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## Microvillus Inclusion Disease. Lessons about the apical plasma membrane.

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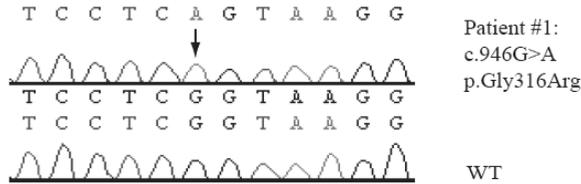
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# **APPENDIX 1**

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## **Supplementary Figures for Chapter 2**

## Appendix 1



### Orthologues

### p.Gly316

<i>Homo sapiens MYO5B</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVKESHQMSIFKIIASILHL
<i>Pan troglodytes</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVKESHQMSIFKIIASILHL
<i>Pongo pygmaeus</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVKESHQMSIFKIIASILHL
<i>Macaca mulatta</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Otolemur garnettii</i>	DAEDFEKTRQAF <sup>11</sup> FALLGVRESHQISIFKIIASILHL
<i>Tupaia belangeri</i>	DAKDFEKTRQAF <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Equus caballus</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Spermophilus tridecemlineatus</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Loxodonta Africana</i>	XXXXFEKTRQAF <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Cavia porcellus</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESHQINIFKIIASILHL
<i>Bos taurus</i>	-----
<i>Ochotona princeps</i>	XXXXXXXXXXXXXXXXXVRESHQMSIFKIIASILHL
<i>Myotis lucifugus</i>	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
<i>Canis familiaris</i>	-----
<i>Erinaceus europaeus</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Rattus norvegicus</i>	DAEDFEKTRQAL <sup>11</sup> TLLGVRESHQISIFKIIASILHL
<i>Mus musculus</i>	DADDFEKTRQAL <sup>11</sup> TLLGVRD <sup>11</sup> SHQISIFKIIASILHL
<i>Echinops telfairi</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESHQMSIF-IIASILHL
<i>Monodelphis domestica</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVRESYQINIFKIIASILHL
<i>Xenopus tropicalis</i>	DAEDFEKTRQAF <sup>11</sup> TLLGVKETHQMGIFKIVASILHL
<i>Danio rerio</i>	DAEDLVKTREAL <sup>11</sup> TMLGVKENHQMSIFKIIASILHL
<i>Takifugu rubripes</i>	DAEDFVKTREAF <sup>11</sup> TLLGIKESTQNNVFKIIASILHL
<i>Tetraodon nigroviridis</i>	DAEDFVKTREGFV <sup>11</sup> FLGIKDSTQNNVFKIIASILHL
<i>Oryzias latipes</i>	-----
<i>Ciona savigny</i>	DKQEPQETVHAFT <sup>11</sup> L <sup>11</sup> GVSSK <sup>11</sup> HQSLIFRLLSAVLHM
<i>Ciona intestinalis</i>	DESEPKETIHAFT <sup>11</sup> L <sup>11</sup> GVSSK <sup>11</sup> HQSLVFRLLSAILHM

### Paralogues

<i>Homo sapiens MYO5A</i>	DAKEMAHTRQACTLL <sup>11</sup> GISESHQMGIFRILAGILHL
<i>Homo sapiens MYO5C</i>	DRAEMVETQKTF <sup>11</sup> L <sup>11</sup> GVKED <sup>11</sup> QMDVFKILAAAILHL

