Supporting material and methods

Ethical considerations

This study was approved by the medical ethical committee of the University Hospital of Leuven. Informed consent was obtained for all patients, controls and parents.

Immunoblotting

Proteins were separated by SDS-PAGE electrophoresis and blotted onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences UK limited, Bucks HP79NA, UK), according to the routine protocols. Incubations were performed overnight at 4°C using the described antibody dilutions. The signal was generated using the ECL Plus detection kit (Amersham Biosciences, UK), according to the manufacturer's instructions. Signal detection was performed with the Fuji Image Reader miniLAS-3000 (Fujifilm, Düsseldorf, Germany) and quantified with the Aida Image Analyzer v.4.22 software package (Raytest GmBH, Straubenhardt, Germany).

Mutation analysis

Total RNA was isolated from the primary fibroblasts of the patient, parents and control cells using the RNeasy kit (Qiagen, Hilden, Germany), cDNA was prepared using oligo-dT priming and Superscript II RNase-H reverse transcriptase (Invitrogen, Carlsbad, CA, USA), using 3µg of RNA in a total volume of 20µl. This cDNA was subsequently used for PCR, amplifying the *COG4* sequence with the primers COG4_F (5'- AGA TGG CGG ACC TTG ATT C -3') and COG4_R (5'- GTC TGG GCT GTC AGA TCT CC -3'). Genomic DNA was extracted from the primary fibroblasts from the patient, his parents and a control. PCR primers used for confirmation of the mutation were COG4_EXON18_F (5'- GAA GCC GTA GAG CAA ACA -3') and COG4_EXON18_R (5'- GTG GGG TTA TTC TGA AAG -3'). In

both PCR reactions 1µl cDNA or gDNA was used in a total volume of 50µl, containing 0,75µl 'Expand long template DNA polymerase' (Roche Applied Science, Indianapolis, IN, USA). For amplification of *COG4* from cDNA, reaction conditions were: 2 min at 95°C, 10 cycles of 10 sec at 95°C, 30 sec at 65°C (-1°C each cycle), 2 min at 68°C, followed by 30 cycles of 10 sec at 95°C, 30 sec at 55°C and 2 min at 68°C, the reaction was finished with an incubation of 7 min at 68°C.

For COG4 amplification from gDNA, reaction conditions were 3 min at 95°C, 10 cycles of 30 sec at 95°C, 45 sec at 65°C (-1°C each cycle) and 1 min at 68°C, followed by 30 cycles of 30 sec at 95°C, 45 sec at 55°C and 1 min at 68°C, and finishing the reaction with 7 min at 68°C. For the sequencing of the resulting PCR product the BigDye Terminator Ready reaction cycle sequencing kit v.3.1 (Applied Biosystems, Carlsbad, CA, USA) was used. Analysis of the results was performed on an ABI3100 Avant (Applied Biosystems, Carlsbad, CA, USA).

Indirect immunofluorescence staining

Cells were grown on glass coverslips for 48h, until 75-90% confluency. Coverslips were washed once with phosphate-buffered saline (PBS) before fixation with 4% paraformaldehyde in 0,1M sodium phosphate buffer pH7.2, during 30 minutes at room temperature. For COG staining the coverslips were washed twice with 0,1M glycine in PBS for 15 minutes. After blocking the cells for 30 minutes in 0,1% Triton 1% BSA 2% normal goat serum in PBS, the coverslips were treated with 6M urea in PBS. Subsequently the cells were washed 4 times during 5 minutes each with PBS and again incubated in the blocking solution for 15 minutes. The cells were then incubated overnight with the antibody solutions. After 3 washes of 5 minutes with PBS the Alexa488 and/or Alexa 568 conjugated secondary antibodies (Molecular Probes – Invitrogen, Carlsbad, CA, USA) diluted in blocking solution, were

applied for 1h. After extensive washing of the coverslips in PBS and distilled water they were placed on a glass slide using 6µl Mowiol.

For all other stainings, the cells were rinsed 3 times during 5 minutes with PBS after fixation and subsequently permeabilized for 5 minutes in 0,1% Triton in PBS. Next the coverslips were incubated in blocking solution (2% Fetal Calf Serum v/v, 2% Bovine Serum Albumin w/v and 0.2% gelatine w/v supplemented with 5% normal goat serum) during 1 hour, after which the antibody solutions were applied to the cells. The rest of the protocol is identical to that for the COG staining.

