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Deficiency in COG5 causes a moderate form of congenital disorders of glycosylation

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The conserved oligomeric Golgi (COG) complex is a tethering factor composed of eight subunits that is involved in the retrograde transport of intra-Golgi components. Deficient biosynthesis of COG subunits leads to alterations of protein trafficking along the secretory pathway and thereby to severe diseases in humans. Since the COG complex affects the localization of several Golgi glycosyltransferase enzymes, COG deficiency also leads to defective protein glycosylation, thereby explaining the classification of COG deficiencies as forms of congenital disorders of glycosylation (CDG). To date, mutations in *COG1*, *COG4*, *COG7* and *COG8* genes have been associated with diseases, which range from severe multi-organ disorders to moderate forms of neurological impairment. In the present study, we describe a new type of COG deficiency related to a splicing mutation in the *COG5* gene. Sequence analysis in the patient identified a homozygous intronic substitution (c.1669-15T>C) leading to exon skipping and severely reduced expression of the COG5 protein. This defect was associated with a mild psychomotor retardation with delayed motor and language development. Analysis of different serum glycoproteins revealed a CDG phenotype with typical undersialylation of N- and O-glycans. Retrograde Golgi-to-endoplasmic reticulum trafficking was markedly delayed in the patient's fibroblast upon brefeldin-A treatment, which is a hallmark of COG deficiency. This trafficking delay could be restored to normal values by expressing a wild-type COG5 cDNA in the patient cells. This case demonstrates that COG deficiency and thereby CDG must be taken into consideration even in children presenting mild neurological impairments.

INTRODUCTION

Glycosylation is a ubiquitous form of post-translational modification that is essential in most living organisms. In humans, defects of glycosylation cause diseases classified under the generic name of congenital disorders of glycosylation (CDG). The features of these diseases reflect the broad physiological relevance of glycans, thereby ranging from multi-organ failures, dysmorphisms and skeletal malformations to hormonal disorders and coagulopathies (1–3). The clinical manifestations depend also on the type of glycosylation affected. Defects of N-glycosylation are rather associated with neurological disorders, whereas defects of O-mannosylation are linked to congenital muscular dystrophies (4) and defects of mucin-type O-glycosylation for example with anemia (5) and tumoral calcinosis (6).

Diagnosis of CDG is usually reached by isoelectric focusing (IEF) of the serum proteins transferrin and apolipoprotein CIII (ApoCIII) (7) followed by specific enzymatic assays and mutation analysis (8,9). Most forms of CDG can be assigned to mutations in genes participating in glycan biosynthesis including glycosyltransferases, glycosidases, sugar transporters and enzymes involved in the production of substrates for the glycosylation reactions. Only recently, defects in genes involved in vesicular trafficking have been shown to cause CDG. Mutations in the genes encoding subunits of the conserved oligomeric Golgi (COG) complex were the first group of CDG defects to be found outside of the glycosylation

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pathway (10–12). The COG complex has been described as a cytosolic protein complex that is peripherally associated with the Golgi serving as a tethering factor for retrograde vesicular transport.

To date, mutations in genes encoding four of the COG subunits, namely COG1, COG4, COG7 and COG8, have been described as causing CDG (13–17). In COG-deficient cells, the intra-Golgi retrograde transport is disturbed, which affects the distribution of so-called GEAR proteins (18). The GEAR protein family includes SNARE proteins, the golgin and giantin matrix proteins and glycosyltransferases and glycosidases, such as α-mannosidase II, β1,2-*N*-acetylglucosaminyltransferase I, α-2,3-sialyltransferase I (13,18,19). Because of the abnormal distribution of several glycosylation enzymes, COG deficiency is usually associated with underglycosylation of proteins and thus with typical CDG phenotypes. Besides leading to glycosylation defects, COG deficient cells have previously been shown to respond slowly to brefeldin-A (BFA) treatment (20). This drug blocks the GDP/GTP-exchange factor of ADP-ribosylation factor 1 and inhibits the formation of COPI vesicles on the Golgi membrane. It leads to a rapid redistribution of Golgi proteins into the endoplasmic reticulum (ER) (21), a process that can be visualized by immunofluorescence microscopy of Golgi resident proteins. Considering the combined impairment of vesicular trafficking and protein glycosylation, it is expected that COG deficiency leads to severe diseases. However, as reported here, it appears that COG deficiency can also be associated with relatively mild clinical features.