**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 orthologues), and in myosins Va and Vc.



## Appendix 1

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### Patient 2 and 3

c.2330\_delG  
p.Gly777AsnfsX6

#### WT

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

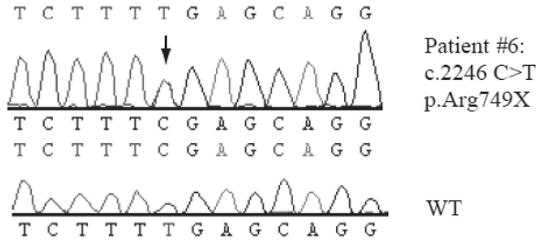
#### Mutated

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

---

**Supplementary Figure 3. Deletion in exon 19 of MYO5b 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.

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Orthologues

p.Arg749

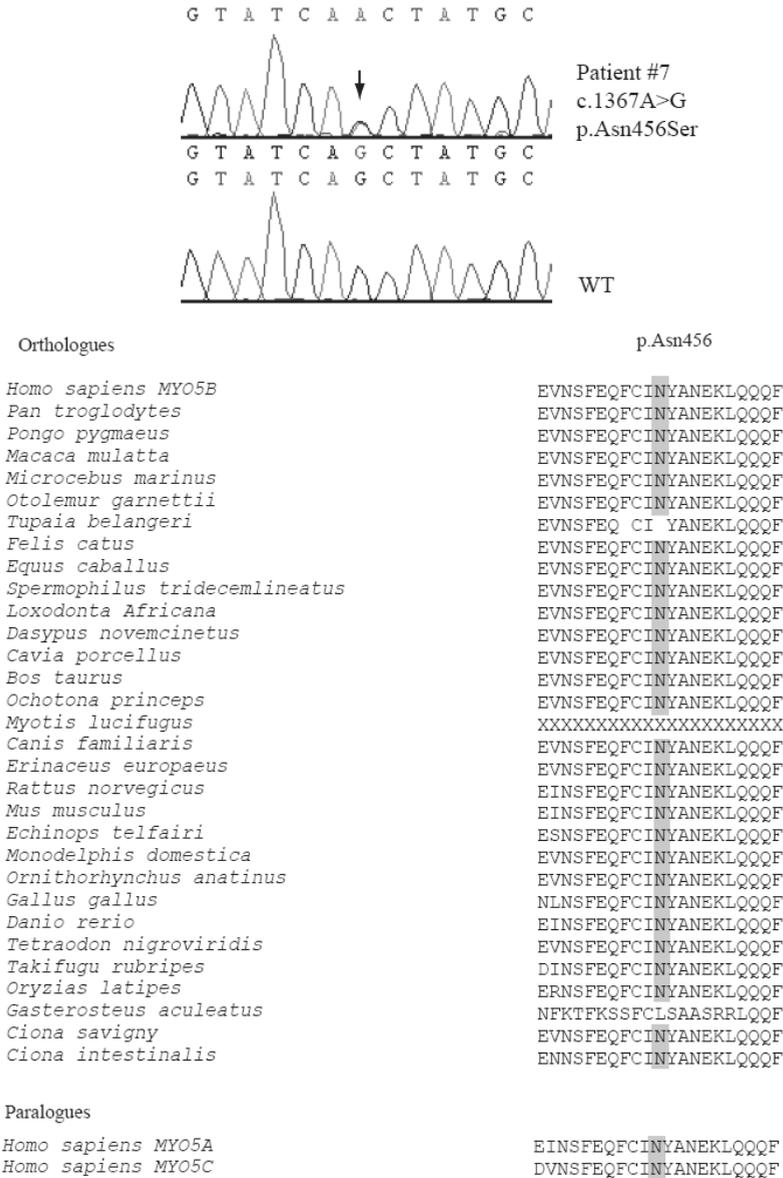
<i>Homo sapiens MYO5B</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Pan troglodytes</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Pongo pygmaeus</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Macaca mulatta</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Otolemur garnettii</i>	XXDPDKFQFGRTKIFFRAGQVAYLEKLRADRFRAAT
<i>Tupaia belangeri</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Equus caballus</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Spermophilus tridecemlineatus</i>	XXDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Loxodonta Africana</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Cavia porcellus</i>	IRDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Bos taurus</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Ochotona princeps</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Myotis lucifugus</i>	IKN-DK-QFGRTKI-FFRAGQVAYLEKLRADKFRAT
<i>Canis familiaris</i>	-----
<i>Erinaceus europaeus</i>	XXDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Rattus norvegicus</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFREAT
<i>Mus musculus</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFREAT
<i>Echinops telfairi</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADRFRAAT
<i>Monodelphis domestica</i>	IKDPDKFQFGRTKIFFRAGQVAYLEKLRADKFRAT
<i>Xenopus tropicalis</i>	-----
<i>Danio rerio</i>	IKDPDKFQFGKTKIFFRAGQVAYLEKLRADKFRFAC
<i>Tetraodon nigroviridis</i>	IKEDPMFQFGKTKIFFRAGQVAYLEKLRADKFRAC
<i>Takifugu rubripes</i>	IKGTRHVQFGKTKIFFRAGQVAYLEKLRADKFRAC
<i>Oryzias latipes</i>	-----
<i>Ciona savigny</i>	IPEADKYQPGKNIFFRAGQVAYLEKLRADKLRSCA
<i>Ciona intestinalis</i>	IPEADKYQPGKNIFFRAGQVAYLEKLRANKLRACA