The stainings were visualized through an inverted Diaphot 300 (Nikon) microscope connected to a confocal microscope (MRC 1024, Bio-Rad). Data collection was achieved using LASERSHARP 3.0 (Bio-Rad, Hercules, CA94547, USA). Processing of the obtained results was performed using the ADOBE PHOTOSHOP 7.0 software package (Adobe Systems, San Jose, CA, USA).

RNAi

"ON-TARGETplus SMARTpool" oligo's were obtained from Dharmacon. Target sequences of oligo's were as follows, Oligo 1: 5'-GUG CUG AAA UCC ACC UUU A-3'; Oligo 2: 5'-GCA AAG UUC GUC AGC UUG A-3'; Oligo 3: 5'-UAA CCG GCC UCA UGA CUA G-3' and Oligo 4: 5'-UCG GAG AGC UGC AGU CAU C-3'. The GL2 luciferase RNAi oligo was used as a non-specific control. HeLa cells were plated onto coverslips and transfections were performed 24 hours later, at a confluency of 50-60%. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as transfection agent according to the manufacturer's instructions. Cells were passaged the next day and retransfected 24h later at a confluency of 50-60%. Cells were left in the incubator for 48h after which they were scraped, as described

above, or subjected to BFA treatment and processed for confocal microscopy, as described above.

Supporting figure legends

Supplemental figure 1

Isoelectric focusing of serum transferrin of a control and the COG4-deficient patient. *The number of sialic acid residues attached to transferrin molecules is indicated, normal glycosylated transferrin carries 4 residues.*

Supplemental figure 2

The full COG complex is mainly membrane associated. *Quantifications of the gradients of both cytosolic and membrane fractions of all three cell lines showing the percentage of the indicated protein present in each fraction (A: COG3 / B: COG4 / C: COG7/ D: COG8). The control results are averages of three independent experiments, while the results of both patients are averages of two. Only the results of the COG4 protein in the membrane fractions of the COG4 and COG8 patients and the COG7 protein in the cytosolic and membrane fractions of the COG8 patient were based on single experiments.*

Supplemental figure 3

Normal intracellular localization of the COG proteins in the COG4-deficient patient. *Confocal staining for the COG proteins was performed on cells of a control and the COG4 patient. Both an overview and a zoomed (indicated on the overview by a square) representative images are shown for the COG3 and COG8 stainings.*

Supplemental figure 4

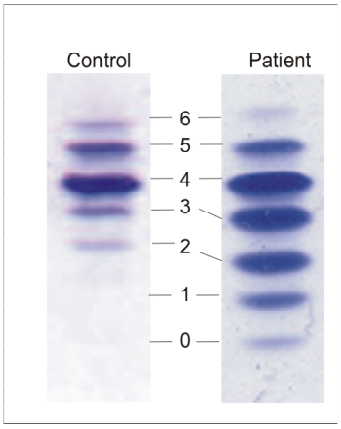
Analysis of the Golgi morphology at the submicroscopical level. *Cells of all patients and one control were subjected to electron microscopy, in order to visualize the defective Golgi structure into more detail. Both the COG1 and COG4 deficient patient cells still show some stacks with a normal morphology, in contrast to the COG7 and COG8 patients. The most striking finding in all cells, but mainly in the COG7 and COG8 patients, was an undulated appearance of the stacked cisternae, as indicated by the arrowheads. Furthermore, several stacks have a fragmented and/or vesiculated appearance, indicated by arrows, a phenotype which is most severe in the COG8 patient.*