RESULTS

The index patient is the first child of remotely consanguineous parents of Iraqi origin. At her first neuropsychological examination at the age of 8 years, she showed global developmental delay with moderate mental retardation (IQ around 50–55). She showed no dysmorphic features, growth retardation or ocular motor apraxia. However, her speech was slow and inarticulate. She had a tendency to tumble and displayed truncal ataxia and slight muscular hypotonia and showed a slight coordination deficiency in the finger-nose test. Magnetic resonance imaging analysis showed pronounced diffuse atrophy of the cerebellum and brain stem (data not shown). The supratentorial brain parenchyma was without pathological findings. Karyotype analysis, routine laboratory tests, such as measurement of liver enzymes, alkaline phosphatase, lactate and ammonia in blood as well as organic and amino acids in urine were without pathological findings. Furthermore, screens for inborn errors of metabolism including thyrotropin-releasing-hormone, α-fetoprotein, very-long-chain fatty acids and vitamin E were normal. At the age of 12, IEF of serum transferrin was performed, leading to the first indication for CDG (Fig. 1A). Now at 14 years, she has a good speech perception, even tough her speed of speech is still slow. Her language skills improved significantly and her education is bilingual (Arabic/German). She remains mildly hypotonic, with normal reflexes, good strength and truncal ataxia without extrapyramidal signs. Blood for genomic DNA analysis was drawn from all family members, whereas a skin biopsy was taken only from the index patient.

The IEF of serum transferrin in the patient showed increased levels of trisialo-transferrin that clearly differed from the pattern of a control subject or a patient with a N-glycosylation defect caused by a phosphomannomutase2 (PMM2) deficiency (Fig. 1A). This accumulation of trisialo-transferrin is usually a sign of normal N-glycosylation site occupancy but incomplete N-glycan structures. To investigate the N-glycan structure, we have analyzed serum transferrin using electrospray ionization mass spectrometry (Fig. 1B). The observed transferrin peaks were assigned according to the calculated molecular masses (22). In the patient sample, an elevated peak at 79 248 Da was observed. The mass shift of about 300 Da indicated the loss of a single sialic acid residue in the glycoprotein. This finding was compatible with the result obtained from the IEF analysis.

Considering the modest underglycosylation of serum transferrin, we assessed additional serum glycoproteins such as haptoglobin and α1-acid glycoprotein (AGP). Haptoglobin normally carries four N-glycans (23), whereas haptoglobin from a PMM2-deficient patient shows underglycosylated protein forms missing complete N-glycans (Fig. 1C). However, haptoglobin from the present patient did not show any loss of N-glycans, although it appeared to migrate slightly faster in the SDS-PAGE compared with the control lane, suggesting truncated glycan structures on the protein of the patient (Fig. 1C). Enzymatic release of N-glycans with the endoglycosidase PNGaseF led to identically deglycosylated haptoglobin in all samples (Fig. 1C). The glycosylation status of AGP was investigated by 2-D gel electorphoresis. AGP contains five highly sialylated complex-type N-glycans (24), and, due to its low pI narrow-range IEF it can be analyzed readily without purification steps (25). The molecular masses of the AGP glycoforms were comparable in both control and patient samples. However, a shift to more basic pI values was observed in the patient sample (Fig. 1D). This shift was compatible with reduced terminal sialylation of the patient AGP. To determine whether reduced sialylation was limited to N-glycans, we also analyzed the sialylation of the serum O-glycoprotein ApoCIII. The IEF of control and patient serum ApoCIII revealed the presence of unsialylated ApoCIII in the patient sample (Fig. 1E).