Paralogues

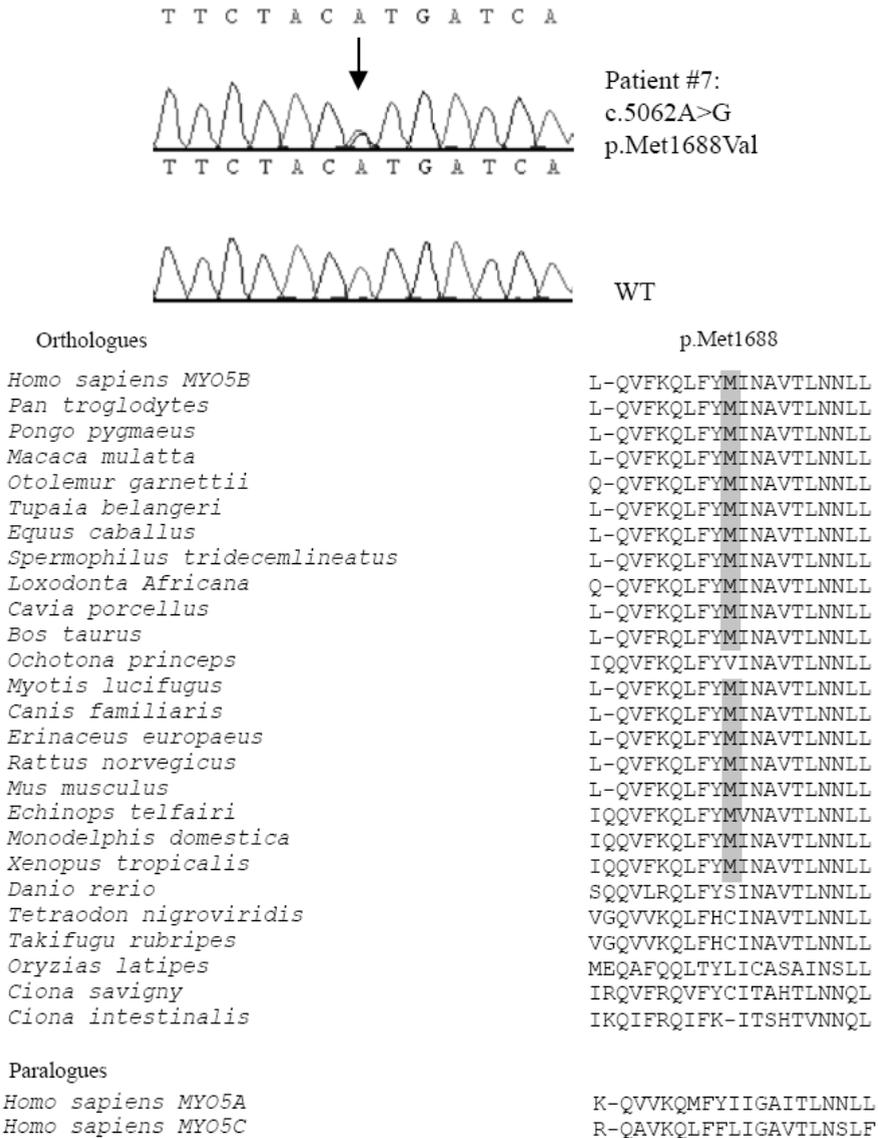
<i>Homo sapiens MYO5A</i>	ILDLDKYQFGKTKIFFRAGQVAYLEKLRADKLRAC
<i>Homo sapiens MYO5C</i>	IQDSNQYQFGKTKIFFRAGQVAYLEKLRDLKLRQSC

