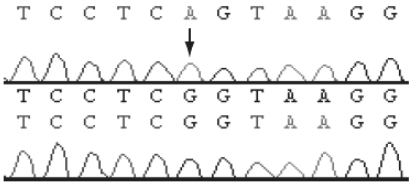
Microvillus Inclusion Disease. Lessons about the apical plasma membrane.

Golachowska, Magdalena Renata

APPENDIX 1

Supplementary Figures for Chapter 2



Patient #1:
c.946G>A
p.Gly316Arg

WT

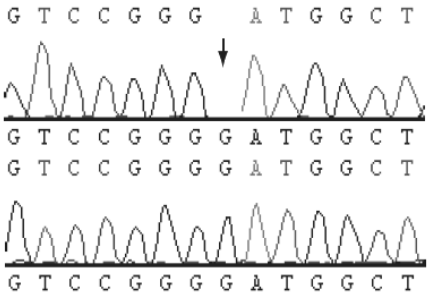
Orthologues p.Gly316

Homo sapiens MYO5B	DAEDFEKTRQAFTLLGVKESHQMSIFKIIASILHL
Pan troglodytes	DAEDFEKTRQAFTLLGVKESHQMSIFKIIASILHL
Pongo pygmaeus	DAEDFEKTRQAFTLLGVKESHQMSIFKIIASILHL
Macaca mulatta	DAEDFEKTRQAFTLLGVRESHQISIFKIIASILHL
Otolemur garnettii	DAEDFEKTRQAFALLGVRESHQISIFKIIASILHL
Tupaia belangeri	DAKDFEKTRQAFTLLGVRESHQISIFKIIASILHL
Equus caballus	DAEDFEKTRQAFTLLGVRESHQISIFKIIASILHL
Spermophilus tridecemlineatus	DAEDFEKTRQAFTLLGVRESHQISIFKIIASILHL
Loxodonta Africana	XXXXFEKTRQAFTLLGVRESHQISIFKIIASILHL
Cavia porcellus	DAEDFEKTRQAFTLLGVRESHQINIFKIIASILHL
Bos taurus	----------
Ochotona princeps	XXXXXXXXXXXXXXXXVRESHQMSIFKIIASILHL
Myotis lucifugus	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Canis familiaris	----------
Erinaceus europaeus	DAEDFEKTRQAFTLLGVRESHQISIFKIIASILHL
Rattus norvegicus	DAEDFEKTRQALTLLGVRESHQISIFKIIASILHL
Mus musculus	DADDFEKTRQALTLLGVRDSHQISIFKIIASILHL
Echinops telfairi	DAEDFEKTRQAFTLLGVRESHQMSIF-IIASILHL
Monodelphis domestica	DAEDFEKTRQAFTLLGVRESYQINIFKIIASILHL
Xenopus tropicalis	DAEDFEKTRQAFTLLGVKETHQMGIFKIVASILHL
Danio rerio	DAEDLVKTREALTMLGVKENHQMSIFKIIASILHL
Takifugu rubripes	DAEDFVKTREAFTLLGIKESTQNNVFKIIASILHL
Tetraodon nigroviridis	DAEDFVKTREGFVFFLGIKDSTQNNVFKIIASILHL
Oryzias latipes	----------
Ciona savigny	DKQEFQETVHAFTLLGVSSKHQSLIFRLLSAVLHM
Ciona intestinalis	DESEFKETIHAFTLLGVSSKHQSLVFRLLSAILHM

Paralogues

Homo sapiens MYO5A	DAKEMAHTRQACTLLGISESHQMGIFRILAGILHL
Homo sapiens MYO5C	DRAEMVETQKTFTLLGFKEDFQMDVFKILAAILHL

Supplementary Figure 1. Mutation in MYO5 gene found in Patient 1. Patient 1 carries a homozygous non-conservative missense mutation in exon 8 (c.946G>A, p.Gly316Arg), which replaces a small aliphatic glycine with a large and charged arginine in the protein's conserved head domain region. The p.Gly316 residue is evolutionary conserved in 20 species (myosin Vb orthologes), and in myosins Va and Vc.