A defect of terminal sialylation of both N- and O-glyans has been previously described in patients with deficiency of the COG complex (11,26). COG deficiency is associated with delayed BFA-mediated collapse of the Golgi apparatus into the ER (20), which represents a simple test to assess a possible COG involvement in the underglycosylation of the present patient. Treatment of control and patient fibroblasts with BFA and monitoring of the Golgi structure by β1,4 galactosyltransferase-I immunofluorescence confirmed the possibility of a COG deficiency, as shown by the BFA-resistant phenotype in the patient cells (Fig. 2).

To detect possible mutations in COG genes, we first amplified the corresponding transcripts by RT–PCR. In this survey, we noticed an unexpectedly short cDNA besides the normal *COG5* cDNA. Using various primer sets allowing the amplification of partial stretches of the *COG5* mRNA, we localized the cause of the shorter transcript to a region of the *COG5* gene encompassing exons 13–18 (Fig. 3A and B). Direct sequencing of both *COG5* cDNAs revealed the skipping of

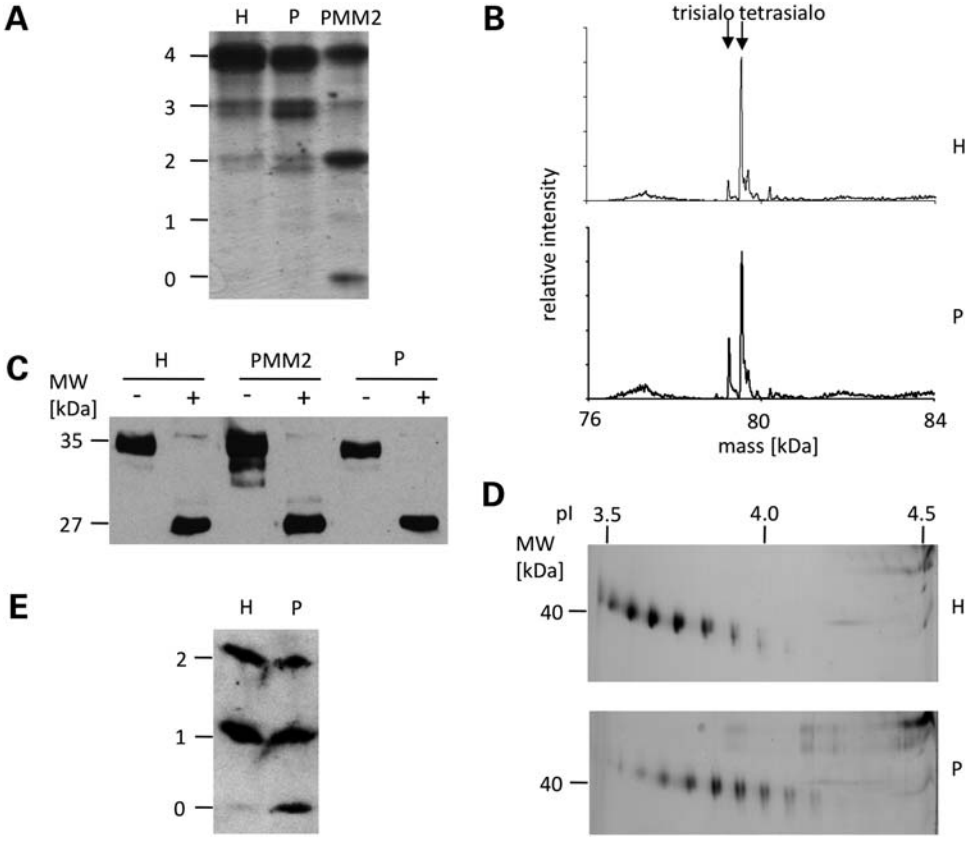

Figure 1. Glycosylation of serum glycoproteins. (**A**) IEF of transferrin from healthy control (H), patient (P) and PMM2 deficient patient (PMM2). The number of sialic acid residues is given on the left. (**B**) Electrospray ionization mass spectrometry analysis of transferrin from healthy control (H) and patient (P). The deconvoluted mass spectra with the molecular masses (kDa) and the relative intensity are indicated. The fully glycosylated transferrin (tetrasialo) and the lighter trisialo-transferrin are marked with arrows. (**C**) Western blot analysis of haptoglobin in healthy control (H), PMM2 deficient patient (PMM2) and patient (P), before (−) and after (+) PNGaseF treatment. (**D**) Narrow range 2-D gel electrophoresis of AGP from healthy control (H) and patient (P). Molecular weight (MW) and isoelectric point (pI) are indicated. (**E**) IEF and immunoblotting of ApoCIII of healthy control (H) and patient (P). The number of sialic acid residues is marked at the left.