**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 orthologues) and in myosin Va and Vc.

## Appendix 1



**Supplementary Figure 5. Mutation in paternal allele 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.



**Supplementary Figure 6. Mutation in maternal allele 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*.

## Appendix 1

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**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](http://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/membraancelbio/onderzoekslijnen/cell\\_polarity\\_and\\_membrane\\_trafficking/dissertations/golachowska-m-suppinfo/index](http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbio/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index)

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### Intron 33/exon 34 splice site mutation in P9

Original reading frame (1 – 0)

```
tggggaatattgaaaaacacgctcattcagatgaggattctgacagcctccattgtccdc
catagatcctaacttggcatttgcctgctctcgttgcccttacACTTGAAGCCCAGATGCT
GTGGGCACAGTGCCCTGTCTCCCGCTACATCCTCTACATGTGCATCCGGCACGCGGA
CTACACCAACGACGATCTCAAGGTGCACCTCCCTGACTCCACCATCAACGGCATTAA
GAAAGTCCTGAAA
```

Alternative reading frame 1 (0 - 2)

```
tggggaatattgaaaaacacgctcattcagatgaggattctgacagcctccattgtccdc
catagatcctaacttggcatttgcctgctctcgttgcccttacACTTGAAGCCCAGATGCT
GTGGGCACAGTGCCCTGTCTCCCGCTACATCCTCTACATGTGCATCCGGCACGCGGA
CTACACCAACGACGATCTCAAGGTGCACCTCCCTGACTCCACCATCAACGGCATTAA
GAAAGTCCTGAAA
```

Alternative reading frame 2 (2 - 1)

```
tggggaatattgaaaaacacgctcattcagatgaggattctgacagcctccattgtccdc
catagatcctaacttggcatttgcctgctctcgttgcccttacACTTGAAGCCCAGATGCT
GTGGGCACAGTGCCCTGTCTCCCGCTACATCCTCTACATGTGCATCCGGCACGCGGA
CTACACCAACGACGATCTCAAGGTGCACCTCCCTGACTCCACCATCAACGGCATTAA
GAAAGTCCTGAAA
```

**TAA**; **TAG**; **TGA**; stopcodons

C; splice acceptor site mutation IVS33+3753G>C

tggggaatattgaaaaacacg; primer g.Ex\_34F

Capital letters indicate exon 34

---

**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.

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**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](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	GGCTGACCAACGAGAATCTG	464	60°C
c.Ex35R	GCGGCAGGTGTTGGATAAC		
c.Ex31F	GGCTGACCAACGAGAATCTG	575	60°C
c.Ex36R	CTGACGGTATTCGGTGAGGT		
g.Ex34F	TGGGGAAATATTGAAAACACG	307	60°C
c.Ex35R	GCGGCAGGTGTTGGATAAC		

**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](http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbiologie/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index)

## Appendix 1

Gene	Variation	Amino acid	ID in SNP database	Minor allele frequency in NCBI, db SNP build 129	Comments
<i>RAB11A</i>	c.237-19 A>G c.1627 C>T c.3892 C>T	- - -	- - rs1469184	- - T- 0.058	Intron 3'UTR 3'UTR
<i>RAB11FIP1</i>	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
<i>RAB11FIP3</i>	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
<i>RAB11FIP5</i>	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.