Orthologues	p.Gly777
Homo sapiens MYO5B	RADKFRTATIMIQKTVRGWLQKVKYHRLKGATLTL
Pan troglodytes	RADKFRTATIMIQKTVRGWLQKVKYRRLKGATLTL
Pongo pygmaeus	RADKFRTATIMIQKTVRGWLQKVKYRRLKGATLTL
Macaca mulatta	RADKFRTATIMIQKTVRGWLQKVKYRRLKGATLTL
Otolemur garnettii	RADRFRAATIMIQKTVRGWLQKVKYHRLKGATLTL
Tupaia belangeri	RADKFRAATIMIQKTVRGWLQKVKYRRLKKATLTL
Equus caballus	RADKFRAATIMIQKTVRGWLQRVKYRRLKWATLTL
Spermophilus tridecemlineatus	RADKFRAATIMIQKTVRGWLQKVKYRRLKRATLTL
Loxodonta Africana	RADKFRAATIMIQKTVRGWLQKVKYRRLKRATLTL
Cavia porcellus	RADKFRAATIMIQKTVRGWLQRVKYNRLKKATVTL
Bos taurus	RADKFRAATIMIQKAVRGWLQKVKYRRLKGATLIL
Ochotona princeps	RADKFRAATIMIQKTVRGWLQRVKYRRLKGAALTL
Myotis lucifugus	RADKFRAATITIQKTVRGWLQRVKYRRLKGATLTL
Canis familiaris	----------
Erinaceus europaeus	RADKFRAATIMIQKTVRGWLQRVKYQRLKGATLTL
Rattus norvegicus	RADKFREATIMIQKTVRGWLQRVKYRRLRAATLTL
Mus musculus	RADKFREATIMIQKSVRGWLQRVKYRRLRAATLSL
Echinops telfairi	RADRFRAATIMIQKTVRGWLQKVKYRRLKRATITL
Monodelphis domestica	RADKFRAATIMIQKTVRGWLQKVKYRRLRGATLTL
Xenopus tropicalis	----------
Danio rerio	RADKFRFACIKIQKTVRGWLQRIRYRKIRKSAITL
Tetraodon nigroviridis	RADKFRAACIKIQKTVRGWLQRVRYRKIYRAAVTL
Takifugu rubripes	RADKFRAACIKIQKTVRGWLQRIRYRKICKAAITL
Oryzias latipes	----------
Ciona savigny	RADKFRTATIMIQKNVRMWLYRNKYIRMKRSAVII
Ciona intestinalis	RANKLRACAVIIQKNTRMWLQYKRYIRMKQSAIVV

Paralogues	
Homo sapiens MYO5A	RADKLRAACIRIQKTIRGWLLRKKYLRMRKAAITM
Homo sapiens MYO5C	RLDKLRQSCVMVQKHMRGWLQRKKFLRERRAALII

Supplementary Figure 2. Mutation in MYO5 gene found in Patient 2 and Patient 3. Patients 2 and 3 (siblings) share a homozygous deletion in exon 19 (c.2330_del G), which disturbes the reading frame and leads to a premature stop codon (p.Gly777AsnfsX6) in the first calmodulin-binding IQ1 motif of myosin Vb. The p.Gly777 residue is evolutionary conserved in 21 species (myosin Vb orthologes), and in myosins Va and Vc.

Patient 2 and 3

c.2330_delG
p.Gly777AsnfsX6

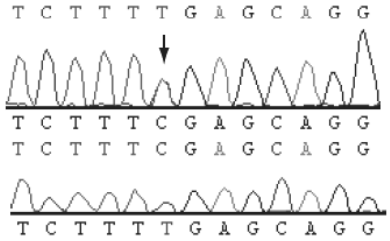
WT

```
2300 CCACCATCATGATCCAGAAAACTGTCCGGGGATGGCTGCAGAAGGTGAAATATCACAGGC
 767 A--T--I--M--I--Q--K--T--V--R--G--W--L--Q--K--V--K--Y--H--R—
```

Mutated

```
2300 CCACCATCATGATCCAGAAAACTGTCCGGGATGGCTGCAGAAGGTGAAATATCACAGGC
 767 A--T--I--M--I--Q--K--T--V--R--D--G--C--R—-R--X-
```

Supplementary Figure 3. Deletion in exon 19 ofMYO5b shared by Patients 2 and 3. The c.2330_del G variant of exon 19 in Patients 2 and 3 disturbs the reading frame and leads to a premature stop codon in the first calmodulin-binding IQ1 motif of myosin Vb.