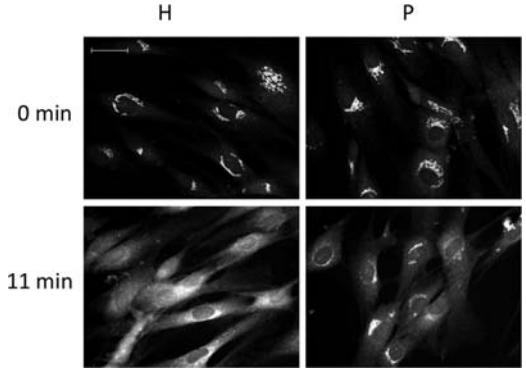

Figure 2. Retrograde transport in BFA treated cells. Healthy control (H) and patient (P) fibroblast were treated with BFA for the indicated time. The Golgi apparatus was stained with β1,4 galactosyltransferase-I antibody. The scale bar corresponds to 20 μm.

exons 15 and 16 in the shorter transcript. Sequence analysis of the patient genomic DNA identified a homozygous mutation in the intronic region 15 bp upstream of exon 15 (c.1669-15T>C), which is presumably leading to the observed altered splicing. To exclude common single nucleotide polymorphisms, we screened 200 unrelated alleles using a MALDI-TOF-MS-based procedure for polymorphism genotyping (27). Fifty of these alleles were derived from samples of similar ethnical and geographical region to the patient's family. None of the alleles analyzed carried the COG5 mutation c.1669-15T>C (data not shown). Haplotype analysis of the family validated our findings since the parents both carry the intronic mutation (Fig. 3C). The expression of the shorter transcript was calculated to create a loss of 58 amino acids (6.4 kDa) in the COG5 protein since the loss of exons 15 and 16 does not lead to a frameshift during translation. To examine this finding, we analyzed the size and amount of the COG5 protein by western blotting in control and patient fibroblasts. As shown in Figure 3D, the low level of full length COG5 protein detected in the patient fibroblasts confirmed the disease-causing effect of the splicing mutation. However, no shorter protein was observed, suggesting that the truncated protein was unstable and prone to degradation.

To address whether the detected *COG5* mutation was uniquely responsible for the trafficking defect, we introduced a wild-type *COG5* cDNA by lentivirus-mediated transfection in the patient fibroblasts. After confirming the stable expression of the wild-type *COG5* construct in the fibroblasts