		COG1	COG1	COG4	COG7	COG8	COG8
Mutation(s)		c.2659-2660insC p.P888fsX900	c.1070+5G>A	- c.2185C>T p.R729W - Submicroscopical deletion 16q22	c.IVS1+4A>C altered splicing leading to a frame shift and stop codon	c.1611C>G p.Y537X	- c.IVS3+1G>A p.N290_V452delX453 - c.1687-1688delTT p.F543insHHGX547
Number of families reported (references)		1 (Foulquier *et al.*, PNAS, 2007)	2 (Zeevaert *et al*., Hum Mol Genet, 2008)	1 (this publication)	4 (Wu *et al.,* Nat Med, 2004 Wopereis *et al.,* Eur J Hum Genet, 2007 Ng *et al.,*Mol Genet Metabol, 2007)	1 (Foulquier *et al.,* Hum Mol Genet, 2007)	1 (Kranz *et al.*, Hum Mol Genet, 2007)
General symptoms[a]	Lethality in first year of life	-	-	-	+	-	-
	Feeding/intestinal problems	+	-	-	+	+	+
	Growth retardation	+	+	-	+	+	+
	Unexplained hyperthermia	N.D.	N.D.	N.D.	+	-	-
Dysmorphia	Loose/wrinkled skin	-	-	N.D.	+	-	-
	Micro/retrognathia	-	+	N.D.	+	-	-
	Short neck	-	+	N.D.	+	-	-
	Finger/toe abnormalities	-	+	N.D.	+	+	-
	Other dysmorphies	-	CCSM (Cerebrocosto-mandibular) syndrome	N.D.	+	+	-
Neurological abnormalities	Psychomotor retardation	mild	moderate	N.D.	severe	moderate	severe
	Epilepsy	-	-	+	+	+	+
	Hypotonia	+	+	-	+	+	+
	Microcephaly	+	+	+	+	+ >4 years	+
	Brain MRI abnormalities	+	+	+	+	+	+
	Other neurological features	-	-	ataxia	behaviour	ataxia, contractures, periphiral neuropathy	
Other organ systems	Ocular involvement	-	strabismus	-	-	strabismus, oculomotor apraxia	strabismus
	Cholestasis	-	-	-	+	-	-
	Hepato(spleno)megaly	+	-	-	+	-	-
	Increased serum AST/ALT	N.D	-	N.D.	+	+	N.D.
	Cardio(myo)pathy	+	+	-	+	-	N.D.
	Coagulopathy	N.D.	N.D.	+	+	+	N.D.
	Recurrent infections	N.D.	+	+	N.D.	-	N.D.
	Renal involvement	N.D.	+	N.D.	N.D.	-	N.D.
Included in this study		yes	no	yes	yes	yes	no