Orthologues

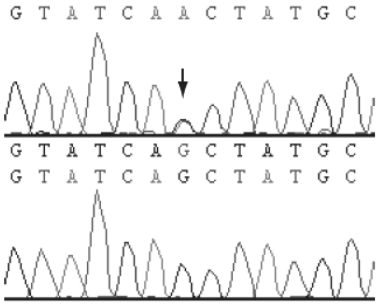
p.Arg749

Homo sapiens MYO5B	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRTAT
Pan troglodytes	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRTAT
Pongo pygmaeus	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRTAT
Macaca mulatta	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRTAT
Otolemur garnettii	XXDPDKFQFGRTKIFFRAGQVAYLEKLRADRFRAAT
Tupaia belangeri	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Equus caballus	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Spermophilus tridecemlineatus	XXDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Loxodonta Africana	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Cavia porcellus	IRDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Bos taurus	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Ochotona princeps	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Myotis lucifugus	IKN-DK-QFGRTKI-FRAGQVAYLEKLRADKFRAAT
Canis familiaris	----------
Erinaceus europaeus	XXDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Rattus norvegicus	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFREAT
Mus musculus	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFREAT
Echinops telfairi	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADRFRAAT
Monodelphis domestica	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAAT
Xenopus tropicalis	----------
Danio rerio	IKDPDKFQFGKTKIFFRAGQVAYLEKLRADKFRFAC
Tetraodon nigroviridis	IKEPDMFQFGKTKIFFRAGQVAYLEKLRADKFRAAC
Takifugu rubripes	IKGTRHVQFGKTKIFFRAGQVAYLEKIRADKFRAAC
Oryzias latipes	----------
Ciona savigny	IPEADKYQPGKNKIFFRAGQVAYLEKLRADKLRSCA
Ciona intestinalis	IPEEDKYQPGKNKIFFRAGQVAYLEKLRANKLRACA

Paralogues

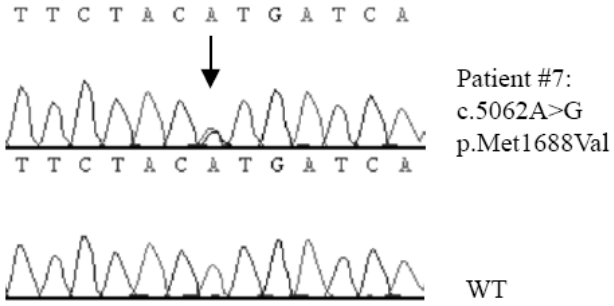
Homo sapiens MYO5A	ILDKDKYQFGKTKIFFRAGQVAYLEKLRADKLRAAC
Homo sapiens MYO5C	IQDSNQYQFGKTKIFFRAGQVAYLEKLRLDKLRQSC

Supplementary Figure 4. Mutation in MYO5 gene found in Patient 6. Patient 6 carries one heterozygous mutation in exon 19 which results in a premature stop codon (c.2246C>T, p.Arg749X) in the head domain of myosin Vb. The p.Arg749 is conserved in 23 species (myosin Vb orthologs) and in myosin Va and Vc.



Orthologues	p.Asn456
Homo sapiens MYO5B	EVNSFEQFCINYANEKLQQQF
Pan troglodytes	EVNSFEQFCINYANEKLQQQF
Pongo pygmaeus	EVNSFEQFCINYANEKLQQQF
Macaca mulatta	EVNSFEQFCINYANEKLQQQF
Microcebus marinus	EVNSFEQFCINYANEKLQQQF
Otolemur garnettii	EVNSFEQFCINYANEKLQQQF
Tupaia belangeri	EVNSFEQ CI YANEKLQQQF
Felis catus	EVNSFEQFCINYANEKLQQQF
Equus caballus	EVNSFEQFCINYANEKLQQQF
Spermophilus tridecemlineatus	EVNSFEQFCINYANEKLQQQF
Loxodonta Africana	EVNSFEQFCINYANEKLQQQF
Dasypus novemcinetus	EVNSFEQFCINYANEKLQQQF
Cavia porcellus	EVNSFEQFCINYANEKLQQQF
Bos taurus	EVNSFEQFCINYANEKLQQQF
Ochotona princeps	EVNSFEQFCINYANEKLQQQF
Myotis lucifugus	XXXXXXXXXXXXXXXXXXXXX
Canis familiaris	EVNSFEQFCINYANEKLQQQF
Erinaceus europaeus	EVNSFEQFCINYANEKLQQQF
Rattus norvegicus	EINSFEQFCINYANEKLQQQF
Mus musculus	EINSFEQFCINYANEKLQQQF
Echinops telfairi	ESNSFEQFCINYANEKLQQQF
Monodelphis domestica	EVNSFEQFCINYANEKLQQQF
Ornithorhynchus anatinus	EVNSFEQFCINYANEKLQQQF
Gallus gallus	NLNSFEQFCINYANEKLQQQF
Danio rerio	EINSFEQFCINYANEKLQQQF
Tetraodon nigroviridis	EVNSFEQFCINYANEKLQQQF
Takifugu rubripes	DINSFEQFCINYANEKLQQQF
Oryzias latipes	ERNSFEQFCINYANEKLQQQF
Gasterosteus aculeatus	NFKTFKSSFCLSAASRRLQQF
Ciona savigny	EVNSFEQFCINYANEKLQQQF
Ciona intestinalis	ENNSFEQFCINYANEKLQQQF
Paralogues	
Homo sapiens MYO5A	EINSFEQFCINYANEKLQQQF
Homo sapiens MYO5C	DVNSFEQFCINYANEKLQQQF