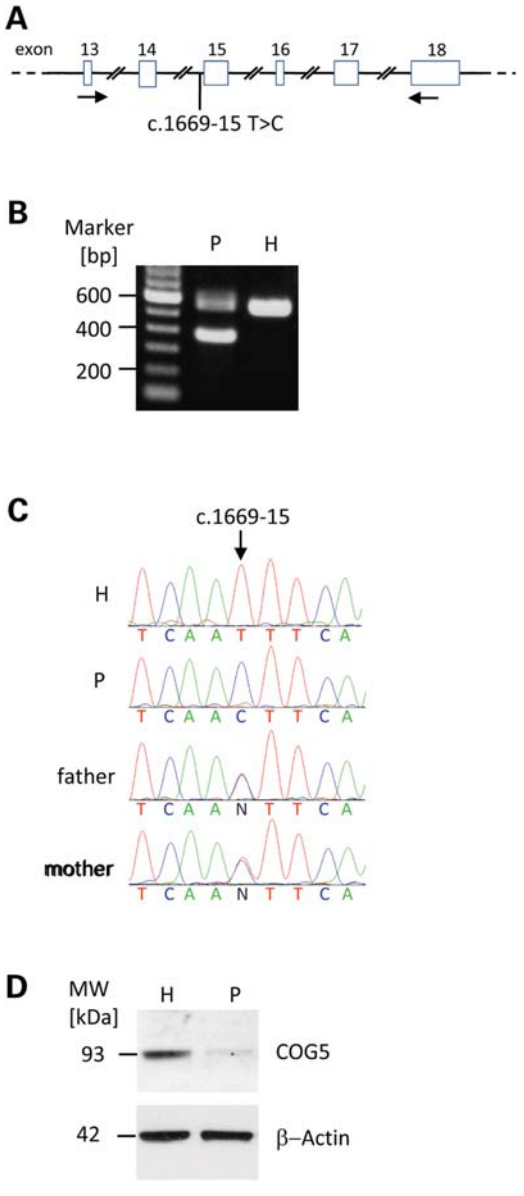

Figure 3. *COG5* mutation analysis. (**A**) Schematic representation of the *COG5* gene organization surrounding the mutation. The positions of the primers used in the RT–PCR and of the mutation are indicated. (**B**) RT–PCR analysis of mRNA isolated from healthy control (H) and patient (P) fibroblasts. The primers used span exons 13–18. The healthy control cDNA fragment is 557 bp-long. The RT–PCR product from the patient sample showed an additional, shorter fragment of 383 bp. The bp-marker is shown at the left. (**C**) Electropherograms corresponding to the stretch of *COG5* genomic DNA encompassing the c.1669-15T>C mutation as detected from healthy control DNA (H), patient DNA (P) and DNA from the parents of the patient. The mutation is indicated by an arrow. (**D**) Steady-state levels of the COG5 protein detected by western blotting from healthy control (H) and patient (P) fibroblasts. β-actin was used as loading control.

(data not shown), we treated the cells with BFA and monitored the collapse of the Golgi apparatus as done before. Whereas the patient cells transfected with a mock lentivirus construct showed the previously described delayed collapse of the Golgi apparatus, the patient cells expressing the normal *COG5* cDNA showed the same rapid collapse of the Golgi apparatus as normal cells, thereby confirming the causality

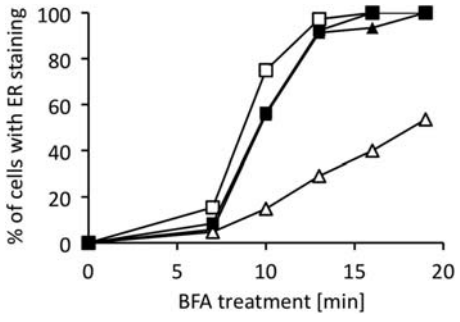

Figure 4. Retrograde transport in fibroblast cells expressing a wild-type COG5 cDNA. Healthy control (squares) and patient (triangles) fibroblasts were lentivirally transduced with either a mock (opened) or a *COG5* cDNA (closed) construct. The Golgi apparatus was stained with β1,4 galactosyltransferase-I antibody. For each time point 250 cells were counted and the values expressed in percents of cells with an ER-staining pattern.

of the COG5 deficiency in the observed phenotype (Fig. 4). As a control, we also showed that expression of either a mock or a *COG5* construct in normal fibroblasts did not significantly affect the speed of the collapse.

DISCUSSION

The identification of forms of CDG caused by COG deficiency marked a new era in CDG pathogenesis, since these defects affect glycosylation indirectly through altered trafficking of glycosyltransferases. To date, deficiencies in the COG1, COG4, COG7 and COG8 subunits have been related to a form of CDG. The first case identified, namely COG7 deficiency, was described for two siblings who died in their first months of life (13). Subsequently identified COG7 patients were also severely affected so that it has been concluded as COG7 deficiency is mainly a lethal multi-systemic disorder (28,29). Two patients are known to be affected at the *COG8* locus (15,16). The clinical features were not as severe as those observed in COG7 deficiency, but both COG8 patients were strongly retarded. They showed obvious neurological abnormalities and required hospitalization. Up to now, the COG1 patients and the recently published COG4 case have represented the group with the mildest clinical features (14,17,21,26,30). However, the present case of COG5 deficiency was even milder, since her clinical status allowed her to live a regular life and to attend a college with therapeutic support.