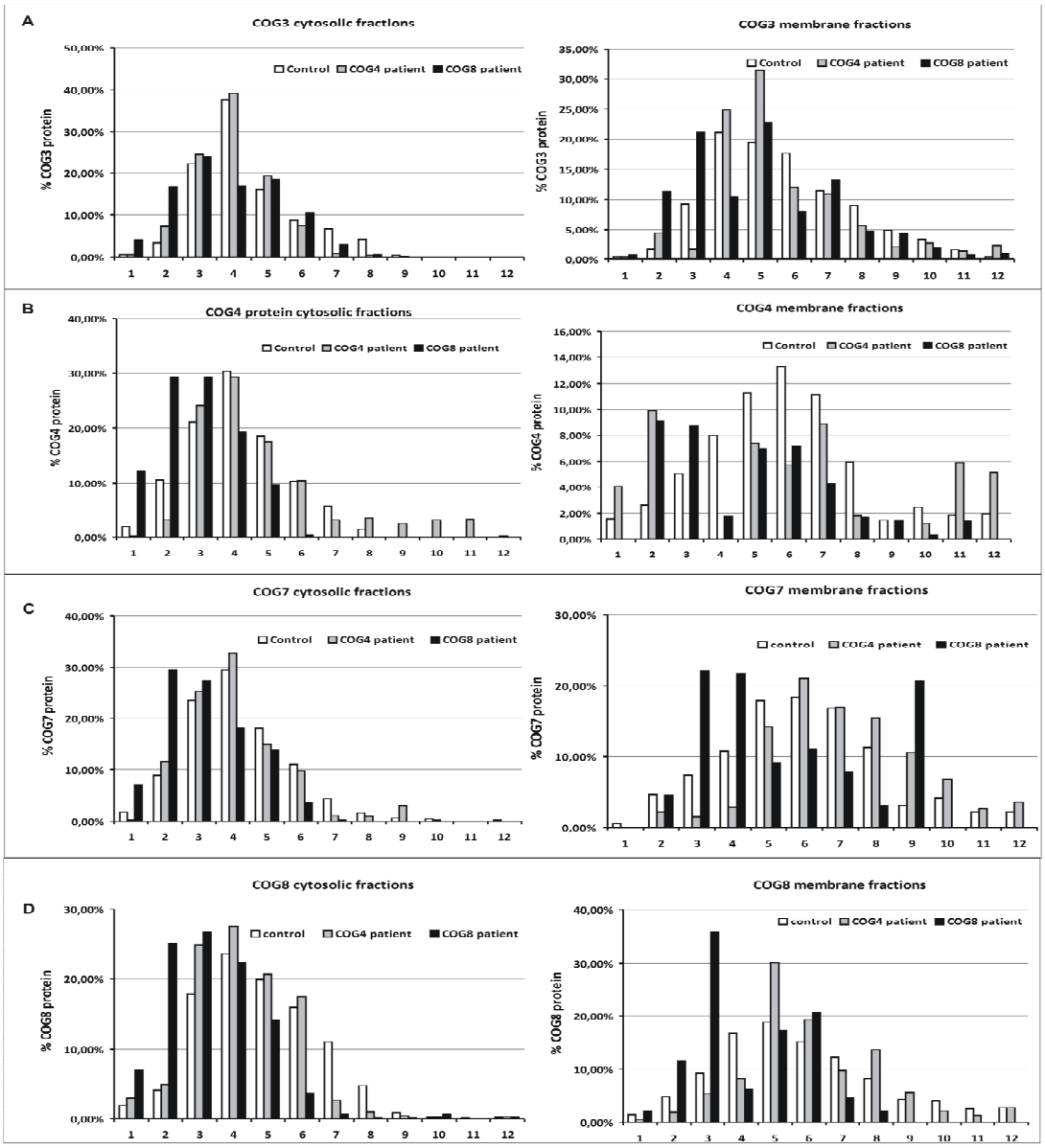
[a] Presence and absence of symptoms is indicated by respectively "+" and "-", N.D. indicates no data was available.

Supplemental table I: ***A clinical comparison of all COG patients known up-to-date.***