Supplementary Figure 5. Mutation in paternal allel of MYO5 gene found in Patient 7. Patient 7 reveals a compound heterozygous mutation, which includes a paternal allele with a non-conservative asparagine-to-serine (c.1367A>G, p.Asn456Ser) substitution in exon 11 of the head domain. The p.Asn456 is evolutionary conserved among 28 species (MyosinVb orthologs), and in myosin Va and Vc.

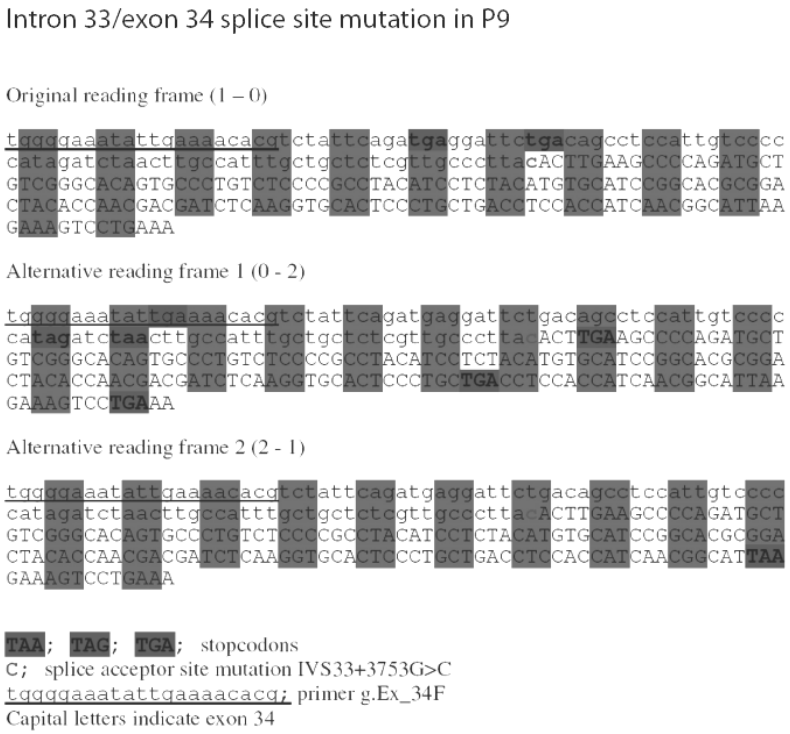


Orthologues	p.Met1688
Homo sapiens MYO5B	L-QVFKQLFYMINAVTLNNLL
Pan troglodytes	L-QVFKQLFYMINAVTLNNLL
Pongo pygmaeus	L-QVFKQLFYMINAVTLNNLL
Macaca mulatta	L-QVFKQLFYMINAVTLNNLL
Otolemur garnettii	Q-QVFKQLFYMINAVTLNNLL
Tupaia belangeri	L-QVFKQLFYMINAVTLNNLL
Equus caballus	L-QVFKQLFYMINAVTLNNLL
Spermophilus tridecemlineatus	L-QVFKQLFYMINAVTLNNLL
Loxodonta Africana	Q-QVFKQLFYMINAVTLNNLL
Cavia porcellus	L-QVFKQLFYMINAVTLNNLL
Bos taurus	L-QVFRQLFYMINAVTLNNLL
Ochotona princeps	IQQVFKQLFYVINAVTLNNLL
Myotis lucifugus	L-QVFKQLFYMINAVTLNNLL
Canis familiaris	L-QVFKQLFYMINAVTLNNLL
Erinaceus europaeus	L-QVFKQLFYMINAVTLNNLL
Rattus norvegicus	L-QVFKQLFYMINAVTLNNLL
Mus musculus	L-QVFKQLFYMINAVTLNNLL
Echinops telfairi	IQQVFKQLFYMVNAVTLNNLL
Monodelphis domestica	IQQVFKQLFYMINAVTLNNLL
Xenopus tropicalis	IQQVFKQLFYMINAVTLNNLL
Danio rerio	SQQVLRQLFYSINAVTLNNLL
Tetraodon nigroviridis	VGQVVKQLFHCINAVTLNNLL
Takifugu rubripes	VGQVVKQLFHCINAVTLNNLL
Oryzias latipes	MEQAFQQLTYLICASAINSLL
Ciona savigny	IRQVFRQVFYCITAHTLNNQL
Ciona intestinalis	IKQIFRQIFK-ITSHTVNNQL
Paralogues	
Homo sapiens MYO5A	K-QVVKQMFYIIGAITLNNLL
Homo sapiens MYO5C	R-QAVKQLFFLIGAVTLNSLF

Supplementary Figure 6. Mutation in maternal allel of MYO5 gene found in Patient 7. Patient 7 reveals a compound heterozygous mutation, which includes a missense variant p.Met1688Val (c.5062A>G) in exon 37. The p.Met1688Val represents an infrequent polymorphism, as it was found in Polish and Dutch controls with allele frequencies of 5.8% (6/104) and 1.7% (2/116), respectively. The p.Asn456 is evolutionary conserved among 19 species (*MYO5B* orthologs), but is substituted in *MYO5A* and *MYO5C*.