The moderate clinical outcome of the present patient might be explained by the rather mild glycosylation defect observed in the COG5-deficient cells, since only terminal sialylation was affected. This mild glycosylation phenotype yielded only minor changes in the IEF profile of serum transferrin (Fig. 1A), meaning that complementary tests, such as an ESI-MS analysis, were required to confirm the pathological nature of the finding. Considering the moderate clinical features of the COG5-deficient patient and the nearly normal transferrin IEF profile, we would recommend the application of secondary tests in similar putative cases of CDG. A similar weak glycosylation phenotype was also seen in COG5-depleted HeLa cells (31) and a mild phenotype was

also noted in loss of COG5 function in *Drosophila melanogaster*, which only affected spermatogenesis (32). In contrast, analysis of patient cells with COG1 and COG7 defects revealed that β1,4-linked galactose residues were also partially missing in addition to decreased terminal sialylation (17).

COG subunits may each mediate interaction with different set of proteins, thus explaining varying severity of defects. Defect at a single subunit does not obligatorily destabilize the whole complex. Along this line, it has been shown that deficiency of the TRAPPC2 subunit of the TRAPP complex, another tethering factor, leads to a spondyloepiphyseal dysplasia tarda, a disease affecting the secretion of some extracellular matrix proteins (33).

The intronic mutation in the *COG5* allele is leading to partial skipping of exons 15 and 16 and sequence analysis of the shorter transcript suggested the expression of a truncated COG5 protein in the patient cells, especially because the shorter transcript was more abundant.

However, only the full length protein was detected upon western blot analysis. We assume that the shorter COG5 protein is unstable and thus leading to its degradation.

The description of additional cases of COG deficiency will eventually establish whether the severity of COG deficiency relates to the affected COG subunit or rather to the level of inactivation conferred by individual mutations irrespectively of the subunits themselves.

Since the splicing mutation described here enabled the expression of full-size COG5 protein, yet to low levels, we would assume that other mutations may lead to more severe forms of COG5 deficiency. The identification of a novel mutation in the COG complex is important, since only 11 patients with defects in four of the subunits of the complex have been described so far. It emphasizes that probably all the subunits are essential for correct Golgi trafficking and glycosylation and it is likely that mutations in all subunits of the COG complex could be identified soon among untyped CDG cases. The present description of the COG5 defect in this patient calls for a revision of the concept that COG deficiencies represent rather severe forms of CDG. Accordingly, we would recommend testing for possible COG defects also among cases of mild neurological disorders.

MATERIALS AND METHODS

Glycosylation analysis of serum glycoproteins

IEF of transferrin was performed as described (7) using Immobiline DryPlates (pH 4–7, GE Healthcare) and the Phast System (GE Healthcare). Two-dimensional SDS-PAGE for AGP analysis was carried out as described by Kleinert *et al.* (25). Transferrin was isolated from serum samples and analyzed with electrospray ionization mass spectrometry as reported (22).

Antibodies and western blotting

Anti-haptoglobin was purchased from Rockland (Gilbertsville, PA, USA) and anti-ApoCIII from ANAWA (Wangen, CH). Anti-COG5 was a gift from Dr D. Ungar (University of York, York, UK) and was used at a dilution of 1:100. Proteins were separated by SDS-PAGE and immunoblotted. Signals were detected using the ECL detection kit (Amersham Biosciences). Enzymatic deglycosylation of serum haptoglobin was performed using PNGase F (New England Biolabs).

