclusions are not unique to MVID and they can be seen also in epithelial cancers (Eusebi et al, 1977; Alroy et al, 1979; Remy, 1986; Hagiwara et al, 1997) and in cell-cell adhesion-deficient epithelial cells in culture (Vega-Salas et al, 1987; Vega-Salas et al, 1988; Vega-Salas et al, 1993). Likely, microvillus inclusions are a more general yet extreme trait reflecting impaired epithelial cell differentiation/polarization (Low et al, 2000). Nevertheless, the similarities in the CDC42 KO intestine and MVID/MYO5B mutant intestine, and the notion that cdc42, rab11a, rab8a and myosin Vb are all components of an intricate molecular pathway involved in apical plasma membrane development (Bryant et al, 2010) (Figure 4), advocate a screening for mutations in CDC42, RAB8A or other components of this machinery in (heterozygous) MVID patients.

**Diagnostic and clinical relevance**

The diagnosis of MVID on the basis of biopsy histology can be challenging (Mierau et al, 2001; Weeks et al, 2003; Iancu et al, 2007). MYO5B mutation analysis can be of value in the differential diagnosis of the expanding collection of rare congenital diarrheal disorders. The identification of MYO5B gene mutations allows for reliable genetic counselling
and prenatal screening, which is currently offered at different medical centres world-
wide including the University Medical Center Groningen. Successful prenatal screening
for MYO5B has been reported in MVID (Chen et al, 2011). The expansion of the MVID/
MYO5B registry will facilitate the future association of distinct MYO5B mutations to extra-
intestinal clinical manifestations which, in turn, will be invaluable in the management of
the course of the disease.

**Future prospects**

Different homozygous and heterozygous MYO5B mutations have been identified in MVID
patients that can be categorized into distinct structural and functional properties of the
myosin Vb motor protein. Myosin Vb, in turn, can interact with multiple components
of recycling endosome-associated machinery involved in apical plasma membrane
development. Together with the progressing knowledge of the workings of this machinery,
understanding how the different MYO5B mutations precisely interfere with this machinery
is expected to provide the necessary insights in genotype-phenotype relations and
improve the diagnosis, prognosis and genetic counseling of this devastating disease.
We hope that this prospective registry for microvillus inclusion disease patients and their
MYO5B mutations and accompanying review contribute to this goal.

**Acknowledgements**

This work was supported by The Dutch Digestive foundation (MLDS) (S.v.IJ., E.R.), FRM
équipes (A.H.), ANR blanche BLAN 2010 1504 01 (A.H.) and the ARC subvention fixe
(SFI20121205398) (A.H.). This study has been performed by a team belonging to the
Labex Deep: 11-LBX-0044. MAS and KJV acknowledge and thank the EU-GEN2PHEN
and EU-BioSHARE projects (FP7-HEALTH contracts 200754 and 261433) and the RP-2
project of BBMRI-NL, a Research Infrastructure financed by the Dutch government (NWO
184.021.007)
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