Supplementary Figure 7. Identification of the cryptic splice acceptor sites in the intronic sequence upstream of the c.4460-1G>C mutation and exon 34 in Patient 9. The upper panel shows the manually annotated consensus splice acceptor (SA) sites, while the lower panel indicates those selected by the software tool from www.fruitfly.org. Upper panel: SA dinucleotide AG (yellow), consensus SA (pink), sequence primer g.E_34F (green). Lower panel: Software selected consensus SA (yellow) with hing ranking scores in bold.

Supplementary Figure 7. is available at:
http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbiologie/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index



Supplementary Figure 8. The sequence of the cDNA of Patient 9 with the splice site mutation c.4460-1G>C, downstream of the sequence primer g.Ex_34F in intron 34 and including exon 34. Since the start position of the inclusion of intron 33 was not known, all possible reading frames were examined for stop codons. In total, nine stop codons were present (indicated in red), with premature termination affecting exon 34 in all possible three reading frames.

Supplementary Table 1. Sequences of primers used for sequencing of myosin Vb gene, designed with Primer3 software.

Supplementary Table 1. is available at:
http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbiologie/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index

Name	Sequence(5'-3')	Original length (bp)	Annealing temperature
c.Ex31F c.Ex35R	GGCTGACCAACGAGAATCTG GCGGCAGGTGTTGGATAAC	464	60°C
c.Ex31F c.Ex36R	GGCTGACCAACGAGAATCTG CTGACGGTATTCGGTGAGGT	575	60°C
g.Ex34F c.Ex35R	TGGGGAAATATTGAAAACACG GCGGCAGGTGTTGGATAAC	307	60°C

Supplementary Table 2. Primers used for *MYO5B* cDNA amplification.

Supplementary Table 3. The homozygosity mapping of Patient 8.
Homozygous regions over considerable genetic distances and physical sizes were located.