Sequencing and mutation analysis of *COG5* gene

Total RNA was isolated from fibroblasts using Qiagen RNeasy Kit (Qiagen Inc.). Reverse transcription was performed using QIAGEN OneStep RT–PCR Kit. Mutation screening of the *COG* genes *COG1* to *COG8* was carried out by fluorescent sequencing of cDNA-amplified PCR products on an ABI 3100 automated sequence detection system (Applied Biosystems). PCR conditions and primer sequences are available from the authors upon request. Genomic DNA was isolated from peripheral blood leukocytes using DNeasy Blood & Tissue Kit (Qiagen Inc.). For amplification of the *COG5* cDNA region that was used to examine the skipping of exons 15 and 16, respectively, the following primers were used: 5′-GATTATGATCCAGAAAAGGCT-3′ (forward) and 5′-GGATAATGACCCAGAAAAGTC-3′ (reverse). These primers span the exons 13–18. For amplification of the genomic region that includes the mutation identified in this study, the following primers were used: 5′-TAACATTCTC TTTGTCAGA-3′ (forward) and 5′-GCCAGAGTCAAAGAC TGT-3′ (reverse). The exact PCR protocols are available on request.

Single nucleotide polymorphism analysis

The genomic DNA region containing the mutation was amplified using the forward and reverse primer pair 5′-TAACATTC TCTTTGTCAGA-3′ (forward) and 5′-GCCAGAGTCAAAG ACTGT-3′ (reverse). The PCR product was purified using the QIAquick PCR purification kit (Qiagen Inc.) and used as template for the allele-specific primer extension. The extension reaction was performed with the extension primer 5′-TCATTCTGACACATTCTLTTTGAA-3′ containing a photocleavable linker. The exact PCR protocols are available on request. The extended product was purified using the genopure oligo kit (Bruker Daltonics) according to the manufacturer's instructions. The sample was transferred to an AnchorChip sample target (400 μm, Bruker Daltonics) preloaded with 3-HPA matrix. After UV-cleavage of the linker, molecular masses of the extension products were determined with an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics) in the linear mode.

Cell culture

A punch biopsy of skin from the upper arm was taken from the index patient, minced and incubated in DMEM medium with 10% fetal calf serum, 0.5% penicillin and streptomycin (Invitrogen) at 37°C under 5% CO_2. Primary fibroblasts cultures were obtained by expansion of fibroblast cells growing out of skin explants after 7–14 days. The fibroblasts were further cultured in DMEM with 10% fetal calf serum and 0.5% penicillin and streptomycin at 37°C.

BFA assay and immunofluorescence microscopy

Fibroblasts were grown over night on glass cover slips to ~60% confluence. Medium was then changed with pre-warmed medium containing 2 μg/ml of BFA (LC laboratories). The assay was stopped at different time points by fixing the cells with 3% paraformaldehyde. The cells were permeabilized with 0.1% saponin in PBS followed by immunostaining using a mouse monoclonal antibody to the human β1,4 galactosyltransferase-I protein in a 1:50 dilution (gift from Dr E.G. Berger, University of Zurich, Switzerland). Anti-mouse Alexa Fluor-488 antibody was purchased from Molecular Probes (Invitrogen) and pictures were taken with a fluorescence microscope (Axiovert 200, Zeiss).

Cloning of *COG5* and transfection of fibroblasts

The vector encoding *COG5* cDNA was obtained from Open Biosystems (MHS1010-97227871). The terminal 113 bp of *COG5* missing in this construct were introduced as a *BamH*I–*Xba*I PCR fragment amplified from fibroblast cDNA with the following primer: 5′-TTGGATCCTCCAGCTCAGG AAAACCTGATG-3′ (forward, including a *BamH*I restriction site) and 5′-CGTCTAGAGGGTTAGCACAAAGTGGAGAT G-3′ (reverse, including a *Xba*I restriction site). The complete *COG5* cDNA was then subcloned into pLenti6 vector (Invitrogen) using *BamH*I and *Sac*II restriction sites. Transfection of fibroblasts using lentiviral vectors was carried out as described previously (34).

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Conflict of Interest statement. None declared.

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