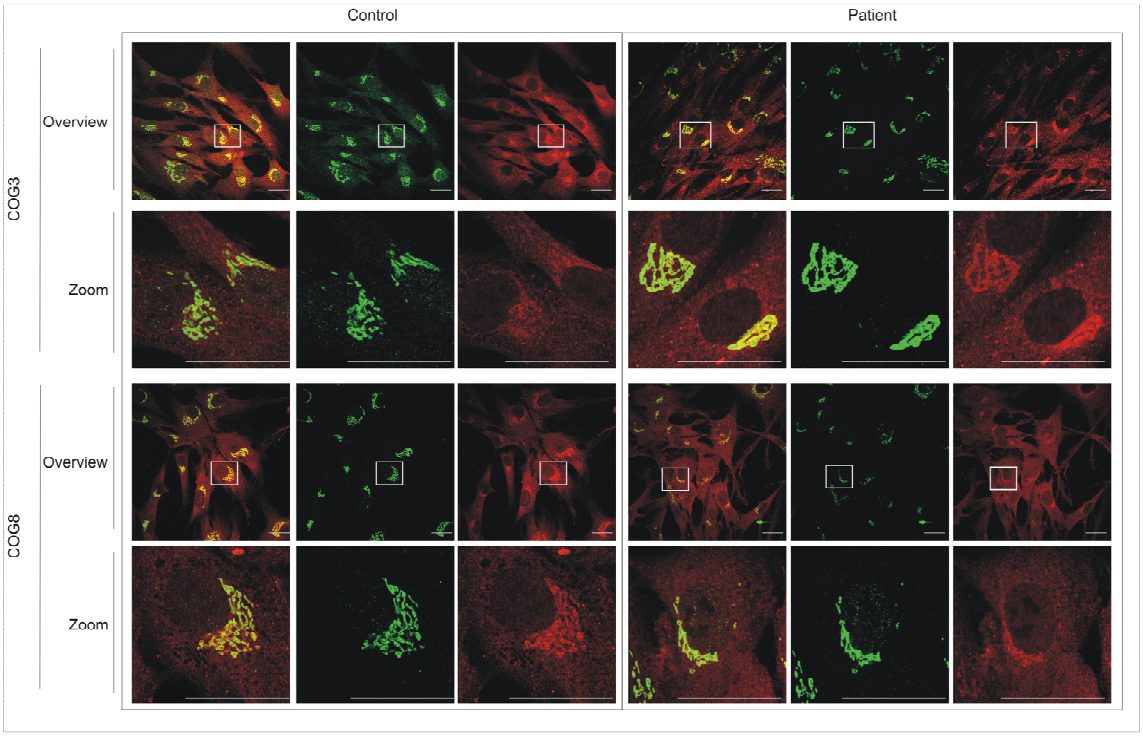
Figures



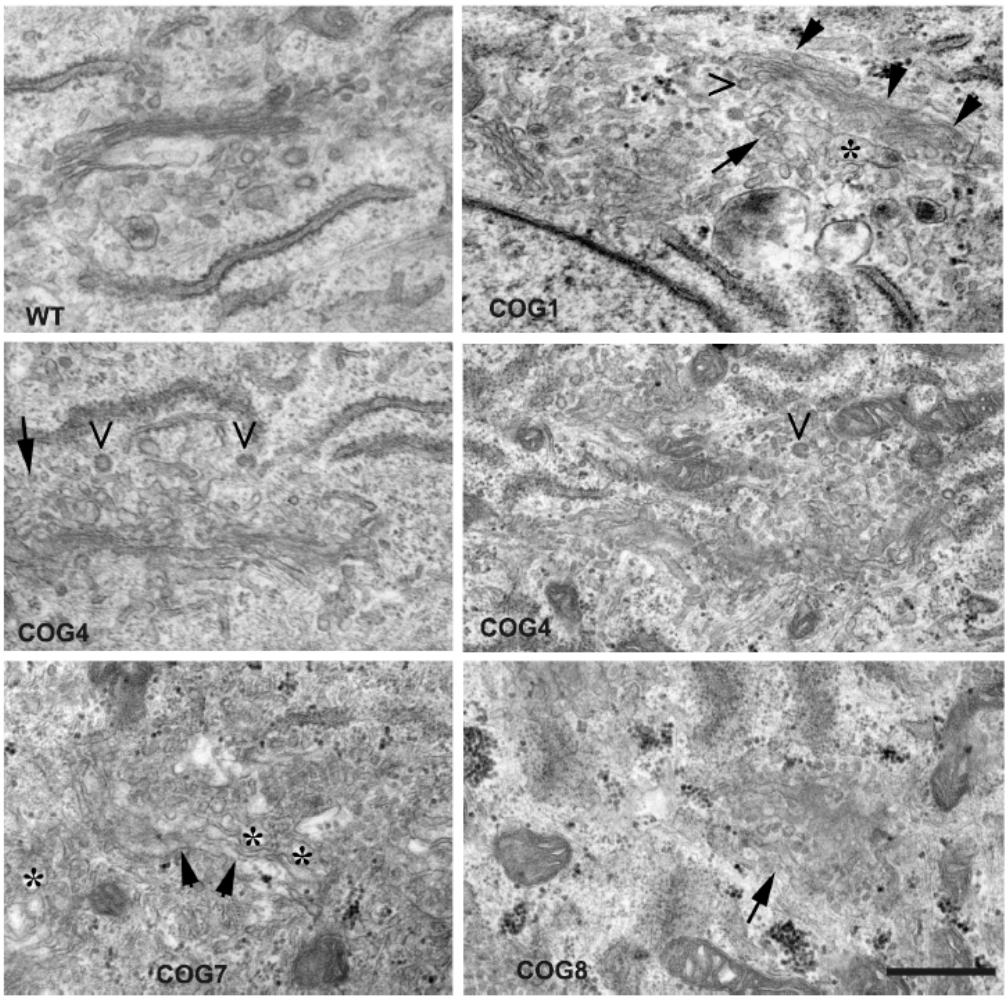
Reynders et al.: Supplemental figure 1



Reynders et al.: Supplemental figure 2



Reynders et al.: Supplemental figure 3



Reynders et al.: Supplemental figure 4