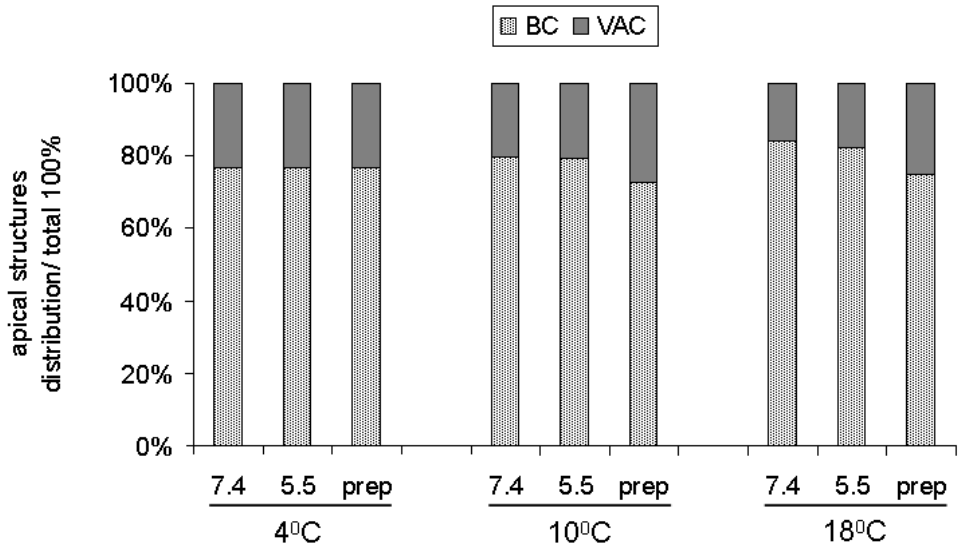
Supplementary Table 3 is available at:
http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbiologie/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index

Gene	Variation	Amino acid	ID in SNP database	Minor allele frequency in NCBI, db SNP build 129	Comments
RAB11A	c.237-19 A>G c.1627 C>T c.3892 C>T	- - -	- - rs1469184	- - T- 0.058	Intron 3'UTR 3'UTR
RAB11FIP1	c.1952 C>T c.3552 C>A c.3554 T>C c.7088 G>T	p.A651V p.A1184A p.M1185T -	rs12541651 rs7820872 rs7817179 rs3192596	C- 0.3 A- 0.25 T- 0.23 G- 0.25	Tiny>small Synonymous Nonpolar >polar 3'UTR
RAB11FIP3	c.2017- 4 T>C c.2115 T>C c.2157+23 G>A	- p.S705S	rs11863276 rs11537754 rs2071978	- T- 0.34 G- 0.41	Intron Synonymous Intron
RAB11FIP5	c.4761 A>G c.4881 C>T c.5011 A>G c.5027 C>A	- - - -	rs1046183 rs1046186 rs1992133 rs17008654	G- 0.117 - G- 0.23 A- 0.08	3'UTR 3'UTR 3'UTR 3'UTR

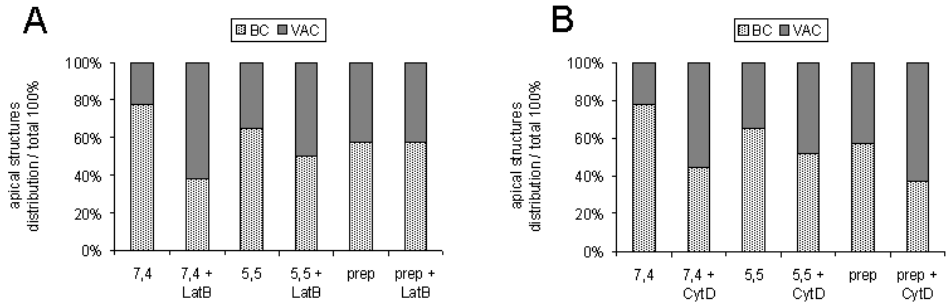
Supplementary Table 4. Results of the gene sequencing of several apical recycling-related proteins. The sequence analysis of the genes *RAB11A*, *RAB11-FIP1*, *RAB11-FIP3,* and *RAB11-FIP5* in Patients 7, 8 and 9, revealed no functional mutations in the coding regions. Several single nucleotide polymorphisms (variations) were detected, of which two in the *RAB11A* were not reported previously in the database.