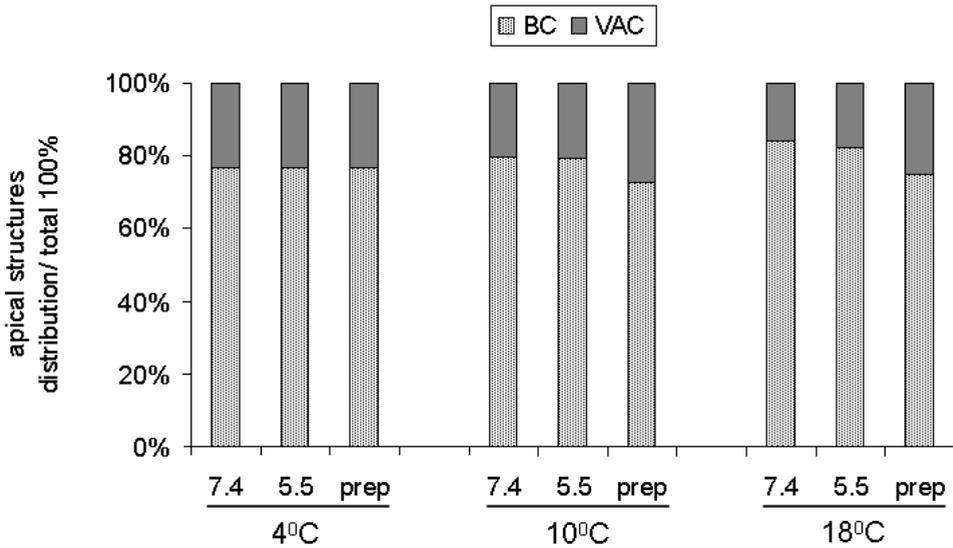
## **APPENDIX 2**

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### **Supplementary Figures for Chapter 4**

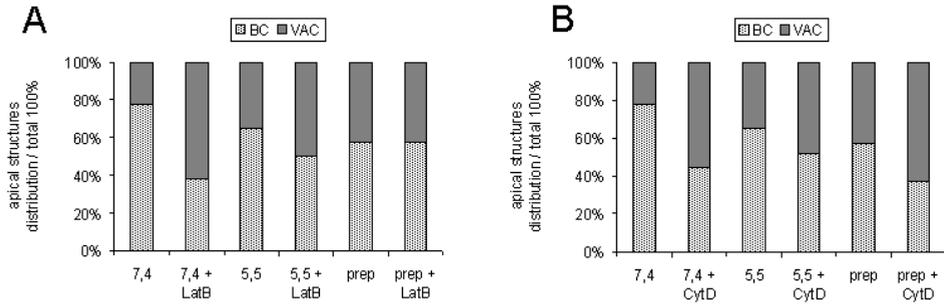
## Appendix 2

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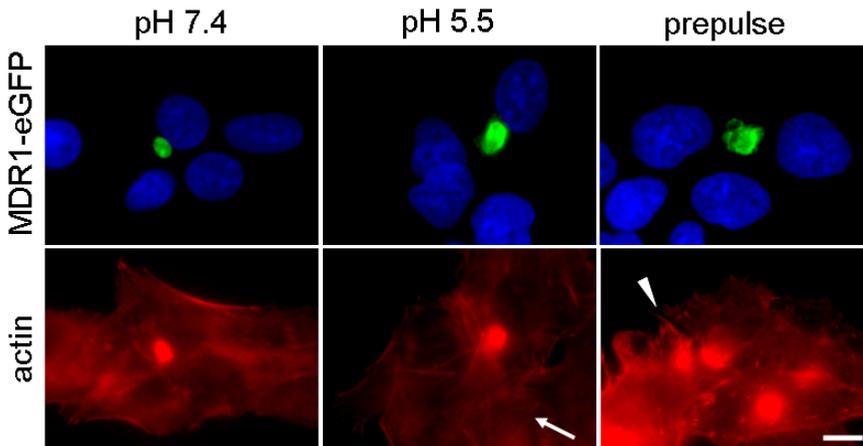
**Supplementary Figure 1. The acid-induced internalization of apical membrane does not occur in temperatures below 18°C.** The HepG2-MDR1-eGFP cells were cultured on glass coverslips for 72h in normal conditions, followed by 2h-long acidification (pH 5.5 and ammonia prepulse) at 4°C, 10°C and 18°C. Cells 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.

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**Supplementary Figure 2. The actin disrupting agents, latrunculin 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) latrunculin B (20 $\mu$ M) and (B) cytochalasin D (20 $\mu$ g/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 5 $\mu$ m.

## Appendix 2

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### 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°C, 5% CO<sub>2</sub>), 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 rise 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](http://www.rug.nl/umcg/faculteit/disciplinegroepen/celbiologie/membraancelbiologie/onderzoekslijnen/cell_polarity_and_membrane_trafficking/dissertations/golachowska-m-suppinfo/index)