APPENDIX 2

Supplementary Figures for Chapter 4

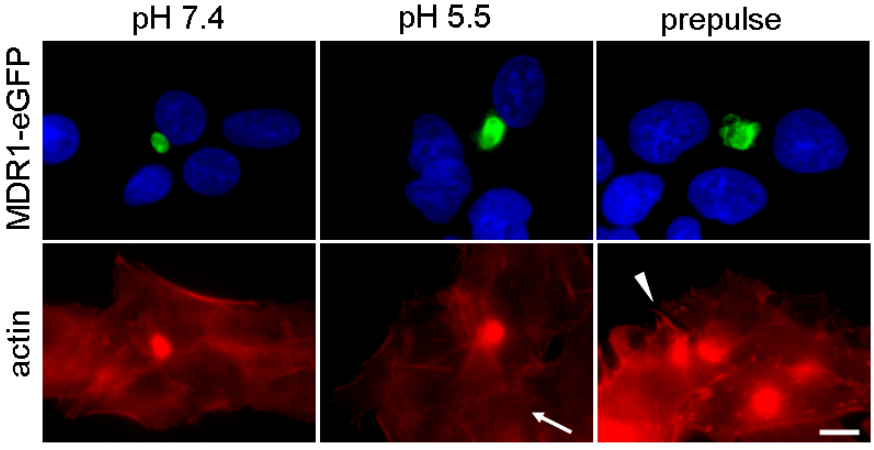


Supplementary Figure 1. The acid-induced internalization of apical membrane does not occur in temperatures below 18°C. The HepG2-MDR1-eGFP cells were cultured on glass coverslips for 72h in normal conditions, followed by 2h-long acidification (pH 5.5 and ammonia prepulse) at 4°C, 10°C and 18°C. Cells were then fixed, stained for actin and nucleus, and analyzed under fluorescent microscope. Dependence on temperature indicates the acid-induced internalization of apical membrane as an active process.



Supplementary Figure 2. The actin disrupting agents, latrunkulin B and cytochalasin D enhances the apical membrane internalization in neutral pH 7.4 and in acidic conditions.

The HepG2-MDR1-eGFP cells were cultured on glass coverslips for 72h, and acidified for 2h in pH 5.5 and by ammonia prepulse in the presence of (A) latrunkulin B (20µM) and (B) cytochalasin D (20µg/ml). Cells were then fixed, stained for actin and nucleus, and analyzed under fluorescent microscope. Compounds that affect actin dynamics (also jasplakinolide, see Fig. 7) enhance the apical membrane internalization in acidic conditions (pH 5.5 and prepulse) and in neutral pH 7.4.



Supplementary Figure 3. The acidification of cytoplasm affects the cortical actin organization.

The HepG2-MDR1-eGFP cells were cultured on glass coverslips for 72h, and acidified for 2h in pH 5.5 medium and in ammonia prepulse, fixed and stained for actin (phalloidin-TRITC) and nucleus (DAPI), and analyzed under fluorescent microscope. The acid treatment induces the formation of stress fibers (arrow) and spikes (arrowhead). Scale bar 5µm**.**

Appendix 2

SUPPLEMENTARY MOVIES

The HepG2 cells stably expressing the apical protein MDR1-eGFP were cultured on LabTek II-chambered Coverglass for 60h in normal medium. Then the medium was refreshed (control, Movie A) or changed to pH 5.0 (Movies B-E), chambers were placed under confocal microscope suited for life imaging (37^oC, 5% CO_2), and the cells were photographed every 5 minutes during 16 hours long incubation.

Movie A
HepG2-MDR1-eGFP cells kept in control conditions in pH 7.4. Note the pulsating behavior of the bile canaliculi that represents the cyclic activation of actin and myosin II (ref). Scale bar 5μm.

Movie B
HepG2-MDR1-eGFP cells incubated in pH 5.0 show thinning and inward expansion of the bile canaliculi apical surface. Note the heterogeneity of the cells' response – one cell show dramatic changes in apical membrane structure (upper cell), while the other cell, participating in the same BC (bottom cell), seems to be less affected. Scale bar 5μm.

Movie C
The acidic environment (pH 5.0) of the HepG2-MDR1-eGFP cells promotes apical membrane flattening that, in 54% cases, is followed by invaginations of apical-positive membrane towards the cell interior. Note that the invaginating apical-marker-positive membrane stay continuous with apical domain, the formation and "growing" of the vacuole is relatively slow and may be spread over several hours (up to 9h). Scale bar 5μm.

Movie D
In some cases the acid-induced (pH 5.0) invagination of the apical membrane of HepG2-MDR1-eGFP cells may "grow" into a vacuole detaching from the apical domain, and form a separate VAC. Scale bar 5μm.

Movie E
The vacuoles formed by detachment from the apical membrane upon the acid treatment (pH 5.0) might undergo a homotypic fusion, giving rinse to larger apically-derived VACs. Scale bar 5μm.

Supplementary MOVIES are available at:
http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbiologie/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index